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Cancer Nanotechnology Plan 2015

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Preface

Nanotechnology offers the capability to unlock new avenues in the patient specific prevention, early diagnosis, control and treatment of cancer. As such, nanotechnology is expected to offer a significant improvement as compared to the current standard of care in oncology. To capitalize on its potential, the U.S. National Cancer Institute (NCI) in 2004 launched the NCI Alliance for Nanotechnology in Cancer. The Alliance is a large multidisciplinary effort involving researchers and clinicians, who have being working tirelessly in developing new nanotechnological approaches to develop new, and improve upon existing, therapeutic modalities, and similarly for diagnostic and detection techniques. The collective focus has remained on one thing; *a decrease in societal cancer-related morbidity/mortality of multiple tumor types via nanotechnology*. In as much, the Alliance has made very significant progress over the last 10 years producing many scientific discoveries and forming multiple companies, which are commercializing the technologies developed in academia.

Since the beginning of the program, the field of cancer nanotechnology has continually evolved and matured. Recognizing this constant evolution, we publish the *Cancer Nanotechnology Plan* (**CaNanoPlan**) to acknowledge these changes and to attempt charting the path forward for this dynamic field. The authors of this book include clinicians and researchers from the academic, industrial and government sectors. Of importance to notice, is that the number of covered topics has grown substantially since the last edition of CaNanoPlan published in 2010—this is a direct result of the ever-expanding number of areas in the cancer research space that nanotechnology solutions are being effectively used for. Our hope is to deliver to you, the reader, a *current and future state of the cancer nanotechnology field*, without bias, and, more importantly, to impart the numerous areas in which nanotechnological discoveries will impact the future of medical approaches to cancer care.

Nanomedicines: Are they a platform for drug delivery common to many cancer types or a new approach to design drugs for specific tumor types?

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Simply stated, nanomedicines are both. The NCI Alliance for Nanotechnology in Cancer is now entering the third phase of its existence (Phase I and II funding from 2005-2010 and 2011-2015, respectively), and it is an appropriate time to assess where nanomedicines have been and where they are going. Nanomedicine is the medical application of nanotechnology[1] (specifically for cancer see Chow and Ho[2]), so I consider nanomedicines to be nanoparticle-based therapeutics for the treatment of human disease. At this time, the term nanomedicine is used more liberally in that it is employed to categorize nanoparticle-based, therapeutic entities whether or not they are used for the treatment of humans. Petros and DeSimone[3] provide an excellent historical timeline for the development of nanoparticle-based therapeutic entities, while Davis et al.[4] describe how nanoparticle-based, experimental therapeutics distinguish themselves from previous anticancer therapies. Here, I will address the title question by discussing the transition from the "so called" first generation of nanoparticles (Petros and DeSimone, 2008) to the current application of nanoparticle-based, investigational therapeutics for the treatment of cancer.

First generation nanomedicines such as Doxil® (~ 100 nm nanoparticle - liposome encapsulated doxorubicin; approved in 1995) and Abraxane® (albumin-based nanoparticle formulation (~ 120 nm) containing paclitaxel; approved in 2005) are the most referenced nanomedicines that currently are being used to treat cancer patients. These commercial products have provided benefits to patients. For example, Doxil® greatly assists in mitigating the heart damage that can occur with doxorubicin, and Abraxane® does not have the classic hypersensitivity issues due to the cremophor component of paclitaxel formulations. However, these products do have properties that are undesirable. For example, nanoparticle formulations have the potential to create new toxicities that are not observed with the naked drug molecules,

and this phenomenon is observed with Doxil® (causes a form of skin toxicity that is due to the liposomal formulation, while free doxorubicin does not reveal this side effect). Additionally, Doxil® shows changes in pharmacokinetics (PK) upon multiple-cycle dosing in patients[5]. Abraxane® does not function as a true nanoparticle, and should be called a nanoparticle formulation because it dissolves upon administration due to contact with the blood[6]. As such, the control over drug properties, such as release rates, is not possible with these formulations. While Doxil®, Abraxane® [7,8] and other first generation nanomedicines have certain features that modern nanoparticles strive to eliminate or improve upon, these pioneering therapeutics have provided the field of nanomedicines a legitimate starting point. Additionally, they have generated a baseline of human therapeutic data to learn from and for which modern nanomedicines must strive to exceed[9].

Nanomedicines are evolving platforms for continually improving drug delivery that is common to many cancer types

Nanomedicines can be used to deliver drugs to many cancer types. As the field of nanomedicine has progressed, due in part do to increased knowledge of nanoparticle synthesis (better homogeneity is important[10]) and nanoparticle properties (though improved measurement techniques and methodologies), better understanding of how nanoparticles behave in animals[11,12] and humans[13,14] is occurring. This information is enabling nanomedicines to evolve to the point of providing increased functionality that improves the delivery of drug molecules to cancer patients. Nanomedicines seek to improve PK properties (enhanced solubility of the drug, tunable circulation times, tunable release of the drug, even at the site of active in the tumor) and alter biodistribution; in order to have low amounts of drug in non-target tissues and increased drug in tumors for greatly diminished side effect profiles (and most importantly, no new side effects due to the nanoparticle) in patients. These properties can: (i) enable drug combinations formerly inhibited by toxicity limits, (ii) enable new classes of drug delivery (for example, siRNA), and (iii) provide cell specific targeting within a tumor (all illustrated below).

Liposomal formulations such as those used with products like Doxil® have been improved upon, and now can provide new types of nanomedicines. For example, CPX-351 (Celator Pharmaceuticals) is a liposomal formulation of cytarabine and daunorubin in a 5:1 ratio for the treatment of high-risk AML patients. In

this case, the liposome acts to maintain the two drugs in a ratio that creates a synergistic efficacy of the target cancer. This product showed enhanced efficacy in Phase II clinical trials, and is currently being tested in a Phase III trial (NCT01696084). In addition to delivering drug molecules, lipid-based nanoparticles are now used to deliver small interfering RNA (siRNA)[15,16] and other nucleic acids[17]. Tabernero et al.[15] have published the first-in-human clinical results for simultaneously delivering siRNAs against two different gene targets to cancer patients.

...CRLX101 has now been shown in clinical trials to be combinable with other drugs as well as radiation therapy.

Polymer containing nanoparticles are also being developed as nanomedicines for cancer, and they are showing new and interesting behaviors in animal studies and human clinical trials. For example, Schluep et al.[18] showed that a polymeric nanoparticle containing the tubulysin peptide can be an effective antitumor agent while the tubulysin alone is so toxic that there is no therapeutic window for it, even in mice. These types of data show how nanomedicines can open new opportunities with compounds that are not viable on their own (due to toxicity and/or other issues). Polymeric nanoparticles have also been used to deliver siRNA, and in fact, were the first example of siRNA delivery to cancer patients[19]. Additionally, there are situations where the therapeutic agent need not be delivered to the cancer cells, but rather to other cell types within the tumor (like macrophages or stromal tissue). Ortega et al. recently showed how a polymeric nanoparticle could deliver siRNA to tumor-associated macrophages[20].

Polymer containing nanoparticles are progressing in clinical studies. Examples of this type of nanomedicine are the polymeric micelles Genexol-PM (approved in South Korea) and NK105[21], and the homogeneous polymeric nanoparticles CRLX101[13] and BIND-014[22]. NK105 is currently in Phase III clinical testing (NCT01644890), and both of the polymeric nanoparticles are currently in Phase II clinical studies. Of importance to the field of nanomedicine, CRLX101 has now been shown in clinical trials to be combinable with other drugs as well as radiation therapy. This is an important point, as nanomedicines should produce an efficacious therapy with low side effects that they can be used in typical combination therapy regimens. As it is well understood, that combinations of therapeutic agents are ultimately the desired goal in treating cancer patients, in order to provide efficacy and suppress resistance mechanisms from emerging. Pham et al.[23] recently described how CRLX101 (containing the drug molecule, camptothecin) could be used in combination with bevacizumab in ovarian

(both animal and human results) and kidney (human results) tumors. In refractory, metastatic renal cell carcinoma, the combination therapy significantly outperformed a monotherapy of bevacizumab or topotecan (FDA approved analog of camptothecin). A key point is that in the human clinical trials, the doses of CRLX101 or bevacizumab when used in combination did not have to be lowered from the amounts administered when they are used as monotherapies.

Overall, current investigational nanomedicines are showing interesting behavior in animal and human studies. They are providing new properties that have not previously been available (for example, CRLX101 can provide durable inhibition of HIF-1alpha that can be used in combination with anti-angiogenesis therapeutics[23]), and are enabling new types of therapeutic entities like siRNA.

Nanomedicines are a new approach to design drugs for specific tumor types

In essence, nanomedicines are small chemical systems, so they can consist of several components that are designed to provide multiple functions, such as the targeting of specific tumor types. A clear example of this approach is in the delivery of siRNA. Since siRNA can be used to inhibit essentially any gene, and multiple targets can be simultaneously inhibited, specific tumor types can be targeted and treated using this approach. Recently, Yuan et al. showed that four different siRNAs could be delivered to tumor xenografts using a nanoparticle delivery system[24]. Additionally, improved therapeutic efficacy was observed when simultaneously delivering siRNAs against KRAS and PIK3CA/B. This study nicely demonstrates the power of siRNA therapeutics for cancer by showing that multiple gene targets can be simultaneously inhibited (without increased toxicity like would be the case with combining other therapeutic molecules) to produce greater anti-tumor efficacy. This is the goal for the clinical application of siRNA treatments of cancer, and if achievable, could be a "game changing" way to treat cancer. Information from three finished Phase I trials with siRNA are available to guide future studies[14–16,19]. At this time, all of the clinical trials that have employed siRNA do not attack a specific tumor type. However, it is expected that this approach will be used to treat cancer patients with specific cancer types in the near future.

...four different siRNAs could be delivered to tumor xenografts using a nanoparticle delivery system.

Another approach for creating specific tumor targeting nanomedicines involves the inclusion of a so-called "targeting agent" to the nanoparticle to provide for "active targeting"[25]. These targeting agents engage cell surface receptors to not only provide for active targeting, but also to enable a number of other biological functions. CALAA-01 contains the human transferrin protein (Tf) on its surface to engage transferrin receptors (TfR) that are upregulated on the surface of many cancer cell types[26]. The Tf enhances the amount and rate of nanoparticle uptake into the cancer cells. Thus, in this case and others that target the TfR[27], these nanoparticles are appropriate for treating the limited number of cancer cell types that have upregulated TfR. The targeting agents can have biological functions in addition to providing cancer cell uptake, e.g., antibodies and antibody fragments can block signaling effects. An example of this type of nanoparticle, that has been tested in a Phase I clinical trial, is a liposome encapsulating doxorubicin and containing the Fab' fragment of the antibody cetuximab (binds to EGFR)[28]. This nanoparticle is appropriate for treating cancers with overexpressed EGFR. The inclusion of targeting agents adds complexity to the nanoparticles, and the costs versus benefits of these agents have been discussed[29]. However, this type of additional functionality in nanoparticles can clearly be used to create nanoparticles that are designed to treat specific cancer types, e.g., those with upregulated surface proteins like Her2, EGFR, etc. Historically, it has been difficult to achieve functions from the targeting agents. Although recently, investigators have learned how to construct nanoparticles that can have multiple functions, including those of a targeting agent, where the functions work at the appropriate time and place along the delivery process rather than annihilating each other like in the past[30].

Within the next 5 years it is most likely that a number of new nanomedicines will become FDA approved.

What does the future hold for cancer nanomedicine?

Within the next 5 years it is most likely that a number of new nanomedicines will become FDA approved. The cancer nanomedicines that are nearing final clinical testing and approval are those carrying small molecule drugs. Additionally, within this time, there should be the first of several approved siRNA-based nanomedicines. These nanomedicines will not be to treat cancer, but rather for the treatment of liver diseases. However, they will lead the way for siRNA-based nanomedicines to be approved for cancer at a latter time (say within 10 years).

Because of the safety of nanomedicines, once they are approved, it is expected that they will be combined with numerous other therapeutics (including new immunotherapeutics) to provide more individualized and potent therapies to cancer patients. Thus, nanomedicines will be utilized in combination therapies to treat a broad spectrum of cancer types AND to treat specific tumor types, where the mode of deployment of the nanomedicine will depend only upon their specific designs and chemical configuration.

Mission of the NCI Alliance for Nanotechnology in Cancer Program

Nanotechnology is the application of materials, functionalized structures, devices, or systems at the atomic, molecular, or macromolecular scales. At these length scales, approximately the *1-100 nanometer range* as defined by the U.S. National Nanotechnology Initiative (NNI), unique and specific physical properties of matter exist, which can be readily manipulated for a desired application or effect. Furthermore, nanoscale structures can be used as individual entities or integrated into larger material components, systems, and architectures. Nanotechnology-based structures and devices are already enabling a large number of novel applications in various fields – including medicine.

Currently, scientists are limited in their ability to turn promising molecular discoveries into cancer patient benefits. Nanotechnology can provide technical control and tools to enable the development of new diagnostics, therapeutics, and preventions that keep pace with today's explosion in knowledge.

The Office of Cancer Nanotechnology Research (OCNR) within the Center for Strategic Scientific Initiatives (CSSI) at the National Cancer Institute (NCI) of the National Institutes of Health (NIH), develops and implements programs with and for the extramural research community related to the use of nanotechnology in medicine and cancer. The overarching goal of these initiatives is to discover and develop innovative nanotechnologies for application(s), ranging from discovery through to clinical translation phases, for the delivery of innovative clinically relevant technologies aimed at cancer prevention, diagnosis, control, and treatment. These initiatives include a programmatic effort known, collectively, as the NCI Alliance for Nanotechnology in Cancer, which aligns to several key areas of the National Cancer Institute's existing priority areas as displayed in **Figure 1**.

The OCNR's *NCI Alliance for Nanotechnology in Cancer* was designed to develop research capabilities for multidisciplinary team research, with the goal of advancing basic science, prevention, diagnostic, and/or treatment efforts from the research discovery to preclinical and early clinical development stages. The Alliance's development model calls for the most promising strategies discovered

and developed by its grantees to be handed off to potential for-profit partners for effective clinical translation and commercial development. Furthermore, to expedite translation into the clinical setting, it calls for the technologies to be characterized by the Nanotechnology Characterization Laboratory (NCL) in Frederick, MD.

The *Alliance for Nanotechnology in Cancer* is engaged in efforts to harness the power of nanotechnology to radically change the way we diagnose, treat and prevent cancer. As such, the *NCI Alliance for Nanotechnology in Cancer* is a comprehensive, systematized and multidisciplinary initiative encompassing the public and private sectors, designed to accelerate the application of the best capabilities of nanotechnological developments into the realm of contemporary oncology[31].

Purpose of Cancer Nanotechnology Plan 2015

The primary purpose of the *Cancer Nanotechnology Plan 2015* is to serve as a strategic document to the *NCI Alliance for Nanotechnology in Cancer* as well as a guiding document to the cancer nanotechnology and oncology fields, as a whole. Now in its third incarnation, this ***CaNanoPlan 2015*** has increased in scope, mostly, due to the fact that the field has significantly matured and expanded over the last decade. It includes contributions from researchers, clinicians, policy makers, and industrial experts in order to give a broad perspective on where the field is now and where it is heading in the future.

Figure 1. Graphical depiction of NCI Alliance for Nanotechnology in Cancer research areas (colored only) relative to the overall NCI priority areas.

Current State of the Program

In its first round (*Phase I, 2005-2010*), the Alliance focused on translational research (e.g., clinically worthy technologies) and developmental efforts to set the framework for the future. During this period, the program focused on multifunctional therapeutics, *in vivo* molecular imaging (imaging systems and contrast agents), and reporters of efficacy as well as on the areas of early detection, prevention, and control. The research covered a broad spectrum of cancer-specific targets[32]. The awards made during this period included, ***eight*** U54 (formally called ***Centers of Cancer Nanotechnology Excellence*** or ***CCNE***) and ***twelve*** R01 (formally called ***Cancer Nanotechnology Platform Partnerships*** or ***CNPP***) grants. The Alliance was overseen by the Coordination and Governance Committee (CGC), which consisted of its principle investigators and the National Cancer Institute program staff. Near the conclusion of the first round, strategies were re-assessed from lessons learned by the NCI, CGC, and the extramural communities to determine the best path forward for the next round[33,34].

In its second round (*Phase II, 2010-2015*), the Alliance re-balanced itself while maintaining translational research for its CCNEs with more basic research for its CNPPs. Also, the training and developmental efforts to proliferate the preparation of the next generation of multidisciplinary researchers in the field of cancer nanotechnology were expanded. This training component was viewed as an increasingly critical element to developing the multi- and trans-disciplinary scientists necessary to the future implementation of nano-enabled interventions in the practice of clinical oncology. In an attempt to emphasize cancers with the poorest survival rates and explore successful use of nanotechnology in therapies and diagnostics for them, Phase II of the program focused on brain, lung, pancreatic, and ovarian cancers. The awards made during this period included, ***nine*** U54 (*CCNEs*), ***twelve***

U01 (*CNPPs*), ***six*** R25 (formally called ***Cancer Nanotechnology Training Center*** or ***CNTC***), and ***seven*** K99/R00 *Pathway to Independence Award* grants. Nearing the expiration of this second phase in 2013, again a reevaluation was performed in order to formulate a path forward for the program, guided by similar principles as before[35,36].

To date, the communal output from the Alliance members has been substantial. Beginning with the output of robust science, the Alliance has published over *2,750* peer-reviewed journal articles that have been collectively cited over *83,500* times across the scientific literature spectrum generating an average impact factor of *7.7*. From the perspective of clinical translation, the Alliance researchers have filed over *220* patents/disclosures, filed many applications to the FDA with over *18* clinical trials approved, and formed over *85* companies that have collectively commercialized multiple products. This collective

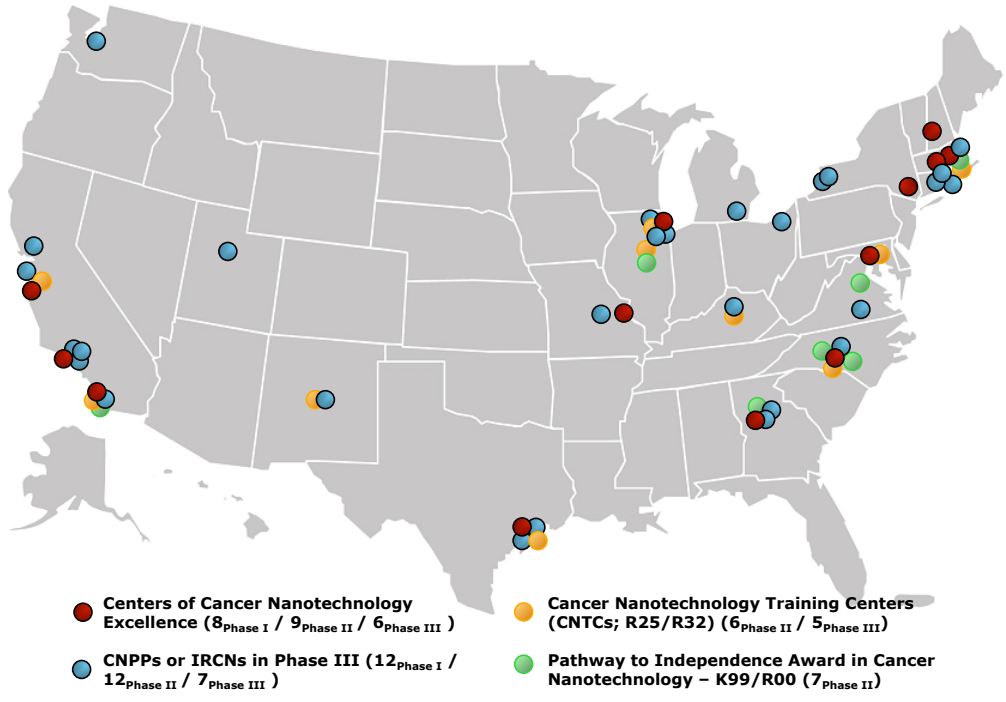

Figure 2. Map of United States as a geographical depiction of the locations of the NCI funded institutions (past and present, all represented) within the Alliance as of Fall 2015. CCNEs (red dots), CNPPs/IRCNs (blue dots), CNTCs (orange dots) and Pathway to Independence (green dots) all displayed circa their actual location in U.S.

output has come by way of NCI funding of over 1250 individual researchers and trainees. All of these statistics are direct results from work completed on Alliance-specific funded projects during only the 10-year period of the first two phases and are compiled in the **Infographic**.

Presently, the *NCI Alliance for Nanotechnology in Cancer* program is beginning its third round (i.e., Phase III), which began Fall 2015. The academic institutions that have been awarded grants during all three rounds to date are displayed, geographically, on the map in **Figure 2**. Although, this third round is similar overall to the previous, there are still several key differences. In this third phase, ***six*** U54 (CCNEs) have been awarded and the U01 granting mechanism has been altered from an RFA to a PAR for recurrent acceptance of applications including two application receipt dates per year through 2017. U01 grants are now formally termed Innovative Research in Cancer Nanotechnology (IRCNs) under this FOA, which reflects a shift in program focus towards addressing major barriers in cancer biology and/or oncology using nanotechnology and with an emphasis on fundamental understanding of nanomaterial interactions with biological systems and/or mechanisms of their *in vivo* delivery. CNTCs have also been transitioned to continual submission and are now funded via a T32 granting mechanism albeit through recurrent receipt dates. Although, the focus on training the next generation cancer nanotechnology experts has remained effectively unchanged. As of Fall 2015, ***seven*** U01 (IRCN) and ***five*** (CNTC) awards have been funded, although it is anticipated that more could be made over the course of next several years as more applications come in for the upcoming submission dates.

Nanotechnology Characterization Laboratory

In an effort to help advance the clinical translation of novel nanomedicines designed to improve therapeutic outcomes and enhance diagnostic capabilities, the National Cancer Institute, in concert with the Food and Drug Administration (FDA) and the National Institute of Standards and Technology (NIST), created the Nanotechnology Characterization Laboratory (NCL). The NCL has been pursuing preclinical characterization and development of these oncology-directed therapies and diagnostics for more than ten years now. In this time, NCL's multi-disciplinary team has worked with more than 100 of the world's foremost nanotechnology research organizations and evaluated

more than 300 different nanomaterials. Nearly a dozen NCL collaborators are now in human clinical trials with novel treatment strategies afforded through nanotechnology. NCL's unique setup has afforded an extraordinary opportunity to explore the biocompatibility trends and advantages and disadvantages of a vast array of nanoplatforms, cytotoxics, and targeting strategies in a relatively limited time span. Through sustained research and extensive educational outreach, the NCL strives to continually improve the pursuit of these much needed therapies, speeding their progression to clinical trials.

caNanoLab

The cancer Nanotechnology Laboratory (caNanoLab) is a web-based portal and data repository that allows researchers to submit and retrieve information on well-characterized nanomaterials including their composition, function, physical properties, and *in vitro / in vivo* experimental characterizations. Furthermore, information on the protocols used for these characterizations and links to any related publications may be similarly accessed. Initiated in 2006 by the National Cancer Institute as a collaborative effort between the NCI Center for Biomedical Informatics and Information Technology (CBIIT) and the NCI OCNR, caNanoLab serves as an established resource with an infrastructure supporting the structured collection of nanotechnology data to address the needs of the cancer biomedical and nanotechnology communities. While the majority of caNanoLab data has been entered through an in-house curator, individual users can submit data via web-based forms and an established, simple workflow. Submitters can customize the visibility of their data which ranges from private, sharable within a collaboration group, to open for public consumption. caNanoLab can also be used for discovery purposes by searching the results of all the publicly available data, protocols, and information about publications using webform-based queries. These results can be downloaded in spreadsheet-based reports for re-use and additional analyses. caNanoLab software is open source and available for download for local installation. Currently, the NCI instance of caNanoLab has information on 1,090 curated nanomaterial samples, 46 protocols , and 1,901 publications. Users are primarily from the U.S., but have grown to include users from several other countries such as Great Britain, Germany, China, the Netherlands, Spain, and Japan. In 2014, the number of unique portal visitors numbered over 3,000.

TONIC Consortium

The Alliance for Nanotechnology in Cancer established the Translation Of Nanotechnology In Cancer (TONIC) consortium in October 2011 to bring together public, private, and academic sectors interested in nanomedicine drug development, with the mission of accelerating the translation and development of nanotechnology solutions for the early detection, diagnosis, and treatment of cancer. TONIC members organized to combine their expertise to identify and evaluate the most promising technology candidates to develop a robust translational roadmap for the development of nanotechnology-based cancer products. The main goals of this partnership model include providing Alliance researchers insight into industry needs in technology platforms and drug targets, promoting collaborations between Alliance investigators and industry partners on promising pre-competitive and late-stage programs, and serving as a sustained forum for nanotechnology idea exchange. The partnership further provides TONIC members the opportunity to interact with regulatory authorities and the Nanotechnology Characterization Laboratory to promote the qualification, development, and regulatory acceptance of nanotechnologies in cancer. TONIC also encourages the sharing of consortium project results with the scientific community and independent verification opportunities to ensure data reproducibility and robustness.

Membership to the TONIC consortium remains free of charge, and for companies is limited to those that (1) have a successful track record of translating diagnostics and drug formulations and reaching their regulatory approval and, (2) are engaged in the development of nanotechnology-based formulations with application to imaging, diagnostics and therapy. In addition, these companies are expected to have a corporate structure with centralized operations and the capability and resources to effectively move along translational efforts. Currently, membership includes 14 corporate partners, and three patient advocacy groups, with participation by NCL and the FDA.

TONIC has organized several meetings and presentations at various venues over the past three years to educate Pharma and enhance awareness of nanotechnology platform opportunities in developing cancer solutions. It continues to participate in the annual Alliance principal investigators' meetings to promote networking and collaborations between industry and academic groups, and encourages the evaluation of external opportunities and platforms. The consortium has been credited with facilitating interactions

with NCL for TEVA and Astra Zeneca, two TONIC members. TEVA and NCL signed an agreement to initiate a collaborative study. Cytimmune credits TONIC for facilitating a research agreement with AstraZeneca to create a new nanomedicine using an AstraZeneca proprietary drug mounted on Cytimmune's PEGylated TNF gold nanoparticle platform. Moving forward, TONIC continues to take advantage of new opportunities to accelerate the consortium's mission of translating nanotechnologies to the clinic, and enhance academic-industrial partnerships.

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Section I: Emerging Strategies in Cancer Nanotechnology

Early-to-Late Stage Diagnosis: Nanotechnology-Based Interventions

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Introduction

The best chance of winning the war against cancer is to detect the disease at its earliest possible stages prior to there being increased cellular heterogeneity and physical spread of cancer cells from the primary site of origin. Finding cancer early is particularly challenging, as there are fewer numbers of cancer cells, and therefore lower concentrations of biomarkers at the cancer site and in bodily fluids, at an early stage along the natural progression path of the cancer. Furthermore, since most cancers are detected relatively late we often lack the ability to ideally characterize the true properties of early cancers, which are likely quite different than late cancers. Simply put, as there are more cancer cells present in advanced stage disease, in a similar fashion there are likely to be more changes in the genome, epigenome, proteome, and transcriptome when characterized *ex vivo*, as well as more protein targets for molecular imaging probes *in vivo*. All of these challenges can ideally be addressed by nanotechnology-based medical diagnostics as part of the *Nanomedicine* field. For its part, *Nanomedicine* promises unprecedented innovations for early diagnosis, staging, and therapy. It offers capabilities to perform simultaneous cancer detection and treatment in ways unachievable with other strategies. For example, nanotechnology has the potential to greatly impact *in vivo* diagnostics through molecular imaging for early cancer detection, even if, this approach must first be validated through the more tractable problem of impacting the management of later stage cancers. With its capacity to provide enormous sensitivity, multiplexing, throughput, and flexibility, nanotechnology has the potential to profoundly impact cancer patient management in the upcoming years.

Surgery is still the mainstay in medical management for both early and late stage cancers. Preoperative molecular diagnostic screening using both *in vitro* nano-enabled diagnostics tools and nanoimaging can detect and localize the tumor, exclude the patients who have metastasized beyond eligibility for a resection, identify the molecular signatures which can

be used to guide surgical procedure, screen the suitable cases whose biology is surgically most relevant, and orientate the surgeons to enable surgery planning.

Nanotechnology offers many other benefits for cancer early to late stage detection such as detailed single molecule and single cell analysis possibilities instead of 'bulk' measurements (**Figure 1**). Nanotechnology offers: (1) analytical sensitivity, (2) massive biomarker/analyte multiplexing ability, (3) low clinical sample volume operability, (4) capability to continuously monitor health and detect any deviation from it via implantable sensors, (5) capability for simultaneous cancer detection and therapy (theranostics), (6) solutions to visualize oncologic pathogenesis and its response to medical intervention in animal models via intravital fluorescence imaging, bioluminescence, and magnetic resonance imaging (MRI) and finally (7) cost benefits to the patients and the healthcare system at large.

Current Trends in Nanotechnology-Based Intervention for Early to Late Stage Diagnosis

A myriad of preclinical research grade nanobiosensors have already been developed, however, the ultimate goal of multiplexed, low-cost, high-throughput, reliable diagnostic devices for the clinic has yet to be fully realized. Having this capability in the clinic would undoubtedly allow for the improved detection of cancer with potential significant benefits to patients and the health-care system at large.

Often the vast majority of long-term cancer survivors have resectable tumors seemingly confined to the primary site at the onset of diagnosis and hence, they can benefit significantly from curative surgery, supporting that early cancer detection and intervention will increase the overall survival of patients. From a technological perspective, we have great nano-centric tools within our arsenal; disappointingly there are currently no reliable serum biomarkers with the sensitivity and specificity to accurately detect early pre-cancerous lesions. In many ways our technologies are ahead of our understanding of the underlying cancer biology. Furthermore, the heterogeneous nature of cancer and the inherently

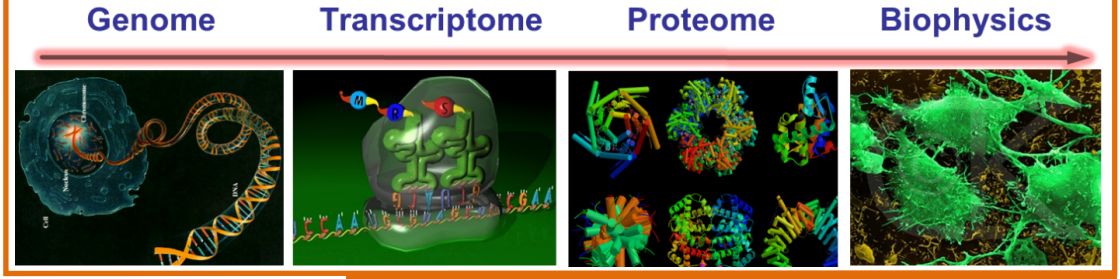

Figure 1. Nanotechnologies for comprehensive cancer cell analysis, ideally at single cell and single molecule sensitivity levels.

complex stromal microenvironment also present a challenge for identification of potential biomarkers. Hence, early diagnosis of tumors requires the simultaneous use of a panel of biomarkers for greater accuracy. In a recent mathematical modeling study[1] it was found that a tumor could grow unnoticed for more than 10 years and reach a spherical diameter of about 25 mm, before becoming detectable by current clinical blood assays. Further complicating it, the shedding rates of most current clinical blood biomarkers are found to be 10^4-fold too low to enable detection of a developing tumor within the first decade of tumor growth. These predictions well-align with clinical observations. Thus, currently there are no biomarkers suitable for screening of healthy general populations for possible occurrence of precancerous events. Routine surveillance of cancer is currently performed through classical cancer detection technologies, such as x-ray imaging based mammography for breast cancer, visible light colonoscopy for colorectal cancer, histo-pathological evaluation of Pap smears for uterine and genital cancers, and skin lesions by microscopic pathology, etc., none of which are presently enabled via nanotechnology. Currently, several preclinical diagnostic imaging tools are going through evaluation for their suitability as adjunctive technologies to the existing contemporary cancer diagnostic approaches. Some of these technologies are magnetic nanoparticle or gadolinium chelate-functionalized nanoparticle-enabled for high resolution MRI[2–4], nanoparticle and intrinsic contrast-based photoacoustic imaging[5,6], surface enhanced Raman spectroscopy-based endoscopy[7], cancer triggered self-assembling smart optical and MRI nanoimaging agents[8–10], micro-Nuclear Magnetic Resonance imaging[11], dual (*e.g.*, PET-Near Infrared fluorescence and PET-MRI)[12,13] and nano-enabled triple modality imaging (*e.g.*, MRI-Photoacosutics and Raman)[14]. A recent review summarizes the status of nanoimaging agents and the clinical trials associated with these approaches[15].

Currently, in the field of cancer nanotechnology-focused diagnostics, two very broad groups of devices and tools are emerging and there is strong and ongoing research in both. These groups are (1) benchtop or larger scale medical diagnostic devices and (2) miniaturized nano-based or nano-enabled diagnostic assays/devices that are designed and suitable for point-of-care or for patient's use at home directly or suitable for implantable, wearable, ingestible, inhalable uses. The medical expectations from the first group of devices is that they will be extremely robust, sensitive and specific as such they are suitable for confirmatory decision making that can both inform and guide clinical management of cancer. Nanoparticle-based imaging agents (*e.g.*, paramagnetic iron oxide or gold or silica-based nanoparticles, carbon nanotubes, surface enhanced Raman nanoparticles, etc.) and their associated detection/analysis instrumentation and nanoimaging devices (*e.g.*, nanoparticle assisted MRI, photoacoustic imaging, Raman spectroscopy) are examples of this category. On the other hand, the second group of cancer nanodiagnostic tools includes: nanocantilever, nanopore, nanowire, quantum dot, plasmonic nanoparticle-enabled micro/nanofluidic

devices, among many others. The medical expectations from these second group of point-of-care devices is that they will be cheap, produce rapid and reliable results, often during the same office visit and yield actionable results for seeking further medical evaluation. The first category of nanodiagnostic tools that are typically more suitable for later stage cancer and the second category of diagnostic tools are more applicable to early stage detection of cancer, recurrence, therapeutic efficacy monitoring, as well as general surveillance. There is a continued cancer nanotechnology research need for the improvement of and innovation in both of these categories of the medical diagnostic tools, which are inherently synergistic in principle from a medical benefits perspective.

Even with the progress resulting from early detection, the long-term prognosis of cancer patients is still limited by the occurrence of distant secondary metastases via circulating tumor cells (CTCs). Clinically occult micrometastases caused by these cells cannot currently be detected at primary diagnosis even by high-resolution diagnostic imaging approaches. The presence of CTCs in blood and bone marrow has shown to have therapeutic and prognostic impact for cancer[16–20]. It is postulated that CTCs could escape from chemotherapy by maintaining a dormant non-proliferating cell state (senescence) until the conditions are optimal to start expansion to manifest metastases[21]. Thus, the detection, enumeration and characterization of CTCs and their clusters (*i.e.*, 'liquid biopsy') remains as a viable candidate to investigate its potential to increase survival benefit for cancer patients, in particular, due to its ease of access and amenability for repeat sampling. A multitude of micro- to nano-scale technologies are now available to isolate an enrich CTCs[22,23], as well as highly sensitive and specific immunological and molecular assays[24,25] to characterize these cells at the single cell level in bone marrow and peripheral blood. These studies are providing insights into the critical steps of the initiation of the metastatic cascade.

...CDs offer significant potential as replacements for toxic metal-based quantum dots that have had difficulty with clinical translation.

Similar to CTC capture and characterization, extracellular vesicles released/secreted by cancer cells and loaded with cellular signals such as microRNAs and proteins, are emerging as important oncologic clues that can be obtained from clinical cancer samples (reviewed in Zocco *et al* 2014 and Webber *et al* 2015)[26,27]. The nondestructive isolation, enrichment, enumeration and intra-vesicular content analyses of these particles via the use of nanotechnology, such as nano-mechanical filters[28,29], nanoflare-based diagnostics (reviewed in Heuer *et al*, 2013, Prigodich *et al* 2012)[30,31], nanoproteomics analysis[32], bio-barcode-based analysis (reviewed in Pritchard, *et al* 2012)[33] are emerging as important

tool for cancer diagnosis, response to therapy and for prognostic surveillance. This field is currently expanding and it is expected to play a major role in cancer medical management in the near future.

Luminescent carbon dots (CDs) are emerging as new medical diagnostic tools as alternatives to quantum dots and other carbon-based nanomaterials such carbon nano tubes and graphene. These nanoparticles have well-defined, tunable surface functionalities, and their manufacture involves simple, fast, and cheap synthetic routes. Because of good biocompatibility, hydrophilicity, non-toxicity, resistance to photobleaching and -blinking, CDs offer significant potential as replacements for toxic metal-based quantum dots that have had difficulty with clinical translation.

Another novel development in the cancer nanotechnology field is the use of mass-encoded synthetic biomarker libraries for multiplexed monitoring of cancer in bodily fluids[34]. These exogenously administered 'synthetic biomarkers' are composed of mass-encoded tandem peptides conjugated onto nanoworm nanoparticles that leverage the intrinsic features of human disease and physiology for noninvasive urinary monitoring. These protease-cleavable peptide-based cancer sensors can target sites of disease, sample dysregulated protease activity and emit mass-encoded reporters into patient urine for multiplexed detection by mass spectrometry. It was shown that these agents can noninvasively monitor disease without the need for invasive core biopsies and the respective blood biomarkers.

The Future of Nanotechnology-Based Intervention for Early-to-Late Stage Diagnosis

Nanoscience applied to cancer research is proving to be a critical and encouraging approach for the eventual elimination or at least chronic control of cancer. Nanotechnology has been making a significant impact on cancer diagnosis and therapeutic management in revolutionary ways as exemplified in the NCI's 2010 Cancer Nanotechnology Plan (http://nano.cancer.gov/about/plan). Nanotechnology will continue to advance both *in vitro* diagnostics through genomic, cellomic, transcriptomic, proteomic and circulating tumor cell enumeration as well as exosome and microRNA analysis based nanosensors and for *in vivo* diagnostics via nanoparticles for molecular imaging. Moreover, *in vitro* diagnostics used in conjunction with *in vivo* molecular imaging is expected to markedly impact future cancer patient management by providing a synergy that neither strategy alone can offer. Indeed, the areas of earlier cancer detection and the prediction and monitoring of patient response to anti-cancer therapies could be impacted by this synergetic approach. Both represent very important applications for nano-enabled diagnostics with near-term clinical translational potential.

Specifically, the earlier detection of relevant cancers that are aggressive is still a major challenge for the cancer community. Earlier intervention of potentially aggressive cancers can greatly improve patient survival, quality of life and financial outcomes. These could be achieved via the synergistic use of highly sensitive and specific *in vitro* diagnostic devices to interrogate easily accessible clinical sample sources such as blood, urine, feces, sweat, tears, and saliva for multiple biomarkers (both protein and nucleic acid-based) and verify the presence and location of the tumor with nano-/molecular imaging *in vivo* using novel nanoparticles that allow signal amplification and multiplexing. As example, a cancer patient has cancer detected at much earlier stage through use of biomarkers derived from blood or other non-invasive samples and results from these *in vitro* tests are then verified by molecular imaging that simultaneously localizes tumor(s) prior to treatment. Additionally, post-treatment and potentially during treatment, the patients' response to therapy is measured to ensure the accurate differentiation of responders from non-responders can, which could be continually evaluated by blood analysis, without necessitating another tumor biopsy and/or molecular imaging.

The application of the above two approaches (combination of *in vitro* diagnostics with nanoimaging and the combination of *in vitro* diagnostics with benchtop ultrasensitive, specific nanodiagnostic technologies) in particular to the current unsolved oncologic challenges of detection of distant micrometastases, prognostic evaluation of tumor aggressiveness and its predicted response to a given therapy, differentiation of indolent tumors from the ones that have metastatic potential, tumor border demarcation during surgery are areas where there are significant gaps in our diagnostic abilities, hence, further and significant cancer nanotechnology efforts need to be spent on these critical areas to improve cancer patient outcomes within the next 5-15 years. Ideally, nanotechnology could make a huge impact in cancer by virtue of pre-emptive interventions to detect cancer early through continuous health monitoring via wearable, ingestible and implantable nanodiagnostics to detect deviation from health to pre-neoplastic conversion as early as possible. However, being able to get there will involve not only further nanotechnological advancements, but also, further improvements in the toxicological, biocompatibility and immunological concerns related to nanoparticles' use as cancer *in vivo* diagnostics. With appropriate level and timely financial commitments for nanoscience and nanotechnology research, the future of the *Cancer Nanotechnology* field is bright and full of opportunities as well as tremendous near-term rewards for patients.

Early-to-Late Stage Diagnosis: Detecting and Analyzing Circulating Tumor Cells

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Circulating Tumor Cells (CTC)

The tissue-based evaluation of biopsy samples remains the gold standard for diagnosis and prognosis in clinical care and research. The bulk of published research focuses on tissue samples obtained by surgical excision or radiographically directed needle extractions. While these approaches have driven a tremendous amount of research, they are complicated by several issues. First, these extractions are both invasive to the patient and costly overall. Typically, serial biopsies are avoided for fear of complications from the procedure, but are essential in obtaining dynamic insight. Second, in cancers where metastatic tissue biopsies are problematic, research has relied upon historic primary tissues. Third, there is growing focus and concern for the impact of the tumor tissue's temporospatial heterogeneity.

As a measure to address these problems, circulating tumor cells (CTCs) have been proposed as they provide a means to sampling tumors across all present disease sites (they are perfused systemically in blood), including the primary tumor and metastases[35]. In addition to conventional diagnostic imaging and serum marker detection in cancer, the detection and characterization of CTCs in patients over the course of therapy creates new possibilities for personalizing cancer care by: (i) monitoring cancer progression, (ii) understanding the pathogenic mechanisms driving lethal disease and the dynamics of this evolving biology, and iii) guiding the implementation of the most effective treatment interventions and re-strategizing upon the emergence of resistance. Over the last decade, significant progress has been made in the areas of CTC detection, isolation, and characterization that has largely been driven by collaborative and interdisciplinary research efforts spanning across chemistry, materials science, bioengineering, and oncology. Recent technological advances in the field of nanotechnology offer powerful microfluidic systems and unique nanomaterials, which will enable a diversity of in-depth characterizations of CTCs with drastically reduced costs and ultimately bring the field of oncology closer to the goal of personalized care.

Conventional CTC Assays

The most widely used CTC detection assays include: (i) Immunomagnetic separation: these methods utilize capture agent-labeled magnetic beads to either positively select CTCs using a cell surface marker (*e.g.*, anti-EpCAM) or negatively deplete white blood cells (WBCs) using anti-CD45. The CellSearch™ Assay is the only FDA-cleared CTC diagnostic technology for metastatic breast, prostate, and colorectal cancers[36]. CellSearch™ Assay harvests CTCs with anti-EpCAM-coated magnetic beads, and the subsequent immunocytochemistry (ICC) process helps to identify CTCs (DAPI+/cytokeratin, CK+/CD45-) from nonspecifically captured WBCs (DAPI+/CK-/CD45+). Recently, several new systems (*e.g.*, MagSweeper, IsoFlux, Cynvenio, magnetic sifters, VerIFAST and AdnaGen/Qiagen) have been developed to further improve detection speed and efficiency. (ii) Flow cytometry: In conjunction with the use of fluorescent markers, flow cytometry is one of the most mature technologies for analyzing and sorting subpopulations of cells. However, this flow-based methodology is unable to provide the CTCs' morphological information to meet the gold standard set by pathologists. An improved method, known as ensemble-decision aliquot ranking, was developed to address this weakness[37]. (iii) Microscopy imaging. Microscopy imaging of ICC-treated blood samples allows for highly sensitive detection of CTCs, accompanied with their morphometric characteristics and protein expression. Currently, Epic Sciences is one of the leaders in the commercial sector, now providing CLIA-certified laboratory tests for both CTC enumeration and characterization. In contrast to the previous three approaches, which require the use of CTC markers, the following two approaches are recognized as label-free methods. (iv) CTC filters: Filter-based approaches have been established to trap CTCs according to their sizes. A wide collection of commercial kits/systems from Rarecells, ScreenCell, Clearbridge, and Creatv MicroTech etc. are now available to support research utility. Nevertheless, concerns regarding overlooking small-sized CTCs have been raised. (v) Dielectrophoresis: CTCs can be sorted from WBCs in the presence of a dielectrophoretic field, since the CTC's dielectric properties (depending on their diameter, membrane area, density, conductivity and volume) are different from those of WBCs. ApoCell's technology leverages these differences in a microfluidic flow channel to isolate CTCs. Silicon Biosystems' DEPArrayTM combines the use of microscopy imaging and dielectrophoresis sorting to identify and isolate pre-sorted CTCs, paving the way for downstream single-CTC molecular characterizations. (vi) Other methods: There are several outstanding review articles where side-by-side comparisons of a wide collection of CTC detection technologies are presented[38,39].

Microfluidics-enabled CTC Assays

The microfluidic affinity-capture devices demonstrated by the Massachusetts General Hospital team kicked off the research efforts devoted to the development of

nanotechnology-enabled CTC assays[40]. Their 1st-generation (gen) device (*i.e.*, CTC-Chip) featured chemically etched microposts on a silicon substrate, on which anti-EpCAM antibodies were covalently functionalized. These embedded microposts maximize the contact between the device surfaces and the flow through cells. Following CTC capture, ICC was conducted to identify CTCs from background WBCs. The CTC-Chips demonstrated significantly more gains in CTC enumeration performance than most of the conventional CTC assays. Thereafter, similar device configurations were adapted to create new microfluidic chips (*e.g.*, geometrically enhanced differential immunocapture, GEDI approach and Biocept's CTC assay), where different antibody capture agents were employed. Recently, a unique "Ephesia" approach based on microposts of capture agent- coated magnetic beads self-assembled in a microchip demonstrated combined advantages of both microfluidic and immunomagnetic cell sorting[41]. The MGH's 2nd-gen device (*i.e.*, herringbone-chip, HB-Chip) was made from an imprinted PDMS component on a glass slide[42]. Microscale herringbone patterns were engineered into the PDMS component to introduce microvortices, leading to enhanced contact between the CTCs and the antibody- coated chip surfaces. In addition to the commonly used ICC technique, the transparent nature of the HB-Chip allowed for imaging of the captured CTCs by standard clinical histopathological stains (*i.e.*, H&E stain),. Although the microfluidic setting improves CTC- capture performance, the majority of the microfluidic CTC assays suffer from depth of field issues when performing microscopy imaging due to the vertical depths of 3-dimensional device features. Time-consuming multiple cross-sectional imaging scans that generate large image files are required in order to avoid out-of-focus or superimposed micrographs. By coupling a pair of microelectrodes at the terminal of a plastic microfluidic chip, enzymatic release of the captured CTCs can be electrically counted without the issue of microscopy imaging[43]. In contrast to MGH's 1st and 2nd-gen devices, their 3rd-gen iChip represents a groundbreaking label-free approach, which combines negative immunomagnetic depletion processes with an inertial focusing setting in an integrated microchip[44]. Most importantly, this approach allowed for the recovery of unmanipulated CTCs with desired molecular integrity and viability, paving the way for downstream expressional profiling[45], as well as *ex vivo* culture and drug susceptibility tests[46]. Other microfluidic CTC assays based on unique principles, including micro-nuclear magnetic resonance (μNMR) platform[47], cell rolling[48], and Vortex technology[49] have also been developed and demonstrated. In addition to the microfluidic assays developed for the enumeration, molecular characterization, and *ex vivo* expansion of CTCs, a microfluidic device with designated sections for selectively capturing CTCs according to the amount of magnetic beads grafted on their surfaces has been created[50]. The device was employed to dissect CTCs into subpopulations according to EpCAM expression levels of individual CTCs.

Nanomaterials-enabled CTC Assays

It has long been documented that nanoscale components present in the tissue microenvironment, including extracellular matrix and cell-surface structures provide structural and biochemical support that regulates cellular behaviors and fates. Inspired by the nanoscale interactions observed in the tissue microenvironment, the UCLA team pioneered a unique concept of "NanoVelcro" cell-affinity substrates in which CTC capture agent-coated nanostructured substrates were utilized to immobilize CTCs with high efficiency[52]. The working mechanism of NanoVelcro cell-affinity substrates mimics that of Velcro™ – when the two fabric strips of a Velcro fastener are pressed together, tangling between the hairy surfaces on two strips leads to strong affinity between cell and nanosubstrates. Through continuous evolution, 3 generations of NanoVelcro CTC Chips (**Figure 2**) have been established to achieve different clinical utilities. The 1st-gen NanoVelcro Chip, composed of a silicon nanowire substrate (SiNS) and an overlaid microfluidic chaotic mixer, was created for CTC enumeration. Side-by-side analytical validation studies using clinical blood samples suggested that the sensitivity of the 1st-gen NanoVelcro Chip outperforms that of FDA-approved CellSearch™. In addition to SiNS, the general applicability of the NanoVelcro cell-affinity assay is supported by extensive research endeavors devoted to exploiting different nanomaterials, *e.g.*, polymer dots/nanotubes, TiO2 nanowires/nanoparticles, layer-by-layer-assembled nanostructures, gold clusters on silicon nanowires, Fe3O4 nanoparticles, and graphene oxide nanosheets to achieve high affinity capture of CTCs and other types of rare cells[53]. It is worth noting that NanoVelcro-like approaches allow immobilization of CTCs onto a relatively flat and small surface area, thus allowing subsequent microscopic imaging/identification of CTCs to be conducted quickly. Moving beyond CTC enumeration, UCLA's 2nd-gen NanoVelcro Chip (*i.e.*, NanoVelcro-LMD) was developed by replacing SiNS with a transparent substrate covered with polymer nanofibers[54]. The transparent NanoVelcro substrate retains the desired CTC capture performance, and allows for seamless integration with a laser microdissection (LMD) technique to isolate immobilized CTCs with single-cell resolution. The individually isolated CTCs can be subjected to single-CTC genotyping (*e.g.*, Sanger sequencing and next- generation sequencing, NGS) to verify CTC's role as a tumor liquid biopsy. Most CTC enrichment and isolation methods yield purified CTCs that are either fixed before isolation, damaged during the cell purification process, or irreversibly immobilized on an adherent matrix. Similar to MGH team's iChip, UCLA's 3rd-gen Thermoresponsive NanoVelcro Chip has demonstrated the feasibility to capture and release CTCs at 37 and 4°C, respectively[55]. By grafting thermoresponsive polymer brushes onto SiNS, the temperature-dependent conformational changes of polymer brushes can effectively alter the accessibility of the capture agent on SiNS, allowing for rapid CTC purification with desired viability and molecular integrity. The team has been exploring

the use of Thermoresponsive NanoVelcro Chips to purify viable CTCs for downstream molecular and functional analyses.

Future Scientific and Clinical Developments

Moving forward, future research endeavors in developing the Nanotechnology-enabled CTC assays will be driven by the needs of: i) acquiring a fundamental understanding of the nanointerfaces between CTCs (*e.g.*, how the underlying physical/chemical

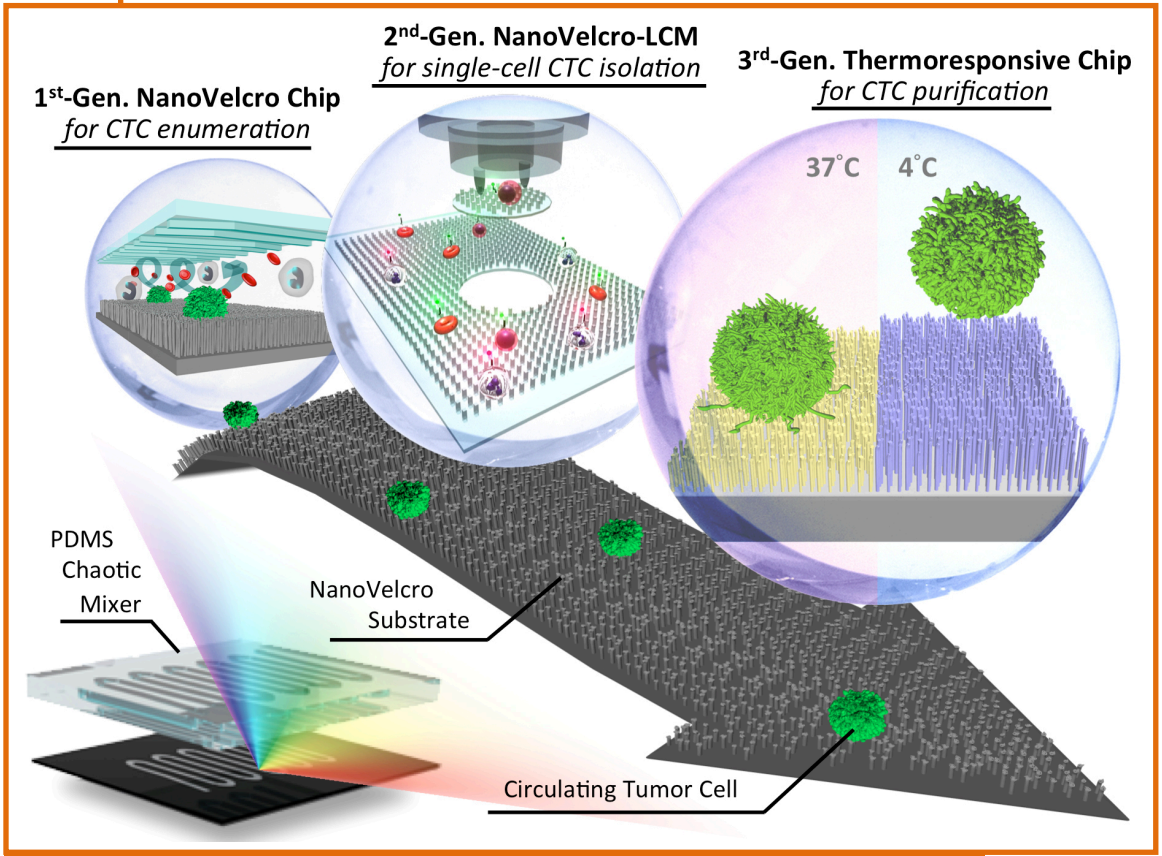

Figure 2. Conceptual illustration of the three generations of NanoVelcro CTC Assays developed by the UCLA team to achieve different clinical utilities. 1st-gen NanoVelcro Chip, composed of a silicon nanowire substrate (SiNS) and an overlaid microfluidic chaotic mixer, was created for CTC enumeration. In conjunction with the use of the laser microdissection (LMD) technique, 2nd-gen NanoVelcro-LMD technology, was developed for single-CTC isolation. The individually isolated CTCs can be subjected to single-CTC genotyping. By grafting thermoresponsive polymer brushes onto SiNS, 3rd-gen Thermoresponsive NanoVelcro CTC Chips were developed for purification of CTCs via capture and release of CTCs at 37 and 4°C, respectively. The surface-grafted polymer brushes were responsible for altering the accessibility of the capture agent on NanoVelcro substrates, allowing for rapid CTC purification with desired viability and molecular integrity. (*Reprinted with permission from Tseng et al, 2014*)[51]

properties of any given nanosubstrate affect their CTC-capture performance, as well as the viability and molecular integrity of captured CTCs); ii) developing new CTC-capture/release mechanisms governed by physiologically compatible stimulations for instant isolation/purification of CTCs with desired viability and molecular integrity in order to set the stage for conducting downstream *ex vivo* characterization, as well as molecular analysis; iii) exploiting a broad diversity of multi-omic analytical technologies (that could be from other research initiatives within NCI Nanotechnology Alliance Program) with single-cell resolution to characterize the heterogeneous CTC pool; iv) exploring the use of rare-cell culture techniques that will enable *ex vivo* expansion of purified CTCs for in-depth studies (*e.g.*, xerograph models and drug susceptibility tests); v) studying other types of circulating rare cells (*e.g.*, tumor associated macrophage and stromal cells) and non- cellular particles (*e.g.*, exosomes), which also carry information about the tumor microenvironment.

Following development of these technologic advances, challenges remain in utilizing these new assays to address unmet needs in the areas of cancer biology and, most importantly, clinical oncology. Research endeavors should be devoted to: i) performing multi-omic molecular characterizations on CTCs together with concurrent tumor tissues (including primary and metastatic sites if available) to establish CTC-tumor relationship that will become the foundation for using CTCs as liquid biopsy[35]. Consequently, CTCs can then be used as surrogate tumor tissue for providing relevant information to guide implementation of cancer treatment; ii) dissecting CTC subpopulations according to their distinct phenotypes (*e.g.*, molecular fingerprints, morphological characteristics, and behaviors) in order to address the issue of heterogeneity in tumor/CTC pool. For instance, a subpopulation of CTCs with defined small nuclei (*i.e.*, vsnCTCs) was discovered to strongly correlate with the presence of visceral metastasis in prostate cancer, offering a new way to detect the onset of the most lethal disease progression[56]; iii) conducting analyses on serial CTC samples through monitoring the dynamic change of CTC subpopulations and their multi-omic molecular signatures to better understand the evolution of cancer, which is currently limited by the difficulty of obtaining tumor tissues; iv) effectively generating and applying CTC-derived cell lines as well as xerograph models to better understand the oncogenic/resistant mechanism, and evaluate a wide range of treatment options that can poetically benefit individual patients. Validation in appropriately powered studies will be needed as these ideas translate directly into the clinical setting. Ultimately, the regulatory and commercial efforts will be required to bring these tools to the population at large.

Conclusion and Outlook

Early successes in the field of nanotechnology have shown great promise for addressing the existing unmet needs in clinical oncology. As the scientific understanding of the dynamic and complex biology of cancer evolves, it has become clear to clinical scientists and cancer biologists that characterizing this dynamic biology may add an important dimension to clinical data. Oncologists practicing cancer care in this evolving biologic environment are already accustomed to handling temporal variation of data. Monitoring the dynamic alterations of biological variables, which themselves follow a distinct and biologically relevant rhythm, is a fundamental part of clinical medicine. Given the limitations of performing serial biopsies or the limited data obtainable in single biomarker panels, to date, this type of dynamic characterization has been possible only in animal models or in limited biomarker panels. The promise that the analysis of CTCs and other circulating entities holds is in the ability to study the dynamic biology that bares the greatest relevance: that of the individual patient. In this era of molecular medicine that has brought us beyond the cell to the level of DNA, RNA, and proteins, it has become exceedingly clear that no two patients are identical and no two cancers are identical. Having a non-invasive means of dissecting these differences bridges the gap between the laboratory and the clinic. While these ideas are young, the successes seen in this field provide ample cause for continued work and fuel the enthusiasm for launching integrated transdisciplinary research in this transformative field.

The promise that the analisis of CTCs and other circulating entities holds is in the ability to study the dynamic biology that bares the greatest relevance: that of the individual patient.

Early-to-Late Stage Diagnosis: Nanoflares for Intracellular mRNA Detection

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Spherical nucleic acids (SNAs)[57] have recently emerged as a powerful tool in biomedicine with far-reaching implications in the fields of cancer research and oncology. SNAs are typically composed of nanoparticle cores (*e.g.*, gold[58], silver[59], iron oxide[60], infinite coordination polymers[61], silica[62]), densely functionalized with highly oriented oligonucleotide shells (*e.g.*, single- or double-stranded DNA[58], siRNA[63], mRNA[64], PNA[65], LNA[66], RNA/DNA hybrids[67]) (**Figure 3**). Core-less or hollow versions of these structures have also been synthesized (*e.g.*, crosslinked alkyne polymers[68], liposomes[69]), some of which are composed purely of biologically compatible components. Many of the novel chemical and physical properties that make these materials useful in cancer research and oncology stem from the unique architecture of the oligonucleotide shell and are core-independent. Indeed, SNAs are recognized by Class A scavenger receptors and enter cells (over 60 tested to date) as a single-entity without the use of ancillary transfection agents[70–72]. They also are resistant to enzymatic degradation and show no apparent toxicity or immunogenicity[73–75]. SNAs also exhibit a high affinity for complementary DNA strands (100 times higher than that of free DNA of the same sequence in solution)[76]. SNAs are highly modular and the composition of their cores as well as the sequence, length, and density of their oligonucleotide shells can be tailored; in the context of cancer research and oncology, this means that SNAs can be designed to target almost any gene, including those associated with a wide variety of cancer types, in extracellular and intracellular biodetection and therapeutic schemes. SNAs were first synthesized in the Chad Mirkin laboratory at Northwestern University in 1996, and they were first formulated as nanoflare constructs in 2007 by the same lab.

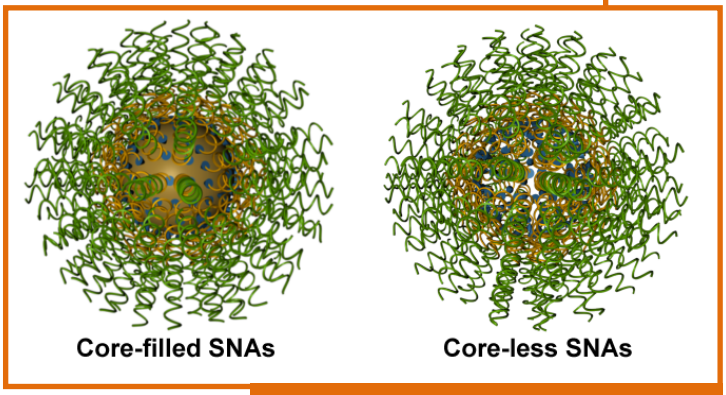

Figure 3. Gold nanoparticle-filled (left) and core-less (right) spherical nucleic acid (SNA) structures.

Based upon SNAs, these new constructs, termed NanoFlare, possess many of the

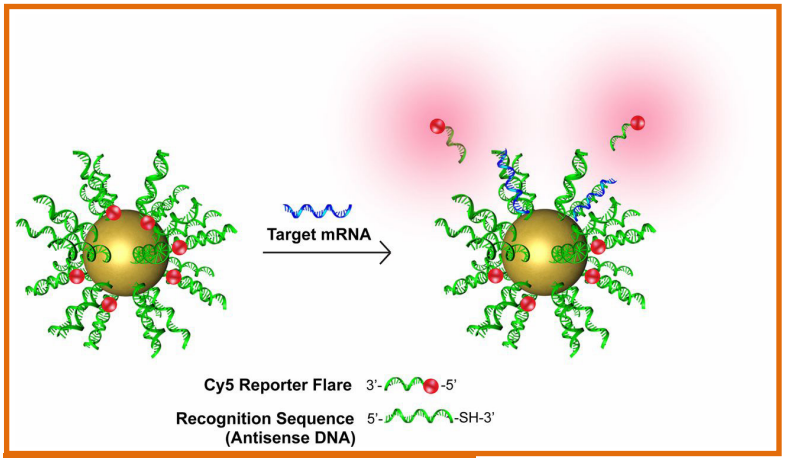

Figure 4. Schematic of Nanoflare structure and function. (*Reprinted with permission from Halo et al, 2014*)[79]

aforementioned useful chemical and physical properties[77]. Specifically, NanoFlares are gold nanoparticle-based SNAs that are hybridized with short, fluorophore-labeled complementary DNA strands (**Figure 4**). Their usefulness as a diagnostic is simple, when hybridized the fluorophores are held in close proximity to the gold nanoparticle and their respective fluorescence output is quenched. However, when a nanoflare encounters a longer, complementary target (*e.g.*, mRNA strand) in a cellular environment, it displaces one of the shorter "flare" strands and the fluorescence signal is observed. As such, these novel nanomaterials have proven to be highly useful probes for intracellular mRNA detection with exceptionally low limits of detection (*e.g.*, sub-pM). When coupled with flow cytometry, NanoFlares currently constitute the only means of interrogating the genetic content of live cells and sorting them based on such content. NanoFlares are also capable of engaging in gene regulation as potent antisense, siRNA, and microRNA delivery vehicles; indeed, these structures have been proven to have theranostic potential as they could be used to both detect and treat cancer, simultaneously[78].

In initial proof-of-concept studies, it was demonstrated that NanoFlares could be used to detect oncogenes – specifically survivin, an anti-apoptotic gene that is up-regulated in a range of cancer types – for example, in a breast cancer cell line (SKBR3) in a highly sensitive and sequence-specific manner[77]. Indeed, increased fluorescence was observed when NanoFlares targeting survivin were added to SKBR3 cells expressing survivin compared to when either NanoFlares bearing a non-complementary sequence were added or cells that did not express survivin (C166 cells) were used (**Figure 5**). These results demonstrate how researchers can use NanoFlares to distinguish cancerous cell populations based on the expression of an mRNA target of interest. Further, in the context of cancer research and oncology, it would be useful to track the up- or down-regulation of multiple genes at once. Thus, more advanced nanoflare systems have been developed that allow a single nanoflare to target multiple genes (*e.g.*, two[31], three[80], or four[81]) in cervical and breast cancer cell lines. These multiplexed NanoFlares also allow quantitative information to be obtained, the signal-to-noise level to be reduced, and to mitigate the effects of cell-to- cell variability.

More recently, NanoFlares were designed to target markers (*i.e.*, vimentin and fibronectin) of the epithelial-to-mesenchymal transition (EMT), an integral part of cancer metastasis. Coupled with flow cytometry, they also were used to capture live breast cancer circulating tumor cells (MDA-MB-231) from human whole blood samples and from an orthotopic murine model of metastatic triple negative breast cancer[79]. Furthermore, these NanoFlares were used to retrieve GFP-positive cells in a HER2+ mouse model of breast cancer and subsequently cultured into mammospheres (**Figure 6**), which are spherical clusters formed only from cancer stem cells. These results suggest that it may be possible to isolate and further culture live CTCs from human patients *ex vivo*, providing the opportunity to study cancer cell heterogeneity and its relation to patient outcomes. Simultaneously, these results demonstrate the ability of NanoFlares to survey the metastatic potential of cells in the blood stream. This approach provides an unprecedented opportunity to isolate cancer stem cells based on the presence of genetic markers and may improve cancer diagnosis and prognosis.

In 2012, nanoflares were commercialized by AuraSense, LLC, a company founded by Chad Mirkin. Two years ago, AuraSense entered into a multi-million dollar partnership with EMD Millipore to commercialize them under the trade name SmartFlares™ for use in *in vitro* cell assays. SmartFlares™ are now available as research tools to investigators with over 1,700 different versions sold in over 230 countries. Over the next 5-15 years, the number of flares available through EMD Millipore is expected to increase, and subsequently nanoflares will move beyond the research setting to the clinic to be used for medical diagnostic purposes. Concurrently, there is an initiative to quantify and track the spatial location of mRNA in cells, as this is highly related to cellular function. As such, it is anticipated that drugs coupled to nanoflare systems

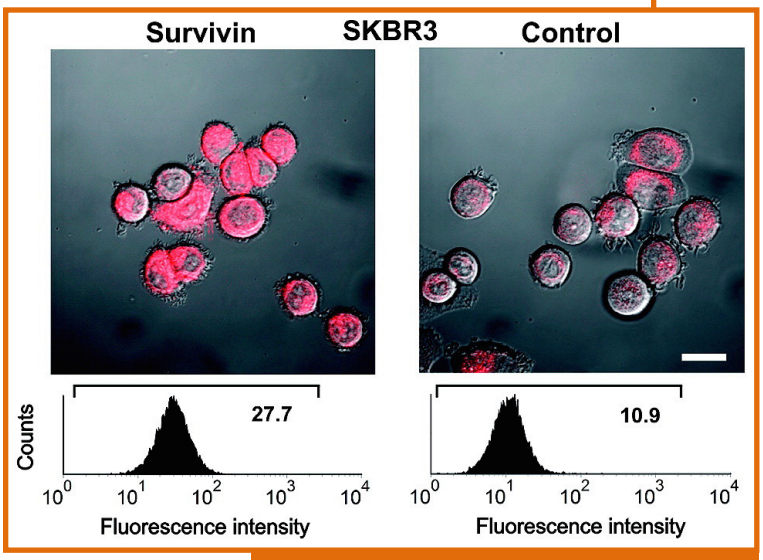

Figure 5. Intracellular testing of nano-flares. Differential contrast and fluorescence image of survivin-expressing SKBR3 cells treated with survivin-specific nano-flares (top left panel) and noncomplementary nano-flares (top right panel). Scale bar is 20 µm. Flow cytometry data are shown below each image. The bold numbers to the right of the histogram are the total mean fluorescence of the cell populations. (*Reprinted with permission from Seferos et al, 2007*)[77]

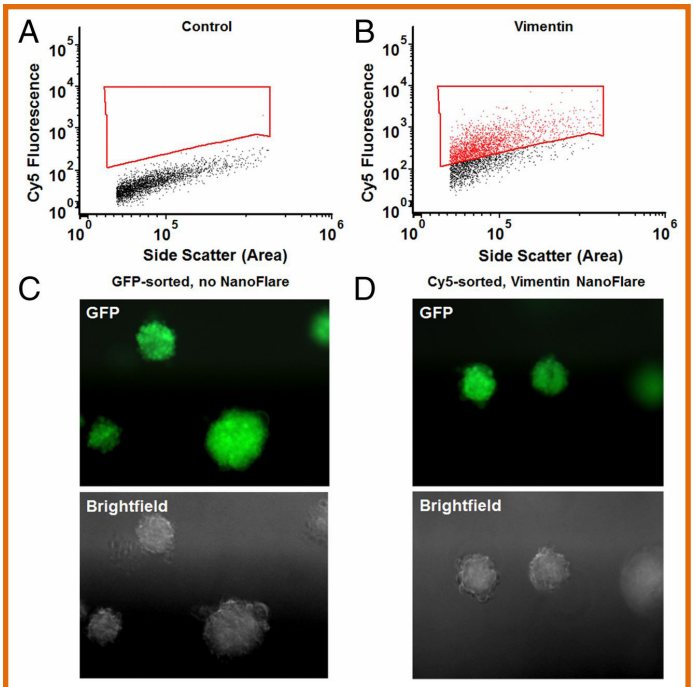

Figure 6. Cell isolation and mammosphere formation post NanoFlare treatment and flow cytometry analysis. Representative scatter plots show Cy5 fluorescence (NanoFlare) of GFP recurrent cells spiked into (A) untreated human whole blood or (B) Vimentin NanoFlare-treated blood. Upon treatment with NanoFlares, Cy5 fluorescence of GFP-positive cells increases 5.4-fold. Cells in the red gate in the Vimentin sample were sorted for mammosphere culture. Cells retrieved from blood form mammospheres (C) untreated or (D) Vimentin NanoFlare-treated. (*Reprinted with permission from Halo et al, 2014*)[79]

will allow therapy to be administered based on the genetic content of the cell, in a highly targeted manner. These research directions are already underway and will have significant implications for the field of cancer research and oncology.

Intraoperative Imaging

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Introduction

In the operating theatre, there is an urgent need for implementing new image-directed visualization tools that will enhance surgical vision, facilitate minimally invasive surgical procedures, and dramatically alter surgical outcomes of oncological patients. Early detection, staging, and treatment of cancer are essential to minimizing morbidity and mortality. Each year, nearly 13 million new cancer cases and 7.6 million cancer deaths occur worldwide[82]. The cornerstone of clinical cancer care rests on surgical management. However, intervention is often limited to tumors diagnosed in an early stage as outcomes are notably poorer when surgery is no longer a treatment option[83]. Adjuvant radiation and/or chemotherapy are typically added for specific indications including locally invasive tumors and/or spread to regional lymph nodes. The challenge has been in the lack of clear 'surgical vision,' which impacts the ability of the operating surgeon to accurately and specifically identify the extent of malignancy[83,84], macroscopic/microscopic tumor burden[85–88], or remnant disease, notably at the site of surgical removal (*i.e.* surgical margin). Complete assessment of surgical margins will be based upon the quality and extent of tissue sampling[89]. Collectively, these factors will affect therapeutic outcome, prognosis, and treatment management. Moreover, despite technical advances that have enabled large-scale imaging instruments, such as PET-CT and MRI, to meaningfully impact preoperative cancer diagnostics and staging, they are either not practical for intraoperative settings or offer limited utility in terms of achievable spatial resolution and/or sensitivity. Alternatively, newer molecular imaging probe designs (*i.e.*, engineered optically- active nanomaterials), coupled with state-of-the-art device technologies, may enhance cancer care, provide real-time imaging guidance, and lead to new, more efficient approaches for early-stage detection and treatment.

A key goal of cancer surgery is to reliably distinguish cancer from normal tissues at an early stage to pursue a surgical cure while maximizing safety, limiting damage to vital structures, preserving cosmesis, and increasing throughput. The current standard of care relies upon palpation and visual inspection[90]. Although anatomic structures can be efficiently identified, such evaluations depend on successful discrimination of a narrow range of spectral features (*i.e.*, contrast) or subtle textural differences, rather than elucidating molecular processes

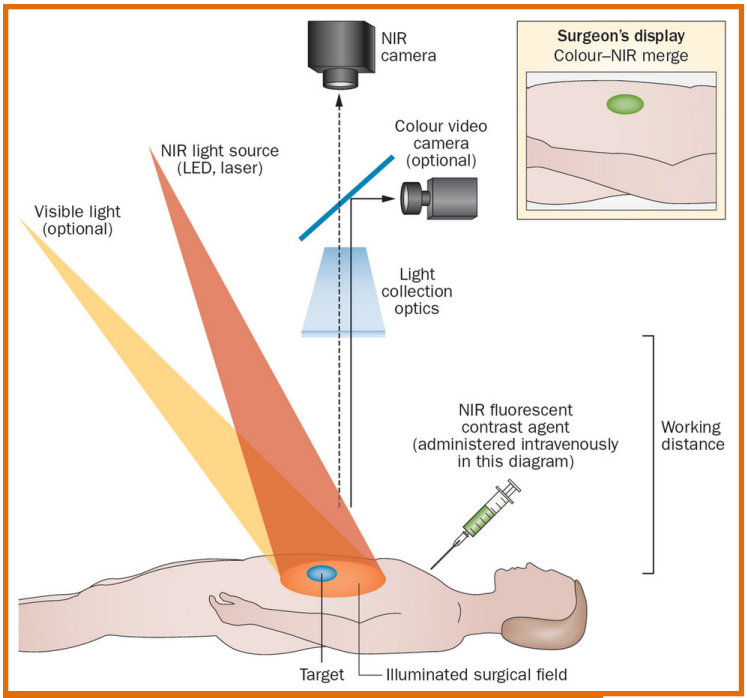

Figure 7. Mechanics of NIR fluorescence imaging. During surgery, an NIR optically-active agent is visualized using a fluorescence camera system. All systems must have adequate NIR excitation light, collection optics, filtration and a camera sensitive to NIR optical emissions. Optimal imaging systems include simultaneous visible (white) light illumination of the surgical field, which can be merged with NIR optical images. The display can be a standard computer monitor, goggles, or a projector. Current imaging systems operate at working distances that enable illumination of a sizable surgical field. LED, light-emitting diode (*Reprinted with permission from Vahrmeijer et al, 2013*).

defining a given disease stage[91]. This leads to a higher risk of incomplete surgical resection and/or soft tissue injury.

These limitations may be overcome by the application of improved intraoperative optical imaging approaches, which have traditionally been hampered by (1) the small number of imaging agents available in the near-infrared (NIR) spectrum, (2) high background autofluorescence that restricts depth and detection sensitivity, (3) large spectral overlap between optical agents preventing concurrent detection of multiple targets (*i.e.*, multiplexing), and (4) rapid photobleaching that reduces the imaging duration[15]. However, significant progress is being made on a number of fronts. Fueled by the emergence of an increasing number of new, diverse, and clinically promising NIR fluorescence probes, including particle-based agents, that can enhance soft tissue contrast, detection sensitivity, and depth penetration, some of these key drawbacks are being addressed, noting that these probes require an intraoperative optical imaging system with clinical grade accuracy (**Figure 7**). In addition to offering exquisitely sensitive real-time detection sensitivities, the higher resolution offered by these systems has enabled lesions to be detected down to sizes smaller than 10 µm, which truly revolutionizes imaging capabilities by dramatically increasing the sensitivity and specificity of detection over human vision[92]. Such tools can be seamlessly integrated with minimally invasive, robotic-assisted surgical equipment to enable navigation to target sites deep within the body. Unlike other imaging modalities, the combination of optically-active, disease-targeting probes and state-of-the-art multichannel camera systems offers

the possibility of interrogating real-time biological processes and identifying one or more novel biomarkers for (1) imaging (*i.e.*, cancerous nodes, surgical margins, remnant tumor); (2) staging; and (3) treatment response (**Figure 8**). Such markers can be further validated in the clinical trials setting. Collectively, the potential of these technologies to improve patient outcomes, minimize surgical risk, promote clinical throughput, and lower health care costs represents a significant clinical advance, and promises to transform the current practice of surgical oncology.

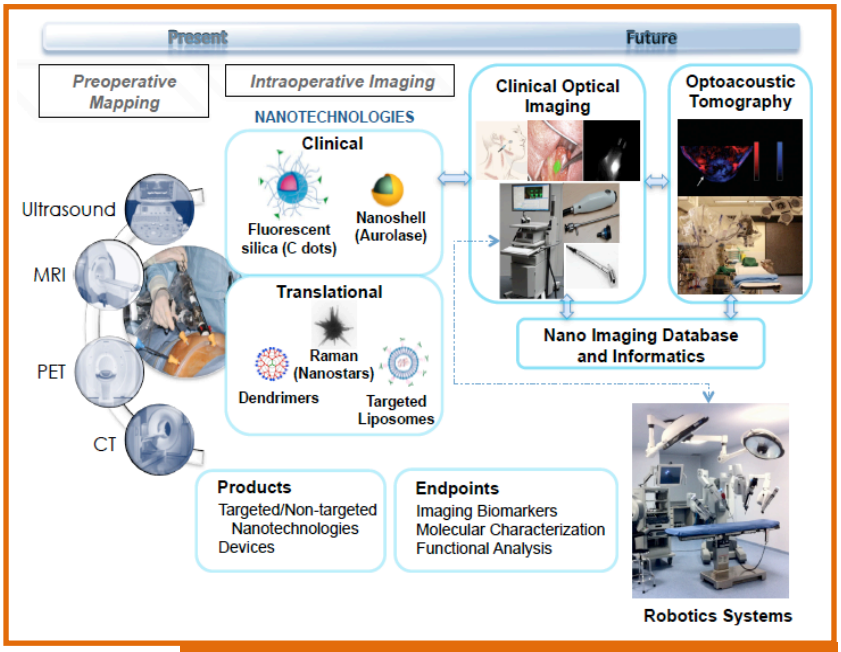

Figure 8. Present and future of NanoOncology Image-guided Surgical Suite. Preoperative conventional imaging tools are used to screen for disease and inform optically-driven minimally-invasive and open surgical procedures. Clinically available particle platforms can be monitored in real-time using portable multichannel camera systems. Representative translational probes and devices for future clinical use are also shown. In the future, the operating surgeon will select suitable probe-device combinations for specific indications, and be provided with structural, functional, and/or molecular-level data regarding tissue status for further treatment management.

Intraoperative Imaging Via Nanotechnology

A significant volume of work, however, has been performed utilizing endogenous tissue contrast, which is restricted to examination of only very small fields-of-view, or by administering non-specific optical agents[93,94]. The latter class of agents have included particle-based probes (*i.e.*, quantum dots)[95] and fluorescent dyes, such as indocyanine green (ICG)[96,97], an FDA-approved NIR dye for selected clinical indications. However, the lack of selective targeting found with these agents limits their utility for many applications aimed at detection of strictly cancer-bearing tissues. Thus, to enhance surgical vision during image-guided procedures, as well as impart labeling specificity, NIR optical probes targeting tumor-selective biomolecules are desired. Towards this end, a number of targeted molecular products, including dye-bound antibodies and peptides, can be applied as visualization tools for improving examination of tumor borders or localization of tumor deposits by attaching to upregulated cancer receptors[98–100]. Although not yet reaching full potential in surgical

practice, early potential benefits of optical imaging have been shown in clinical studies utilizing targeted molecular probes, albeit conjugated to visible dyes. However, such dyes reduce contrast resolution and depth penetration due to higher absorption and scatter in this part of the light spectrum[101,102].

More recently, the emergence of diverse classes of NIR fluorescent nanoparticle platforms, designed to improve the sensitivity, accuracy, and reliability of lesion detection over that of organic dyes, has revealed exciting new possibilities for probing and characterizing new molecular targets and novel biomarkers within human subjects[15]. The ability to tailor and refine the physicochemical and photophysical properties of these materials in a well-controlled and iterative fashion can favorably modulate their biological activities, resulting in one or more characteristics that improve upon those exhibited by simple molecular agents. These characteristics include multivalency enhancement (potency) as a consequence of simultaneous interactions of multiple targeting ligands with cell surface receptors, improved target retention, extended plasma residence time, bulk renal clearance, and improved pharmacokinetic profiles. Moreover, in some cases, the encapsulation of dyes within the particle structure has led to significantly enhanced brightness and photostability relative to the native dye, in addition to increasing tissue penetration depths (up to several centimeters)[103]. Collectively, these adaptations can improve target-to-background ratios and *in vivo* detection sensitivities following particle administration, the ultimate goal being to identify and remove all cancer cells. Finally, the ability to create multimodality platforms by incorporating more than one contrast-producing moiety into the particle design can yield multiparametric imaging data that validates potential biomarkers, potentially altering current standard of care.

Given these diverse, highly versatile, and integrated particle surface designs, coupled with improved state-of-the-art optical clinical camera systems, key surgical indications can be performed more reliably and accurately. Current applications have mainly focused on (1) selective mapping of cancerous lymph nodes, (2) precise identification of surgical borders (crucial landmarks), (3) accurate detection and treatment of remnant disease, and (4) reliable assessment of tissue function (*i.e.*, perfusion). For SLN mapping, the principal aim is to map the lymphatic drainage of exogenous agents and highlight only cancer- bearing nodes for selective resection. The primary factor controlling lymphatic transport is the agent size. An optimal size is one that is small enough to exhibit rapid lymphatic transport to the SLNs and other downstream nodes, yet large enough to be retained, typically around 5–10 nm[87,104]. One such sub-10 nm hybrid (PET-optical) cancer-targeting imaging platform is shown in **Figure 9**. A second surgical indication, the mapping of surgical margins, involves precise delineation of the tumor extent. The presence or absence of tumor cells at the site of resection is a key determinant of treatment success or failure, and is often used

to determine the need for adjuvant therapy. Positive margins are a negative prognostic indicator for many solid cancers[83]. Furthermore, surgical margins are often evaluated by immediate intraoperative analysis of the specimen, which can lengthen operating time and/or lead to incomplete readouts due to suboptimal specimen quality or inadequate sampling, the result being a positive surgical margin and poor outcome[89]. One such triple-modality (*i.e.*, MR-photoacoustic-Raman imaging, MPR) particle has sought to address this issue by efficiently and accurately delineating brain tumor margins (**Figure 10**)[14].

In addition, newer higher resolution whole-body optical imaging strategies, such as multispectral optoacoustic tomography (MSOT) (**Figure 8**), which detects optical absorption by means of ultrasound, have grown in popularity due to the concurrent development of clinical imaging systems[91,95]. These methods utilize multiple optical wavelengths and spectral demixing algorithms to permit imaging at depths greater than those typically achievable with fluorescence imaging. In addition, these methods can detect a broad range of novel light-absorbing nanoparticles (gold nanorods)[105], among other entities (*i.e.*, endogenous chromophores, organic dyes)[91], to yield high resolution optical assessments of targets deep to the tissue surface, as well as provide functional measures of viability and/or perfusion.

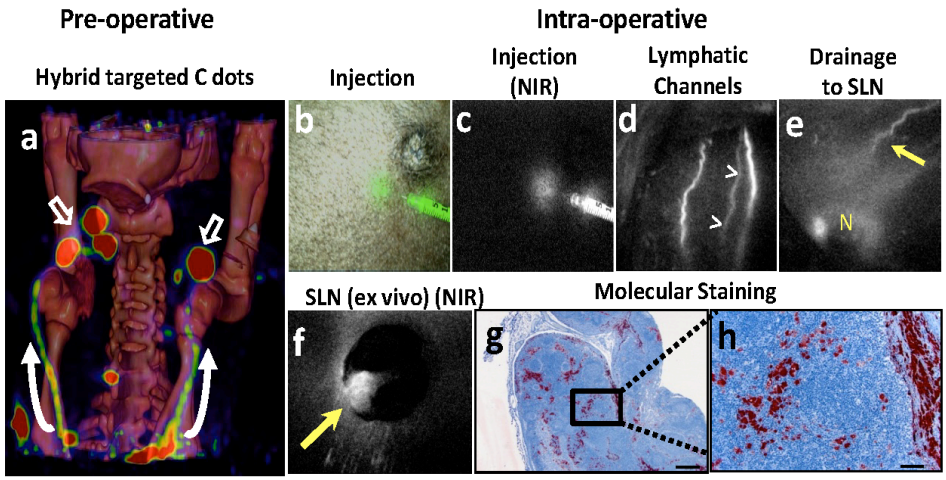

Figure 9. Mapping of Metastatic Lymph Nodes Using a Clinically Translated Hybrid PET-Optical Silica Nanoparticle (C dots). (a) Volume-rendered pre-operative PET-CT fusion images of the neck shows metastatic lymph nodes (red) bilaterally and lymphatic channels after injection of ultrasmall (6 nm diameter) integrin-targeting C dots into melanoma miniswine. (b,c) Intraoperative SLN mapping with two-channel NIR optical imaging of the exposed nodal basin. Local injection of fluorescent C dots displayed in dual-channel model (b) RGB color (green) and (c) NIR fluorescent channels (white). (d,e) Draining lymphatics (arrowheads) distal to the injection site extending toward the node (N). (f) Image of excised SLN in the NIR channel. (g) Low-power view of HMB45-stained (red) SLN confirms the presence of metastases (black box, bar = 500 µm). (h) Higher magnification reveals HMB-45+ expressing melanoma cells (bar = 100 µm) (*Reprinted with permission Bradbury et al, 2013*).

Future of Intraoperative Imaging Via Nanotechnology

It is anticipated that fluorescence-enhanced surgical vision, despite its limitations, will significantly impact and likely transform conventional surgical practice in oncology over the next 5 to 15 years by increasing the sensitivity and accuracy of surgical procedures, such as evaluation of surgical margins, mapping of local and distant cancerous lymph nodes, and detection of microscopic disease. Rather than relying on visual and tactile cues for guiding disease assessment and therapeutic management, the surgeon will utilize a growing array of dedicated intraoperative treatment tools in the form of targeted optically-active particle probes and portable multichannel optical devices. Nanoparticle surface versatility and their unique physicochemical and biological properties will play a key role in this field, providing new opportunities to probe critical cancer targets and identify potential biomarkers that can be validated in clinical trials. Although in its infancy, a variety of particle therapeutic strategies are currently being developed for effectively treating disease in the intraoperative setting. The future implementation of such tools in clinical practice should lead to improved patient outcomes and reduced surgical risks. The foregoing developments are also expected to promote acceptance of optical technologies and, as a consequence, accelerate the growth of minimally invasive surgical procedures, with the intent of maximizing functional outcomes and limiting treatment-related morbidity. Identification of normal tissue markers may also enable particles to be engineered with specific ligands and fluorescent labels for highlighting poorly visualized vital structures (*i.e.*, nerves). In addition to their expected utility for real-time intraoperative procedures, the application of these optical technologies

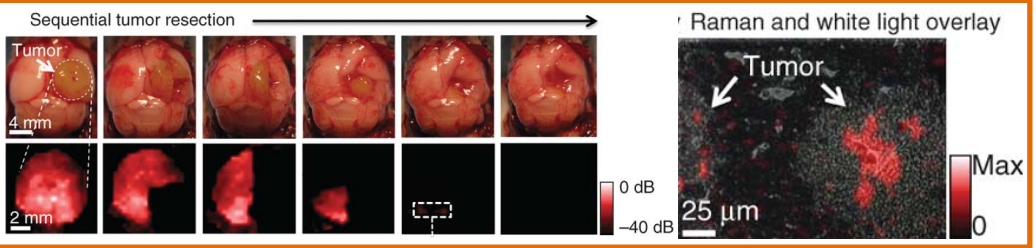

Figure 10. Raman-guided intraoperative surgery using Raman imaging nanoparticles (MPR). (a,b) Living tumor-bearing mice underwent craniotomy. Quarters of the tumor were sequentially removed (photographs, a), and intraoperative Raman imaging was performed after each resection step (b) until the entire tumor had been removed, as assessed by visual inspection. After gross tumor removal, small foci of Raman signal were found in the resection bed (dashed white square). Raman microscopy image (right) of dashed white square depicts Raman signal within an infiltrative tumor, indicating the selective presence of MPRs. Raman color scale (red): –40 dB to 0 dB (*Reprinted with permission from Kircher et al, 2012*).

may additionally aid inspection of resected tissue specimens, leading to less time-intensive evaluations and improved clinical throughput.

Despite the significant data generated to support the translational developments of new, optically-active particle probes for intraoperative cancer treatment, advancing such agents into the clinic has been challenging, particularly those exhibiting molecular specificity[106-108]. Importantly, FDA-IND approvals have been issued for both targeted particle drug[106] and device[109] technologies, and such developments are paving the way for translating additional targeted optically-active technologies to the clinic for use in image-guided surgeries. Furthermore, as tumor heterogeneity is an important consideration for selecting a targeting ligand, 'cocktails' of multiple cancer-targeting particle probes will be increasingly utilized, each probe incorporating a different ligand and optical dye for improving detection and staging accuracy. Enabling simultaneous visualization of these cocktails will require implementation of state-of-the-art multichannel fluorescence camera systems that can detect fluorescence from multiple wavelengths. Several of these camera systems are already in clinical use. As additional novel particle probes are developed and camera systems continually evolved to permit both structural and functional assessments, the true clinical value of these combined technologies will ultimately be realized. Promising higher resolution techniques, such as optoacoustic imaging, may be increasingly implemented to overcome instances where degradation of the emitted fluorescence signal is observed, notably when interrogating complex tissue compositions.

Nanoparticle surface versatility and their unique physicochemical and biological properties will play a key role...

Finally, the need to establish standardized quantitative metrics for intraoperative decision-making is paramount, and is at a very early stage of development. Often these assessments are of a qualitative nature, and the chosen endpoints may depend on many factors, including the nanomaterials probe selected and the device providing the measurements. It is expected that the optical imaging community will address these issues in the near future, as they will significantly hamper efforts to make effective comparisons among different probe-device combinations for a specific indication. Implementation of well-designed outcomes studies will also be critically important for widespread dissemination and acceptance of image-guided optical technologies in standard surgical practice.

Targeting the Tumor Microenvironment

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The Big Picture

Personalized medicine, or precision medicine, relies on the selection of the correct drugs, or drug combinations, based on the disease-specific genetic traits. Selecting the proper drugs is the first step toward precision medicine, but its completion needs effective delivery of the selected drugs to the target (*e.g.*, tumor). Recent progress in nanotechnology has made drug delivery more efficient compared with the control solution formulation, but subsequent effectiveness of the drugs delivered is still in question. Nanoparticulate drug delivery systems are designed and tested for the ultimate goal of developing clinically useful formulations to treat various cancers. Thus, the usefulness of nanoparticle formulations needs to be considered in the context of treating cancers (*i.e.*, improving efficacy and safety) in human patients.

Benefits of Nanoparticle Formulations

Over the last few decades, various nanoparticles have been prepared for treating cancers. One large benefit to using nanoparticle formulations is in the ability to avoid non-aqueous solvents when administering hydrophobic drugs to patients, resulting in fewer side effects, even if the efficacy remains the same. This has been exemplified by the success of Abraxane® (based on nanoalbumin particles) and Doxil® (PEGylated liposome formulation), which in large part, rely on delivering anticancer drugs without using organic solvents. Although, nanoparticle formulations, or for that matter any formulation, can deliver drugs to the area near target tumors, but the subsequent delivery to the tumor cells is hindered by the complex microenvironment of tumors. Drug efficacy occurs only after the drug is absorbed into target tumor cells. Thus, it is important to understand the tumor microenvironment (TME) to achieve or improve upon the desired drug efficacy.

Understanding the Tumor Microenvironment (TME)

The tumor microenvironment comprises a highly heterogeneous mixture of tumor and stromal cells embedded in an extracellular matrix with many cytokines, growth factors, inflammatory cells and macrophages[109]. The current difficulty of developing new anticancer

drugs and drug delivery systems partly stems from the lack of a clear understanding of the delicate interplay between tumor and stromal cells in the complex TME[111]. Here, pancreatic ductal adenocarcinoma (PDAC) is used as the fundamental, albeit extreme, example of this in order to portray the importance of improved targeting to TME.

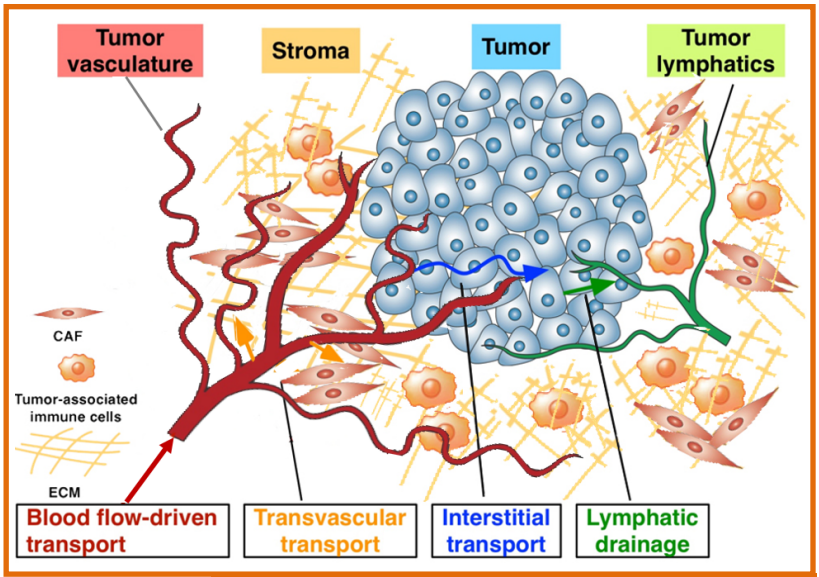

Figure 11. Transport of drug molecules and nanoparticles in the TME of PDAC. Drugs and nanoparticles can only reach the target tumors via multiple transport processes in the TME. PDAC has a very complex TME with dense stroma composed of cancer-associated fibroblasts (CAFs), tumor-associated immune cells, and dense ECM structure.

PDAC consists of two components, the malignant epithelial cell population and a complex, large stromal compartment. **Figure 11** describes a highly desmoplastic PDAC tumor which is infiltrated with activated cancer-associated fibroblasts (CAFs) and inflammatory cells. CAFs release collagens, laminin, and fibronectin. The complex extracellular matrix (ECM) includes dense collagen types I and III bundles, hyaluronic acid (HA), fibronectin, desmin, cytokines, growth factors, and the matrix metalloproteinase family of proteases. The exact roles of the stromal compartment are still not clearly established, but it certainly provides an immense physical barrier to the multiple transport steps for effective drug delivery. Overcoming the transport barriers presented by both stroma and tumor for effective delivery requires ingenious design of nanoparticles, at least beyond the nanoparticle design paradigms currently in clinical use due to their size and surface functionalities. Moreover, interactions between tumor cells and various cell types in the stroma may affect the drug response of tumor cells. The outcome of these interactions is highly context-dependent, and further understanding of dynamic cancer biology and oncology is critical. The current idea of targeted drug delivery using nanoparticles addresses only a very small portion of this complexity. As such, any new paradigm should comprise tools for overcoming the enormous complexities of the TME.

Future Needs to Efficient Delivery of Anticancer Drugs Through Priming of the TME

The TME has enhanced stiffness, increased HA content, and elevated hydrostatic pressure, all of which are known to reduce effective intratumoral drug delivery. For drugs to be effective, they must reach the target tumor cells through the TME or the stromal surrounding. Thus, solid tumor priming, *i.e.*, modulating the abnormal TME, is promising idea for enhancing the antitumor efficacy. The strategies of solid tumor priming includes vascular normalization using anti-angiogenic treatment, solid stress alleviation by induced apoptosis and stromal normalization, and using tumor-penetrating peptides[112]. Of these stromal normalization is attractive because it can be achieved by using relatively benign components.

Stromal HA is known to be a key factor making the too TME dense for proper diffusion of drug molecules, not to mention nanoparticles. This provides a means to enhance the permeation of nanoparticles through TME by treating PDAC first with hyaluronidase[113]. Calcipotriol, a synthetic, highly potent derivative of vitamin D that does not cause hypercalcemia, was recently reported to reduce the activation of pancreatic stellate cells and their conversion to CAFs by activating the vitamin D receptors that are expressed in these cells, thereby decreasing desmoplasia[114]. When used in combination with gemcitabine, calcipotriol prolonged survival in a genetically engineered mouse model (GEMM) of PDAC by decreasing fibrosis, increasing intra-tumoral vasculature, and enhancing gemcitabine delivery into the tumor. Importantly, Calcipotriol has been shown to exert anti-proliferative and pro-differentiation effects, as well as immune-modulating effects[114]. Interpretation of these results is complicated by a very recent finding that vitamin D may also promote tumor chemoresistance to gemcitabine, *underscoring the need to improve our knowledge on how to target the stroma*[115].

While the stroma-targeting approach has been successful in GEMMs of PDAC, it did not work in clinical trials. The successful treatments observed in mouse models seldom translate into clinical success. There may be several reasons for this discordance between findings in humans and in GEMMs of PDAC. The TME in mouse is likely to be very different from that in human. In addition, the amount of a drug delivered after HA priming was simply not adequate in clinical trials. Disrupting stromal layer alone may not be sufficient to kill tumor cells without delivering sufficient drugs. Since tumors are highly heterogeneous, delivering a single drug might have not been effective. Indeed, the heterogeneity of gene alterations in the cancer cells and the complexity of the stromal components mandate the design of novel multi-targeted and multi-drug dosing approaches.

Future Needs for New In Vitro Test Methods

Effective tumor treatment requires testing various priming agents in combination with delivery of multiple drugs, either simultaneously or sequentially. This involves a very large number of studies, and it makes animal testing expensive and time consuming. Moreover, small animal data may not be good predictors of clinical outcome. Thus, it is essential to develop *in vitro* test methods that can represent the microenvironment of human tumors.

Recent advances in tissue engineering and microfluidic technologies present an opportunity to realize *in vitro* platforms alternative to animal testing.

Recent advances in tissue engineering and microfluidic technologies present an opportunity to realize *in vitro* platforms alternative to animal testing. These platforms enable mimicking complex and multiple transport processes of drug delivery systems including circulation in the blood, extravasation from blood vessels to the tumor region, and diffusion of drug to the target tumor[116]. Tumor cells can be grown in 3D matrices with other relevant stromal cells to more closely recapitulate the complexity of solid tumors in patients. The current ability of forming 3D perfused tumor tissue needs to be advanced further to create an accurate TME, which accurately represents that of human tumors. This requires the design of 3D co-culture systems in which cancer cells, CAFs, and other stromal cells are grown within the necessary ECM components, yielding a delicate balance of biological, chemical and physical parameters relevant to human tumors.

Exact duplication of the human TME in microfluidic systems may not be feasible in the near future, but the TME-on-Chip can be used to systematically study the significance of given biological, chemical and physical parameters on the efficacy of nanotechnology-based drug delivery system and priming agents. Eventually, it should serve as a useful screening system for testing a large number of priming agents and drug combinations for personalized medicine.

Overcoming Specific Biological Barriers: Stromal

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the 4th leading cause of cancer-related deaths in the United States and its 5-year survival rate has remained unchanged (6%) over the past decades (*Cancer Facts & Figures 2014*, www.cancer.org). Due to the inevitable late diagnosis and early metastasis, chemotherapy is the only approved option for the majority of PDAC patients, with the standard of care involving the use of nucleoside analog gemcitabine or a more potent (but more toxic) four-drug regimen, oxaliplatin, irinotecan, 5-fluorouracil, and leucovorin (a.k.a FOLFIRINOX). Chemotherapy failure can be partly explained by the presence of an abundant dysplastic stroma, serving as a physical and biological barrier for drug access and unfavorable pharmacokinetics. It is appropriate, therefore, to consider the important stromal contribution to drug delivery and chemoresistance and sidestepping this barrier to improve survival outcomes[117]. This short overview will address the inhibitory role of the stroma in the treatment of PDAC, including the consideration for the use of nanocarriers to potentially engineer past this obstacle. We provide a perspective and guidance towards the implementation of nanotherapeutic approaches that could prove useful to improve therapeutic delivery and efficacy of gemcitabine and FOLFIRINOX.

Overcoming Tumor Stroma is Important to Cancer Nanotherapeutics

Because the stromal volume in PDAC is the highest among solid tumors (~70% of the total tumor volume), this requires special consideration in the treatment of this deadly disease[117]. Not only is the stroma poorly vascularized, but the existing vessels exhibit low permeability due to a high pericyte coverage, which blocks the extravasation of drugs, molecular therapeutics, and even nanocarriers to the tumor site (**Figure 12A**)[118]. The stroma also contributes to chemo-resistance and an unfavorable pharmacokinetic/pharmacodynamic (PK/PD) profile[117], including the expression of a high content of cytidine deaminase (CDA), which leads to gemcitabine inactivation, limiting its half-life to as little as 0.28 hours

(**Figure 12A**)[119]. Moreover, the intracellular activation of gemcitabine is dependent on phosphorylation by the rate-limiting kinase, deoxycytidine kinase (dCK) to generate the active metabolites, dFdCDP and dFdCTP (**Figure 12A**)[120]. It is believed that chemo-resistance to gemcitabine in PDAC is due in part to decreased expression of dCK. Another important stromal contribution is its pro-tumorigenic effect through supportive cell types that promote cancer cells proliferation and metastasis via complicated cross-talk mechanisms. Given this background, it is important to consider overcoming the challenges of the stromal barrier to address drug delivery and unfavorable PK/PD to the cancer site, including the improvement of intratumoral distribution, bioavailability, and overcoming drug resistance.

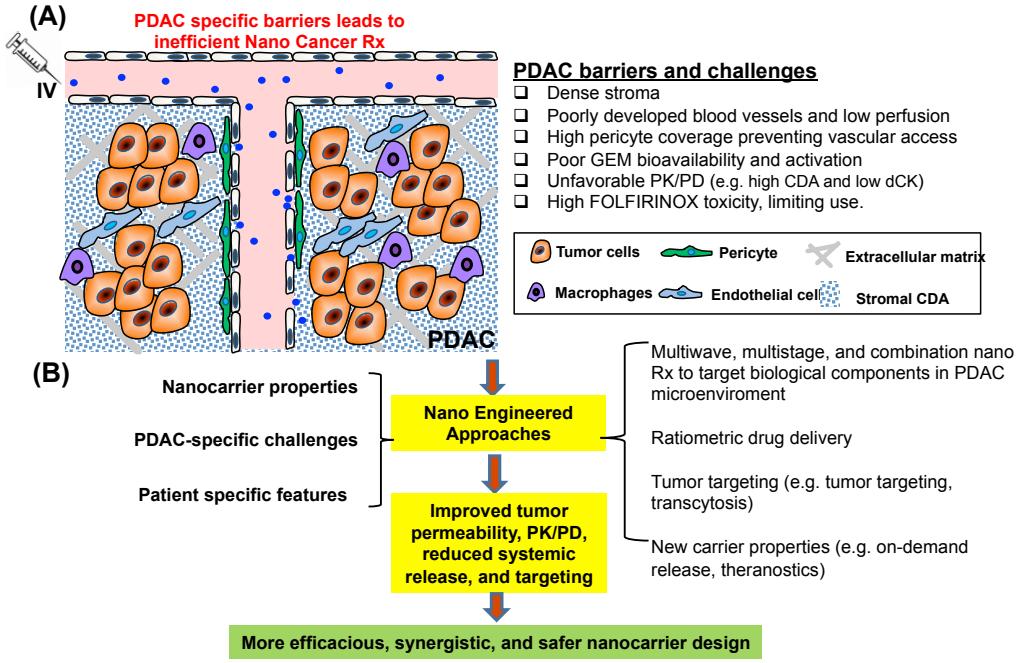

Figure 12. (A) Schematic to show the barriers and challenges that are responsible for failed chemotherapy in PDAC, including as a result of an abundant dysplastic stroma, which serves as a physical and biological barrier. This includes interference in vascular access and the presence of a high local concentration of deaminase activity, which leads to in activation of GEM. **(B).** We propose an engineered approach using nanocarriers, which can overcome stromal vascular gate or suppress the stromal abundance by the delivery of drugs that suppress pericyte coverage or decreases the stromal volume and abundance of deaminase activity. Moreover, a combination of these features could be used in synergistic designed nanocarriers. It is also a possible to include tumor targeting or the use of peptides that induce transcytosis across the stromal barrier.

The Current State of Overcoming Stromal Barriers in Cancer Nanotherapy

A number of stromal treatment strategies are currently being considered to improve PDAC treatment. These efforts have involved the use of enzymatic degradation, pharmacological suppression, tumor vasculature modification/intervention, and stromal targeting peptides. The first approach is the introduction of stromal-directed agents that obliterate the dense stromal microenvironment to improve drug delivery[113]. An ongoing clinical trial has demonstrated that the combination of gemcitabine with PEGylated hyaluronidase (PEGPH20) can ablate hyaluronan and overcome the stromal barrier, allowing chemotherapeutic drug access to the cancer site[121]. While PEGPH20 showed promising results pre-clinically and in some clinical studies, success is dependent on the dosing schedule as well as the specificity of this treatment[122]. In April 2014, FDA announced a clinical hold due to dosing and safety (*e.g.*, induction of thromboembolic event) concerns about the use of PEGPH20 in a Phase II clinical trial (www.halozyme.com). Although the clinical study resumed in September 2014, no update is available at this time. The second approach is to consider the use of pharmacokinetic suppression, as illustrated by the FDA granting approval for the use of the albumin-bound paclitaxel nano-complex, Abraxane®, in PDAC; co-administration of this therapy promotes gemcitabine survival outcome by 1.8 months. The proposed mechanism of Abraxane® action is the suppression of stromal density and reduced expression of CDA at the tumor site[123,124]. While the efficacy of this treatment is premised on using conventional therapeutic doses of each drug, it is not designed to deliver a ratio-dependent drug combination, which is an important consideration due to differences in the PK, distribution and elimination of the synergistic drug combination. This provides the opportunity to consider the ratiometric design of a single gemcitabine/Abraxane carrier to achieve *in vivo* synergy. The third approach is to use vasculature modification to improve drug delivery. In this category, there are a number of options, including targeting of the transforming growth factor beta (TGF-β) pathway, which promotes pericyte coverage of vascular fenestrations, among its pluripotent biological effects[125]. Intervention in the TGF-β signaling pathway using receptor kinase inhibitors or monoclonal antibodies have shown promising results to enhance vascular access and delivery of cancer drugs and nanocarriers to the tumor site[126,127]. However, the use of free inhibitor or antibody may require relatively high-dose/frequency and/or "off-target" effects due to the limited tumor targeting of these agents. Vasculature access can also be improved by stromal depletion through the use of antifibrogenic drugs, such as losartan (a clinically approved angiotensin II receptor antagonist)[128] and Hedgehog inhibitors[129], leading to decreased contractile elements, lowering of the interstitial fluid pressure[130] or a transient increase in intratumoral vascular density. While it has been shown that small 30 nm drug-loaded polymeric micelles can

permeate the stromal barrier to deliver antitumor drugs in PDAC without the need for targeting, the use of small particles may come at the expense of a reduced drug loading capacity[131]. The last approach is to develop stromal targeting therapy. This includes the recent discovery that iRGD peptides can increase PDAC vasculature access[132]. The exposed "CendR" motif, upon cleavage from the iRGD peptide, interacts with NRP-1 kinase receptor, which is capable of triggering transcytosis of macromolecules and liposomes, without the need of covalent conjugation of the peptide to the nanocarrier. This pathway is likely analogous to the vesiculo-vacuolar organelle, which has been observed in tumor vasculature during performance of electron microcopy[133].

Future Perspective in Overcoming Stromal Barriers

Because of the challenges of conventional chemotherapy for PDAC and the realistic expectation that there are no imminent changes in the treatments for metastatic disease, there is a unique opportunity for the use of nanotechnology in the treatment of this disease over the next 5-15 years. This is evidenced by the introduction of classic (*e.g.*, liposome and polymer) as well as novel (*e.g.*, inorganic-based) nanocarriers for this purpose. Although the use of small particles that rely on size-exclusion principles has shown promising results, nanotherapeutics are poised to make an even bigger impact because nanocarriers can be designed to deliver single or synergistic drug combinations, target, image and deliver, as well as allowing for engineered approaches to treatment. We define an "engineered approach" as the dynamic integration of the drug delivery properties with additional nanocarrier properties that address tumor-specific challenges, such as the stromal barrier (**Figure 12B**). Such an engineered approach could be particularly relevant to stroma-rich cancers in which the tumor stroma and other inferring biological components result in heterogeneous treatment effects in the tumor microenvironment. It is possible to design stromal targeting nanocarriers to enhance the efficacy of existing cancer drugs such as small molecules, peptides and proteins. One example is the introduction of a proof-of-principle "two-wave" platform in which a small molecule inhibitor of the TGF-β receptor kinase was used to decrease pericyte coverage at PDAC vascular fenestrations, allowing 2nd wave access of gemcitabine-laden liposomes, which could enter the tumor site to enhance gemcitabine tumor killing[134]. We postulate that the use of multiwave, multistage, and combination nanotherapeutics could have a translational impact on PDAC therapeutics in the clinic[135–137]. Another approach would be to design nanocarriers that can deliver synergistic drug combinations in a ratiometric fashion. In this sense 'ratiometric delivery' is defined as the *in vivo* release of a drug combination from a nanocarrier, with the purpose of providing a fixed drug ratio at the target site[138]. One example is the combination of a drug that exerts therapeutic effects on the suppression of the stroma (*e.g.*, paclitaxel) and a drug that kills PDAC cancer cells (*e.g.*, gemcitabine). In this regard, we have recently demonstrated

the design of a lipid bilayer supported mesoporous silica nanoparticle that can achieve ratiometric delivery of gemcitabine (trapped in the porous interior) with a sub-cytotoxic dose of paclitaxel incorporated into the lipid bilayer[139]. This synergistic combination resulted in the suppression of the tumor stroma and CDA expression in subcutaneous and orthotopic PDAC models in mice, providing more effective tumor shrinkage than free gemcitabine plus Abraxane. This type of nanocarrier could also be useful for treatment of other cancers with the same drug combination. Moreover, we envisage that this carrier can be further improved through the addition of incremental design features, such as on-demand release, theranostics, and promotion of transcytosis with iRGD peptides[132]. It is important, however, to consider the design complexity against the cost of each component and the ability to achieve GMP level manufacturing production volumes.

...nanocarriers could prove useful for addressing the toxicity of FOLFIRINOX.

It is possible to develop nanocarriers for precision medicine and addressing patient-specific response differences for treatment with gemcitabine and FOLFIRINOX. This could include the use of drug profiling, PK, drug uptake and metabolic effects in treatment design (*e.g.*, consideration of the delivery of a diphosphorylated version of gemcitabine to patients that have a relative low expression of dCK enzyme) leading to intracellular gemcitabine activation. To achieve this integration of nanotherapeutics with clinical-based approaches for PDAC, we have assembled a multidisciplinary team to advance the clinical tools, infrastructure and imaging approaches for delineating gemcitabine-responsiveness in PDAC patients (*e.g.*, PET scanning and intratumoral drug profiling)[120]. This could constitute the basis of future translational studies that build on the development of nanocarriers that can address patient-specific disease characteristics in orthotopic implant models in animals.

In addition to influencing the stromal barrier, nanocarriers could prove useful for addressing the toxicity of FOLFIRINOX. While this regimen has an increased response rate compared to gemcitabine (31.6% *versus* 9.4%), FOLFIRINOX is far more toxic and therefore restricted to patients with good performance status[140]. Encouraged by the promising results of MM-398 (an irinotecan liposomal formulation in Phase III trials)[141], single and multi-drug nano formulations are being developed to provide toxicity reduction, while maintaining efficacy. This could lead to FOLFIRINOX usage in more patients, with the ability to enhance the efficacy by combining this treatment with the “engineered approaches” described in the foregoing section. It is possible to envisage the use of engineered and targeted approaches (**Figure 12B**) to stromal therapy in preclinical studies over the next 5 years, assisted by the use of the transgenic KPC model and patient-derived orthotopic tumors. GMP-level

manufacturing, quality control and initiation of Phase I into clinical studies are achievable within 10 years. FDA approval and the introduction of at least one nanocarrier platform are envisaged after 15 years.

Overcoming Specific Biological Barriers: The Blood-Brain Barrier to Target Primary and Metastatic Brain Tumors

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Clinical Problems in Glioma Treatment

Gliomas are the most common primary brain tumors; grades III (*anaplastic astrocytoma*) and IV (*glioblastoma multiforme, GBM*) are characterized by increased cell and vessel density, cellular atypia and high mitotic activity. Malignancy grade is directly related to endothelial proliferation[142]. Despite considerable clinical and scientific efforts, patient survival still remains at 15.8 months on average. Little progress in pharmacological brain cancer treatment is due to the inability of many drugs to cross the blood-brain barrier (BBB) mostly formed by brain vascular endothelium. The BBB was discovered by Edwin E. Goldman more than 100 years ago. It protects the brain from environmental "noise", but, when the pharmacological treatment is needed, the same barrier prevents the brain influx of most drugs useful for the brain cancer treatment. Over a century-long scientific effort to circumvent the BBB has failed to answer many questions about drug delivery through the most powerful biological barrier in the body.

Nanomedicine Advances in Overcoming the Blood Brain Barrier

Glioma-derived signals triggering an intense angiogenesis in the tumor are not completely understood. Importantly, GBM and BBB interactions occur via extracellular proteins. For instance, the imbalance of tenascin and fibronectin in the tumor contributes to vessel formation[143]. We have described a switch of vascular basement membrane protein laminin isoforms in GBM from laminin-421 detected in normal brain to laminin-411, which may lead to higher rate of recurrences and shorter patient survival (Ljubimova *et al.* 2004, Cedars-Sinai Medical Center, clinical trial). The overexpression of laminin-411 in gliomas may contribute to increased glioma invasion (**Figure 13**). One clinical complication is the development of vasogenic brain edema, which dramatically increases the intracranial pressure (ICP) due to the BBB leakage[144]. Brain tumor-related edema can be a life-threatening complication of glioma growth, and so far, its treatment has relied on the use of corticosteroids.

Using systemically administered novel nanobiopolymer, Polycefin, anti-laminin drugs were delivered through the BBB, which dramatically reduced GBM size and normalized

brain cancer vasculature[145]. After the BBB crossing, polymeric nanobioconjugate release molecular inhibitors into the cytoplasm of glioma cells *in vivo* preventing the syntheses of laminin-411. Inhibition of this ECM protein decreased the tumor size by 90%. It has further been shown that the molecular mechanism of action of the endosomal drug releasing unit trileucine peptide (Leu-Leu-Leu) is based on pH sensitivity[146]; nano drug toxicity was found to be negligible and scale-up production has already begun. These nano drug treatments may significantly protect the brain from edema developing (**Figure 13**).

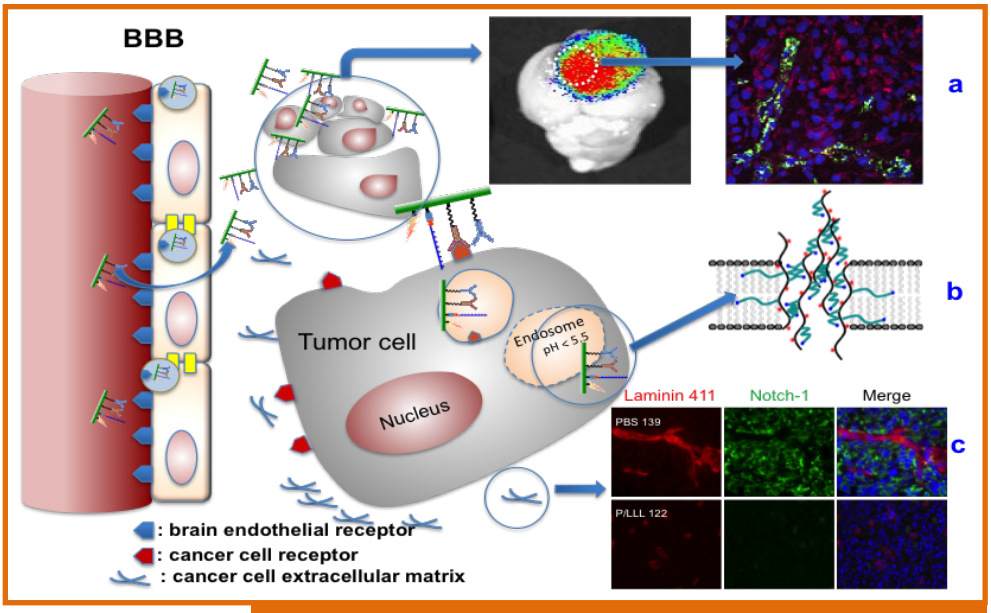

Figure 13. Multifunctional nanoconjugates for drug delivery into brain tumors. a, The nanoconjugates specifically target and accumulate in brain tumor (left), and cross BBB through receptor mediated transcytosis confirmed by confocal microscopy (right); b, Nanoconjugates are delivered into the cytoplasm by pH-dependent endosome membrane disruption and antisense oligonucleotide drugs are released; c, Successful inhibition of brain cancer stem cell marker Notch-1 as a result of inhibition of glioma-overexpressed vascular laminin-411.

Recently, the combination treatment of glioma-bearing animals with polymeric nano drugs showed significant life prolongation[147]. The polymeric nanoparticles were used for convection-enhanced intratumoral delivery of herpes simplex virus type I thymidine kinase DNA combined with the prodrug ganciclovir. An obstacle in brain tumor treatment is the limited ability for the delivery of a number of therapeutic and immunoregulatory molecules. For instance, therapeutic monoclonal antibodies, such as trastuzumab for breast and ovarian cancer, cetuximab for lung and breast cancer, and rituximab for lymphoma are effective for primary tumor treatment however cannot penetrate the BBB to reach the brain, and thus fail to treat their respective metastases in the brain. However, these antibodies can be used for brain drug delivery when they are part of 'nano-vehicles' capable of crossing

the BBB. Nanotechnology can master these problems with nanomedicines designed to cross the BBB and deliver drugs and/or immunostimulatory agents directly to a brain tumor and the respective immune cells in its microenvironment. Taking these possibilities into consideration Polycefin nano drug variants were engineered to treat human EGFR-positive triple negative breast cancer[148] and HER-2/neu positive breast tumors[149] in nude mice. The same nano drugs were similarly used to treat brain metastases from triple negative and HER2/neu positive breast cancer metastases to the brain). Furthermore, primary HER2/neu positive breast cancer has been successfully treated with a combination nanodrug that blocked HER2/neu synthesis and provided an immune system boost by directly targeted IL-2 at the same time. In this case, IL-2 was delivered as part of fusion monoclonal antibody against HER2/neu positive breast cancer[150].

Overall, the development of versatile biodegradable and non-toxic nanobioconjugate based on naturally derived polymalic acid[151] with its ability of targeting brain and breast human tumors in preclinical cancer models, inhibiting the expression of tumor-specific markers, normalizing vasculature, reducing invasion, and blocking their growth, resulted in significantly increased tumor-bearing animal survival. Additional recent nanodelivery systems/methods studied to deliver drugs across the BBB, include: focused ultrasound (FUS) disruption, SR-mediated endocytosis, and targeted adsorptive-mediated transcytosis among several others[152–158].

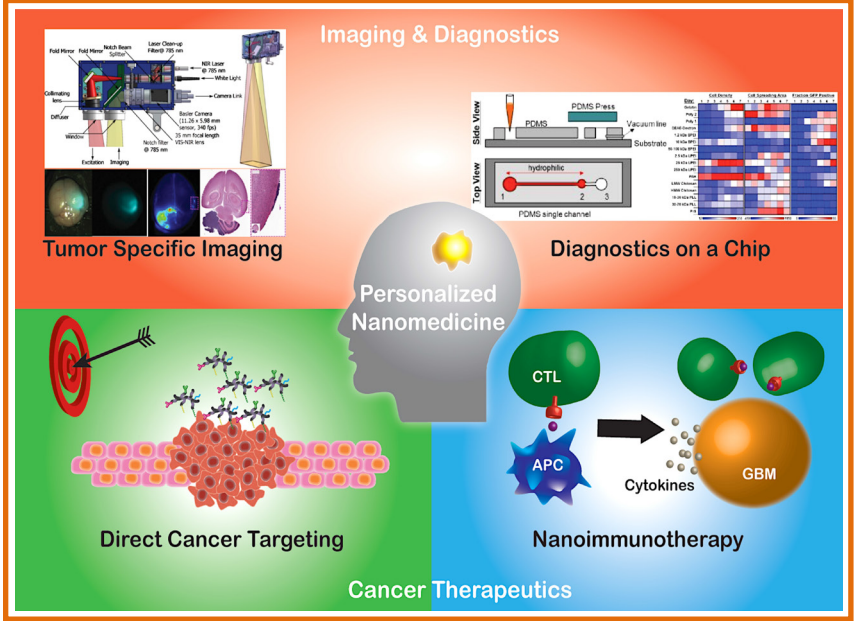

Figure 14. Brain tumor diagnostics and treatment.

Future Scientific and Clinical Developments

Treatment of brain metastases

Progress in treatment of primary cancers has led to improved patients' survival but has also increased the chance of residual tumor cells to metastasize, in particular to the brain. Melanoma, breast and lung cancer form brain metastases in up to 50% of cancer cases, with 3 to 6

months median survival. Therefore, brain metastasis treatment becomes a major issue for brain cancer management.

Personalized nanomedicine

During the last two decades, the dominant model of cancer based on genetic changes has been the chief conceptual foundation for developing targeted therapies. However, cancer immunology is currently coming back and may soon provide new mainstream cancer therapies[159]. We believe that tumor-targeted nano drugs can combine cancer genetics providing tumor cell markers, and immunotherapy providing anti-cancer immune response to treat each cancer patient individually (**Figure 14**).

Diagnostic and targeting

Current targeting strategies of nano drugs and imaging agents are based on monoclonal antibodies that will be substituted by peptides in the future to reduce immunogenicity and production costs. Significant advances of nanotechnology in cancer treatment give hope for the use of its achievements to treat a variety of other human diseases. Notable examples include neurodegenerative disorders, such as Alzheimer's and Parkinson's disease, which are on the rise due to the aging of the world population.

Significant advances of nanotechnology in cancer treatment give hope for the use of its achievements to treat a variety of other human diseases.

Non-Intravenous Routes of Delivery: Aerosol Therapy for Cancer Management

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Nanoparticle-based inhalation drug delivery holds several advantages over intravenous drug delivery. First, inhalation is less invasive and drug administration is more rapid than intravenous. Second, inhaled therapeutics enter circulation directly and avoid the first pass through hepatic clearance. Lastly, nanoparticles allow for tunable drug release in the lung that can provide long-term treatment with fewer administrations[160]. Additionally, nanoparticles can be used to program the local mucosal immune response and re-purpose resident immune cells for tumor immunotherapy[161,162]. Historically, aerosol delivery of nanoparticles has been considered inefficient due to the low particle mass impacting aerodynamic properties and airway deposition. However, recent advances in particle fabrication and inhaler designs are changing this outlook[163]. This document will discuss the existing science and future directions for aerosol cancer treatment using nanoparticle chemotherapy, chemopreventatives, and cancer vaccines (**Figure 15**).

Aerosol Chemotherapy

Inhalation chemotherapy offers the potential for higher drug concentrations in the lung[163–166]. Additionally, aerosol delivery allows for enhanced access to the intra-thoracic lymphatic system either through direct drainage or intra-cellular transport. Preclinical studies have suggested that there may be benefits to aerosol chemotherapy. Inhaled liposomal formulations of chemotherapies have demonstrated superior efficacy over traditional routes for the treatment of lung metastases in preclinical models[167]. Other formulations such as aerosol particles of 5-fluorourcil (5-FU), paclitaxel, carboplatin, and gemcitabine have also been studied preclinically[164,168–174]. Clinically, chemotherapeutic drugs have been delivered to the lungs through the use of nebulizers for both free drug and liposome formulations. The liposome formulations have encapsulated 9-nitrocamptothecin, doxorubicin, and cisplatin[175–177]; however, clinical trial results to date are inconclusive and suggest utilizing caution with this approach.

Delivery of Chemopreventatives to the Lung

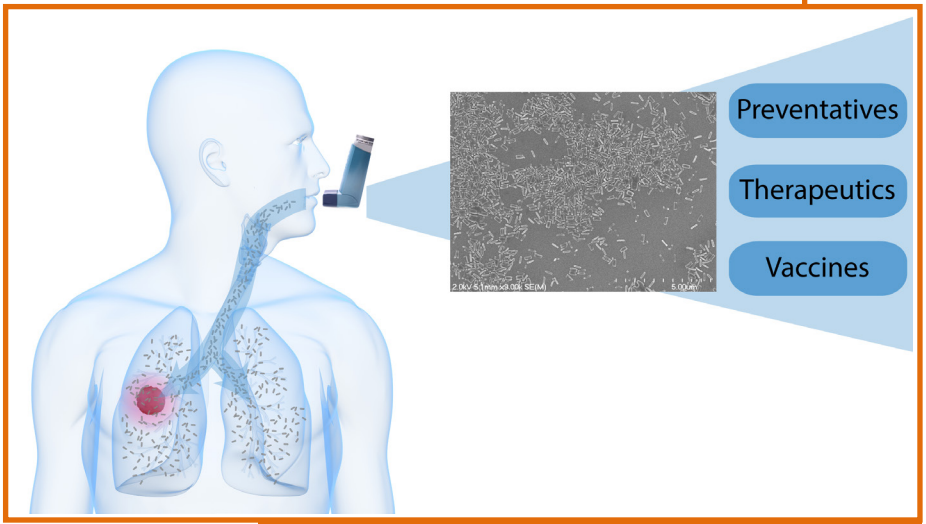

Figure 15. Depiction of aerosol based delivery of chemopreventitives, chemotherapeutics, or cancer vaccines via nanotechnology delivery with SEM image (inset) of nanoparticles designed for aerosol delivery route.

While chemotherapeutics are intended to alter disease progression following tumor establishment, chemopreventative agents are pharmaceutical interventions aimed at halting, or reversing disease progression[178–181]. Chemopreventatives can be given at a tumors' primary stage to high-risk patients, a secondary stage to patients with an identified pre-malignancy state, or a tertiary stage to prevent a secondary occurrence of the tumor[178]. To date, there have been numerous clinical trials targeting lung cancer, with minimal, or even negative, impact on disease progression. These trials have included mainly dietary supplements including various anti-oxidants, vitamins, and retinoids. Pre-clinical studies administering inhaled corticosteroids as a chemopreventative reduced cancer formation in mouse models; however, these findings did not translate to humans[182–185]. Despite these negative data, there is cause for optimism in this approach. There have been considerable successes in preclinical models involving aerosol delivery of selenium and cyclooxygenase inhibitors delivered at the primary stage[178–181]. Aerosol liposomal formulations of interleukin-2 (IL-2) have resulted in disease remission or maintenance in canine cancer models, and a number of clinical trials using nebulized IL-2 show slightly decreased tumor occurrence in humans[166,182]. Inhaled delivery of interferon, granulocyte-macrophage colony-stimulating factor (GM-CSF), and cyclosporine have also demonstrated efficacy in pre-clinical studies and, to some extent, in humans with no adverse systemic effects. Furthermore, use of oral iloprost in a randomized Phase II, placebo controlled trial for heavy smokers, has demonstrated the ability to decrease endobronchial dysplasia[186].

Lung Targeted Nano-Based Cancer Vaccines

Modulating the local immune environment of the tumor and surrounding tissue to enhance tumor eradication may be further achieved through a cancer vaccine. An ideal cancer vaccine would direct the power and precision of the patient's own immune system toward tumor elimination while providing immunological memory for rapid elimination of subsequent malignancies. The biggest challenge for cancer vaccine development is convincing the immune system that the tumor is harmful and needs to be eliminated while minimizing collateral damage in healthy tissues[183]. Achieving tumor specific immune responses requires immune targets that are exclusively (or at least preferentially) expressed by tumors, termed tumor associated antigens (TAA). The hope is that vaccines combining TAAs and immune modulating adjuvants will instruct the immune system to eliminate tumor cells.

Recent clinical trials for lung cancer vaccines incorporating non-small cell lung cancer (NSCLC) TAAs and strong immune modulators have shown measurable increases in patient survival (~3 month increase OS versus placebo control); however, none were curative[183]. Potential explanations for modest efficacy include patient selection and vaccination timing; however, another major consideration is the route of vaccine delivery. Some vaccines required multiple injections via parenteral routes[184]; however, recent pre-clinical studies using lung targeted nano-based vaccines suggest that pulmonary vaccine delivery may provide more robust immune responses with implications for targeting cancer[162,185].

Pre-clinical infectious disease models using a variety of nano-based vaccines provide protection from subsequent pathogen challenge[162,185–189]. Two of these studies directly compared pulmonary and parenteral vaccine administration and found that direct immunization of the lung provided better protection than injection at distal sites[162,188]. Part of the protective immune mechanism works through activation of cytotoxic T cells (CTLs) that seek out and eliminate cancerous cells. In addition to CTL activation, several of these vaccines also promoted TNFα and IFN-γ cytokine production, which are known to promote an anti-tumor environment by inhibiting suppressive tumor associated macrophages[162,185,190]. The added benefit of an efficacious cancer vaccine is that these immune cells roam the body and have the capacity to target sites away from the primary tumor, which has major implications for metastatic control. Support for this hypothesis includes a study in which a nano-vaccine delivered to the lung was able to eliminate melanoma in the flank and establish long-term tumor rejection and survival[162].

Future Directions for Aerosol Delivery of Nanoparticles in Cancer Management

Nanoparticle therapeutics in the lung represent an area of great potential, especially for treating cancer. To date, most aerosol therapies have involved delivery of 1-5 µm sized particles, due to their aerodynamic properties and their assumed deposition in the lung[191]. Indeed, even the chemotherapy liposome formulations evaluated in clinical trials were on the order of ~1 µm[164,167,192]. More recent nanoparticle formulations (<200 nm) could offer tremendous benefits to the three aspects of cancer management mentioned here: drug delivery (including enhanced tumor uptake), mucosal diffusion, and lymph trafficking[160]. However, delivery concerns will need to be addressed in order for nanoparticles to deliver and deposit at high efficiencies in the airways. Controlled aggregation or a "Trojan horse" approach may be required for effective delivery, with independently tunable aerodynamic properties for controlled deposition in the region of interest within the lung[173]. Additionally, advancement of particle-based lung therapies will require continued optimization of inhaled delivery devices[165,193].

Of the potential applications for aerosol cancer management, nanoparticle delivery of cancer vaccines may be best situated to make the greatest impact within the next decade. The extensive research and success in particle formulations for intravenous nanoparticle therapies can be readily translated to lung administration with minimal reformulation, while current clinical evaluations of aerosol liposome formulations establish precedence for use of a particle approach for direct vaccine delivery. The biggest challenges moving forward will be choosing the most specific TAA's, overcoming immune tolerance mechanisms and avoiding immune pathology in an already vulnerable patient population. Overcoming immune tolerance may require co-administration of therapeutic antibodies to disrupt normal lymphatic checkpoint mechanisms (anti-CTLA4, anti-PD1, anti-PDL1) and allow the vaccine to establish an immune response[194]. Another challenge will be establishing the safety of the nanoparticle platforms, especially in combination with immune adjuvants with a goal of inducing strong immune responses without damaging lung tissue. Ultimately, studies assessing patient tolerance to pulmonary-targeted nano-vaccines will be critical to the use of safe adjuvant combinations.

Aerosol chemotherapy faces a steep uphill battle to fruition. There are two deeply rooted schools of thought regarding inhaled chemotherapeutics and it is likely to remain a controversial issue. Most clinicians believe the direct delivery of highly toxic chemotherapeutics to the lungs exposes the patient to unacceptable risk, and could inflict further damage to an already susceptible tissue. The opposing argument points to the urgent need for alternative approaches for lung cancer treatment. Thus moving forward,

nanoparticle aerosol delivery of chemotherapeutics will require substantial and strategic preclinical and clinical research to discern the practical application of these therapies.

Chemopreventative agents have demonstrated success in preclinical models...

Chemopreventative agents have demonstrated success in preclinical models, but the difficulties in identifying target patient populations makes widespread chemoprevention in a primary stage cancer challenging. Evaluation of lung specific biomarkers and further characterization of the lung cancer progression will help identify patient populations likely to benefit from chemoprevention; however, dosing at a secondary or tertiary stage following the identification of pre-malignant lesions or prevention of a secondary occurrence may be more tractable. Winterhalder *et al.* suggest that cell surface receptors, such as EGFR and HER2, may be important targets to halt progression of epithelial lung cancer; given the history of systemic nanoparticle formulations targeting these pathways, this may be a tractable first nanoparticle approach[181]. Finally, there are many genetic factors in lung cancer that could be potential targets for gene therapy that are considered "undruggable" using conventional approaches, which are also ideally suited for nanoparticle formulations[195,196].

The nanoparticle approaches discussed here represent novel lung cancer management strategies that may also apply to other cancers. Additionally, topics discussed here may be better suited as combination therapies with more traditional approaches including surgical resection, chemotherapy, and radiation. We anticipate that many of these approaches will be first investigated in recurrent or late-stage disease following alternative interventions. Success in these situations may ultimately lead to a paradigm shift that utilizes aerosol-only based approaches.

Milestones to address these critical areas that researchers should be able to be achieve over the next 3-10 year time frame include many aspects. In the next 3 years, researchers will conduct further preclinical studies on direct lung chemotherapeutics use and efficacy; develop chemopreventatives to better establish effects on lung cancer progression; and identify and validate drug targets for local lung cancer vaccine therapy. Looking further ahead over the next 5 years, researchers will identify tumor associated antigens and adjuvant combinations that target lung related tumors for nano-based cancer vaccines; and carry out perspective studies on effects of direct lung therapy, positive or negative. In the next 10 years, researchers will establish a clinical development program for aerosol treatment of lung cancer, utilizing chemotherapy, chemopreventatives, and nano-based cancer vaccines.

Non-Intravenous Routes of Delivery: Oral

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Introduction

Nanoparticles (NPs) have the potential to make a tremendous impact on the treatment of cancer. Combining biological understanding with engineering and materials science principles has led to the development of nanomedicines for the treatment of cancer that are now entering clinical trials[197–199]. However, NPs are currently limited to parenteral methods of administration. In addition, many chemotherapeutic agents and biological therapeutics are limited to parenteral administration because of low bioavailability. Injection-based therapies can suffer from poor patient compliance and reduced efficacy due to the pain and inconvenience associated with the treatment regimens. Therefore, alternate routes of administration, such as transdermal, nasal, buccal, pulmonary, and oral, are under investigation as a means to improve these therapies. Of these alternate routes, oral is considered the most desirable, especially for long-term treatment of diseases, because of the convenience and improved compliance[200].

In clinical studies with cancer patients, most favored oral over intravenous chemotherapy because of the increased convenience as long as efficacy was not compromised[201–203]. The convenience of taking medications at home was especially convenient for patients that lived far from hospitals and clinics[204]. Several trials have demonstrated that oral-based therapies can be as efficacious as parenteral administration, but offered additional advantages. In one trial, oral administration of Tegafur-uracil (UFT) was compared with intravenous administration of 5-fluorouracil (5-FU) for the treatment of metastatic colorectal cancer[205]. The oral administration was associated with decreased incidence of drug-related adverse effects without compromising efficacy. Other studies have shown that intravenous methods required more frequent hospitalizations that were expensive, time intensive, and required intravenous access[206]. Oral formulations have advantages for physicians as well, providing flexibility and adaptability to tune dosing schedules to individual patients based on efficacy and toxicity[204]. Without the intensive demands on staff required by intravenous administration, studies in the United Kingdom showed that switching from intravenous to oral chemotherapy allowed a 7-fold increase in patients treated[207]. Finally, reducing hospital or clinic visits as well as costs associated by using oral formulations could reduce overall costs for cancer treatments[208–210]. Indeed, cost-benefit studies conducted in Europe and Canada examining oral versus standard intravenous regimens for colorectal cancer suggested

significant savings with the oral route despite the higher cost of the orally formulated therapies[211].

While oral delivery is highly desirable, it presents many challenges due to the number of barriers presented by the gastrointestinal tract before therapeutics are absorbed and enter the bloodstream. These barriers include extreme pH environments ranging from 1 to 8[212] and enzymatic degradation, which limit the absorption of biologic therapeutics such as proteins and nucleic acids. In addition, there is a transport barrier presented by the intestinal epithelium, which is a polarized cell monolayer that tightly regulates the transport of material from the external environment (intestinal lumen) to the *lamina propria*[213]. This intestinal epithelium is covered by a mucus layer, which protects the epithelial surface by trapping pathogens and foreign particulates and rapidly clearing them[214]. Therapeutics that reach the intestinal cell surface and enter the cells must than bypass the cells metabolic systems and P-glycoprotein (P-gp) drug efflux pumps, which can cause low bioavailability for many small molecule drugs such as chemotherapeutic agents[215]. Finally, if the therapeutics cross the intestinal transport barrier, they must avoid immune cells that patrol the *lamina propria* in order to reach the bloodstream and the mononuclear phagocyte system of the liver in order to reach other organs in the body.

Polymeric NPs are a well-studied option for oral delivery that can aid in overcoming many of the intestinal barriers

Polymeric NPs are a well-studied option for oral delivery that can aid in overcoming many of the intestinal barriers. The NPs are stable in the GI environment and can protect encapsulated therapeutics from the pH environment, enzyme degradation, and drug efflux pumps[200,216]. However, intestinal absorption of NPs is highly inefficient because the physicochemical parameters, particularly size, of NPs prevent their transport across cellular barriers such as the intestinal epithelium. To improve the absorption efficiency of NPs and make oral administration practical in the clinic, additional strategies are necessary to overcome the intestinal epithelial barrier.

Oral Delivery Strategies

There are several pathways across the intestinal epithelial barrier that could be used for oral delivery[217]. One option is the paracellular pathway, which is a major passive permeation pathway across the intestines and allows diffusion of small molecules in the space between epithelial cells. The tight junctions between epithelial cells regulate the permeability of this pathway based on the size and charge of the molecules[218,219]. Another option is the

transcytosis pathway, which is an active transport pathway that relies on receptors specific for a molecule to guide the molecule through the cell in endosomes without entering a degradation pathway. Because of their large size, NPs are restricted to this pathway.

One approach for oral delivery that has been extensively evaluated is the use mucoadhesive materials (**Figure 16A**). These are polymers such as chitosan[220], polyacrylic acid (PAA)[221], and poly(fumaric-co-sebacic) anhydride[222] that interact with the mucus layer covering the epithelial cells. Adherence to the mucus layer increases the residence time and contact of released drug with the underlying epithelium, resulting in increased drug concentrations at the site of absorption[223]. In addition to increasing the concentration of therapeutics near the epithelium, many mucoadhesive polymers increase intestinal absorption by acting as permeation enhancers, reversibly opening tight junctions between epithelial cells to allow enhanced paracellular transport[224]. Since the tight junctions are less than 20 nm in diameter, NPs are unable to pass through this pathway, but small molecule therapeutics can cross the epithelium[225]. One disadvantage of this approach is that the permeation enhancer activity

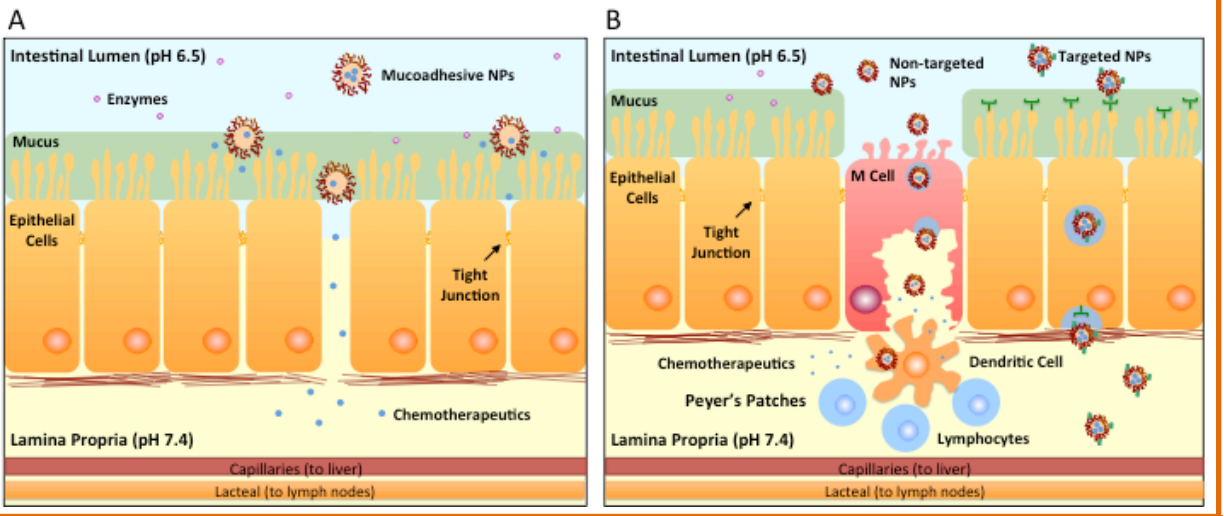

Figure 16. Schematic illustration of strategies for oral delivery. (A) Mucoadhesive materials used to form NPs adhere to the mucus layer above the epithelial cells and release therapeutics at high concentrations near the surface of the epithelial cells. In addition, they are able to reversibly open tight junctions to allow paracellular transport of therapeutics between the cells and across the epithelial barrier into the *lamina propria*. (B) The transcytosis pathway is an active transport pathway that transports material across cells in endosomes while evading degradation pathways in the cell. Examples of transcytosis pathways include M cells, which are responsible for transporting antigens across the intestines for immune surveillance and are associated with Peyer's Patches. Other examples include the vitamin B12 receptor pathway and the FcRn pathway, where NPs targeted to the specific receptors are trafficked across the epithelial cells and released in the *lamina propria*.

is non-specific, potentially allowing toxins and other pathogens present in the intestines to cross the intestinal barrier once the tight junctions are open[226,227]. Another limitation is that the surface area for absorption through the paracellular pathway is less than 0.1% of the total intestinal epithelium surface area, which could limit the capacity for absorption of therapeutics[228].

Targeting NPs to natural transcytosis pathways is another approach used for oral delivery (**Figure 16B**). It offers a way to cross the intestinal barrier without affecting the intestinal epithelium barrier integrity. There are several mechanisms that have been studied for transcytosis of NPs. The most extensively studied is the M cell transcytosis pathway. M cells are associated with Peyer's Patches, which are organized components of the gut-associated lymphoid tissue (GALT). The role of M cells is to transport antigens across the intestines through a non-degradative pathway for immune surveillance[229,230]. This pathway is attractive because M cells have reduced protease activity, lack mucus secretion, and have a sparse glycocalyx[231]. One potential problem with this approach is that since M cells are closely associated with immune cells in the *lamina propria*, NPs crossing the intestines through this pathway may be engulfed by immune cells before reaching the bloodstream and releasing their cargo[232]. Absorption by M cells may also be limited because M cells only make up a small percentage (5-10%) of the non-absorptive epithelium in humans[233,234].

Other strategies have focused on targeting NPs to receptor-mediated transcytosis pathways that are not associated with the GALT, which may help NPs evade immune cells after crossing the epithelium. One example is the vitamin B12 receptor, which traffics vitamin B12 across the intestinal epithelium[235]. NPs targeted to this pathway have been shown to successfully deliver biologic payloads to the bloodstream, although transport of NPs has not been demonstrated yet[236,237]. One potential drawback of this approach is that vitamin B12 absorption does not occur until the distal section of the ileum, requiring NPs to maintain stability and not release their cargo while traveling through most of the small intestine. Another example is the neonatal Fc receptor (FcRn), which transports IgG antibodies across the intestinal epithelium[238,239]. This receptor is expressed throughout the intestines. NPs targeted to the FcRn were able to cross the epithelium and circulate in the bloodstream to several different organs, including the liver, spleen, lungs, and kidneys, along with releasing a therapeutic payload[240].

Clinical Impact

While oral delivery has been extensively studied and many strategies have had success in animal models, there has not been much success translating the research into practical clinical solutions. Most of the effort has focused on developing technologies for oral delivery

of insulin. However, NPs are flexible in terms of the molecules that can be encapsulated and changes to formulations could easily result in NPs capable of delivering chemotherapeutic molecules. In addition, NPs can encapsulate protein therapeutics and small interfering RNA (siRNA), which are emerging treatment modalities for cancer. The major limitation to translation is that the technologies developed are not efficient enough to make them practical for the clinic. More recent technologies such as NPs targeting the B12 receptor and FcRn have demonstrated higher efficiencies, but only in animal models at this point.

There are currently several technologies that are entering early-stage clinical trials for oral delivery of therapeutics. These include Oramed's oral formulation consisting of permeation enhancers that is now entering Phase II clinical trials. Novo Nordisk is developing an absorption enhancer technology that is entering Phase I trials. Entrega is developing a mucoadhesive technology that is still in early stage development. Each of these technologies is focused on enhancing transport through paracellular pathways, which would enable drugs, but not NPs, to cross the intestinal epithelium.

As nanomedicines are shown to be effective for cancer therapy in clinical trials, future efforts should focus on translating technologies to the clinic that utilize the transcytosis pathway. These technologies could enable the NPs carrying chemotherapeutics to cross the intestinal epithelium and reach circulation. In this case, the advantages of NPs in the bloodstream could be utilized for the treatment of cancer, such as passive or active targeting of tumor cells, delivery of multiple therapeutics in a controlled or triggered release manner, and selective biodistribution of the therapeutics to the tumor to reduce side effects. Future research should also focus on discovering other natural transcytosis pathways that could be used to transport NPs across the intestines. This could include studying how some bacteria are able to cross the intestines and the subsequent rational design of NPs that could mimic those processes. In addition, new technologies such as microneedle-based pills have shown promise in improving bioavailability of biologics in initial animal studies, but need further study to determine clinical feasibility[241].

...researchers will develop NP delivery vehicles targeted to transcytosis pathways that specifically encapsulate and deliver chemotherapeutic agents...

Milestones to address these critical areas that researchers should be able to be achieve over the next 3-10 year time frame include many aspects. In the next 3 years, researchers

will optimize the physicochemical parameters of NPs targeted to transcytosis pathways to maximize bioavailability after oral administration; and conduct research into alternate transcytosis pathway receptors and alternative technologies such as microneedle-based pills. Looking further ahead over the next 5 years, researchers will develop NP delivery vehicles targeted to transcytosis pathways that specifically encapsulate and deliver chemotherapeutic agents; and evaluate the performance of permeation enhancer and mucoadhesive technologies currently entering clinical trials. In the next 10 years, researchers will gain FDA approval for permeation enhancer and mucoadhesive technologies that are successful in clinical trials; conduct clinical trials on NP delivery vehicles targeted to transcytosis pathways for cancer treatments; and study how patient-to-patient variability, diet, fasting states, and disease states affect the performance of these technologies in humans in order to determine the robustness of these technologies.

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Section II: Unique Modalities for Nanotherapeutics

Optimizing Nanoparticle Delivery of Chemotherapeutics

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Chemotherapeutics in Cancer Therapy

Chemotherapy can be defined as the use of cytotoxic drugs that attack or interfere non-specifically with critical components of the cell. Chemotherapeutic drugs include at least 3 well-known categories: agents that damage the DNA template directly or indirectly; agents that damage microtubules; and, agents that inhibit DNA, RNA, or protein synthesis (antimetabolites). In addition to their lack of specificity, various pharmacologic factors seriously limit drug distribution and penetration to tumors and neutralize the activity of chemotherapy. This group of agents could tremendously benefit from a delivery system to improve its tumor specificity and reduce its toxicity to normal tissues. However, it is now often questioned whether chemotherapy will be abandoned and replaced entirely with biological and immunological therapies in the near future. While important advances have been made in the areas of biological therapy and immunotherapy of cancer, chemotherapy remains a critical tool of cancer treatment with a large contribution to cancer cures in the adjuvant setting and an important contribution to life extension in the metastatic setting. Improvements in safety and efficacy of chemotherapy are definitely a worthy endeavor since they will have a dramatic effect on the well-being of our patients, their quality of life during treatment, and their ability to face the hardship of therapy and complete successfully the protocol regimes. Moreover, chemotherapy is also likely to remain an important component of a multimodality therapeutic approach, together with biological therapy and immunotherapy, to improve the antitumor response rates in a broad array of cancer types. There are many examples of the continuing role of chemotherapy and its critical added value to biological therapy. One of them is exemplified by the combination of chemotherapy with anti-HER2 antibodies (Trastuzumab) in HER2-positive breast cancer, which is required for optimal antitumor response. From a tumor response rate of only 12% for single agent Trastuzumab, the response rate climbs to 56% when doxorubicin and cyclophosphamide are combined with Trastuzumab[1]. While this combination of doxorubicin with Trastuzumab was problematic because of a major rise in cardiac complications, a number of subsequent studies have shown that replacing doxorubicin with liposomal

doxorubicin can avoid or minimize cardiac toxicity[2]. This example emphasizes the valuable contribution of chemotherapy to targeted therapies and the need to refine the formulations of chemotherapy for optimal results.

Towards "Smart" Chemotherapy with Nanoparticle Delivery

Nanomedicine is a platform to allow sophisticated and smart drug delivery within the size window of a submicroscopic system that enables delicate and complex interactions with cancer cells and their biological milieu. Nanoparticles and some macromolecules are the main tools of nanomedicine[3]. Pegylated liposomal doxorubicin (PLD) was the first nanoparticle-based cancer chemotherapeutic approved by the FDA. PLD together with nanoparticle albumin-bound paclitaxel (NAB-paclitaxel) are probably the cancer nanomedicines that have made, so far, the most important clinical impact[4,5], excluding antibody-drug conjugates, generally considered to be a separate group of complex drugs.

Transforming the administration of a drug in free form, several angstroms across, into a 100-nm diameter nanoparticle loaded with thousands of drug molecules and with ~1 million-fold greater volume is a formidable pharmaceutical challenge that will have major pharmacological implications. However, from the clinical point of view, the only questions that have any significance when using nanopharmaceuticals are: Is the safety profile of the drug improved? Is the efficacy of the nano-engineered drug superior to the standard treatment or best performing comparator? To achieve these objectives, the nanoparticle-based approach should ideally fulfill two critical parameters:

a. Stable association of drug and carrier in circulation, and release of active drug in tissues, at a satisfactory rate, for anti-tumor activity. This parameter appears to have been satisfactorily met by pegylated liposomal doxorubicin (PLD)[6].

b. Enhanced drug delivery to tumors via the nanoparticle formulation. For this to occur, first, the nanodrug or nanopharmaceutical must have a long circulation time to increase the number of potential passages through the tumor microvasculature. Second, the nanoparticle physical size has to be in the optimal size regime to allow extravasation across tumor blood vessels, which usually display higher permeability than normal blood vessels. The size window that will exploit the difference in permeability between normal and tumor blood vessels appears to be between 20 to 200 nm.

Successful control of these two parameters in the drug nano-formulation allows sparing normal tissues from toxicity and in boosting the antitumor effect with an overall increase of the therapeutic index. Some nanomedicines have failed to meet these requirements because

of either short circulation time, poor drug retention, or insufficient drug release[7–9]. Yet, other nanomedicines have been able to make a positive clinical contribution despite only minor changes in drug pharmacokinetics. This is the case of NAB-paclitaxel which avoids the acute toxicities associated with Cremophor EL® vehicle used in solvent-based paclitaxel, and has been found useful in various indications.

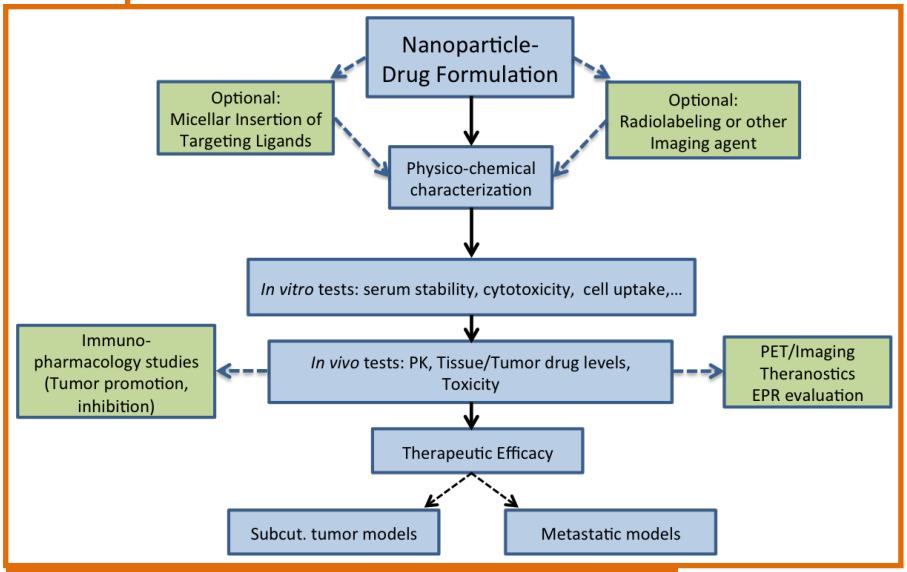

Figure 1. Schematic model of a work plan for rational development of nanoparticle-based chemotherapeutics.

High microvascular permeability is an important and frequent feature of tumors usually referred to as Enhanced Permeability and Retention (EPR) effect, and is a key component for nanoparticle transport into tumors[10]. EPR appears to be a particular feature of tumor-driven neoangiogenesis. While EPR is observed in most models of implanted experimental tumors, large variations have been observed in human cancer depending on tumor type, tumor size, tumor site, and other factors, such as previous chemotherapy, antiangiogenic therapy, and radiotherapy. EPR may also be modulated by pharmacologic mediators. In some instances, tumors or their metastases derive their blood supply by a process known as co-option of normal blood vessels which results in blood vessels less permeable and less responsive to anti-angiogenic treatments and, consequently, less likely to display the EPR effect[11]. The high response rate of Kaposi Sarcoma, a tumor with high vascular permeability, to relatively low doses of PLD suggests that EPR is critical for the antitumor activity of nanodrugs. While this hypothesis has a strong pharmacologic rationale, it has not been tested rigorously, and we cannot discard that tumors with low EPR will still respond to nanodrugs better than to free drugs.

Smart delivery of chemotherapeutics may be simply achieved by controlling release rate of the active agent and by changes in tissue distribution, without necessarily including a targeting component specific for cancer cells. In fact, all the nanopharmaceuticals approved for clinical use belong to the non-targeted category. A scheme for development of nanoparticle-based chemotherapeutics is shown in **Figure 1**.

Targeted Nanomedicines

Our understanding of the molecular processes underlying the pathologic behavior of cancer cells has progressed enormously in the last decade. Overexpressed receptors in the membrane of tumor cells, may offer a potential Trojan horse for targeting specific ligands or antibodies and delivering a cytotoxic drug cargo. Probably, the best example of a successful clinical translation of this approach is the antibody-drug conjugate known as T-DM1 which combines Trastuzumab, an anti-HER2 antibody, with emtansine, a potent and highly toxic chemotherapeutic, and has conferred a significant disease-free survival advantage to patients with HER2-positive breast cancer[12].

Targeted delivery of a large payload of drug via ligand-directed nanoparticles to cancer cell-specific receptors is probably the most valuable objective of nanomedicine. A comprehensive and in-depth review of this subject has been recently published[13]. Indeed, the most logical improvement of nano-based drugs is the coupling of a ligand to the surface of the nanoparticle to target to a specific cell-surface receptor. This would be followed by internalization and intracellular delivery of the small-molecule drug cargo. Examples in this direction are the targeting of PLD to HER2-expressing or folate-receptor expressing cancer cells using respectively a specific anti-HER2 scFv or a folate conjugate anchored to the liposome surface, or the targeting of polymeric nanoparticle of docetaxel to PSMA, a marker of prostate cancer[14–16]. Yet, another example is the tumor vascular targeting of liposomes with endothelium-specific peptides associated to liposomes[17]. A major advantage of targeted nanocarriers over ligand-drug bioconjugates is the delivery-amplifying effect of the former, which can deliver to the target cell at a ratio of ~1000 drug molecules per single ligand-

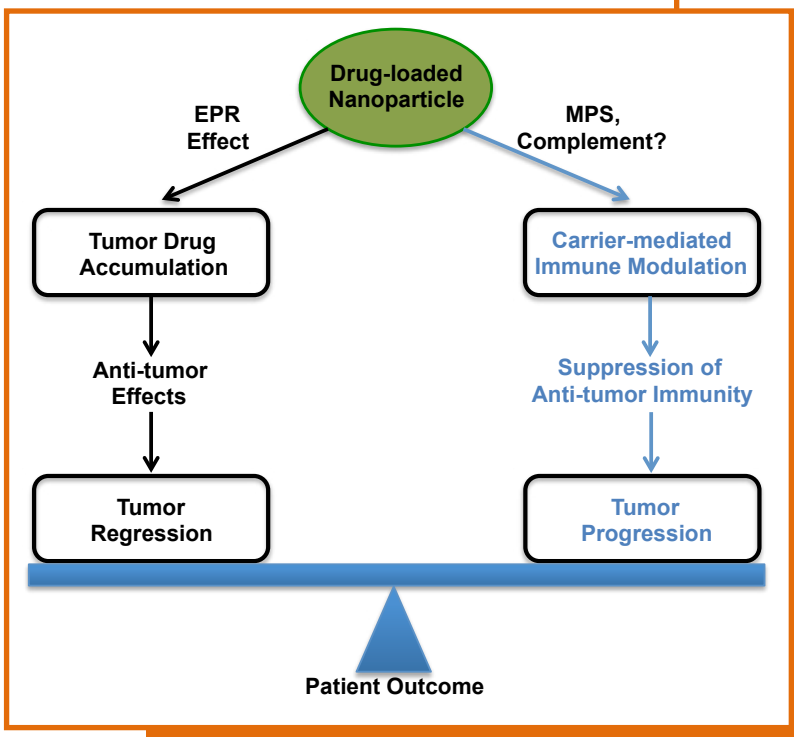

Figure 2. Nanoparticle carrier interactions with the immune system may suppress antitumor immunity, thereby attenuating the antitumor effects of the drug cargo. A mechanistic understanding of the mechanisms of carrier-induced immune modulation will enable the development of systematic tools that may help to realize the full clinical potential of nanoparticle-based therapies.

receptor interaction. In addition, the multivalent conjugation of targeting ligands on the surface of nanoparticles is presumed to enhance binding to the desired target. Targeting ligands, particularly small molecule ligands, can significantly enhance target-specific avidity of nanoparticles by several orders of magnitude through multivalent interactions[13].

Interaction of Nanoparticles with the Host

Nanoparticles, including liposomes, are known to interact with the immune system to varying extents[18]. These interactions can affect drug pharmacokinetic parameters and may have significant clinical consequences. The majority of intravenously administered nanoparticles are rapidly cleared by the mononuclear phagocyte system (MPS) through internalization by phagocytic cells such as hepatic Kupffer cells and splenic macrophages. Notably, peripheral blood monocyte count and phagocytic function have been shown to correlate with PLD clearance rates in patients[19], and similar correlations have been observed with other pegylated liposomal formulations (S-CKD-602, and SPI-077) in preclinical rodent and canine models[20]. Thus uptake and sequestration of nanoparticles in cells and organs of the MPS is a major barrier limiting the circulation half-life and, hence, tumor accumulation of carrier-mediated drugs.

In addition to interactions with the MPS, it is well established that nano-carriers interact with serum proteins such as IgG, IgM and the blood complement proteins, which contribute to opsonization of the carrier and enhance clearance by the MPS. Importantly, activation of complement proteins also generates anaphylatoxins (C3a, C4a, C5a) which can stimulate release of inflammatory mediators by immune cells leading to complement activation-related pseudoallergic reactions (CARPA) in swine and canine models, and several formulations of nanoparticles in clinical use (Doxil, DaunoXome, AmBisome, Abelcet, Amphocil) have been shown to cause hypersensitivity reactions consistent with CARPA. Clinically, it was shown that PLD activates complement in the peripheral blood of cancer patients and that the extent of complement activation correlated with the development of acute infusion reactions[21]. Therefore, undesired interactions with circulating serum proteins can also affect the pharmacokinetics and tolerability of carrier-mediated drugs.

Coating of nanoparticles with poly-ethylene glycol (PEG) (“pegylation”) has become widely used to reduce opsonization, improve stability in plasma, and prolong circulation time which are important requirements for effective tumor targeting. However, these approaches may not abolish immune reactions to nanoparticles. In addition, recent evidence suggests that PEG is not immunologically inert. Several groups have demonstrated that the initial systemic administration of pegylated nanoparticles induces production of anti-PEG IgM antibodies that enhance immune recognition and clearance of the second dose of nanoparticles in

preclinical models. Interestingly this "accelerated blood clearance" (ABC) phenomenon has not been reported in patients and its clinical relevance is currently unclear. In fact, the opposite has been observed in patients treated with PLD, where clearance rates decrease with repeat administration, up to 30% by the third cycle[22].

Recently, it was shown that nanoparticle-induced complement activation could promote C5a-dependent tumor growth in tumor bearing mice, presumably through the recruitment and activation of immunosuppressive leukocytes. Yet, the nanoparticles used in these studies were intentionally designed to activate specific complement pathways[23]. It is not known whether clinically relevant nanoparticulate carriers, which activate complement in the peripheral blood, also induce complement activation in the tumor tissue, or how this impacts tumor growth. However, new evidence with a pegylated liposomal carrier similar to the PLD carrier, showed that these liposomes significantly enhanced tumor growth in an immune competent murine tumor model[24]. This was associated with suppression of antitumor immunity as indicated by blunting of cytokine production in tumor-associated macrophages and cytotoxic T cells, and diminished tumor antigen specific immune responses. Moreover, tumor microvessel density was significantly increased, consistent with enhanced angiogenesis. Collectively, these findings suggest that carrier-induced immune modulation could attenuate therapeutic efficacy of the nano-encapsulated drug (**Figure 2**), which may partially explain why there has been an insufficient improvement in anticancer efficacy in many of the clinical studies with nano-drugs despite their major pharmacologic advantages over free drugs[25].

It is possible that during preclinical development, the prevalent use of rodent models with immune defects and the dearth of *in vivo* immune functional studies may have downplayed the consequences of the interactions between drug carriers and the immune system. It is also possible that manufacturing of the nanomedicines themselves were not as pure as initially thought with various solvents left behind in the formulations. Either way, incorporation of fully immune competent tumor models along with systematic immune functional studies may yield more accurate insight and analytical tools, that may help to realize the full clinical potential of nanoparticle-based therapies[26].

Cancer Nanodrugs in Clinical Use or Clinical Testing

Table 1 shows a list of nanoparticle-based drugs approved for cancer treatment by the FDA and/or the EMA. As seen in Table 1, the number of nanopharmaceuticals in clinical use has been slowly albeit steadily rising and includes chemotherapeutics of various classes, such as anthracyclines, taxanes, vinca alkaloids, and DNA topoisomerase-1 inhibitors. Most of these formulations are liposome based. Two of them, Depocyt and Mepact, are large

liposomes above the ultrafilterable range and probably should not be considered *bona fide* nanomedicines. Also included in Table 1 is NaL-Iri, which has not yet been approved although it has completed phase 3 trials for the 2nd line therapy of pancreatic cancer and met its primary objective of improved survival rates.

The early and positive preclinical and clinical experience with liposomal delivery of anthracyclines is probably one of the reasons for the dominance of liposomes in the field. Liposomes still remain as one of the most attractive particulate systems for cancer nanomedicine applications. A liposome formulation of doxorubicin, PLD (known as Doxil/Caelyx or Lipodox in generic version), is currently approved for various indications and in wide clinical use[4]. PLD has significantly reduced acute toxicity, as well as cardiac toxicity as compared to free doxorubicin precisely because of its unique pharmacokinetic characteristics. Probably the most significant clinical value added of PLD is the evidence of a major (~3-fold) risk reduction of cardiotoxicity as compared to free doxorubicin enabling risk-free, extended treatment[2].

In addition, many other promising nanochemotherapeutic products are under clinical testing or about to be clinically tested. These include: polymeric nanoparticles of docetaxel in targeted and non-targeted form which have a significantly different pharmacological profile from the solvent-based docetaxel formulation; pegylated liposomal formulations of various cytotoxic drugs including eribulin and a prodrug of mitomycin C; a HER2-targeted version of PLD (MM-302); a low-temperature, release-sensitive, liposomal doxorubicin formulation; and a liposome formulation of co-encapsulated cytarabine and daunorubicin at fixed molar ratio[16,27–32].

Table 1: Nanoparticle-based products for cancer approved by FDA and/or EMA

Product	***Indication in cancer***
Pegylated Liposomal Doxorubicin	Kaposi Sa., Ovary, Breast, Myeloma
Liposomal Daunorubicin	Kaposi Sa.
NAB-Paclitaxel (Abraxane)	Breast, Lung, Pancreas
Liposomal Doxorubicin	Breast
Liposomal Vincristine (Marqibo)	Adult A.L.L.
Low-pegylated Liposomal Irinotecan (NAL-IRI)	Pancreas (Phase 3 completed, awaiting NDA)
Liposomal Cytarabine (DepoCyt)	Lymphomatous meningitis
Liposomal Mifamurtide (Mepact)	Osteosarcoma

The Future of Nanoparticle-Based Chemotherapeutics - Quo Vadis?

Two fundamental aspects of nanomedicines remain to be clarified in upcoming years: we need an improved understand of the interaction of nanoparticles with the immune system and to learn how to manipulate it for the benefit of the patient; and, we need to understnad how relevant is the EPR effect in human cancer, particularly in metastases, and what role does it play in the performance of nanopharmaceuticals.

It is likely that we will witness a more extensive use of the currently approved nanotherapeutics at the expense of conventional use of chemotherapeutics. In addition, other nanodrugs in clinical development may be approved in the coming years, expanding the classes of drug available in nanopharmaceutical form. Nanodrugs designed to exploit the EPR effect best, with optimal stability and drug release profiles, are likely to perform better although safety improvements will remain a key aspect dictating clinician preference. The use of targeted nanomedicines is probably going to be on the rise, particularly when there is a need to improve the cell uptake of a specific pharmaceutical agent.

Two fundamental aspects of nanomedicines remain to be clarified in upcoming years:...

The use of nanoparticles to deliver therapies, other than chemotherapeutic drugs, is also foreseeable, especially for agents with problematic *in vivo* delivery. In the case of siRNA, the nanoparticle protection is crucial. Recently published studies suggest that for some biologic agents such as tyrosine kinase inhibitors[33], or, immunomodulators such as aminobisphosphonates[34], nanoparticle-based delivery may also improve their *in vivo* performance in combination with chemotherapy or adoptive lymphoid cell therapy respectively.

Another area where nanoparticles could have a future impact is co-encapsulation of drugs[35]. Synchronized co-delivery of drugs co-encapsulated in the same particle or encapsulated separately in particles with identical physico-chemical and pharmacokinetic characteristics. Ideally, the drugs chosen should have synergistic or complementary anti-tumor effects with minimal overlap of toxicity profiles.

The co-administration, on the same nano delivery platform, of a therapeutic and a diagnostic or tracking agent, such as a PET-emitting radionuclide, is referred to as a Theranostic. This approach could enable real-time monitoring of the fate of a nanoparticle and its drug

payload. In essence, providing an insight as to the degree of cancer targeting achieved in each specific cancer individual. By imaging the nanoparticle, the EPR effect can then be predicted in each specific case and correlated with clinical response. This would provide direct clinical data to determine whether selecting patients based on their EPR tumor activity could lead to improved therapeutic benefit of nanoparticle based therapy[36].

Finally, the use of nanomedicines in conjunction with loco-regional approaches to therapy (e.g., hyperthermia, radiofrequency ablation, radiotherapy) is a small niche, but has potential opportunities in specific applications that will increasingly attract clinical testing and adoption[37].

RNAi Therapeutics

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RNAi as a Tool for Precision Cancer Medicine

Precision cancer medicine, i.e., the design of therapeutic regimens informed by tumor genotyping, continues to be a central paradigm in modern cancer research. The most recent FDA approval of crizotinib and vemurafenib for the treatment of ALK-translocated lung cancer and BRAF-mutated melanoma, represents the latest proof-of-concept that oncogenomics-driven drug design can improve cancer prognosis[38,39]. High-throughput interrogations of cancer genomes have evolved with unprecedented pace. Bioinformatics, functional cancer biology and genetics continue to identify oncogenes and tumor suppressors that drive or contribute to the pathogenesis of cancer. The design and clinical testing of small molecules inhibiting 'druggable' targets, such as BRAF or ALK, embodied the initial promise of precision medicine, but the vast majority of the dauntingly complex oncogenome has yet to be translated into meaningful therapeutic strategies. How can the activity of multiple unprecedented, non-enzymatic targets with unknown *modi operandi* be modulated?

RNA interference (RNAi) comes to mind, as a potent mechanism to silence aberrant oncogene expression by blocking the translation of their encoding mRNAs. Without prior knowledge of oncogene function, sequence-specific microRNAs (miRNAs) or small interfering (si) RNAs can be designed to selectively target oncogenic pathways, which drive unabated growth, apoptosis resistance, neo-angiogenesis and enhanced migration/invasion of tumor cells. siRNAs are generated by cleavage of long double-stranded (ds) RNAs into ~20 nucleotide-containing siRNAs by the enzyme Dicer. Unwinding of siRNAs into two single-stranded (ss) RNAs, incorporation of the guide strand into the RNA-induced silencing complex (RISC), and binding of siRNAs to complementary mRNAs triggers the degradation of endogenous mRNA by Argonaute, the catalytic component of the RISC complex (reviewed by Hannon and Rossi 2004)[40]. Structurally similar to siRNAs, mature miRNAs are non-coding RNAs, which typically exhibit incomplete base pairing to the target mRNA, and inhibit translation of multiple mRNAs via binding to their untranslated regions (reviewed by Di Leva et al. 2014)[41]. Thus, the level of expression of single miRNAs can influence multiple biologic processes. In contrast, siRNAs bind the coding portion of the mRNA with complete base-pair match and induce mRNA cleavage only in a single, specific target. Due to the negative charge of the RNA backbone, siRNA or miRNA oligonucleotides require delivery systems to

overcome negatively charged membranes, and to prevent rapid renal and hepatic clearance, the degradation of si/miRNAs by nucleases, and toxicity and immunogenicity of the RNA payload.

Preclinical Evaluation of RNAi-Based Therapeutics – Recent Developments Utilizing Nano-Enabled Approaches

The first clinical proof-of-concept that systemically delivered siRNA reduce oncogene expression via an RNAi mechanism in humans[42] motivated the development of several RNAi delivery platforms, which target a wide array of oncogenes in many different cancers.

Spherical nucleic acids (SNAs) (i.e., 13 nm polyvalent gold nanoparticles functionalized with siRNAs or miRNAs) were preclinically evaluated to deliver Bcl2-Like12 (Bcl2L12)-targeting siRNAs (**Figure 3**) and mature miR-182 sequences to intracranial glioblastoma[43,44]. Bcl2L12 is potent caspase and p53 inhibitor with near ubiquitous expression in primary GBM specimens[45–49]. miR-182 is a tumor suppressive miRNA, which regulates apoptosis, growth and differentiation programs via transcriptional repression of Bcl2L12, c-Met, and Hypoxia Inducible Factor 2 alpha (HIF2α) to enhance therapeutic susceptibility, and to decrease expansion and multipotency of glioma-initiating cells[44]. siBcl2L12 and miR-182-based SNAs robustly penetrated glioma-initiating cells via scavenger receptor-mediated endocytosis. In an *in vitro* blood-brain barrier (BBB) model involving the co-culture of human primary brain microvascular endothelial cells separated from astrocytes by a semi-permeable filter insert, Cy5.5-labeled SNAs passed through the endothelial cell layer and filter, and rapidly entered the astrocytes. Systemic administration into Sprague-Dawley rats and non-human primates have not resulted in SNA-related differences in body or organ weight, nor in an inflammatory response in the brain

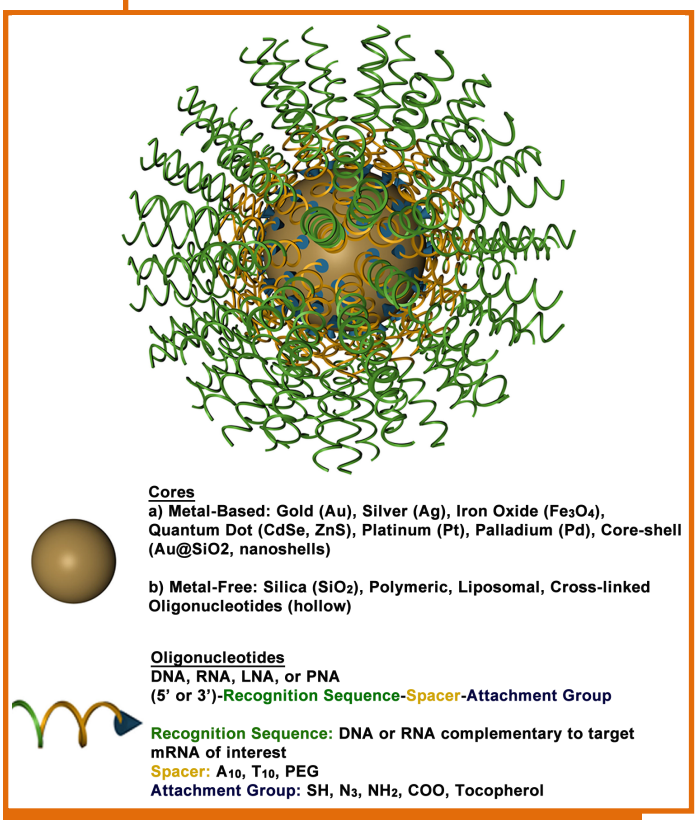

Figure 3. Schematic representation of a Spherical Nucleic Acid (SNA) nanoconjugate. The surface of a variety of different core materials including metal nanoparticles (e.g., Au, Pt), liposomes and polymers, can be functionalized with highly oriented nucleic acids (*Reprinted with permission from Barnaby et al., 2015*)[54].

or in reticuloendothelial system (RES) organs, as shown in published[43], and unpublished data. Importantly, si/miRNA-based SNAs crossed the blood-tumor barrier and accumulated in glioma elements relative to normal brain tissue likely via enhanced permeability and retention of the tumor-associated vasculature. Accumulation and pervasive dissemination into extravascular tumor parenchyma translated into robust intratumoral protein knockdown, increased intratumoral apoptosis, impaired tumorigenicity, and prolonged survival of GIC-derived xenogeneic mice[43,44].

Jacks and colleagues developed a combinatorial RNAi regimen using lung-targeting polymeric nanoparticles made of low-molecular-weight polyamines and lipids to deliver siRNA and miRNA mimetics to lung adenocarcinoma cells *in vitro* and to tumors in a genetically engineered mouse model (GEMM) driven by KRas activation and p53 deletion[50]. The lead compound is a nanoparticle with multilamellar structure, which was synthesized by reacting with a 15-carbon lipid tail in ethanol[51], mixed with $C_{14}PEG_{2000}$. Delivery of miR-34a and siRNAs targeting KRas reduced lung cancer progression more effectively than either small RNA alone, and synergized with cisplatin-based chemotherapy to prolong survival of animal subjects[50].

Accumulation and pervasive dissemination into extravascular tumor parenchyma translated into robust intratumoral protein knockdown...

Bhatia and colleagues developed a tumor-penetrating nanocomplex (TPN) with siRNAs specific for the ovarian cancer oncogene inhibitor of DNA binding 4 (ID4)[52]. For tumor delivery, the nanoconjugate was co-functionalized with a tandem tumor-penetrating and membrane-translocating peptide, which enabled robust and pervasive delivery of siRNA to the tumor parenchyma. Subsequently, treatment of ovarian tumor-bearing mice with ID4-specific TPN suppressed growth of the established tumors and significantly improved survival. Similar to TPN-mediated ID4 knockdown, inhibition of the DNA repair enzyme poly(ADP-ribose) polymerase 1 (PARP1) with siRNA-based lipoids is an effective treatment for ovarian cancer. Intraperitoneal (i.p.) administration of siPARP1 lipoids promoted apoptosis, and increased animal subject survival in BRAC1-deficient, but not the wildtype allografts *in vivo*[53].

Using a genetically engineered breast cancer model, driven by SV40-large T antigen under the control of the C3(1) component of the rat prostate steroid binding protein (PSBP) to direct SV40 expression to the mammary gland, computational gene network modeling identified HoxA1 as a putative driver of early breast cancer progression. RNAi-mediated

suppression of HoxA1 in mammary tumor spheroids increased acinar lumen formation, reduced tumor cell proliferation, and restored normal epithelial polarization. *In vivo*, intraductal delivery of siRNA-based lipoid nanoconjugates targeted to HoxA1 into FVB C3(1)-SV40TAg mice triggered robust reduction of breast cancer progression associated with reduced cell proliferation rates, and sustained expression of estrogen and progesterone receptors[55].

Future Challenges and Directions

The confluence of progress in many different areas of cancer research, i.e., high-throughput oncogenomics, the development of physiologically relevant cell and animal models as testing platforms for gene function and gene-specific therapeutics, and the emergence of RNAi-based nanotechnological strategies, have positioned the field well to implement precision cancer nanomedicine into clinical practice. With currently 24 different RNAi-based therapeutics in 43 different clinical trials, critical questions and challenges for the next 5 to 10 years have become very apparent, i.e., to identify the most critical target genes that drive or contribute to cancer initiation, progression, metastasization and therapy refractoriness, as well as to further improve and comprehensively evaluate efficacy, specificity, and biocompatibility of RNAi nanotherapeutics in the most relevant cell and animal models. Specifically, several important areas for development include the following.

RNAi Nanoconjugates as Tools for Discovery Sciences

With the number of gene aberrations ranging from thousands to hundreds of thousands, the genomic and genetic landscape of cancer is complex. Only a subset of genes drive the initiation and maintenance of cancer. In addition, tumors show specific, spatially and temporally controlled genetic changes, which are influenced by cooperative oncogenic and tumor suppressive signatures, and further modulated by heterotypic tumor-stroma interactions, and patient-specific germline mutations. Genome-wide RNAi and cDNA complementation screens are constantly evolving to determine cancer gene function and their genetic context, and will continue to provide lists of candidate genes that require further in-depth testing in cell and animal models. For preclinical evaluation, established or patient-derived cancer cells, together with murine cancer cell lineages are engineered to over- or underexpress the gene of interest, and these cell systems are then channeled into a variety of functional assays determining the impact of gene dosage on cellular transformation, growth, apoptosis sensitivity and migration/invasion. By orthotopically injecting these cell systems into immunocompromised or syngeneic hosts, subsequent *in vivo* experiments then evaluate the impact of cancer gene overexpression and knockdown on tumor progression. Nano-RNAi should be developed as a tool for discovery science to

evaluate gene function and its impact on cancer progression in cells *in vitro* and in animal models *in vivo*. Instead of generating cell transfectants stably or transiently expressing small hairpin (sh) RNAs and siRNAs, or engineering cells with a gene-specific knockout harnessing the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 technology, RNAi-based nanoconjugates can be administered to cells, graft and genetically engineered cancer models, to determine cancer gene function *in vivo*.

Further Developing RNAi-Based Nanotherapeutics

While a plethora of RNAi-based nanoconjugates have emerged in the past 10 years as fundamentally novel classes of therapeutics that can robustly and safely delivery RNAi to tumor sites, structure-activity relationships that dictate nanomaterial activity (RNAi delivery to cells, target gene knockdown) are only beginning to emerge. This incomplete understanding is based in part on the difficulty in generating structurally defined materials, and in rapidly evaluating the cellular impact of these nanomaterials in a massively parallel fashion. Design rules have to be determined that optimize the development of RNAi nanoconjugates for therapeutic applications. Unlike small molecule-based therapeutics, where millions of compounds are surveyed in an initial high-throughput screen, and thousands are tested under optimized conditions in various cell culture models, nanomedicinal evaluations typically focus on a defined subset of candidates only. Furthermore, deep mechanistic and biological studies are required to fully understand some of the fundamental properties underlying gene knockdown (is gene knockdown truly mediated by an RNAi mechanism, or is it due to rather unspecific toxic effect of the conjugate?) cellular entry, endosomal escape, tissue dissemination, and low-level cellular and organismal impact. With more comprehensive screenings of cancer cell-specific surface markers, the modification of RNAi nanoconjugates with ligands or antibodies to facilitate tumor-specific uptake, beyond the EPR effect, has to be optimized to further increase conjugate efficacy while reducing the potential for adverse side effects associated with systemic administration. Due to the dependence of the cancer phenotype on multiple deregulated pathways, co-extinction strategies have to be developed that concomitantly silence multiple oncogenes and oncogenic pathways. In particular, the concept of therapeutic synergy between siRNAs and miRNAs has to be exploited further, as recent study in ovarian and lung cancer showed significant cooperativity in reducing tumor progression when compared with either monotherapy alone[50,56]. The design of such combination therapies, and the development of multimodal si/miRNA nanoconjugates have to be optimized, and evaluated *in vivo* for efficacy, pharmacokinetics, pharmacodynamics,

...nanomedicinal evaluations typically focus on a defined subset of candidates only.

and toxicology in the relevant grafts and GEMMs. Finally, we have to understand and harness synthetic lethal interaction of si/miRNAs with conventional chemotherapy (e.g., DNA-damage-inducing agents), targeted pharmaceuticals that inhibit critical driving oncogenes, such as (receptor) tyrosine kinases, and possibly immunotherapies. It will be critical to determine the molecular mechanisms that act as roadblocks preventing chemo- and RTK-targeted therapies from inducing tumor-specific apoptosis and regression, and enabling cancers to escape immune surveillance. We then can target these roadblocks using RNAi-based nanomaterials, and can envision using hybrid conjugates co-functionalized with chemotherapeutics, small molecules, biotherapeutic antibodies and si/miRNA sequences to concurrently target driving oncogenes and their downstream signaling.

Milestones to address these critical areas that researchers should be able to be achieve over the next 5-10 year time frame include many aspects. In the next 5 years, researchers will comprehensively determine structure-function relationships of RNAi nanoconjugates with high-throughput methods; determine the potential synthetic lethal interaction between cancer genes and extant chemo-/targeted therapies to identify those genes required for therapy resistance; develop and preclinically evaluate multimodal nanoconjugates for the concurrent delivery of small RNAs and chemo-/targeted therapies; preclinically develop combination regimens of immunotherapies and RNAi-based nanomaterials; and develop RNAi nano-conjugates as tools for discovery sciences to characterize oncogene function in cells and animal models. Looking further ahead over the next 10 years, researchers will perform clinical testing of multiple RNAi-based nanoconjugate combinations, in conjunction with established therapies; and potentially there should be FDA approval of several RNAi conjugates and RNAi-based combinatorial regimens.

X-ray Induced Photodynamic Therapy

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Introduction to X-PDT and its Importance to Oncology

Photodynamic therapy (PDT), as a relatively new cancer treatment methodology, has attracted wide attention. PDT uses a photosensitizing drug that is activated by exposure to light of a specific wavelength. While they display minimal toxicitiy in the dark, photosensitizers, upon light activation, produce cytotoxic reactive oxygen species such as singlet oxygen ($^1O_2$) and hydroxyl radicals, leading to cancer cell death. PDT is minimally invasive and highly selective. Unlike ionizing radiation, PDT can be applied repeatedly to the same diseased sites without causing incurred resistance. PDT can also be applied in conjugation with other treatment modalities to facilitate tumor management. For instance, PDT is being evaluated in the clinic to treat prostate cancer patients who have failed radiotherapy.

One major limitation to PDT, however, is the shallow penetration depth. Even with new generations of photosensitizers, it is challenging for PDT to treat tumors of large volumes (> $1cm^3$) or ones located deep under the skin. This restraint is a major cause behind the limited impact and current role of PDT in the clinic. To address the issue, there have been many efforts on developing two-photon PDT and upconversion nanoparticle-mediated PDT. However, because the excitation source is near-infrared light, their potential therapeutic outcomes are still heavily surface-weighted.

Very recently, our group and others have exploited the possibility of using X-ray as an energy source to activate PDT. We termed this methodology X-ray inducible PDT, or X-PDT. Unlike visible or near-infrared light, X-ray affords excellent tissue penetration ability and is widely used in clinical diagnosis and therapy. X-PDT can thus, to a large degree, transcend the depth limitation of conventional PDT (~ 1 cm), permitting deep-tissue therapy[57]. For X-PDT to work, there are several requirements. First, a scintillating transducer, which converts X-ray photons to visible photons. Second, a photosensitizer, whose excitation wavelength is well matched to the emission of the scintillator. Third, a carrier, which can co-deliver the scintillator and photosensitizer, and ensure that the two components are spatially close enough for efficient energy transfer. As simple as it sounds, it is difficult to meet all three requirements using conventional methods.

This puzzle is solved by advances in nanotechnology, which allow for preparation of nanoscale scintillators and carriers. **Figure 4** shows an example of such an integrated nanosystem, consisting of a nanoscintillator core made of $SrAl_2O_4$:Eu (SAO), a photosensitizer merocyanine 540 (MC540), and a silica capsule that encapsulates the two. Upon X-ray irradiation, the SAO core converts X-ray photons to visible photons via a physical phenomenon known as X-ray excited optical luminescence (XEOL). Due to excellent spectral overlap between the emission and the excitation of MC540, the photons emitted by SAO are absorbed by MC540 deposited in the silica matrix. This produces reactive oxygen species, including hydroxyl radicals and singlet oxygen ($^1O_2$), causing death of cancer cells.

Current State of the Art in X-ray Inducible PDT

The number of studies on X-PDT is relatively small but is increasing. In addition to this group's work, other groups have exploited different scintillator materials using similar or different designs. For instance, the Chen group has investigated X-PDT with Cu-cysteine[58], LaF_3:Ce[59], and ZnS:Cu,Co[60]. The Shi group reported that Ce(III)-doped $LiYF_4$@SiO_2@ZnO nanoparticles upon ionizing irradiation can generate hydroxyl radicals to kill cancer cells[61]. Recently, Kotagiri et al. observed that Cerenkov radiation from radionuclides can be harnessed to activate TiO_2 nanoparticles, an oxygen-independent nanophotosensitizer, to produce radicals and kill cancer cells[62].

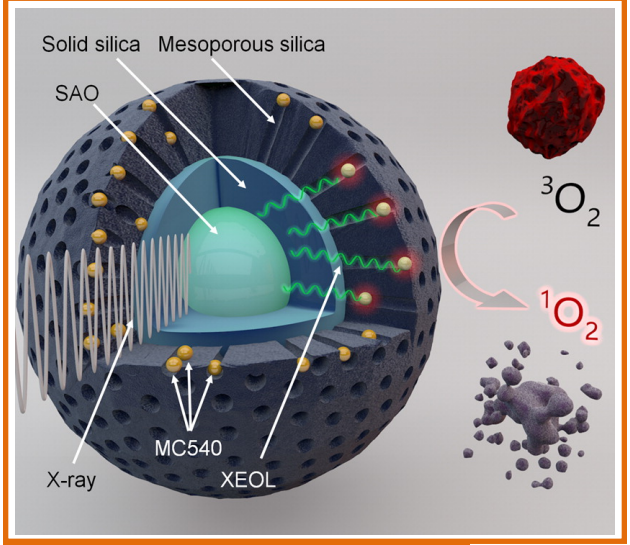

Figure 4. X-PDT, mediated by MC540 loaded and silica coated SAO nanoparticles (or M-SAO@SiO2 nanoparticles). Upon X-ray irradiation, SAO works as a transducer, relaying energy in the form of X-ray excited optical luminescence (XEOL) to MC540 to activate it and produce cytotoxic 1O2. M-SAO@SiO2 nanoparticles can be conjugated with a tumor targeting motif to further enhance the selectivity against cancer cells (*Reprinted with permission from Chen et al, 2015*).

X-PDT treated cells often display blebbing, swelling, and morphology changes, suggesting PDT-induced necrosis as the dominant cell killing mechanism. This is different from ionizing irradiation, in which cell death is often caused by apoptosis. However, it does not mean that there is no contribution of ionizing irradiation in X-PDT. While $^1O_2$ is produced in nanoparticle-rich compartments such as the cell membrane and endosomes/lysosomes, other organelles are under the impact of ionizing irradiation. Hence, X-PDT is essentially a combination therapy of PDT and ionizing irradiation. Previously, several groups

have studied PDT and radiation combination therapy and observed a synergistic effect between the two[63–66]. This is because the two modalities act on different targets: PDT often damages cell membranes whereas ionizing irradiation targets DNA. Due to distinctive cell killing routes, each modality suppresses the cell repair mechanism of the other, leading to enhanced treatment outcomes. The same synergy is believed to play a role in X-PDT.

From this perspective, X-PDT is not only a PDT derivative, but also a type of radiation therapy derivative. It however, affords several benefits over conventional ionizing irradiation. First, X-PDT can kill cells that are resistant to radiotherapy (e.g., glioma cells[57]). This is because the main cell killing mechanism of X-PDT is PDT-induced cell damage rather than radiation caused DNA damage. Second, low irradiation doses. Like PDT, X-PDT achieves good tumor control within in a few or even single treatment sessions[57]. The total irradiation dose is often less than 10 Gy. The dose is much lower than traditional radiotherapy, in which case a total dose of 60-80 Gy is often needed[67,68]. Third, low irradiation dose rates. It is known that irradiation induced toxicities are positively correlated to dose rates[69]. In X-PDT, irradiation doses per fraction are often comparable to conventional radiotherapy (e.g., 2-5 Gy); however, the irradiation is given out over a span of 15-30 min (typical for PDT), as opposed to minutes or even less in radiotherapy. This leads to dramatically lowered dose rates and potentially reduced toxicities. Fourth, high selectivity. In X-PDT, the treatment is mediated by not only irradiation but also the respective nanotransducers. With proper surface coating and by conjugating with a tumor targeting ligand, nanotransducers may accumulate in tumors with high efficiency. This dual selectivity, in conjugation with low irradiation doses and dose rates, are expected to minimize normal tissue toxicities, a major concern in radiotherapy.

Future Scientific and Clinical Developments

While X-PDT has demonstrated good efficacy and benefits, there is a lot that we don't know about this new therapeutic modality. As discussed above, X-PDT is essentially a combination therapy of PDT and ionizing irradiation. However, exactly how the two modalities interplay and whether we can improve the synergy by tuning irradiation parameters and/or changing nanotransducer targets is largely unknown. These need be elucidated in future studies.

The nanoscintillator is the key to X-PDT. It will be important to exploit ways to improve their energy conversion and safety profiles. These include: (1) change scintillator materials to ones that have a larger X-ray absorption cross-section and higher X-ray-to-visible-photon conversion efficiency as well as optimized spatial positioning of the molecular entities involved; (2) reduce the overall size of the nanotransducers; this however, should be balanced against the loss in energy conversion efficiency. It is noted that many of the

One solution to the problem is to use coatings to coat hydrolytic scintillator cores so as to slow down, but not prohibit hydrolysis.

reported nanotransducers in X-PDT have a relatively large size, which is suboptimal to tumor targeting; and (3) strike a balance between short-term stability and fast biodegradation of nanoparticles. Many scintillator materials are hydrolytic, quickly reducing to constituent ions when exposed to water. Water resistant scintillators do exist, but then the issue becomes the too slow degradation *in vivo*. One solution to the problem is to use coatings to coat hydrolytic scintillator cores so as to slow down, but not prohibit hydrolysis. Taking $SrAl_2O_4$:Eu nanoparticles for instance, it was found that after silica coating, the particles can maintain stability in physiological environments for 3-7 days and are then gradually degraded. Other materials/coating strategies should be exploited to modulate the stability and degradation of scintillators *in vivo*.

So far, X-PDT has been demonstrated mostly *in vitro* or with subcutaneous models. In future studies, it is important to evaluate the methodology in more clinically relevant tumor models. X-PDT holds the potential of clinical translation as an alternative to irradiation therapy in the next 10-15 years. It is important to compare the two modalities in the clinic to assess benefits and drawbacks of X-PDT with regard to treatment efficacy and side effects. It is also interesting to evaluate the capacity of X-PDT to treat tumors refractory to or ones that have failed radiotherapy. In radiotherapy, pre-treatment functional imaging (e.g., PET) is often performed to stage tumors and guide irradiation planning. However, functional imaging is not permitted in an irradiation room, and a change in patient position from prescans may occur, leading to setup errors. Many scintillator materials contain high-Z-value elements, making them visible under on-board CT. It is thus possible to use these nanoscintillators to not only regulate PDT but also guide the irradiation so as to minimize normal tissue damage. These possibilities should also be investigated to facilitate clinical translation of X-PDT.

Targeting Undruggable Targets

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The Importance of Targeting Undruggable Targets to Cancer Research/Oncology

Over the last few decades, advances in surgery, chemotherapy, and targeted drugs have led to improvements in progression-free and overall survival increases for many cancer types[70]. However, cure rates have remained largely unchanged. To accelerate the gains in clinical outcomes, large-scale efforts such as the Cancer Genome Atlas (TCGA), Clinical Proteomic Tumor Analysis Consortium (CPTAC), Cancer Target Discovery & Development (CTD[2]), and others were launched. These efforts have produced very high quality data due to the stringent requirements for sample quality and have clearly increased the pace of discovery for novel targets. However, to date, most of the knowledge is correlational in nature and large-functional data are needed. Challenges to rapid translation include the need for rapid, reliable, and effective functional data. While genetically engineered mouse models (GEMMs) remain a key tool in our armamentarium to determine the effects of various molecular pathways on biological processes, such models can have limitations (e.g., lengthy time, expense) and do not always reflect the biology of advanced stage human tumors. Therefore, other approaches such as 3-D, patient-derived xenografts, and orthotopic model systems remain an important component of biological validation and drug development.

The growing knowledge from the large-scale "omics" efforts has produced highly complex maps of genetic dysregulation in cancers. Moreover, these functional and biological systems have produced a plethora of targets that appear attractive for therapeutic development. However, many of the targets are not druggable by conventional strategies. Many important targets are difficult to inhibit with small molecules and furthermore require lengthy development phases that often fail. In addition, many small molecule inhibitors lack specificity and can be associated with intolerable side effects. While monoclonal antibodies have shown substantial promise against specific targets (e.g., VEGF, EGFR), their use is limited to either ligands or surface receptors. Some oncogenic proteins (e.g., Ras) activate pathways leading to altered transcription while others (e.g., Myc) are themselves transcription factors that directly control the expression of genes essential for proliferation, survival, and metastasis. Attempts have been made to develop pharmaceutical inhibitors against some of these factors, but many are still widely considered "undruggable".

Collectively, these and other observations have led many investigators to consider alternative strategies, such as RNA interference (RNAi), for inhibiting these targets.

Current Status in the Targeting of Undruggable Targets

Since the first report of RNAi in the late 1990s, there has been a massive expansion in efforts to apply it for therapeutic applications. Among these, short interfering RNA (siRNA) allows for highly selective silencing of target(s) of interest. Non-coding RNAs such as microRNAs (miRNA) can be used to target a larger array of targets. Moreover, combinations of siRNA and miRNA offer opportunities for "co-extinction" to maximize therapeutic efficacy while avoiding activation of redundant/compensatory pathways. While the promise of RNAi-based therapeutics is enormous, challenges (e.g., potential off-target effects and toxicity, requirement for delivery, endosomal uptake, activation of adaptive pathways) also exist[71]. Among these, perhaps the biggest challenge is achieving efficient systemic delivery. Naked siRNA becomes degraded rapidly and cannot be delivered into the tumor efficiently. However, these are precisely the kinds of concerns that can be overcome with biocompatible nanotechnology platforms. Already, several such platforms have yielded promising results in both pre-clinical and clinical settings for oncological and other clinical needs. For example, Davis and colleagues demonstrated in a landmark paper the ability of a cyclodextrin-based nanoparticle (CALAA-01) to deliver RRM2-targeted siRNA in patients with melanoma[42]. Other studies with delivery of miR-122 for HCV infection[72] and lipid nanoparticles for delivery of siRNAs targeting VEGF and KSP in cancer patients have also demonstrated promising clinical results[73]. The DOPC nanoliposomal platform has already shown promise for delivery of Grb2-targeted anti-sense nucleotides[74] and has also been introduced into phase 1 testing for EphA2-targeted siRNA. Additional platforms are likely to build on these initial experiences and allow for robust delivery of RNAi-therapeutics.

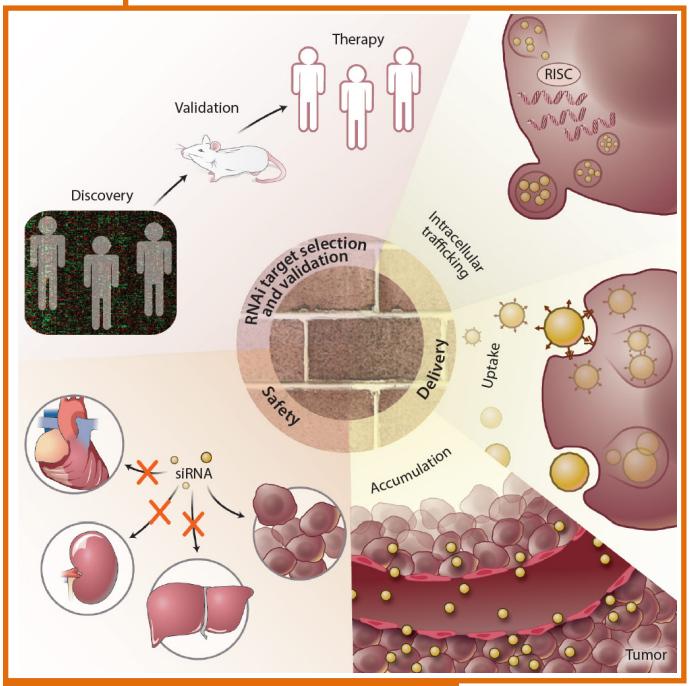

Figure 5. Strategies for targeting undruggable targets that rely on careful target discovery followed by developing nanoparticle systems that allow for highly efficient systemic delivery into the tumor microenvironment while sparing delivery into normal organs such liver, kidneys and heart (*Reprinted with permission from Wu et al., 2014*).

The success of RNAi-therapy depends, in part, on careful selection of targets for such approaches and delivery to the appropriate sites. Several key targets (e.g., KRAS, MYC) are already widely considered to be important. Additional efforts in the selection of targets, have incorporated systems biology approaches where genomic and proteomics screens can be merged with functional and clinical data to identify the highest priority targets[75,76]. In such an approach, following a systematic effort aimed at target selection, validation studies are carefully carried out (**Figure 5**). The biological validation studies are ideally carried out in a portfolio of model systems that can recapitulate human disease and hopefully inform success and potential for toxicity in subsequent clinical studies. The nanoparticle systems should be selected based on several criterial including biocompatibility, efficiency of delivery, safety profile and pharmaceutical feasibility (e.g., ability to scale-up, nucleotide incorporation and cost efficiency).

Future Scientific and Clinical Developments

We are clearly at a crossroads of a massive amount of information and a need to converge disciplines to understand the biological and clinical significance of such data. The ability to convert such data into personalized medicine regimes is still in its infancy. Success will require multi-disciplinary teams that include biomedical engineers, cancer biologists, pharmacologists, and translational as well as clinical scientists.

One major future opportunity will be in improving frequency of dosing and careful planning of clinical trials.

The achievements so far have demonstrated important proof-of-concept studies for RNAi-based therapeutics and have identified opportunities for future work. One major future opportunity will be in improving frequency of dosing and careful planning of clinical trials. Most of the current delivery platforms require frequent dosing to maintain sustained gene silencing. While such therapies are feasible to deliver in clinical trials, sustained delivery methods could ideally reduce the number of clinic visits required for treatment. Some of these delivery methods (e.g., multistage vectors, dual-assembly nanoparticles) have shown preclinical evidence of sustained delivery. But, additional work will be required to refine these approaches for clinical testing.

Given the genomic chaos and instability present in many solid tumors, it is not surprising that bypass or redundant molecular pathways are activated following many of the current therapeutics. Such adaptive mechanisms require an iterative process whereby careful preclinical testing and information-rich early-stage clinical trial designs utilize systems

biology approaches. Either Phase 0 or Phase 1 trials with pre- and post-treatment biopsies are an important avenue to learn about adaptive changes. Moreover, Phase 0 studies offer another unique opportunity for assessing the delivery of nanoparticles directly to the tumor site. Then, using sophisticated model systems, rational combinations could be rapidly developed. Adaptive trial designs can further help to limit the number of patients in the inactive-dose cohorts with the test article and allow faster transition to phase 2 clinical trials. Nanotechnology-enabled RNAi therapies are ideally suited for carrying out "co-extinction" of adaptive pathways. Questions related to packaging multiple RNAi molecules in same nanoparticles vs. loading them separately, but co-administering them is similarly worthy of additional future investigation.

It is unlikely that biologically-targeted drugs will replace the existing therapies such as chemotherapy and radiation. Opportunities exist, however, to identify and block targets that can amplify the anti-tumor response to these traditional therapies. These combinatorial approaches will likely offer new avenues for not only improving response rates, but perhaps even cure rates. Another opportunity resides in enhancing immune therapies. Check-point inhibitors (e.g., anti-CTLA-4, anti-PD-1) have resulted in remarkable efficacy in a fraction of patients with various tumor types, in particular melanoma[77]. There are many reasons why others do not respond to such therapies at present, but silencing "undruggable targets" among others related to immune-tolerance represents an opportunity for expanding the reach of immunotherapies.

Many of the existing delivery methods result in a fraction of the payload being deposited into the tumor with a large fraction going to other organs, especially liver. Understanding the physico-chemical properties that allow for enhanced delivery into the tumor represents an important area of investigation. Moreover, exploiting targeted delivery of nanoparticles decorated with peptides, aptamers or other approaches might enhance therapeutic ratios. Clinical regulatory pathways are needed to allow these targeted delivery methods to move into clinical testing.

Drug Reformulation

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Reformulation via Nanotechnology

Reformulation of legacy drugs offers an efficient pathway for commercialization of nanotechnology platforms. Nanotechnology-based medicine, as a relatively new area of science, does not have the well-defined regulatory path of traditional drugs. Since the development of a new chemical entity utilizing nanotechnology further compounds regulatory scrutiny, the reformulation of existing drugs represents a logical first step toward market. An alternate formulation of an existing drug that is no longer under patent can be developed under the FDA 505(b)(2) regulatory path that utilizes existing safety data, and has less associated development cost and time than that of a new chemical entity under the traditional 505(b)(1) application process. The 505(b)(2) regulatory path was codified in the "Drug Price Competition and Patent Term Restoration Act" (1984) statues with the specific goal of offering cheaper alternatives to the branded products, but has had the, perhaps, unintended consequence of expediting commercialization of new drug formulation technologies that offer therapeutic improvement of existing drugs.

Nanotechnology reformulation can overcome many of the liabilities of current oncology drugs, including insolubility, rapid metabolism, poor bioavailability and off target toxicity. The earliest successful commercialization of nanotechnology was encapsulation of doxorubicin in a nanoscale liposome, approved by the FDA in 1995 (**Figure 6**). Liposomal doxorubicin, Doxil® (Janssen Biotech, Inc.), decreases systemic free doxorubicin concentrations, reducing cardiac exposure and associated cardiotoxicity[78]. The success of this formulation is highlighted by the recent approval of the first Doxil generic, Lipodox® (Sun Pharmaceutical, FDA approval 2013). Liposome reformulation strategies are also being used to deliver synergistic combinations of oncology drugs, an example being Celator's combination cytarabine-duanorubicin liposome (CYT 351) that is currently in phase III clinical trials for treatment of acute myeloid leukemia.

Current Enabling Technologies

Liposomal doxorubicin commercialization was followed by cremophor-free formulations of the highly insoluble drug paclitaxel, initially as an albumin nanoparticle, Abraxane® (Abraxis BioScience), approved in the US 2005, and later a polymeric nanomicelle,

Genexol-PM® (Samyang Genex Company), approved in Korea 2007[79]. Abraxane is a 130 nm nanoparticle composed of human donor-derived albumin, while Genexol-PM is a 25 nm micellar particle composed of monomethoxy poly(ethylene glycol)-block-poly(D,L-lactide) (PEG-PDLLA) copolymer. By removing cremophor from the legacy paclitaxel formulation, Taxol® (Bristol-Myers Squibb), these nanotechnology reformulations demonstrated dramatic improvements in dose tolerability, as cremophor-dependent dose-limiting hypersensitivity reactions were no longer observed. This allows maximum tolerated doses of >300 and 260 mg/m^2 for Cynviloq and Abraxane, respectively, in comparison to 175 mg/m^2 for the legacy Taxol formulation. In addition to eliminating unwanted hypersensitivity side effects, these new cremophor-free formulations are effective against malignancies that the legacy Taxol formulation was not. Abraxane received orphan drug status for treatment of late-stage pancreatic cancer in the US in 2013 and has projected sales of $1.5-2 billion (Celgene Presentation at UBS Global Healthcare Conference, May 19, 2014 pp.9)[80]. Genexol-PM is currently in development in the US under the brand name of Cynviloq™ (Sorrento Therapeutics, Inc.) as an alternate formulation of Abraxane under the 505(b)(2) regulatory pathway for the treatment of advanced pancreatic cancer[81]. This use of the 505(b)(2) pathway for development of an alternate formulation of a marketed nanotechnology formulation is an example of how approval of nanotechnology formulations can further expedite approval of other nanotechnology formulations.

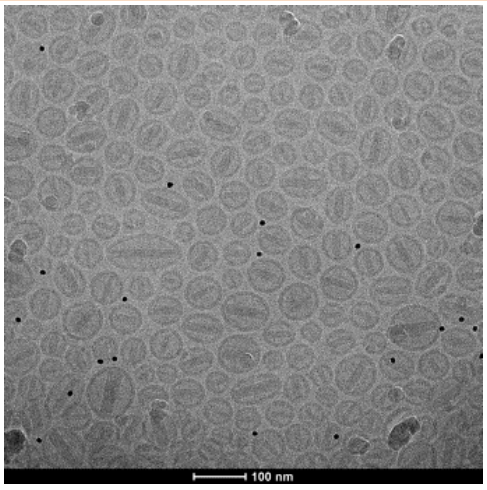

Figure 6. Cryo-transmission electron microscopy image of Doxil liposomal doxorubicin (*courtesy of Dr. Ulrich Baxa, Electron Microscopy Laboratory, Frederick National Laboratory for Cancer Research, 2015*).

The success of these reformulation efforts have solidified the advantages that nanotechnology offers the pharmaceutical industry, driving the implementation of nanotechnology earlier in the discovery phase of drug development. Many pharmaceutical companies now have in house nanotechnology formulation efforts underway, or are partnering with nanotechnology companies to optimize leads and even resurrect failed molecules. For example, a nanotechnology reformulation technique that has become so commercially acceptable that it is now used routinely in development of oral drugs is the Nanocrystal™ technology first developed by the Elan Corporation. The first commercial nanocrystal formulation was a reformulation of sirolimus, Rapamune® (Wyeth Pharmaceuticals, Madison, NJ), approved in 2000[82]. Nanocrystal formulation can increase bioavailability of oral formulations by reducing drug particle size, resulting in a dramatic increase in

surface area, and therefore drug dissolution rate (**Figure 7**)[83]. Other advantages can include enhanced dose linearity and consistency. The Elan nanocrystal technology is also being used for parenteral drug delivery, and an intramuscular nanocrystal reformulation of the schizophrenia drug paliperidone palmitate was approved in 2009.

Future Developments

As described above, the earliest use of nanotechnology to improve oral bioavailability was for incremental increases in the bioavailability of drugs already approved for oral administration through the use of nanocrystal technology. Recent formulation efforts are now focusing on the more difficult challenge of overcoming biological barriers, formulating molecules with little or no inherent bioavailability, such as protein therapeutics. One such example is the work of Robert Langer's lab on oral insulin, utilizing receptor mediated transport to overcome the gastrointestinal mucosal barrier[84]. These researchers utilized a polymeric nanoparticle construct targeting gastrointestinal FcRN receptors to stabilize and deliver insulin to the systemic circulation (**Figure 8**). Optimization of this uptake pathway could revolutionize both protein and small molecule therapeutics, no longer requiring costly and invasive intravenous administrations. Another example of utilization of receptor-mediated transport to cross biological barriers is glutathione-targeted doxorubicin liposome designed to increase uptake across the blood-brain barrier. These glutathione-targeted doxorubicin liposomes developed by BBB Therapeutics are currently in phase II clinical trials for treatment of brain metastasis and glioma[85].

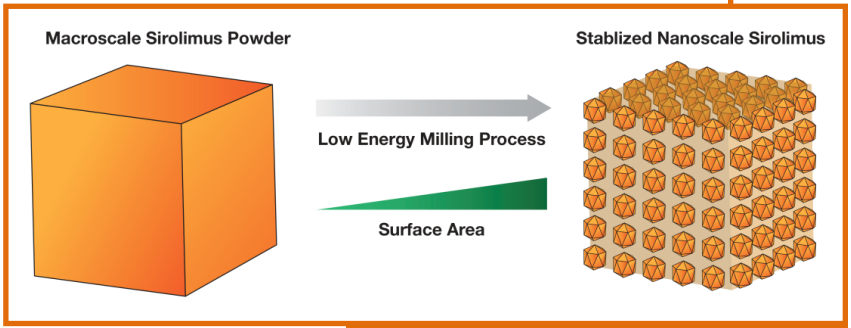

Figure 7. The Elan Nanocrystal™ technology.

Clearly, the future of nanomedicine resides in targeted therapies that allow for exquisite selection of diseased over healthy tissues. This was and continues to be the unrealized potential of this technology. The most notable advance in this area has come from Bind Therapeutics' progression of PMSA-targeted polymeric nanoparticles containing paclitaxel, Bind-014, to the clinic[16]. Bind's Accurin™ platform consists of a PMSA targeting *S,S*-2-[3-[5-amino-1-carboxypentyl]-ureido]-pentanedioic acid small molecule, attached to a mixed pegylated poly(d,l-lactide) (PLA) and poly(d,l-lactide-*co*-glycolide) (PLGA) nanoparticle. In addition to paclitaxel, Bind also has a vincristine formulation under late stage development,

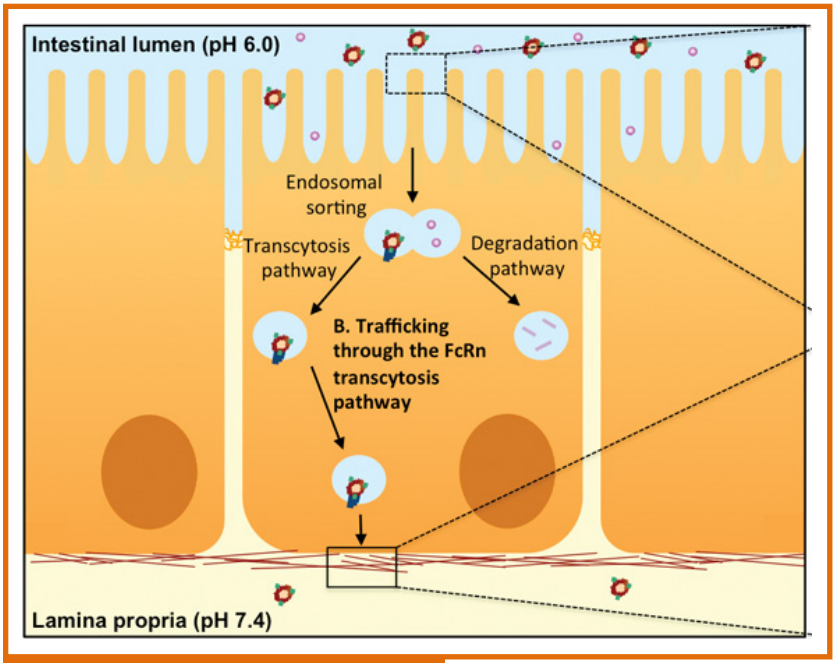

Figure 8. FcRN receptor-mediated nanoparticle uptake. (*Reprinted with permission from Pridgen et al., 2013*).

and is partnering with several pharmaceutical companies, including Pfizer, AstraZeneca, Roche, Merck, and Amgen, for development of their proprietary small molecules. Success of the Accurin platform will undoubtedly lead to further development of targeted therapies and new avenues for targeted reformulation. As has been the case in the past, reformulation will continue to lead commercialization of novel nanotechnology platforms.

With the joint efforts of investigators at academic institutes and within industry, several advances should come to fruition over the upcoming 5-10 year time frame. In the next 5 years, researchers will have begun streamlining of drug reformulation by identification of optimal drug physicochemical properties that result in successful reformulation for each nanomedicine class; and begin commercialization of actively targeted-nanoparticle reformulations. Looking further ahead over the next 10 years, researchers will generate reformulation of intravenously administered small molecule and protein-based therapies for oral and inhalation administration.

Nanotherapeutic Solutions for Metastatic and Disseminated Cancers

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Metastasis Remains the Bane of Successful Cancer Therapy

Cancer metastasis accounts for over 90% of all cancer associated death and suffering, representing the single biggest challenge to the management of cancer[86]. Although the advent of novel therapies and effective combination regimens has increased overall patient survival, many of these interventions are only palliative and an overwhelming number of cancer patients succumb to the disease[87]. Several factors can be attributed to this undesirable outcome, including the inefficiency of using conventional chemotherapeutics to treat small clusters of disseminated malignant cells or therapy-resistant metastases[88]. The three major sites of most cancer metastasis are the lungs, liver, and bone marrow (**Figure 9**).

Although small drugs and nanotherapeutics are readily delivered to the liver and lungs, the protective bone marrow niche provides a conducive environment for metastatic cells to undergo intrinsic genetic and epigenetic cellular changes that eventually lead to drug resistance[88]. When present in small clusters, the small tumor surface area relative to surrounding uninvolved tissue reduces the efficacy of treatment at the typically low concentrations of drugs that reach the metastatic tumor cells. Further complicating the treatment response is the high expression of cell membrane-based efflux transporters, such as P-glycoprotein 1 and multidrug resistance-associated protein 1, which effectively expel the drugs before they can exert therapeutic effects on the cellular machinery[89]. Moreover, the serious side effects caused by conventional chemotherapeutics, particularly to the bone marrow stem cells, are limiting factors. As efforts to uncover the biological mechanisms of cancer metastasis and resistance to therapies continue to provide new insight into the metastatic niche, it is obvious that new therapeutic approaches are needed to increase treatment efficacy, prevent relapse, and provide a cure with minimal off-target toxicity. These goals can be accomplished by harnessing the multivalent and multifunctional attributes of nanoparticles to design novel nanotherapeutics with the capacity to irreversibly trigger cancer cell death.

Cancer Nanotherapeutic Strategies for Metastatic and Disseminated Tumors

Nanotherapeutics have considerable advantages over conventional chemotherapeutics, including the ease of controlling their circulation times in blood, as well as their *in vivo* stability, bioavailability, and bioactivity. These properties can be employed to address some fundamental limitations of small molecule chemotherapeutics in treating metastatic tumors. For example, nanotherapeutics are frequently used to improve the bioavailability and local concentration of existing drugs that are highly effective against metastatic cancer cells via passive targeting. This approach is most effective in large metastases of the liver and lungs, where an enhanced permeability and retention (EPR) effect is achievable. However, EPR uptake is ineffective for small and poorly vascularized micrometastases (tumors <2 mm in size), which are frequently found in the bone marrow and at early stages of metastasis elsewhere. Efforts to address this challenge have focused on nanoparticle formulations designed to target cancer biomarkers selectively. Although the mechanism of tumor uptake is not fully understood at this point, albumin-bound paclitaxel (Abraxane), represents an interesting coupling of EPR and cancer-targeted approaches to deliver drugs to tumor cells. Clinical studies demonstrate that this nanoparticle-bound drug exhibited a blood circulation half-life more than 100 times longer than that of the small molecule paclitaxel alone. Response rate (74% vs 39%) and progression-free survival (14.6 vs 7.8 months) using the nanotherapeutics were higher than for the unbound drug in patients with metastatic breast cancer[90].

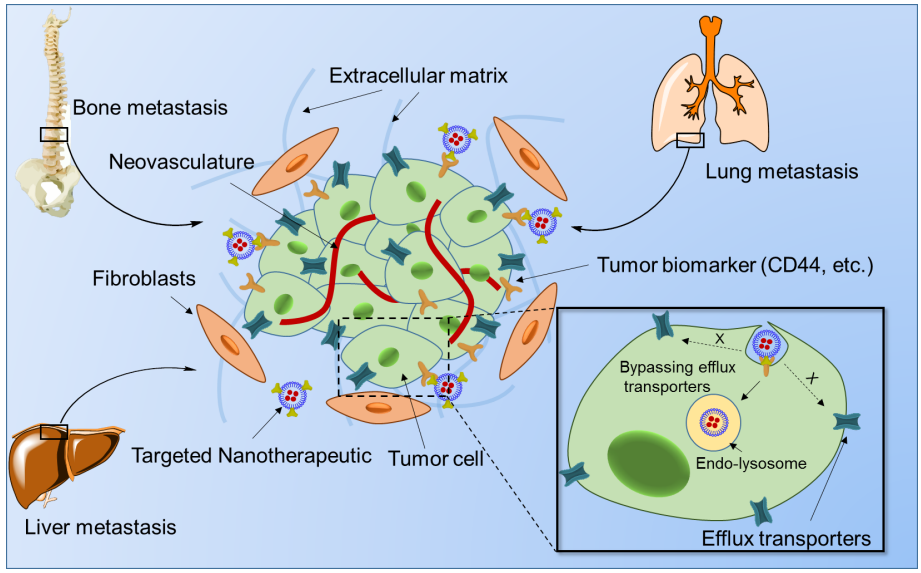

Figure 9. Major sites of cancer metastasis and the respective nanotherapeutic targeting strategies.

Some disseminated tumors, such as multiple myeloma, which can serve as a model of bone marrow metastasis, and particularly drug resistant phenotypes, commonly found in niches such as the bone marrow microenvironment, are not responsive to Abraxane nanotherapy. For example, adhesion of multiple myeloma

cells to the bone marrow stroma results in cell-adhesion-mediated drug resistance (CAM-DR). Thus, a dual-function ligand that simultaneously targets the tumor cells and inhibits adhesion to surrounding stroma would improve treatment outcome. This goal was achieved in a recent study by loading self-assembling micellar nanoparticles with doxorubicin and functionalizing the micelle surface with very late antigen-4 (VLA-4) peptide, which served as an anti-adhesion molecule. This formulation not only selectively delivered doxorubicin to the tumor cells, but also overcame CAM-DR. The micellar nanoparticles preferentially homed to tumors in the bone marrow with ~10-fold higher drug accumulation and tumor growth inhibition with a reduced overall systemic toxicity compared to the small molecule drug alone[91]. An alternative approach incorporates antisense drugs into polymeric nanoparticles for targeting the genes of osteopontin and bone sialoprotein, which are overexpressed in bone metastases of mammary carcinomas. These nanoparticles protect the drugs against nuclease degradation, thereby enabling sustained release of antisense therapeutics and a significant decrease in the incidence of bone metastasis[92].

The effectiveness of some drugs is hampered by the high efflux rate in drug resistant phenotypes of metastatic cells expressing P-glycoprotein 1 and multidrug resistant transporters. Despite several studies demonstrating the efficacy of Vincristine sulfate (VS) in cancer therapy, the high efflux rate by these transporters decreases the intracellular resident time for effective therapy. To overcome this impediment, VS was encapsulated in polymeric nanoparticles, causing it to be taken up through clathrin and caveolae mediated endocytotic pathways and allowing it to bypass the efflux transporters. The ensuing accumulation and retention of VS nanotherapeutics in metastatic cancer cells resulted in a ~21-fold increase in cytotoxicity compared to VS alone[93].

Cancer is a highly heterogeneous disease with distinct cell subpopulations that are phenotypically and biochemically diverse.

Future Challenges

Cancer is a highly heterogeneous disease with distinct cell subpopulations that are phenotypically and biochemically diverse. Given their different capacities to grow, differentiate, develop drug resistance, and form metastases, understanding tumor biology is critical for the development of successful therapies. Biomarker discovery and identification is an important aspect of this progress and an indispensable step in the development of targeted nanotherapeutics. However, significant variations between primary and metastatic cancer from the same patient further complicate the development of a consensus strategy to

treat the disease. The ability to target multiple cancer biomarkers and deliver combinatorial therapy favors the use of nanotherapeutics to maximize treatment outcome. An emerging frontier in cancer therapy is in understanding the contribution of tumor environment to its survival and metastasis. Some studies suggest that several factors alter a secondary site before the homing of migrating tumor cells. Sometimes the metastatic tumor cells remain dormant and undetectable after the primary cancer is removed, leading to relapse. With current knowledge of cancer-type specific metastatic patterns, it will be possible to develop nanotherapeutics that can reside in the secondary tissue for prolonged periods to achieve preventive or augmented nanotherapy. In addition, this treatment paradigm could be enhanced by other forms of therapy, such as gene silencing and immunomodulatory techniques to provide a multipronged strategy to combat cancer, with minimal morbidity effects to the patient. Phototherapy appears to be effective in treating metastasis, but the limited penetration of light has hampered the use of this technique in clinics. A recent study postulates that Cerenkov radiation from radionuclides used in positron emission tomography could serve as a depth-independent light source for cancer therapy in the presence of photo-sensitive nanomaterials that generate cytotoxic radicals upon exposure to light[62]. Application of this concept to the treatment of circulating tumor cells and metastases could improve treatment outcome, especially for chemotherapy resistant metastasis.

Nanotechnology Solutions to Overcome Plasticity and Resistance Using Epigenetic and MicroRNA-Based Reprogramming

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Tumor Plasticity and Therapeutic Resistance

Plasticity is an inherent characteristic of cancer and a plays a vital role in cancer initiation and sustenance. The cellular changes that transition a normal cell into a cancer cell can be defined as cellular plasticity; likewise the perpetual adaptions that cancer cells undergo to survive can be classified as cellular plasticity. In this sense, tumor plasticity enables therapeutic resistance and could be considered a survival response. As cells that continually transform to maintain their immortalization, cancer cells are the ultimate biological representation of "survival of the fittest," through their inherent plasticity they are able to adapt and survive in inhospitable conditions (low oxygen, nutrient deprived) and even evade the effects of cytotoxic drugs and biologics. In 2000 and in a 2011 follow-up review, Hanahan and Weinberg took a comprehensive approach to characterizing cancer and defined the six hallmarks of cancer as; the ability to sustain proliferative signaling, the ability to evade growth suppressors, activation of invasion and metastasis, replicative immortality, induction of angiogenesis, and resistance to cell death[94]. An important feature of solid tumor masses is their cellular heterogeneity, this is caused by survival adaptations of cells (plasticity) and the inherent genome and proteome dysregulation characteristic of cancer cells; tumor heterogeneity undoubtedly contributes to drug resistance. Multi-drug resistance (MDR) can be innate (biologically inherent to the cancer cell) or acquired (after drug exposure); as discussed below, epigenetic factors and microRNA contribute to both innate and acquired MDR as well as to tumor plasticity. Cancer cells employ a variety of mechanisms of MDR including decreasing drug influx into the cell, increasing drug efflux, increasing DNA repair, increasing drug metabolism, and decreasing apoptosis[95]. Tumor heterogeneity is a challenge to the clinical treatment of solid tumors as tumor sub-populations of cells respond differently to treatment, which can increase the development of acquired MDR and metastasis. Tumor plasticity enables drug resistance and cell survival despite aggressive therapeutic treatment.

Epigenetic and Phenotypic Reprogramming

In recent years, the role of epigenetics in genotype expression has been elucidated and we are beginning to understand the significance of epigenetics in cancer development and regulation. Epigenetics refers to a heritable (mitotic and meiotic), stable change in gene expression without a modification of the DNA sequence[96]. The most common epigenetic changes include direct chemical modifications of DNA (methylation), histone modifications, and chromatin remodeling. Epigenetic modifications regulate cell differentiation, maternal and paternal inheritance patterns, gene expression responses to environmental factors and stress, seasonal gene expression, and cancer development[97]. When the human genome project completed in 2003, there were still many questions that the vast "decoding" could not seem to answer; how do our experiences, the food we eat, the environment we are exposed to, and daily stress exert a genetic effect? How can these variables lead to cancer? How does parental imprinting occur? The epigenome has evolved as an answer to these questions. If DNA is thought of as the same set of ingredients that every cell has, the epigenome can be thought of as the recipe – what each cell makes with those ingredients; an old, memorized family recipe that is passed down from generation to generation. Given the governing role of the epigenome in gene expression, the contribution of epigenetic changes to cancer initiation, progression, plasticity, and resistance is not surprising[97]. Although tissue-specific and patient specific epigenetic variations have been noted in tumors, in general, the cancer epigenome displays hypomethylation and hypermethylation at site-specific CpG islands (cytosine clusters) within gene promoters[97].

...the role of epigenetics in genotype expression has been elucidated and we are beginning to understand the significance of epigenetics in cancer development and regulation.

Also in recent years, the powerful contribution of microRNAs (miRNAs) to cancer has been discovered. MicroRNAs are 18-25 nucleotide, noncoding RNAs that negatively regulate gene expression at the post-transcriptional level. RNA polymerase II or III transcribes a primary microRNA (pri-miRNA) in the nucleus, the pri-miRNA is cleaved by a Drosha/DGCR8 complex to form precursor miRNA (pre-miRNA) which is transported into the cytoplasm, then Dicer processes the pre-miRNA into mature miRNA for incorporation with RISC (the Argonaute containing RNA-induced silencing complex)[98]. It is this miRNA-RISC complex that blocks gene expression by either degrading target mRNA or by hybridization to the 3' untranslated region of the target mRNA[98]. Over 2,500 miRNAs have been identified and many have multiple targets; although

many miRNAs are down regulated in different cancers (such as the miR-34 family), miRNAs that are overexpressed in many cancers have been coined "onco-miR's;" these oncogenic microRNAs include miR-155 and miR-21[99]. Validated oncogenic miRNAs such as miR-21 have been demonstrated to contribute to drug resistance, as has miR-19 and the miR-221/222 family[100].

There is a dynamic feedback circuit between epigenetics and miRNAs where the epigenome regulates the expression of miRNAs and certain miRNA's control mediators of the epigenome such as histone deacetylases, DNA methyltransferases, and polycomb group proteins (regulate linage delineation)[101].

Nanotechnology-Based Delivery Strategies for Reprogramming

A recent study validated epigenetic targeting with nanoparticle based therapies as an approach to reverse MDR. The study combined decitabine (a DNA hypermethylation inhibitor) loaded nanoparticles with doxorubicin loaded nanoparticles and demonstrated that combination therapy improved the efficacy of treatment and decreased the expression of DNA methyltransferase isoforms in the tumor bulk and in cancer stem cell populations in an MB-MDA-231 xenograft model in mice[102]. Using nano-based delivery systems to co-administer epigenome modifiers with standard chemotherapeutics has clinical potential as a strategy for reducing tumor plasticity and stem-like properties while reversing drug resistance. Likewise, combination therapy with chemotherapeutics and microRNA mimetics delivered in nanoparticle based formulations have demonstrated reversal of MDR through down regulation of ABC transporters (drug efflux pumps)[103]. MicroRNAs demonstrated to down regulate ABC transporters include miR-451, miR-27a, miR-223, miR-331, miR-326, miR-297, miR-487a, and miR-181a[103]. A variety of nanoparticle platforms have been explored for miRNA mimetic delivery, nanoparticles are ideal for nucleic acid delivery as they offer levels of protection as well as the ability to surface functionalize the vector for active targeting to tumor tissue. In April of 2013, the first clinical trial (phase 1) of a microRNA mimetic began in patients with liver cancer and hematological malignancies[104]. MRX34 consists of a miR-34 mimetic administered in "Smarticles"; pH responsive liposomes that exploit the lower pH of tumors to facilitate uptake[104]. As endogenous miR-34 regulates over 20 oncogenes, pre-clinical studies have demonstrated MRX34's ability to restore tumor suppression[104]. Cationic liposomes have been used to deliver miR-29b in pre-clinical lung cancer models, as miR-29b targets the cyclin dependent protein kinase 6 oncogene in lung cancer, treatment with the liposomes resulted in sixty percent tumor growth inhibition in a mouse model[105]. A variety of lipid and cationic polymer based nanoparticle systems have been developed for miRNA delivery in pre-clinical pancreatic cancer models[106]. More elaborate systems such as a liposome-polycation-hyaluronic acid nanoparticle system surface modified with

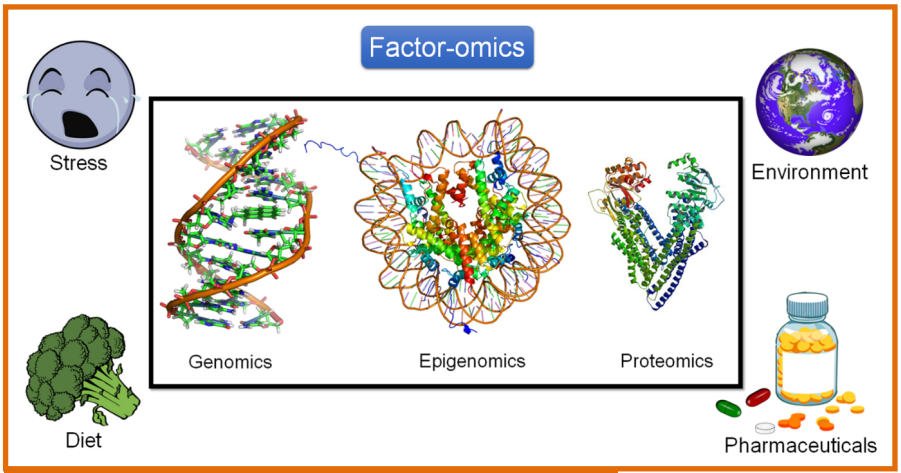

Figure 10. Emergence of "factor-omics" as a field, classifying and studying the environmental, dietary, physiological, and pharmacological factors that influence the epigenome, post-transcriptional gene expression, and the proteome. Genomics is the foundational field, proteomics is the translational product of the genome, the epigenome regulates gene expression (and hence, proteomics), and factor-omics will detail the environmental, nutritional, physiological (such as stress), and pharmacological factors that influence the genome, epigenome, and proteome.

a single chain antibody fragment to actively target GC4 (a metastatic melanoma epitope) for combination delivery of siRNA and miRNA have been developed and have demonstrated efficacy in reducing tumor growth and inhibiting metastasis[107]. Nucleic acids require delivery vectors such as nanoparticles to avoid immune system clearance and degradation and achieve therapeutic concentrations at the target site; the clinical application of microRNA relies on nanotechnology to enable therapeutic delivery. In addition to therapeutic applications, nano-based sensors are also being explored for cancer biomarker detection of circulating microRNAs and circulating tumor DNA[108,109]. In a 2011 article in *Nature Nanotechnology*, Li-Qun Gu and fellow researchers reported the development of a nanopore sensor capable of sub-picomolar detection of target microRNA in the plasma of lung cancer patients[109]. The nanopore used in this study was the α-haemolysin protein pore; synthetic nanoprobes are sure to follow in coming years[109]. More recently, researchers have developed a gold nanoparticle based sensor with peptide nucleic acid probes that exploit localized surface plasmon resonance to detect tumor-specific epigenetic variations in human serum samples[108]. Profiling a patient's disease from their plasma sample is a remarkable advancement in clinical oncology and could provide a powerful means of assessing and tailoring treatment.

Future of the Field

In this era of "omics" we anticipate the development of the next "omics" field; a field we will dub "*factor-omics*" for now (**Figure 10**), a field studying and classifying the factors that affect the epigenome, post-transcriptional gene expression, and the proteome. This field has already begun although has yet to be unified in a cohesive way, as with genomics, proteomics and epigenetics, this will occur naturally as the science progresses. Studies detailing the genetic, epigenetic, and post-translational effects of environmental, nutritional, physiological, and pharmacological factors have been well under way for some time, yet the key to evolving this field will be reviewing the results of the studies and making collective observations that can form the foundational science of the field. A second significant anticipated advancement in this arena will be the clinical application of nanotechnology-based sensors for microRNA and epigenetic cancer biomarkers.

A second significant anticipated advancement in this arena will be the clinical application of nanotechnology-based sensors for microRNA and epigenetic cancer biomarkers.

With the joint efforts of investigators across the spectrum, several advances should come to fruition over the upcoming 5-10 year time frame. In the next 5 years, researchers will have performed scientific studies/reviews to classify and interpret the environmental, physiological, and pharmacological factors that influence the epigenome and proteome; perform clinical evaluations of microRNA nano-sensors for cancer biomarker screening; and research investigational nano-therapeutics that reverse MDR using microRNA and epigenetic approaches. Looking further ahead over the next 10 years, the establishment of "factor-omics"; a field classifying and studying the environmental, physiological, and pharmacological factors that influence the epigenome, post-transcriptional gene expression, and the proteome will occurred. As genomics is the foundational field, proteomics is the translational product of the genome, and the epigenome regulates gene expression (and hence, proteomics), factor-omics will detail the environmental, physiological, and pharmacological factors that influence the epigenome and proteome; clinical application of microRNA nano-sensors for cancer biomarker screening; and clinical testing of nano-therapeutics that reverse MDR using microRNA and epigenetic approaches.

Exosome-Mediated Communication in the Tumor Microenvironment and Metastasis

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Tumor Exosomes and Content

Although exosomes were first discovered in 1987[110], it wasn't until recent years that the importance of exosomes in cellular communication has been elucidated. Exosomes are 30-100 nm vesicles shed by cells as a process of cell signaling and communication. In recent years it has been discovered that cancer cells produce and shed more exosomes than normal cells[111]. Exosomal release is one of three possible fates for multivesicular bodies (MVB). Multivesicular bodies are formed when plasma membrane receptors are marked for recycling or degradation through ubiquitination; early endosomes are formed through plasma membrane internalization and as internal vesicles form within the endosome, the endosome transitions to multivesicular bodies[111]. The three fates for multivesicular bodies are; recycling through the trans-Golgi network, lysosomal degradation, or secreted through exocytosis or through fusion with the plasma membrane (exosome release). Exosome secretion through exocytosis is mediated through intracellular Ca^{2+} levels while factors such as extracellular/intracellular pH gradients can effect release and uptake[112,113]. Much investigation has focused on exosome content and determining if exosome content is a deliberate process in cell signaling; exosome content is rich in enzymes, microRNA, transcription factors, heat shock proteins, MHCs, cytoskeleton components, signal transducers, and tetraspanins (transmembrane proteins). It is most commonly accepted that exosome content is determined non-specifically under multivesicular formation and not through a deliberate sorting and packaging process[111]. But is this really the case? Are most biological processes not deliberate? From a metabolic perspective, it would be a vast waste of cellular energy for exosome content *NOT* to be deliberate. Perhaps there is a missing piece we have not had insight to yet, indeed, the function of the endosomal sorting complex required for transport (ESCRT) in sorting ubiquitinated proteins provides insight to a possible sorting process[114]. Perhaps in healthy cells exosome release is one of three cellular fates for MVB, but in cancer cells, exosome release is exploited as a deliberate means of cell communication and to specifically achieve metastasis. The existence of this missing piece – the confirmation that cancer cells use exosomes as a deliberate mechanism of communication is likely to be proved or disproved within the next five years.

Exosome-Mediated Cell-Cell Communication

Exosomes are taken up by recipient cells through receptor-mediated endocytosis, pinocytosis, phagocytosis, or through fusion with the cell membrane resulting in direct release of contents into the cytoplasm. If cancer cell exosomal content is not selected randomly, but is a deliberate process, then exosomes can be thought of as the cancer cells elevator pitch to the outside world – *this is what I want you to know and why*. On the other hand, if the current paradigm is correct where exosomal content is not selective, and is just a random sample of the cellular content then exosomes can be thought of as an informational press release to the public – *this is the news, this is what I am doing right now*. Either way, it is a powerful means of communication that is utilized by cancer cells more than normal cells. Despite the intent of the message, what is the result of these messages?

Among other effects, such as transferring drug resistance, a demonstrated result of exosomal communication is metastasis. The metastatic process consists of a series of events that include the epithethial-mesenchymal transition (EMT; mobilizing cells) and the mesenchymal-to-epithelial transition (MET; establishing a secondary tumor site). Cancer exosomes have been demonstrated to deliver functional proteins, complexes, and RNA that promote both EMT (such as HIF-1α) and MET (such as miR-200).

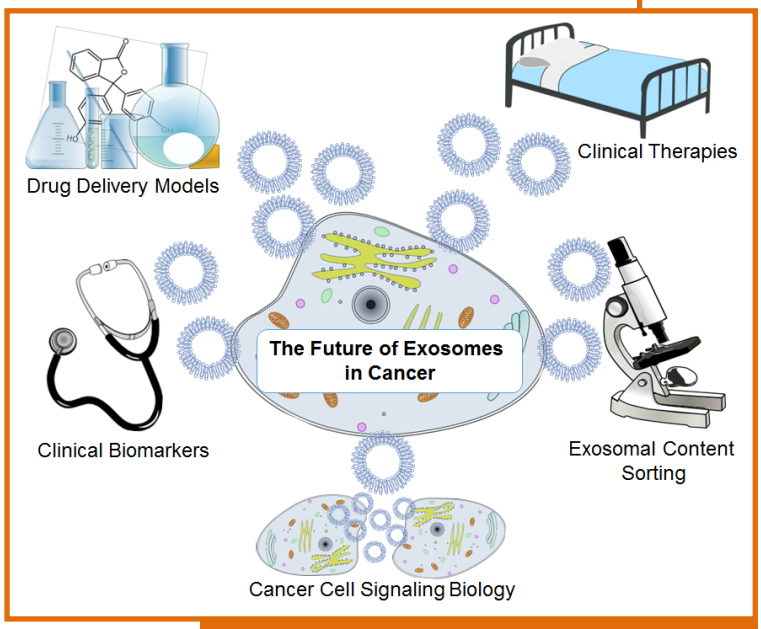

Figure 11. The future of exosomal research in cancer will entail fast-tracked clinical therapies and diagnostics for clinical biomarkers, deeper insight into cancer cell signaling particularly from highly heterogeneous tumors, studying exosomes as a model for drug delivery, and answering the highly debated question of exosomal content sorting and selection as a deliberate or non-selective process.

Metastasis: Epithethial-Mesenchymal Transition (EMT)

Hypoxia Inducible Factor-1α (HIF-1α) has gained attention over the past ten years as a powerful transcription factor contributing to oncogenic, aggressive, and drug resistant phenotypes in cancer. Under hypoxic conditions and under conditions of cell stress HIF-1α translocates from the cytoplasm to the nucleus where it forms an active transcription complex with HIF-1β binding to hypoxia responsive

elements on over fifty target genes including growth factors, drug efflux pumps, glucose transporters, cadherins, and factors that promote invasion and metastasis[115]. Our own studies have demonstrated a correlation between HIF-1α expression, multidrug resistance, and aggressive tumor phenotypes[115]. HIF-1α also contributes to epithethial-mesenchymal transition (EMT)[116]. A recent study by Pagano and Shackelford demonstrated that HIF-1α is excreted in a functional form from nasopharyngeal carcinoma cells infected with Epstein-Barr virus[116]. The study illustrated that transfection of nasopharyngeal carcinoma cells with latent membrane protein 1, the primary oncogene of Epstein-Barr virus, increased HIF-1α in secreted exosomes[116]. Using HA-tagged HIF-1α expression vectors in a series of *in vitro* studies the researchers demonstrated that exosomal HIF-1α was transcriptionally active in recipient cells. This, and similar studies, have demonstrated that exosome content can be altered through genetic and phenotypic modifications in the donor cell and these alterations can have profound effects on cell signaling through exosomal release and uptake.

Metastasis: Mesenchymal-to-Epithelial Transition (MET)

One of the most groundbreaking exosomal studies in recent years was the eloquent investigation conducted by Judy Lieberman at Boston Children's Hospital. Lieberman et al demonstrated that exosomes and ectosomes (larger vesicles formed by cell membrane budding) released from metastatic cancer cells can transfer metastatic capability to non-metastatic cells and this capability appears to be mediated through the microRNA-200 family, known regulators of mesenchymal-to-epithelial transition (MET)[117]. The study used extensive *in vitro* and *in vivo* techniques and through the meticulous selection of experimental conditions, resulted in a foundational exosomal and microRNA study. For example, the study selected cells with distinct metastatic capabilities (metastatic 4T1E mouse cells and metastatic human cells CA1a and BPLER cells and poorly metastatic 4T07 mouse cells and poorly metastatic human mesenchymal MB-231 cells) to study *in vivo* metastatic induction in mouse and human xenograft models. The study optimized the use of fluorescent cell labeling in many experiments; for example, to distinguish between metastatic lesions formed from circulating tail-vein injected cells from primary tumor cells, GFP-expressing primary orthotopic breast cancer tumors were developed in mice and firefly luciferase and mCherry expressing tumor cells were injected via tail-vein-injection[117]. Collectively, the *in vitro* and *in vivo* analysis demonstrated that exosomes and ectosomes from highly metastatic cells can increase the metastatic capabilities of local and distal poorly metastatic cells through the uptake of MET regulating miR-200[117].

Exosome Content Modulation and Application

An interesting phenomena that was noted in the Lieberman study was that micro-RNA's delivered in exosomes are sometimes associated with Ago2, indicating these miRNA's may be contained in RNA-induced silencing complexes (RISC) which results in their immediate activity in recipient cells[117]. In the Pagano and Shackelford's studies of HIF-1α exosomal delivery, HIF-1α was delivered both as an inactive (uncomplexed) and active (complexed) form[116]. Our current understanding of exosomal content is that it is non-specific and dependent on the cellular content. It may be, just as years ago introns were considered to be "junk DNA", that we just do not have a complete understanding of this process yet. It may be that as we learn more about exosome formation and communication that the process is revealed as a deliberate and selective mechanism of cellular communication.

From a drug delivery perspective, exosomes are nature's own nanoparticles delivering an array of functional proteins and nucleic acids. Exosomes are innate "stealth" carriers that can have profound effects on recipient cells. Exosomes can benefit the field of medicine and therapeutics in two ways; studying exosomes as a biological model for "drug" delivery and manipulating exosomes for therapeutic outcomes and as diagnostic tools (**Figure 11**).

...exosomes are nature's own nanoparticles delivering an array of functional proteins and nucleic acids.

The methods for altering exosome content are electroporation, direct chemical transfection of exosomes, transfection of exosome donor cells, activation of exosome donor cells, and direct incubation of exosomes with loading cargo[118]. Elaborate investigational studies, such as Lieberman's miR-200 exosomal study are being conducted, and this exosomal research has been so exciting and promising, exosomes seem to have fast-tracked their way into clinical trials. Several clinical trials have already completed globally to explore the medical promise of exosomes as cancer therapeutics. The most recently completed exosome clinical trial in the United States was a pilot study of an immunotherapy vaccine for malignant gliomas[119]. The Phase I trial was conducted by David Andrews at Jefferson University Hospital and consisted of extracting the patient's own tumor cells, treating them with an antisense oligodeoxynucleotide against insulin-like growth factor type 1 receptor (IGF-1R/AS-ODN), placing the treated cells in a biodiffusion chamber, implanting the device in patients abdomens and relying on exosomes released from the chamber to communicate and initiate an immune response (T-cell activation) against the tumor[119]. A second Phase 1 trial of this therapy is underway as the majority of patients (8/12) in the first trial elicited a positive clinical response[119]. Other clinical trials recruiting

patients in the US include a study investigating the use of plant derived exosomes to deliver curcumin to colon tumors and normal colon tissue and a study evaluating circulating exosomes as prognostic and predictive biomarkers for gastric cancer patients. Exosomes are indeed proving to be effective, innate, *cellular nanoparticles* that can be manipulated for therapeutic applications, used as cancer biomarkers, and studied as ideal models for drug delivery.

Several milestones should come to realization over the upcoming 3-10 year time frame. In the next 3-5 years, researchers will have standardized methods for isolation and study of Exosome communication in the immune/tumor interface, intra-tumoral communication, extracellular matrix composition, and metastasis; should have a definitive answer, is exosomal content deliberately selected in cancer cells as a mechanism of cell communication, invasion, and metastasis?; be studying exosomes as "native" nanoparticles as a model for drug delivery; and clinical trials for therapeutic and biomarker applications of exosomes. Looking further ahead over the next 10 years, the establishment of tools and methods for biomarker screening; began therapeutic intervention at the immune/tumor interface, intra-tumoral communication, extracellular matrix composition, and metastasis; studied exosome signaling from distinct cancer cell populations, MDR cells, cancer stem cells; and clinical approval and marketing of exosomal therapeutics and diagnostic tools.

Measuring Therapeutic Response to Cancer Immunotherapy via Nanotechnology

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Cancer Immunotherapy was the Science Breakthrough for the Year 2013[77], with tremendous promise and excitement surrounding two immunotherapy classes. Class 1 is comprised of immune checkpoint inhibitors[120,121], such as for the programmed death (PD)-1/L1 blockade, or anti-CTLA-4. These drugs can increase the susceptibility of cancer cells to immune system attack. Class 2 is adoptive cell transfer (ACT)[122,123], which seeks to strengthen the anti-tumor immune system function. ACT of chimeric-antigen-receptor (CAR) engineered T cells is now being pursued within a number of major pharmaceutical companies as an effective treatment for leukemias and lymphomas. The clinical testing of PD-1/L1 blockade has been carried out in multiple cancers, but has been led by work in melanoma[124], and has demonstrated a new era in cancer treatment[125,126]. It is fair to say that cancer immunotherapy has, in just the past two years, altered the conversation around cancer therapies from that of 'treatments' to that of 'cures.' However, it is still in its very early days yet, and immunotherapies have only been shown to provide powerful treatments for a subset of cancers, and even within those subsets, only for specific patient populations. Even for those patients who exhibit strong anti-tumor responses to immunotherapies, only a fraction (albeit a large one) exhibit durable responses. Thus, in order for the profound benefits of cancer immunotherapy to be extended to increasingly larger patient populations, there are a number of technological challenges to be addressed, and there are important roles for cancer nanotechnology to play. Here we outline two of many such challenges.

In Vivo Biomarkers

As with any therapy, it is challenging to identify potential immunotherapy responders from non-responders. The most promising prognostic biomarker is that of a pre-therapy anti-tumor immune response, in the form of CD8+ T-cells infiltrating into the growing margins of the tumor. Patients that exhibit such a baseline immune response are significantly more likely to respond to PD-1/L1 blockade therapies[127], and it is an absolute requirement for patients seeking ACT therapies that utilize *in vitro* expanded populations of tumor-infiltrating lymphocytes[122]. For melanoma patients, obtaining tissue biopsies for the analysis of CD8+ T cell infiltrates is straightforward, but for many tumors, such biopsies are not readily obtained. Thus, an *in vivo* imaging probe of CD8+ T cells would provide a powerful diagnostic tool for stratifying patients. If it is a positron emission tomography (PET) probe, then

antibodies are unlikely to serve this purpose, as their retention time in the body provides unwanted competition for the half-life of the ^{18}F-radiolabels commonly used. In addition, commercially available anti-CD8+ monoclonals do not exhibit particularly high affinities for the target. A high affinity, and a low off rate, are both important metrics, because many patients who exhibit a baseline anti-tumor immune response only have a low number of CD8+ T cell infiltrates. Other *in vivo* biomarkers include the emerging list of immune checkpoint molecules that are being explored for expanding immunotherapy to cancers such as prostate or breast. Thus, there is a unique opportunity here for nanotech solutions that can provide for rapid clearance, high target avidity, and tumor penetration.

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Neoantigens and the Design of ACT Therapies

In any cancer immunotherapy, the major tumor cell killers are CD8+ T cells. The killing function of those T cells is activated following a highly specific interaction between the T cell receptor (TCR) and a tumor antigen presented by tumor cells (**Figure 12**). Very recent findings are pointing to the importance of neoantigens in illiciting strong and highly specific anti-tumor T cell responses[128–131]. Neoantigens are fragments of proteins from the cancer cells that contain genetic mutations, and so differ from self-antigens. The very strong implication is that if one knows the tumor antigens present within a patient's tumor, and one knows sequence of the TCR α/β chain gene that encodes a TCR that recognizes those antigens with high avidity, then one can design a personalized, and potentially highly effective ACT therapy for that patient. In terms of guiding this technology discussion, we'll assume that one has access to tumor tissue from the patient. The key information for designing a personalized ACT therapy regimen for the patient is the following:

- *Which T cell populations, as defined by specific TCR receptors, have clonally expanded within the tumor?* That information identifies the cells that have 'seen' tumor antigen.

- *What are the tumor antigens that are promoting this clonal expansion?* If the tumor antigens are neoantigens, then they are likely safe immunotherapy targets. If they are not, then they must be evaluated with great caution.

- *What are the TCR α/β gene sequences that encode recognition for the specific neoantigens?* This is the information that is required for genetically engineering the T cells for the actual ACT.

There has been a recent flurry of activity in this area, but no approach has come close to yielding all three pieces of information, and most only yield one of the three pieces[132,133]. As such, here are the major challenges.

First, the tumor exome may be mined to identify potential neoantigens using existing software, and the number of neoantigens for a given tumor is likely on the order of 20-200. One can build a tetramer library based upon these 20-200 neoantigens[134], but the best cytometry approaches for tetramer-based T cell sorting based are 20-plex, and so barely touch the required range of multiplexing[133]. Even those methods require that the T cells infiltrates from the tumor be expanded *in vitro*. Next, identification of those T cell populations that have clonally expanded within the tumor requires analysis of infiltrating lymphocytes directly from the tumor – i.e., without expansion *in vitro*. One may obtain only 10^4-10^5 T cells from a tumor biopsy. This is not enough for standard cell analysis tools, but may be enough for nanotech tools. Finally, once the T cells that recognize a specific neoantigen are identified, the TCR α/β genes must be sequenced at the single cell level. The TCR gene is very challenging to sequence, but methods for TCR gene sequencing with reasonable (~50%) yield have been reported[135–137]. No existing technology can *simultaneously* solve these three challenges. This should motivate a challenge to the cancer nanotechnology community, specifically, for an analytical/diagnostic modality that can help provide such a solution, in the next 5-10 years.

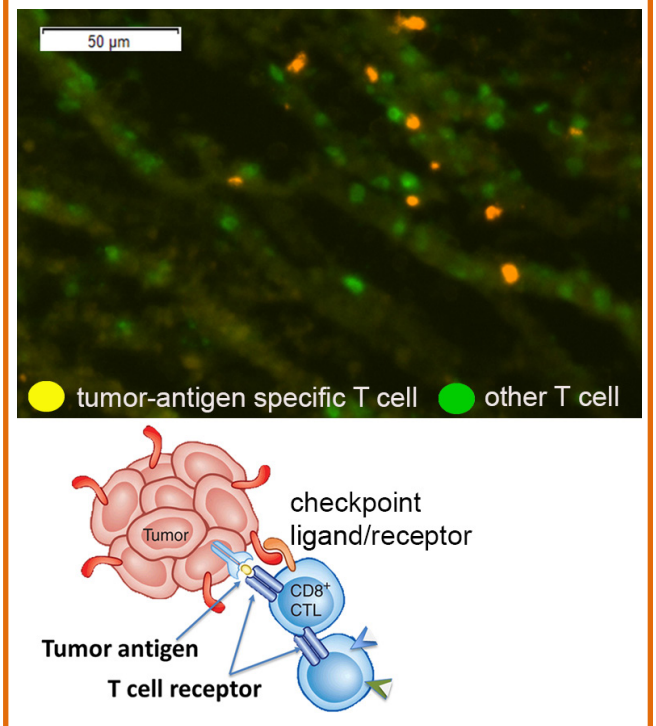

Figure 12. Tumor antigen-specific T cells are imaged in this fluorescence micrograph of a tumor from an in vivo immunotherapy model. Details of tumor/T cell interactions are shown in the drawing below.

Enhancing Cancer Immunotherapy with Nanotechnology

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Cancer Immunotherapy

Cancer immunotherapy utilizes the patient's own immune system to treat cancer, now a powerful novel strategy in cancer treatment. Antibodies blocking negative immune regulatory pathways, such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death 1 (PD-1), have substantially improved clinical outcomes in patients with metastatic melanoma[125,138,139]. Moreover, these agents have been shown to be effective in many other cancers, including head and neck, lung, kidney, bladder, and liver cancer[140]. In addition to checkpoint blockade agents, dendritic cell therapy and chimeric antigen receptor (CAR) T-cell therapies have also achieved clinical success[141,142]. Lastly, recent clinical data suggest that some cancer vaccines may also provide survival benefit. Such successes have generated high interest in developing strategies to further improve cancer immunotherapy.

While highly effective, the major limitation of checkpoint inhibitor therapeutics is the low rate of long-term, durable responses. Most patients eventually develop resistance and progressive disease. CAR-T cells are difficult to engineer and have high toxicity (frequently fatal) if the targeted antigens are also present on normal cells. Lastly, current dendritic cell therapy has low potency and the therapeutic benefit is only realized several years after treatment. Thus, there is ample opportunity for the development of novel therapeutics and strategies to improve cancer immunotherapy.

Nanoparticles and Cancer Immunotherapy

Nanoparticles, because of their virus-like size, readily elicit an immune response upon local or systemic administration. Without pegylation or other anti-fouling surface modification, nanoparticles are rapidly taken up by macrophages and other antigen presenting cells (APCs) and lead to immune activation. While this innate nanoparticle property has been detrimental to drug delivery applications, it is highly favorable for cancer immunotherapy. Taking advantage of this property, nanoparticles can be utilized to deliver tumor antigens to APCs. Moreover, immune responses to NPs can be modulated by adjusting the size and shape of nanoparticles[143,144]. Nanoparticle-bound antigens have been shown to elicit greater

immune responses than free antigens. In addition, nanoparticles can also act as immune adjuvants, enhancing response when given together with cancer vaccines.

Cancer immunotherapy can also capitalize upon the drug delivery property of nanoparticles. Nanoparticles can be formulated to deliver pro-inflammatory/pro-immune molecules with tumor antigens to enhance immune reactions. Such co-delivery is more likely to activate APCs and thus result in robust immune responses.

Current Approaches using Nanotechnology to Enhance Cancer Immunotherapy

...nanotechnology holds great potential in improving cancer immunotherapy.

Despite being a new area of investigation, nanotechnology has been explored by a number of research groups to improve cancer immunotherapy. A common approach has been the use of nanoparticles to improve tumor antigen presentation by APCs *in vivo*[145]. Using mouse tumor cells (such as B16 melanoma cells) overexpressing ovalbumin (OVA) protein, several groups have shown that nanoparticle-delivered OVA is more effective than OVA itself in eliciting immune responses. Such data suggest that nanoparticle-antigen combinations can be effective cancer vaccines. To further enhance immune responses, immune-activating molecules such as CpG have been co-delivered with tumor antigens[146]. The investigators showed that co-delivery of antigen and adjuvant are several-fold more effective than each agent given separately.

Another strategy to improve cancer immunotherapy has been the use of nanoparticles to activate immune cells. Fadel et al. recently reported the use of carbon nanotubes containing immune activating molecules (e.g., IL-2) to activate T-cells[147]. Such activated T-cells were then able to delay tumor growth. In a separate study, Perica et al. engineered nanoparticles that mimic APCs and utilized these nano-APCs to activate T-cells[148]. Nanoparticles have also been used to directly activate dendritic cells (APC)[149]. These studies suggest a role for nanoparticles in cell-based cancer immunotherapy.

In addition to improving antigen presentation, nanoparticles have also been used for their drug delivery properties. Tumor microenvironments are frequently immune suppressive, and nanoparticles can deliver therapeutics to overcome immune suppression. Park et al. demonstrated the proof-of-principle of this approach by delivering a TGF-β inhibitor and IL-2 and showing that these drugs delayed tumor growth and improved survival using a mouse model of melanoma[150]. Xu et al. further demonstrated this approach using nanoparticles

to deliver a TGF-β inhibitor to the tumor microenvironment to enhance tumor vaccine effects[151]. These studies suggest that drug delivery approaches can be combined with vaccine and immune activation approaches described above.

Future Directions

Nanoparticle-based cancer immunotherapy is a new and exciting field. It holds high potential in making direct impact on cancer care. To fully realize the potential of this approach, studies are needed to systematically characterize nanoparticles properties (e.g., size, shape and surface properties) that are optimal for immune activation and cancer immunotherapy.

Immune activation against tumor cells is a highly complex process (**Figure 13**). Because of unique properties of nanoparticles, they can be applied to improve each of these steps. Nanoparticle therapeutics can induce tumor cell death and in turn increase antigen release. They can be utilized to improve antigen presentation and activation by the APCs. Nanoparticles can also deliver pro-immune/pro-inflammatory agents to tumors and tumor microenvironments to enhance the cancer immunotherapy response. Lastly, nanoparticles can be utilized to "train" dendritic and cytotoxic T-cells *ex vivo* for cancer immunotherapy.

Given the exciting clinical data with checkpoint blockade inhibitors, approaches that combine nanomedicine and checkpoint blockade inhibitors are most likely to make immediate clinical impact. Future studies should focus on which checkpoint blockade agents and regimens are synergistic with nanoparticles and how nanoparticle-based agents can be integrated into checkpoint blockade treatments (e.g., timing of nanoparticle administration).

Cancer vaccine is another application where nanomedicine can make immediate impact. Nanoparticles can be formulated using biodegradable and biocompatible GRAS (generally regarded as safe) materials, which enables rapid clinical translation. However, existing clinical literature suggest that cancer vaccines targeting a single tumor antigen have limited benefits. Therefore, future work should focus on the development of multi-antigen cancer vaccines.

Cancer vaccine is another application where nanomedicine can make immediate impact.

Other applications for nanoparticles in immunotherapy include the development of tumor-targeting T cells as well as CAR-T cell treatments. In addition, they can also improve dendritic cell treatments. These applications require better understanding of nanoparticle properties as well as tumor immunotherapy (e.g., which tumor antigens more likely to elicit antitumor responses). As the field of cancer

immunology evolves, nanomedicine approaches will likely become more effective and more clinically relevant.

In summary, nanotechnology holds great potential in improving cancer immunotherapy. There are many known and potential applications of nanoparticles in immunotherapy. We also expect many novel applications for nanoparticles in cancer immunotherapy that have not been discussed given the rapidly evolving field of immunology. Future success in this field will depend on the full integration of cancer biology, cancer immunology and nanomedicine in this research space.

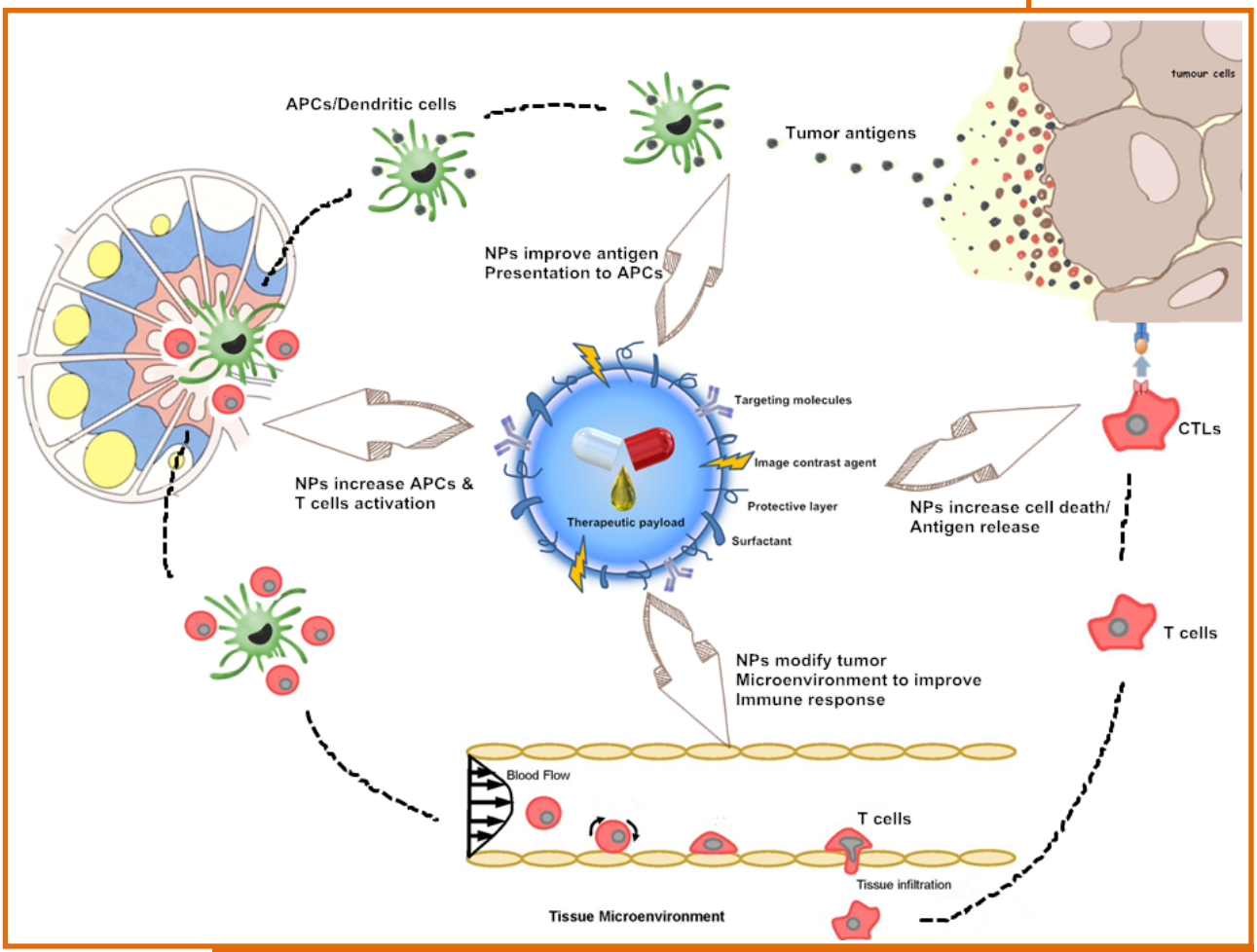

Figure 13. Depiction of the complex pathway involved in cancer immunotherapy. Nanoparticle delivery vehicles can play a role at multiple points along this pathway.

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Section III: Novel Nanomaterials for Diagnosis and Therapy

Mesoporous Silica Constructs

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Introduction

Specific drug delivery is one of the greatest challenges in cancer medicine. Targeted delivery of drugs encapsulated within nanocarriers can potentially ameliorate a number of problems exhibited by conventional 'free' drugs, including poor solubility, limited stability, rapid clearing, and, in particular, lack of selectivity, which results in non-specific toxicity to healthy cells and prevents the dose escalation necessary to eradicate diseased cells and overcome drug resistance. However, the physical and chemical properties of the nanocarrier, including size, shape, internal structure, and surface properties, play major roles in determining biodistribution of the carrier *in vivo*, biological interactions, cargo loading and release, biodegradation, and toxicity[1]. The optimal biodistribution and biological interactions of the nanocarrier can vary between different cancers (and individuals) making the ideal nanocarrier one in which the physical and chemical properties can be controlled and essentially tuned for the specific application[2]. An additional very necessary feature of an effective nanocarrier is the efficient loading and controlled release of the therapeutic cargos, which can range from small molecules to plasmids that have highly variable charge, polarity, and hydrophobic/hydrophilic character. Finally, a nanocarrier's potential to include imaging agents as well as drugs grants the possibility of creating 'theranostics', which allows both drug delivery and the monitoring of the course of therapy to be achieved with a single nanocarrier. In the context of creating a tunable nanocarrier, mesoporous silica nanoparticle constructs, developed over the past decade, have a distinctive *combination of features* that could enable their development as 'universal' nanocarrier platforms, of which, are simultaneously drug and disease agnostic.

Creation of Mesoporous Silica Nanoparticle Constructs

Mesoporous silica nanoparticles (MSNP) are composed of periodic arrangements or uniformly sized mesopores (ranging in diameter from 2 to >20-nm) embedded within an

amorphous silica framework and characterized by exceptionally high internal surface areas ranging from 500 to over 1200 m^2/g^3. MSNP are synthesized by two major routes: solution based synthesis or evaporation-induced self-assembly. Using solution based colloidal self-assembly it is possible to synthesize uniformly sized populations of MSNP with spherical, prismatic, torroidal, rod-like, or hollow shapes[4–8] with dimensions spanning 25-nm to over 250-nm, while in many cases maintaining low polydispersity indices <0.1[9]. Using evaporation induced self-assembly[10], it is possible to generate in a single step spherical MSNP with a predictable power law particle size distribution spanning 25-nm to over 250-nm. The highly tunable synthesis of MSNP allows for the selection of the size, size distribution, and shape most applicable based on the proposed delivery route and target biodistribution (**Figure 1A-D**).

During synthesis, the MSNPs can be modified to increase their functionality, for example their interiors can be constructed in a core/shell manner to introduce metal or metal oxide nanoparticles as imaging agents (**Figure 1E**). Core-shell MSNPs have seen many recent

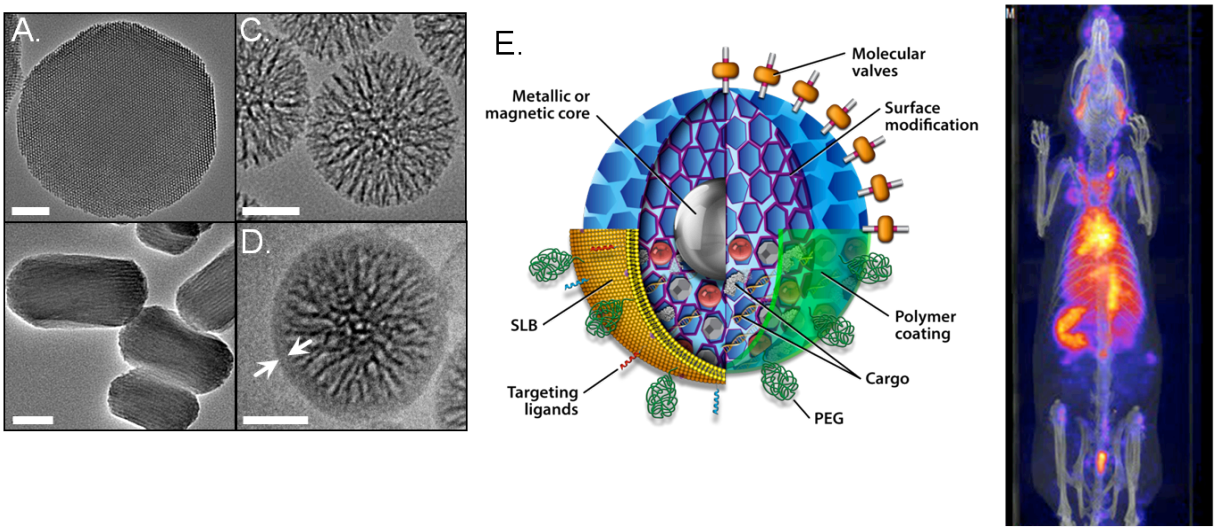

Figure 1. Mesoporous silica nanoparticles shape, pore size, lipid coating, functionalization and use. TEM images of spherical mesoporous silica nanoparticles with 2 nm pores (A), rod shaped mesoporous silica nanoparticles with 2 nm pores (B) and ~150 nm spherical mesoporous silica nanoparticles with 8 nm pores (C). CryoTEM of spherical mesoporous silica nanoparticles with 8 nm pores and a lipid bilayer coating highlighted by the white arrows (D). Scale Bars = 50nm. Schematic of a multifunctional mesoporous silica nanoparticle showing possible core/shell design, surface modifications and multiple types of cargo (E). SPECT image of radiolabeled 50nm mesoporous silica nanoparticles 5 hours post IV injection (F) (*Schematic (E) reprinted with permission from Tarn et al., 2013, TEM and SPECT images courtesy of Paul Durfee, University of New Mexico, Natalie Adolphi, University of New Mexico, and Yu-Shen Lin, Oncothyreon*).

applications in theranostics and allow for combined therapy and imaging simultaneously[11,12]. During or post-synthesis, the MSNP cores can also be loaded with fluorescent dyes with emissions spanning the visual range including; fluorescein isothiocyanate (FITC), rhodamine B isothiocyanate (RITC) and Cy3 as well as near-IR dyes such as AlexaFluor 700 and DayLight 680. The resulting MSNPs are extremely bright and optically stable enabling high-resolution multichannel optical imaging and quantitative multispectral flow cytometry. These labeled MSNPs provide a **unique** opportunity to examine the interaction between cells and nanocarriers along with MSNP biodistribution and delivery to tumors offering a direct measurement of these two important criteria during any regulatory approval[13,14].

Mesoporous Silica Nanoparticle Modification

MSNP functionality can be introduced by modifying silanol groups (ΞSi-OH) present both within the pore interiors and on the outer surface. Silanol groups are chemically accessible and can be easily reacted with alkoxy or chlorosilane derivatives to introduce organic functionality. Modification performed in single step or multi-step procedures provides an almost unlimited ability to 'tune' the charge, polarity, and hydrophobic/hydrophilic character of the pore and exterior particle surfaces, provide sites for further chemical conjugation or chelation with targeting and control ligands, and to couple imaging agents including radio labels for SPECT imaging (**Figure 1F**). Chemical moieties can also be adsorbed onto MSNP, especially facilitated by negatively charged SiO- groups, resulting from deprotonation of surface silanol groups at neutral pH, which result in attractive electrostatic interactions with positively charged moieties.

Introducing functional groups on the MSNP exterior surface gives rise to additional surface properties. They can be further reacted as linkers to attach larger molecules or used to adsorb coatings through noncovalent interactions. For the latter case, polymers are commonly employed on MSNPs[13,15,16]. Due to the intrinsic negative charge of the silica surface resulting from deprotonation of surface silanols, bare nanoparticles can be electrostatically functionalized with a positively charged polymer. Polymers or other surface bound functional groups can also be used to retain cargo within the MSNP and aid in colloidal stability that is required keep MSNPs highly dispersed for biomedical applications. An alternative means of surface coating MSNPs is by fusion with phospholipid bilayers to form a construct referred to as a *protocell*[14,17]. The cryo-TEM image (**Figure 1D**) shows a mesoporous silica particle core prepared by EISA enveloped by a conformal, 4-nm thick supported lipid bilayer (SLB). The properties of the SLB can be varied widely using lipids with differing fluidities or melting transition temperatures and headgroup chemistries that dictate charge and chemical reactivity. Membrane-bound components like cholesterol along with PEG can be introduced to control the fluidity and stability of the SLB, and it can be chemically

conjugated with ligands to effect targeting and internalization (*vide infra*) (**Figure 2**). As with polymer coatings, the SLB can serve to retain cargo introduced into the MSNP interior and aid in colloidal stability for biomedical applications. *Protocells* however have the advantage that acidification, as occurs in a tumor microenvironment or endosome, serves to permeabilize/destabilize the supported lipid bilayers triggering release of cargo[14,18].

Cargo Loading, Targeting and Cargo Delivery

Three major features of mesoporous silica constructs; high surface area, controllable pore size, and the ability to tune the charge of the particle, make them ideal for loading of varied cargo. Small molecule drugs and biological entities such plasmids or mRNA cargo present a large size range, which requires variable pore sizes for cargo loading. Using surfactants or block copolymers as structure directing agents in conjunction with swelling agents, it is possible to control pore size[19] from ~2-nm to over 20-nm, while hollow or toroidal particles provide even larger pore sizes (**Figure 1A-D**).

The tunable surface characteristics in combination with the high surface area allows for the simple loading of high concentrations of diverse classes and combinations of cargos that can be delivered by endocytosis or macropinocytosis[20]. The uniform arrangement, size, and connectivity of the porosity established by self-assembly confer to a MSNP very high BET (i.e., Brunauer–Emmett–Teller theory) surface areas ranging from 500 to over 1200 m^2/g. Surface area is important because it is the drug accessible surface area that dictates the drug loading capacity of an MSNP.

MSNPs can accumulate in tumor targets through both passive and active targeting. Passive targeting schemes rely on the enhanced permeability of tumor vasculature (the so-called enhanced permeability and retention (EPR) effect) to direct accumulation of nanocarriers at tumor sites, but the lack of cell-specific interactions needed to induce nanocarrier internalization decreases therapeutic efficacy and can result in drug expulsion and induction of multiple drug resistance (MDR). In terms of passive targeting, coating of MSNPs with a cationic polymer (e.g., PEI) significantly facilitates their uptake into tumor xenografts[16]. More recently, combining size control of MSNPs and PEI/PEG copolymer coating resulted in enhanced EPR effect in a xenograft tumor model[15].

To limit the degree of nonspecific binding while enhancing specific internalization by the target cell or tissue, MSNPs can be actively targeted toward an intended region (**Figure 2A**). Active targeting employs ligands that bind specifically to receptors overexpressed on the cancer cell surface. Bioactive ligands, such as folate, RGD peptide, and transferrin have been employed due to their respective receptors being overexpressed on many

different cancer cell types[21]. In general, high specificity and binding affinity require a high concentration of surface-conjugated ligands to promote multivalent binding effects, which results in more efficient drug delivery through receptor-mediated internalization pathways. However, high ligand densities can promote nonspecific interactions with endothelial and other noncancerous cells and increase immunogenicity, resulting in opsonization-mediated clearance of nanocarriers via the mononuclear phagocyte system (MPS). In this regard, the MSNP supported lipid bilayer construct (i.e., *protocell*) provides some potential advantages because its fluid SLB enables targeting ligand recruitment to target cell surface receptors, promoting high avidity with a low overall peptide concentration (**Figure 2B**).

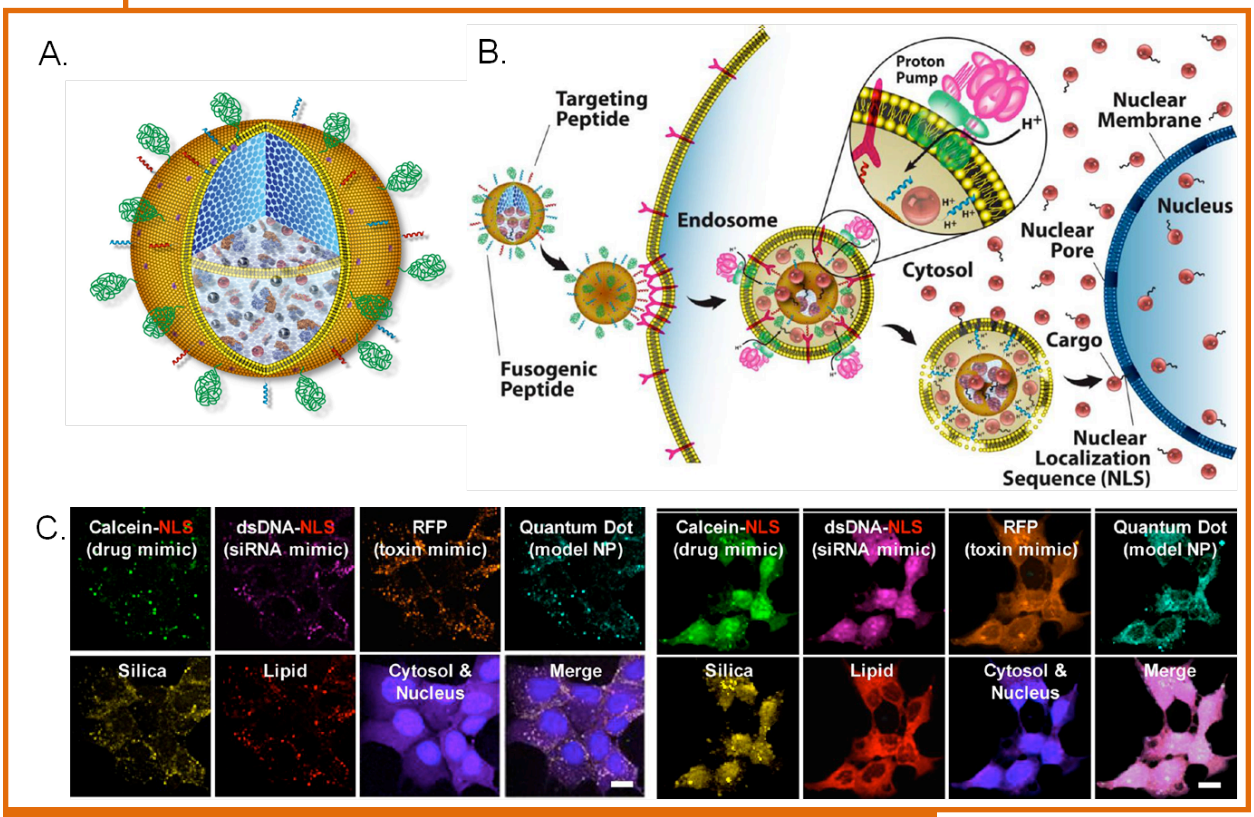

Figure 2. (A) Schematic of the protocell showing the MSNP core containing various cargo; such as drugs, nucleic acids and fluorophores, and coated with a lipid bilayer which has been functionalized by targeting ligands and PEG. (B) Schematic diagram depicting the successive steps of the multivalent binding and interanalization of targeted MSN –supported lipid bilayers, followed by endosomal escape and nuclear localization of MSNP-encapsulated cargo. (C) Hyperspectral confocal imaging of targeted delivery of multicomponent cargos in protocells to Hep3B cells for 15 minutes (left panel) or 12 hours (right panel) at 37°C. Alexa Fluor 532-labeled nanoporous silica cores (yellow) were loaded with calcein (green), an Alexa Fluor 647-labeled dsDNA oligonucleotide (magenta), RFP (orange), and CdSe/ZnS quantum dots (teal). Cargos were sealed in the cores by fusion of Texas Red-labeled DOPC liposomes (red) (*Reprinted with permission from Tarn et al., 2013*).

Thus, simultaneously with porosity, tunable surface and internal chemistry of the MSNP allowing for the inclusion of multiple cargos, MSNPs with lipid or polymer coating and cell type-specific targeting create a very robust single multifunctional nanocarrier platform (**Figure 2C**).

The highly tunable nature of MSNPs has also provided an ideal platform for the development of even more advanced nanocarriers with specific and controlled release of their cargo. The uniform pore size coupled with facile surface chemical conjugation has enabled modification of the pore entrances or interiors with responsive (light, pH, redox, etc.) molecular machines that can serve as gates[22] or 'stir bars' or molecular logic[23] to effect environmentally triggered release and control of the release rate profile.

The highly tunable nature of MSNPs has also provided an ideal platform for the development of even more advanced nanocarriers with specific and controlled release of their cargo.

Biocompatibility and Toxicity

A critical issue for any potential nanocarrier for medical applications is toxicity. The toxicity of silicon dioxide, both crystalline and amorphous, has been studied for more than a century, especially as it relates to *silicosis*, and recently, the toxicity of silica nanoparticles has been extensively investigated, due in part to the high surface-to-volume ratio of nanoparticles that could potentially lead to enhanced cellular interactions and different pathways of toxicity compared with coarse grained silica[15]. There is a general consensus that toxicity of MSNPs and amorphous silica in general is associated in part with the surface silanol groups, which can hydrogen bond to cellular membrane components or, when dissociated to form SiO^- (above the isoelectric point of silica ~pH 2-3), interact electrostatically with the positively charged tetraalkylammonium-containing phospholipids, both processes leading to strong interactions and possibly membranolysis[24].

Based on the high surface-to-volume ratio of silica NPs, it might be anticipated that they would show in general higher toxicity compared with their bulk counterparts (e.g., crystalline or amorphous). However in the case of MSNPs, the intrinsic porosity of the MSNP surface reduces the extent of hydrogen bonding or electrostatic interactions with cell membranes[24]. Considering both former and latter facts about silica in a nanoparticulate form, it would seem unclear as to the potential toxicity that MSNPs would display. With this in mind, many studies have been performed recently to address this.

Although the porosity of MSNPs should decrease their toxicity due to the decreased surface interaction, studies of the toxicity of MSNPs have shown widely variable ranges of toxicity. One potential reason for the variability in toxicity studies is the surfactant used to template the pores is toxic and variable amounts of this surfactant can remain within the pores of the MSNP depending on the processing[25]. A recent study which used FTIR to confirm that the template surfactant had been removed prior to testing MSNPs for toxicity found survival of all mice treated with up to 1000mg/kg by IV injection and followed for 14 days[26]. The survival of all the animals treated with a very high dose of MSNPs that did not retain surfactant shows the lack of toxicity of the silica framework of the MSNP itself. Potential toxicity is further mitigated by the high drug loading capacity of MSNPs, which greatly reduces needed dosages compared with other nanocarriers. Studies of drug loaded MSNPs in mice have shown that they are well tolerated and demonstrated no histological changes in organs at therapeutic doses such as 1mg/kg IV injection[26]. Mice treated with MSNPs with or without a PEG coating at higher doses, such as 20mg/kg IV injection, also demonstrated no signs of toxicity and no organ damage visible by histology[27]. Additionally, the ability to modify the surface of MSNPs with polymers or lipids will alter and potentially reduce toxicity of MSNPs. Finally, the ability to add targeting will further modify and reduce toxicity as the MSNPs are directed specifically to the target cells or tissues of interest and will have reduced nonspecific interactions within the body as a whole. Regardless, it is important to test all proposed nanocarriers in their final form for toxicity as well as to take into account the highly tunable and variable options presented by the MSNP platform. In addition to toxicity, the biocompatibility of the nanocarrier must also be taken into account. In this area, the porous structure of the MSNPs further enhances their biocompatibility as the high surface area and low extent of condensation of the MSNP siloxane framework promote a high rate of dissolution into soluble silicic acid species, which are found to be nontoxic[25]. The breakdown of the MSNPs overtime into nontoxic species supports the potential of repeat and long term use of the MSNPs to deliver drugs as the MSNP can be cleared from a biological system, overtime, in a nontoxic way. Examination of animals treated with both PEG coated and unmodified MSNPs showed excretion of the silica in both feces and urine[27]. The safety of MSNPs is also supported by the fact that amorphous silica is Generally Recognized as Safe (GRAS) by the FDA. Recently amorphous silica nanoparticle ‘C-dots’ (*Cornell Dots*) were FDA approved for diagnostic applications in a stage I human clinical trial[28]. The FDA clearance for a clinical trial of silica nanoparticles should accelerate the acceptance of amorphous colloidally derived silica’s for applications in medicine.

In Vivo Application of Mesoporous Silica Nanoparticles to Cancer Models

The study of MSNP as nanocarriers has advanced in recent years to studying the capacity of MSNPs to successfully deliver cargos to *in vivo* animal models of human cancers. Some of current studies have focused on the use of the enhanced permeability and retention (EPR) effect found in tumors. Meng *et al.* showed that the addition of PEG to the surface of MSNPs loaded with doxorubicin allowed 12% of the particles to accumulate within a tumor xenograft. In this study, the treatment response, of mice bearing squamous cell carcinoma xenografts, to the PEG coated doxorubicin MSNPs were compared to free doxorubicin, which showed an increased efficacy of the MSNPs versus the free drug. The mice in the study also showed reduced side effects, including reduction in weight loss as well as reduced liver and renal injury from the drug loaded MSNPs versus the free doxorubicin treatment[15]. More recent studies have begun to take advantage of the ability to add targeting moieties to the surface of the MSNPs. He *et al.* targeted polymer coated MSNPs to cervical cancer cells by conjugating transferrin to the MSNPs and increased the uptake of the MSNPs by also conjugating TAT cell penetrating peptide to the surface of the MSNPs. These targeted MSNPs were able to successfully deliver selenocysteine as a synergistic chemo- and radiotherapy agent to cervical cancer xenografts. Selenocysteine is a potential anticancer agent whose clinical development has been hindered by low selectivity, solubility and stability issues, which potentially could be overcome by loading the selenocystine into MSNPs. Mice treated with the targeted selenocystine MSNPs had dose dependant decreases in tumor volume at lower doses than mice treated with free selenocystine, showing the increased efficacy of the targeted MSNPs versus free drug[26]. The use of MSNPs has even been explored for increasing vascular access in difficult cancer types such as pancreatic ductal adenocarcinoma (PDAC). PDAC elicits a dense stromal response that limits the vascular access to the tumor and contributes to chemotherapy resistance. Polyethyleneimine (PEI)/polyethylene glycol (PEG) coated MSNPs containing the TGF-β inhibitor, LY364947, were delivered first to decrease pericyte coverage of the vasculature. The MSNPs were then followed by treatment with liposomes containing gemcitabine, a first line chemotherapy agent. The high loading capacity and pH-dependent LY364947 release from the MSNPs facilitated rapid entry of IV-injected gemcitabine containing liposomes and MSNPs at the PDAC tumor site. This two-wave approach provided effective shrinkage of the tumor xenografts compared to the treatment with free drug or gemcitabine-loaded liposomes only[29]. As shown by these studies, the utility and the variety of MSNPs for increasing drug delivery and specificity is increasing rapidly. As such, MSNPs have promise for decreasing toxicity for many chemotherapy agents and potential for increased efficacy in difficult to treat cancers.

Future Developments

The modular design of mesoporous silica constructs promises a new drug and disease agnostic platform technology for customized delivery and controlled release of multiple types of cargos and cargo combinations. Packaging within MSNP will enable the re-purposing of drugs that have to date failed clinical trials due to poor solubility, high toxicity, and/or susceptibility to degradation. MSNP supported lipid bilayers (so-called *protocells*) have the further advantage that the bilayer can retain and protect fragile and/or highly soluble cargos and enable triggered release of the cargo upon acidification within the tumor or tumor microenvironment. The modularity of the MSNP size, shape, pore size and surface chemistry further suggest applications in personalized medicine requiring individualized cargo combinations, targeting, and release profiles. However the modularity and versatility of MSNP may pose difficulties in pursuing FDA approval as new standardized protocols will be needed to establish structure, cargo content, PK/PD, and degradation profiles.

...the utility and the variety of MSNPs for increasing drug delivery and specificity is increasing rapidly.

Milestones to address these critical areas that researchers should be able to be achieve over the next 5-15 year time frame include many aspects. In the next 5 years, researchers will establish standardized procedures to characterize the physicochemical properties of MSNPs including purity, cargo loading and release, and biodegradation; Determine the size, shape, and surface chemistry dependence of the biodistribution, biodegradation and toxicity (e.g. maximum tolerated dose) of non-targeted MSNP depending on the route of administration and cancer model in small animals and dogs; Demonstrate the *in vivo* performance of targeted MSNP for delivery of multiple types of cargo to tumors and circulating and metastatic cancers in small animals; Perform PK/PD studies of select MSNP and targeted MSNP in small animals to correlate therapeutic efficacy with MSNP nanostructure and cargo loading and release characteristics; and conduct Phase 0 clinical trials of select non-targeted MSNP for delivery of small molecule cargos such as doxorubicin, paclitaxel, or cisplatin and cargo combinations. Looking further ahead over the next 10 years, researchers will conduct phase 0, I, and II clinical trials for select MSNP/cargo combinations and optimize MSNP performance (BD and PK/PD) via re-engineering of physicochemical properties; gain FDA approval of at least one MSNP-based therapeutic; and conduct phase 0, I, and II clinical trials for targeted MSNPs and MSNP theranostics and optimize *in vivo* performance. Looking further ahead over the next 15 years, researchers could gain FDA approval of at least twenty MSNP-based therapeutic systems including targeted MSNP, combination cargos, and theranostics; and conduct phase 0, I, and II clinical trials for personalized MSNPs with individualized cargos and targeting.

In Vivo Self-Assembly/Disassembly of Nanoparticles for Cancer Imaging and Drug Delivery

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Introduction

Nanoparticles have been shown to offer great detection sensitivity because of their unique physical, optical, electrical, and magnetic properties. Enormous efforts have been made in designing and synthesizing a variety of nanoparticles and applying them to cancer imaging. However, translation of nanoparticles-based contrast agents to clinical cancer imaging has been challenging, as summarized in a recent opinion paper authored by the NCI Alliance for Nanotechnology in Cancer Imaging working group[30]. Intravenous infusion is the most common delivery strategy for anticancer therapy or imaging applications. Injected nanoparticles have often met hurdles, such as non-specific uptake by the reticuloendothelial system (RES) and long-term retention in the body leading to chronic toxicity. The tools available to mitigate these effects are limited. A commonly used approach to reducing RES uptake and increasing circulation times is steric stabilization of particle dispersions by polyethylene glycol (PEG) coating. However, long circulation times achieved by PEG-coated "stealth" particles do not necessarily lead to enhanced accumulation deep into tumors because the relatively large size of nanoparticles attenuates transvascular transport and interstitial penetration (**Figure 3** left). To overcome these challenges, nanoparticle design and delivery have to be optimized, which is the main focus of the nanoimaging field. We have been exploring a unique approach to developing novel nanotechnology that will have high translational potential to clinical cancer imaging.

Our new, unique approach explores the concept of directly building nanoparticles inside living cells from small molecular weight building blocks taken up by target cells, as outlined in **Figure 3** (right). Small molecules typically have good transvascular transport and interstitial penetration into tumor (**Figure 3** middle), but unfortunately they are poorly retained at the target site and easily washed out. This new strategy seeks to combine the advantages of nanoparticles and small molecules for cancer imaging and drug delivery. More specifically, small molecules are injected through intravenous infusion, so they will diffuse into the interstitial space after crossing through the vascular vessels in the tumor. To enhance their retention in the tumor, they are activated by tumor-specific biomarkers already present and self-assemble into nanoparticles. At other tissue locations, where the cancer-specific biomarkers are absent, activation and the subsequent self-assembly does

not occur. Thus, the injected small molecules are poorly retained relative to the assembled nanoparticles at the tumor site. This new nanotechnology will help provide solutions to many challenges encountered in nanotechnology based drug delivery and cancer imaging.

Current State in the In Vivo Self-Assembly of Nanoparticles

This concept was first demonstrated in fluorescence imaging of the activity of a furin-like convertase in cell culture[31]. The success was enabled by a novel bioorthogonal reaction between an aromatic cyano group and a 1,2-aminothiol group[32]. The amino and thiol groups are conjugated with a masking group, and only after activation by the target enzyme to generate the free cysteine, will condensation take place to form macrocycles. These macrocycles have very affinity for each other and not the surrounding medium, thus readily self-assemble into nanoparticles. The end result being extended signal enhancement and retention in the local region where they assembled. Two modes have been established in the molecular cascade which enable this nanoparticle self-assembly: intermolecular condensation[31,33,34] and intramolecular cyclization[35–39]. Both initial condensations are specific, and with the subsequent intramolecular cyclization, it is free from any potential competition by endogenous free cysteine[35].

Since then, it has been shown that this approach can be applied to image many molecular targets and is compatible with a range of imaging modalities such as fluorescence[37], photoacoustic[34], magnetic resonance imaging (MRI)[33,38,39], and positron emission tomography (PET)[36]. For example, we have successfully synthesized a [^{18}F]-labeled caspase-sensitive nanoaggregation PET tracer ([^{18}F]-C-SNAT), and have validated it for PET imaging of caspase-3 activity with a doxorubicin-induced tumor apoptosis model in nude mice bearing HeLa tumor xenografts[36]. Using a super-resolution fluorophore, we have directly visualized the assembled fluorescent nanoparticles in apoptotic tumors, and thus fully validated the working mechanism *in vivo*[37]. We have shown that different biomolecules such as caspase-3/7[36–38], furin[32,34,35], beta-galactosidase [unpublished], and redox changes[33,39] can specifically remove the masking groups to trigger the condensation reaction and self-assembly.

These studies have clearly demonstrated that this *in vivo* target biomolecule-triggered self-assembly platform could be transformative for clinical cancer imaging. Because the nanoparticles are generated *in situ* at the cancer target site, the small molecule precursors will not encounter the same challenges faced with current injected nanoparticle-based *in vivo* diagnostic contrast agents. Rather, these nanoparticles are selectively synthesized at the tumor site to enhance imaging contrast.

Notably, a group at Brandeis University has developed a different chemical system, albeit based on the same concept, to generate pericellular and intracellular nanofibers for antitumor activity. The monomers used in this system are small peptides that are highly water-soluble. These small peptides are the substrate of a target enzyme such as alkaline phosphatase found in the cell. Upon the enzymatic processing of the small peptides, they will self-assemble into nanofibers through hydrophobic interactions at a site that is near the enzyme. With respect to their potential efficacy, it has been reported that the formation of nanofibers can lead to death of cancer cells *in vitro* through disruption of the dynamics of microtubules[40].

Another group at the University of Toronto has explored this *in vivo* nanoparticle assembly concept through a biotin-streptavidin interaction[41]. In their studies, poly(ethylene glycol) (PEG)-grafted small nanoparticles bearing biotin and streptavidin-conjugated fluorescent probes are injected sequentially. Both are diffusive and permeable to the tumor vasculature, and upon co-localization, they assemble into nanoaggregates, which is mediated via the strong biotin-streptavidin interaction, and enhance retention at the tumor site.

Future Scientific and Clinical Developments

Our current research has established an *in vivo* self-assembly nanoplatform for cancer diagnostics. To further advance this novel platform, one very critical component would be to introduce a novel design element that would allow for a gradual *disassembly* of the assembled nanoparticles into small molecules again, at the end of imaging. The purpose of this would be to allow

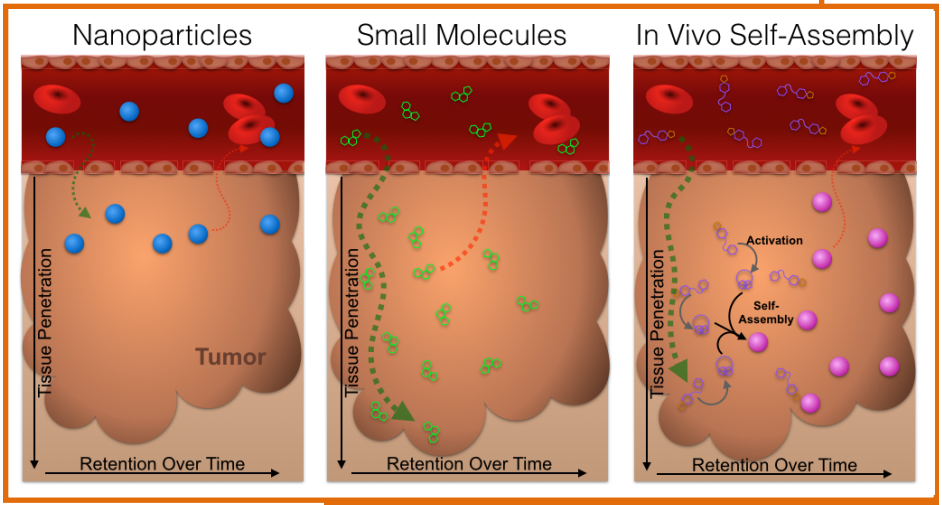

Figure 3. Schematic of transvascular transport and interstitial penetration of three types of intravenously injected materials. Left: nanoparticles cross the leaky tumor vasculature and are trapped well, but poorly penetrate due to its large size. Middle: small molecules (e.g., drugs) diffuse and penetrate deeply, but are poorly retained. Right: a new type of small molecules can be activated to self-assemble into nanoparticles after diffusion and penetration into tumor.

the nanoparticles to be eliminated from the body post-imaging. As such, over the next 5 years, this will be a primary focal point in this field, i.e., to establish *in vivo* disassembling technology and integrate it into the current self-assembling platform for cancer imaging in pre-clinical animal models. This self-assembly/disassembly nanoplatform will be applied to a range of cancer-specific targets and produce a number of imaging probes successfully evaluated in small animals.

In the next 10 years, those most promising Phase 0 candidates should be able to be further translated into human applications in the clinic as they will reach IND stage for clinical testing. It is expected that the unique feature—*in vivo* self-*assembly*/*disassembly* of nanoparticle—of these nanoplatforms should overcome the challenges commonly associated with injected nanoparticles, such as the transendothelial barrier to delivery, and minimize the acute and chronic toxicity, which is the primary reason for an optimistic view of their facile translation to the clinic.

...the small-molecule nature of these agents should present an important advantage for commercialization and large-scale production.

In the next 15 years, some of these agents will gain FDA approval for clinical applications such as cancer diagnosis, patient stratification, treatment monitoring and imaging-guided surgery. Moreover, the small-molecule nature of these agents should present an important advantage for commercialization and large-scale production.

DNA/RNA-Based Nanostructures for Cancer Nanomedicine

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Nucleic Acid Nanotechnology

Over the past several decades, nucleic acid molecules (DNA, RNA and their chemical cousins and derivatives) have emerged as highly programmable building blocks for nano-construction due to the increasing knowledge of their three-dimensional (3D) conformations and intra- and inter-molecular base pairing interactions[42]. A variety of design rules and assembly methods have been developed to engineer self-assembling nucleic acid nanostructures of increasing complexity[43,44]. DNA nanostructures ranging from periodical lattices to discrete objects of various sizes have been constructed using a rich library of DNA nanostructure motifs and different assembly strategies[43]. DNA origami, a method that uses a number of short, single-stranded DNA (ssDNA) oligonucleotides to direct the folding path of a long ssDNA 'scaffold' strand, has enabled the construction of spatially addressable and geometrically sophisticated 2D and 3D DNA nanostructures with near-quantitative yield[45–47]. As the sister molecule to DNA, RNA has also shown great promise in engineering rationally designed nanostructures. The canonical and non-canonical base pairing interactions, as well as the greater diversity of tertiary structures resulting from a rich library of naturally existing RNA structural motifs, have led to an emerging field of RNA nanotechnology[44,48,49]. Nucleic acid analogs such as PNA (peptide nucleic acid), LNA (locked nucleic acid), GNA (glycol nucleic acid) and TNA (threose nucleic acid), and chemical modifications of nucleic acids have all brought useful properties, including improved chemical, biological and thermo-stability to nucleic acid nanostructures. The structural properties of nucleic acid, which allow it to serve as a versatile construction material, have also been exploited to create dynamic nanodevices ranging from small switchable structures to structures that display complex motions[50]. In addition, logic gates and molecular computing based on nucleic acid building blocks have opened up great opportunities to implement sense-compute-actuate mechanisms into nucleic acid based nanosystems[51]. This is highly desirable for developing intelligent molecular devices for biological and medical research.

Nucleic Acid Nanostructures for Cancer Nanomedicine

The ability to engineer designer DNA nanostructures with high programmability and accurate spatial and dynamic control has allowed researchers to explore novel applications

in cancer nanomedicine. Nucleic acid nanostructures are attractive materials for this purpose, not only because of their inherent design modularity, structural programmability and biocompatibility, but also because nucleic acid molecules of a particular sequence can be modified to selectively bind, distinguish and communicate with target cells to trigger controlled delivery of therapeutic agents. With the development of various chemical conjugation methods, it is now technically feasible and convenient to present functional molecules, such as proteins or peptides, nucleic acids (aptamers, anti-sense RNA, siRNA etc.), inorganic nanoparticles (metallic, semiconducting and magnetic nanoparticles) and organic fluorophores at selected sites on nucleic acid nanostructures for making programmed theranostic devices. For example, researchers recently developed a DNA nano-barrel with single stranded aptamer locks that were opened to expose the loaded antibody cargo only in the presence of target cells[52]. Performing molecular computation directly on the surface of cells, or in cellular environments, will facilitate *in vivo* targeting and drug release. Recently, Rudchenko, Stojanovic and colleagues engineered DNA strand displacement cascades that detected the presence of certain biomarkers on the surface of cells[53]. In another report, Hemphill and Deiters successfully engineered oligonucleotide logic gates to detect specific microRNA inputs in live, mammalian cells[54]. As more complex and robust nucleic acid based computing systems are developed, it may be possible to integrate them into cellular systems to control and trigger cellular functions, such as gene expression, or to interfere with the metabolic pathways. By combining nucleic acid computation-based target cell detection with reconfigurable nucleic acid nanostructure-based drug containers, it may be possible to create a nucleic acid-based nanorobot that can interface and communicate with living cells to develop smart cancer therapy.

A critical step in administering effective drug therapy is the initial delivery of the therapeutic agents into cells. It was found that some nucleic acid nanostructures can be directly and efficiently internalized into live cells without transfection agents[55]. Although the underlying mechanisms still remain to be explored, such cell-penetrating nucleic acid nanostructures, in combination with targeted ligand-receptor recognitions, may lead to the development of universal cellular delivery systems. Pure DNA nanostructures have already displayed higher structural stability and resistance to nuclease digestion[56,57], compared to double helical DNA molecules. Recent studies further demonstrated that enclosing DNA nanostructures with PEGylated lipid bilayers leads to enhanced protection against nuclease digestion with decreased immune activation and significantly improved pharmacokinetic bioavailability[58].

There are several studies that have utilized the unique structural and geometric features of DNA nanostructures to deliver DNA or RNA molecules into cells (**Figure 4**). Examples include the delivery of DNA nanostructure-scaffolded CpG oligonucleotides *in vivo* to trigger immune responses[59] and delivery of siRNA both *in cellulo* and *in vivo* for regulation

of protein expressions[60]. DNA nanostructures carrying chemical drugs such as Doxorubicin have demonstrated great value in not only efficient drug delivery, but also simultaneously circumventing the drug resistance problem in chemical therapy[61].

Several unique properties, such as higher thermostability and synthesis scalability through *in vitro* and *in vivo* transcription, have made RNA-based nanostructures appealing molecular scaffolds for cancer therapy applications. In addition, the chemical stability of RNA nanostructures has been greatly enhanced by introducing chemical modifications such as the 2'-Fluro substitution to the 2'-OH group. It has been shown that a RNA-based nano-scaffold displays favorable pharmacokinetic profiles *in vivo* and shows no toxicity in mice[62]. Exemplified by the utility of the phi29 pRNA nanostructure system, RNA nanoparticles carrying various ligands such as siRNA, micro-RNA, and aptamers have shown great promise in targeted delivery of cancer therapeutics[63]. More recently, a multi-module

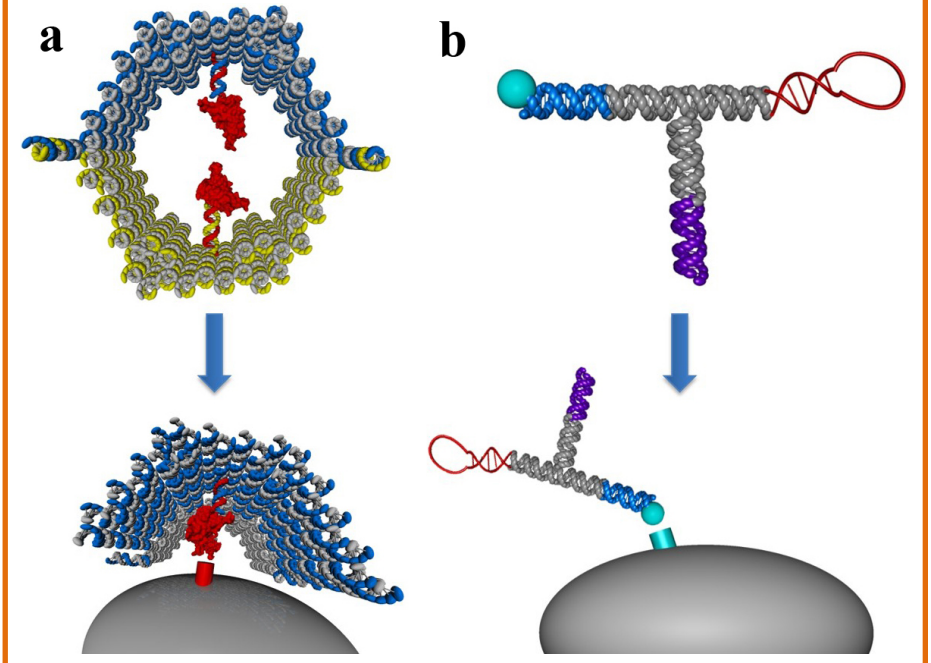

Figure 4. Programmable multi-functional nucleic acid nanostructures for cancer therapeutics. (a) Schematics illustrating the use of a DNA nanocage for targeted recognition of cancer cells. Top: Closed DNA nanocage loaded with an antibody payload. The cage is set to the closed state using structural switching DNA aptamer locks. The aptamers recognize the receptor molecules on the cancer cell surface to trigger the unlocking of the cage to expose the antibody to the target cell. Other payloads, such as chemical drugs, siRNA, and micro-RNA may also be loaded to create multi-functional targeted cancer therapeutics. (b) Illustration of a multi-functional three-way RNA junction motif carrying folate for cancer cell recognition, malachite green dye binding aptamer for cell imaging and siRNA for cancer cell gene expression regulation.

...nucleic acid based nanostructures can also be explored for cancer immunotherapy, ranging from immune activators, tumor-specific vaccines to immunosuppression blockers.

pRNA nanoparticle functionalized with folate acid was constructed to actively target metastatic cancer cells, demonstrating its benefits in treating cancer metastasis[64].

Given the intrinsic adjuvant activity of DNA and RNA molecules, nucleic acid based nanostructures can also be explored for cancer immunotherapy, ranging from immune activators, tumor-specific vaccines to immunosuppression blockers. Initial research in this direction includes the assembly of model vaccines using nucleic acid nanoscaffolds that display multiple immunogenic molecules and deliver immune-stimulating molecules to cells[59]. Yan, Yung and co-workers have demonstrated good immunogenicity of DNA-scaffolded vaccines. With a growing number of immune activators and check-point blockers being identified, one can use nucleic acid based-nanostructures to rationally assemble these molecules for elicitation of stronger and more effective anti-tumor immunity. Thus, the application of nucleic acid based nanostructure platforms for directed assembly of synthetic vaccines and immune modulators has great potential to revolutionize cancer immunotherapy. Furthermore, many chemotherapeutic drugs have been shown to enhance anti-tumor immunity, *via* an induction of immunogenicity of cell death and selective killing of immunosuppressive cells. Thus, programmable nucleic acid based nanostructures are best suited for the development of combined chemo- and immunotherapeutics in our fight against cancer.

Future Developments

To realize the full capability of using nucleic acid nanostructures for cancer research and treatment, several critical issues need to be addressed and carefully investigated. First, although initial studies have shown that some nucleic acid nanostructures (modified or unmodified) do not trigger strong immune responses, the safety of a larger spectrum of nucleic acid nanostructures must be established before practical use in clinical trials, given the adjuvant nature of DNA and RNA. Second, the use of nucleic acid based nanostructures for diagnostic and therapeutic applications rely on the complete clearance or degradation of the nucleic acid nanostructures within a reasonable amount of time. Depending on the type of application, it is important to investigate the bio-distribution, pharmaco-kinetic and dynamic (PK/PD) profiles of the nucleic acid nanostructures so that the nanostructures can be improved to achieve an optimal balance between efficient delivery and sufficient

retention time *in vivo*. Third, a set of design rules and parameters needs to be generalized for the nucleic acid nanostructure geometry, dimension, dynamics of reconfigurability, functionalization and chemical modification to develop the most effective nanodevices for different purposes of cancer therapy (e.g. structures need to be tuned to achieve balanced drug loading capacity and efficient targeted delivery; positions of recognition ligands on the nanoscaffolds need to be optimized to achieve improved affinity with minimized non-specific binding etc.). Fourth, a central obstacle to transforming nucleic acid nanostructures into clinical solutions is the cost of synthetic oligonucleotides. Researchers have made significant progress in producing RNA nanostructures through *in vitro* and *in vivo* transcription[65,66], and replicating small DNA nanostructures *in vivo*[67]. Further efforts are required to develop robust protocols to scale up the production of nucleic acid nanostructures of various designs through transcription, replication or through reducing the cost of nucleic acid oligo synthesis.

Indeed, a great advantage of using nucleic acid nanostructures for cancer nanomedicine is the ability to create multi-functional dynamic nanodevices with high programmability and intrinsic sequence/spatial addressability. There is plenty of room to take full utility of such a unique advantage for cancer nanomedicine. For example, nucleic acid nanostructures hold great potential to design and construct a set of novel, multifunctional, programmable anti-cancer vaccines that are specifically targeted to the tumor and programmed to release anti-cancer therapeutics and immune modulating factors at the tumor site to induce a robust, systemic immune response that will cause a sustained tumor regression. When such designs are integrated with molecular computing and programming, smart molecular doctors and personalized cancer therapeutics are within reach in the foreseeable future. Upcoming breakthroughs would require a multi-disciplinary effort from chemistry, biology, materials sciences, computer science, physics and clinical studies to push the boundaries of this exciting research area.

There is plenty of room to take full utility of such a unique advantage for cancer nanomedicine.

Milestones to address these critical areas that researchers should be able to be achieve over the next 3-10 year time frame include many aspects. In the next 3 years, researchers will evaluate the *in vivo* stability, bio-distribution and pharmaco-kinetics for a wide spectrum of nucleic acid nanostructures; identify optimal nucleic acid nanostructures with predictable behaviors *in vivo*; and develop robust and standard protocols to functionalize nucleic acid nanostructures to display therapeutic functions and targeted *in vivo* delivery properties. Looking further ahead over the next 5 years, researchers will evaluate the safety issue of the nucleic acid nanostructures which have demonstrated optimal *in vivo* behaviors;

develop multifunctional nucleic nanostructures and validate their initial uses in targeted cancer therapy and cancer vaccine development; and develop methods to scale down the cost of nucleic acid nanostructures and standardize protocols to make high yield synthesis of homogenous nucleic acid nanoparticles with designed functionality. Over the course of the next 10 years, researchers will conduct clinical trials of a variety of nucleic acid nanostructure-based cancer therapeutics; and integrate nucleic acid nanostructure-based therapeutics with molecular computing and programming to develop smart therapeutics in response to the cellular and tissue environments of various cancer and cancer matastasis.

Cooperative Nanosystems

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More Than the Sum of Its Parts

Bioengineers are currently designing increasingly sophisticated nanoparticles that can deliver treatments and diagnostics selectively to tumors[68,69]. Much of the field's focus has been on engineering the functionalities of individual nanoparticles to improve their transport[70], to target them to the tumor vasculature[71,72] or extracellular matrix[73], to deliver therapeutics[74,75], diagnostics[76], or heat[77,78] to the tumor environment, and to reprogram cancer cells[79] or the immune system[80]. However, the behavior of each nanoparticle depends not only on its design (size, shape, charge, material, cargo, and coating), but also on the interactions that occur in the body as a result of these design components. Thus, it is the collective, or 'systems' behavior of trillions of such nanoparticles interacting in a complex tumor environment that will define their success as diagnostic or treatment agents[81].

Predicting and engineering these collective nanoparticle behaviors is empirical and not always intuitive. For example, nanoparticles that are optimized to strongly bind and accumulate in cancer cells may mostly build up in the most proximal cells they encounter after leaking into the tumor environment. The resulting collective behavior is poor tissue penetration, leaving deep seeded tumor cells untreated[82–84]. Weaker nanoparticle binding, although detrimental to the function of the individual nanoparticle, could still lead to a better outcome by the system as a whole. Further engineering these behaviors on the level of single nanoparticles could result in emergent cooperative behaviors typically seen in self-organized systems[85].

Self-organized systems in nature, including those formed by social insects, animals, and cells, are able to perform complex behaviors through the local interactions of many simple agents and their environment[86–89]. The field of swarm robotics[90,91] has long taken inspiration from nature to engineer minimal robots that use simple rules to interact with their neighbors and local environment to solve complex real world problems[92–95]. Cooperative behaviors relevant to nanomedicine applications include amplification, optimization, mapping,

structure assembly, collective motion, synchronization and decision-making. By tapping into the field of swarm engineering, we may be able to produce behaviors that go beyond the functionalities of the individual nanoparticles and towards efficient, modular, and predictable system-based outcomes.

State-of-the-Art in Cooperative Nanosystems

Nanoparticles can cooperate implicitly, directly through self-assembly and disassembly, or through stigmergy (**Figure 5**). These behaviors have been useful to improve nanoparticle transport, accumulation, and distribution in tumor tissues towards development of treatment and diagnostic applications.

Most nanoparticle systems implicitly cooperate, in which each nanoparticle is designed to optimize its individual functionality[96]. The collective impact of the nanoparticles as treatment or imaging agents is assumed to be the sum of the independent nanoparticle effects. Understanding the system level behavior of implicit cooperators may add insight that can improve outcome predictions. Emphasis could be placed on studying whether the nanoparticles can collectively distribute throughout a tumor environment or accumulate at effective levels in, or around, targeted cells[70]. Similarly, combination therapies aimed at preventing resistance can be composed of different types of nanoparticles that independently target varied signal pathways, or even subpopulations within the tumor[97–99].

In addition to implicit cooperation, nanoparticles that physically interact harbor a more direct means of cooperation. Nanoparticles in this class of particles typically self-assemble or disassemble to modify their kinetics, or to collectively transport combined treatment and imaging agents to tumors. For example, rapidly diffusing imaging agents are able to anchor in tumors by binding to previously injected gold nanoparticles that have been given time to accumulate outside the vasculature via the EPR effect[40]. Similarly, small (10 nm) gold nanoparticles engineered to release conjugated doxorubicin in acidic tumor environments can subsequently self-assemble to form larger gold aggregates that are then available for use in photothermal therapy[100,101]. *In vitro* experiments reveal that nanoparticles capable of self-assembly in response to enzymatic activity may be able to perform logic computations towards the diagnosis of tumor state[102]. In another example, larger nanoparticles (100 nm) are able to disassemble into smaller nanoparticles once inside the tumor environment in response to enzymatic activity, thereby improving their circulation time, accumulation in the tumor, and ability to penetrate deep in the tissue[103]. Other multi-stage nanoparticles such as nested nanoparticles, mother ships, and nanocells are all able to overcome transport barriers through the release of nano-based components in tumor environments[104–106].

In contrast to collective behaviors mediated by direct interactions between nanoparticles, many swarm systems found in nature communicate by modifying the environment. This concept is called stigmergy[86]. Ants deposit and sense chemical signals to form trails that lead to sources of food[87]. Termites are able to build complex structures by modifying and locally sensing their physical environment[94]. In a similar way, nanoparticles have been designed to modify their physical environment or deposit signals. Gold nanorods that accumulate in a tumor, upon heating to sub-lethal temperatures with NIR light, can improve perfusion of angiogenic vessels and in some cases upregulate receptors used in targeting, which in turn improves the delivery of a second wave of nanoparticles, such as liposomes and magnetic nanoworms, to tumors for treatment and imaging purposes[107,108]. Gold nanorods heated through NIR light can also cause a clotting cascade in tumors[109]. This biological cascade serves as a signal to communicate the location of the tumor to circulating nanoparticles, thereby leading to a 40-fold increase in the amount of chemotherapeutic delivered to the tumor when compared to a non-communicating system[109]. Nanoparticles that aim to normalize the vascular bed, or degrade the extracellular matrix can improve the transport of secondary nanoparticles[110,111]. Nanoparticles can also be designed to release either a cargo or energy, which can directly interact with neighboring nanoparticles. As an example, gold nanorods activated through NIR light emit heat in tumors to trigger the release of chemotherapeutics contained in thermally sensitive drug carriers[112].

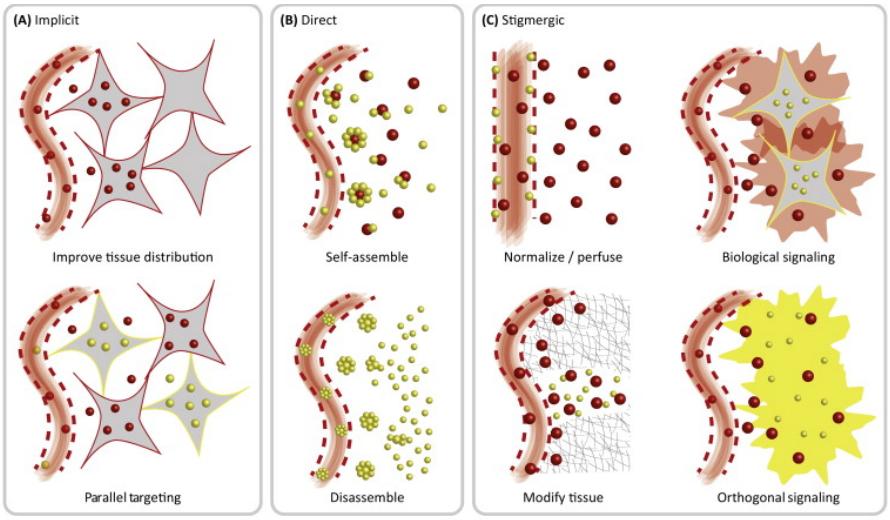

Figure 5. Mechanisms of cooperation in cancer nanomedicine. Nanoparticles can cooperate implicitly to improve their tissue distribution, directly through self-assembly and disassembly to change their distribution, or by communicating through the environment (stigmergy). Using stigmergic interactions, nanoparticles can impact perfusion or tissue density to improve the delivery of secondary nanoparticles. They may also communicate by initiating a biological cascade that can be sensed by other nanoparticles, or send an orthoganal signal (energy, chemicals) to activate secondary nanoparticles. (*Images and text reused with permission, Hauert and Bhatia, 2014*).

Systems Nanotechnology

The practice of engineering and predicting the collective behavior of large numbers of nanoparticles that interact in complex tumor environments is typically non-intuitive, even for simple nanoparticle designs. By harnessing a systems approach, bioengineers could start by automatically exploring potential nanoparticle designs using crowdsourcing (http://nanodoc.org) and machine learning[113], then modeling the resulting collective behavior in simulation[70,82,83,114], followed by testing the best candidates experimentally through fast prototyping of both the nanoparticles[115,116] and their environment[117], and finally validating the collective behaviors *in vivo* with feedback on their outcome provided by high resolution imaging[118]. Through this systems-based process (**Figure 6**), we expect nanoparticles to become more robust in their ability to react to environmental feedback by changing their motion and trajectory, thereby achieving increasingly swarm-like behaviors. Growing expertise in control of nanomaterials, achieving a deeper understanding of cancer biology, and ongoing advances in the modeling and automation of nanosystems are all contributing to the field's first steps in this direction.

More broadly, we anticipate that lessons learned from efforts made to design cooperative nanosystems will also prove useful in the engineering of naturally swarming biological components, such as cells of the immune system[119] or synthetic bacteria[120] in order to improve tumor treatment and diagnostics.

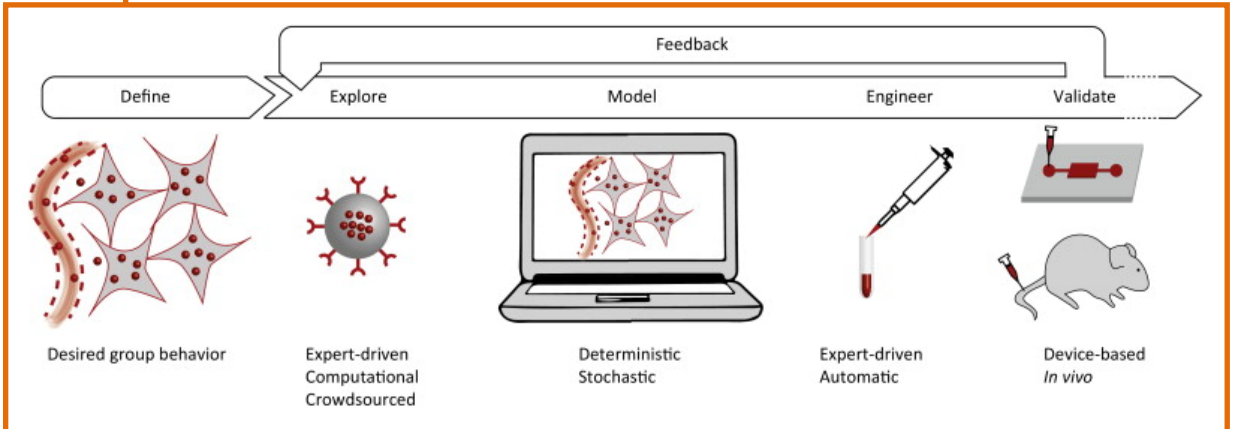

Figure 6. Systems approach to the design of cooperative nanomedicine. Starting from a desired group behavior, tools are needed to explore possible nanoparticle designs, model their resulting cooperative behaviors in simulation, engineer the nanoparticles, and validate them *in vitro*, and *in vivo*, before clinical translation. (*Images and text reused with permission, Hauert and Bhatia, 2014*).

Multimodal Imaging Constructs

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Introduction

With the aim in mind to create molecular imaging beacons that can be "seen" by multiple imaging methods, nanoparticles have several key advantages over small molecule contrast agents: (1) It is possible to integrate multiple contrast agents into the a single nanoparticle, and therefore combine their complementary strengths (e.g., whole body imaging and high resolution during intraoperative imaging). It is not possible, however, to simply mix the contrast agents together and expect reasonable signal to be generated for each modality. Most contrast agents require a particular environment to achieve optimal performance. Nanoparticles are small enough so they can be tuned to reach tissues of interest, but also large enough so that the particular needs of each contrast agent can be met within the same particle. (2) Their size range is ideal so that they can be coated with a variety of surface modifying moieties. These moieties can range from antibodies, affibodies, peptides or small molecules in order to induce binding of the particles to a specific target of interest. Here, the clustering of a large number of such targeting moieties on the relatively small surface of the nanoparticle can amplify their targeting abilities via multivalency effects. Nanoparticle surfaces can also be passivated with other moieties (e.g., polymers), through which one can influence and fine-tune the blood half-life and overall whole body biodistribution. (3) Nanoparticles can also be "armed" with many different therapeutic functions, be it that they deliver drugs at the target site or that they serve as photothermal agents that can destroy tumor cells via heat induction.

Current State for Multimodal Imaging Via Nanotechnology

There has been significant progress in the design and application of multimodal nanoparticles since 2010. One of the first nanoparticles that were in clinical trials for imaging purposes are superparamagnetic iron oxide nanoparticles (SPIONs)[121,122]. While several different versions with slightly different chemical compositions were in clinical trials for lymph node imaging with MRI these never received full FDA approval, and were subsequently taken off the market[121]. It is well known, however, that the iron contained in SPIONs is incorporated into the iron pool of the human body upon degradation of the particles, and the formulation as a nanoparticle can be more efficient than elemental iron in replacing iron in humans. This lead to the FDA approval in 2009 of a modified formulation

(Ferumoxytol) for the treatment of iron deficiency anemia in adult patients with chronic kidney disease. While not yet approved for imaging purposes, this has lead to a renaissance of clinical studies using SPIONs as an MRI contrast agent (e.g., NCT01336803). Given the many preclinical studies that used SPIONs as a platform for multimodal imaging, such as by adding a fluorochrome or radiotracer, this also rekindles the hope that such multimodal nanoparticles will eventually receive approval for diagnostic imaging purposes[123,124].

Several nanoparticle therapeutics made of other materials such as gold, silica or both, are currently in advanced stages of clinical trials[125]. These advances are not only representing milestones in the field of nanotherapeutics, but also increase the likelihood of nanoparticles of similar size and composition to be approved for imaging purposes. In fact, in 2010 the FDA approved an IND for the first in human testing of so-called 'Cornell dots' or C dots (NCT01266096). C dots are silica nanoparticles that are less than 8 nm in size, contain fluorochromes in their core, and can be functionalized with radiotracers for PET imaging for dual modality detection of melanoma metastases[28]. This was the first time that the FDA approved a clinical trial using an inorganic material in the same fashion as a drug in humans.

Major advances have also been made in the

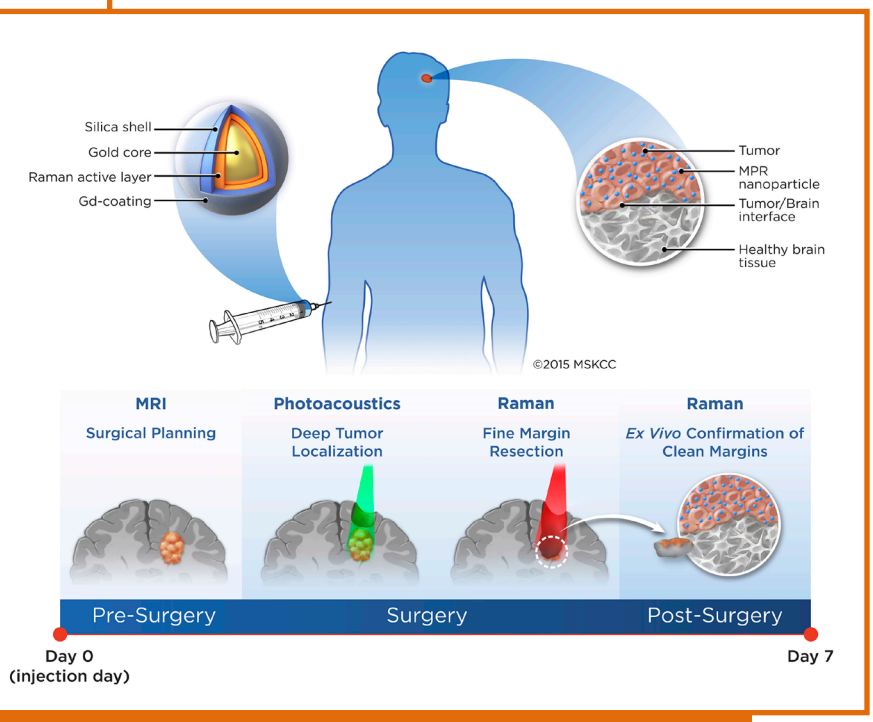

Figure 7. Principle of a triple-modality MRI-photoacoustic-Raman nanoparticle and its envisioned clinical use. The nanoparticle is injected intravenously. In contrast to small molecule contrast agents that wash out of the tumor quickly, the nanoparticles are stably internalized within the brain tumor cells, allowing the whole spectrum from preoperative MRI for surgical planning to intraoperative imaging to be performed with a single injection. T1-weighted MRI depicts the outline of the tumor due to the T1-shortening effect of the gadolinium. During the surgery, photoacoustic imaging with its greater depth penetration and 3D imaging capabilities can be used to guide the gross resection steps, while Raman imaging can guide the resection of the microscopic tumor at the resection margins. Raman could also be used for rapid confirmation of clean margins in the operating room instead of the time-consuming analysis of frozen sections.

preclinical arena, of which only few can be mentioned in this short summary. These comprise improvements to existing modalities, integration of multiple modalities into the same nanoparticle, and the establishment of new imaging modalities. As an example of the latter, "surface-enhanced Raman scattering" (SERS) nanoparticles were shown for the first time to allow imaging of cancer and image-guided tumor resection[126]. It was also shown that such SERS nanoparticles could be transformed into multimodal molecular imaging agents, by adding detectability from both MRI and photoacoustic imaging. This triple-modality approach was developed, with the goal in mind, to perform more precise brain tumor imaging and image-guided resection (**Figure 7**). While the MRI capabilities allow for preoperative planning, intraoperative photoacoustic imaging can provide a surgeon with a roadmap for the gross resection steps, while SERS imaging indicates whether or not the tumor tissue has been completely resected at the microscopic level[126,127]. Because SERS provides such a specific signal (Raman "fingerprint"), it is ideally suited for high precision cancer imaging. This has more recently been demonstrated with a new generation of "surface-enhanced resonance Raman scattering" (SERRS) nanostars that are orders of magnitude brighter and allow imaging of microscopic disease in multiple different cancer types[128,129]. New synthetic protocols now allow the creation of multiple layers of silica, each fine-tuned in thickness and each containing a different contrast agent (patent pending). This principle allows incorporating a large number of contrast agents into the same nanoparticle, while also allowing optimal placement of each contrast agent within the particle architecture. For example, a SERS reporter has to be placed as close as possible to the noble metal core, while a fluorochrome has to be placed at a certain distance to avoid quenching of the fluorescence. An MRI contrast agent is ideally placed at the nanoparticle surface to allow interaction with water molecules. This principle is illustrated in **Figure 8**.

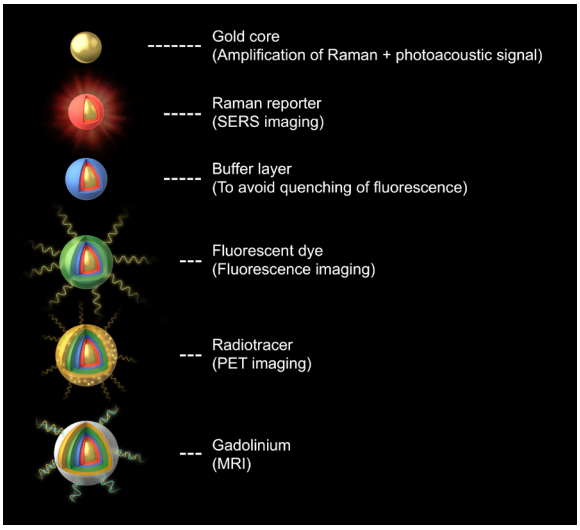

Figure 8. Synthesis of multimodal nanoparticles via a multilayer silication method. Addition of multiple layers of silica with finely tuned thickness as a strategy to incorporate many different imaging modalities into the same nanoparticle, while optimizing the signal intensity of each modality.

Future Challenges in Multimodal Imaging

The main challenge for nanoparticle imaging agents is and remains the regulatory approval by the FDA. Multimodal nanoparticles are facing significantly greater hurdles in the approval process than small molecule agents that would suffice for isolated PET, CT, MRI or fluorescence imaging. The most difficult hurdle for nanoparticles that are not small enough to be cleared via the kidneys is that sufficient proof has to be presented to the FDA that the retention of the nanoparticles in the body does not represent a health risk. Most intravenously injected nanoparticles are cleared from the blood by the organs of the reticuloendothelial system, such as the liver, spleen and lymph nodes, and are retained in these organs for extended amounts of time. In the case of SPIONs, Ferumoxytol has proven to be degraded over time, which facilitated regulatory approval. For those nanoparticle compositions that do not degrade or are eliminated from the body over time, it has to be shown that the retention does not cause any adverse effects. To this end, the recent development of novel artificial organoids that closely recapitulate human organs might offer a great avenue to accelerate such studies without having to risk the health of human patients.

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Milestones to address these critical areas that researchers should be able to be achieve over the next 3-10 year time frame include many aspects. In the next 3 years, researchers will conduct large animal studies of currently available multimodal imaging agents; initiate more clinical trials; and continue the development of next generation nanoparticle imaging agents. Looking further ahead over the next 5 years, researchers will test the newest generations of multimodal nanoparticles in artificial organs, which are expected to exist by then and should facilitate the translation into the clinics; and complete the currently ongoing clinical trials, analyze results and detail the lessons learned. In the next 10 years, multiple clinical trials should have been completed, including those that originated from initial testing in artificial organ systems. This should give a good indication about how well toxicity profiles can be predicted from studies in artificial organ systems, with the hope that parts of the current phases of the FDA required clinical trials can be replaced with testing in those novel model systems.

Theranostics: Smart, Multi-Functional Materials for Diagnosis and Therapy

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Overview

Current orthodox in the treatment of cancer involves surgical resection of large tumor areas followed by non-selective radiation therapy or chemotherapy. Such procedures can cause severe side effects from their non-specificity for tumor cells and concurrent damage to the immune system, rendering patients susceptible to other diseases. Moreover, the cancer frequently returns in refractory forms, resistant to current therapeutic approaches. Owing to the lack of effective late-stage cancer therapies, early detection and appropriate treatment is critical.

For the past two decades, the interesting and unique nanoscale delivery model and its respective tools have proven to be effective in medicine, especially in the field of cancer research and oncology. There has been much work to harness the tunable physicochemical properties of nanomaterials for diagnosis and therapy, such as real time visualization of cells/tissues and the precise delivery of therapeutic molecules to the targeted area. The diagnostic properties of nanomaterials (e.g., high plasmonic effect, enhanced MRI contrast effect, strong fluorescence, etc.) can enable early detection of small-sized tumors with exceptionally high sensitivity[130,131]. Furthermore, the multivalent characteristics of various nanomaterials allow for accurate tumor-specific imaging with the aid of a targeting moiety and synergistically integrated multi-modalities[132,133]. The improved targeting ability has also been advantageous from a therapeutic perspective, by which nanomaterials can selectively deliver therapeutic molecules to the tumor site, thereby increasing the therapeutic efficacy and reducing required dosages to minimize unwanted side-effects[71].

The distinct advantage of nanomaterials over conventional small molecules is their tunable physicochemical properties. Their size, shape, composition, and surface control can be adjusted to optimize their application in diagnosis and therapy. For example, rationally designed nanomaterials with specific dimensions and appropriate surface characteristics (e.g., neutral PEG and zwitterion) can circulate in blood vessels for a long time without opsonization by evading detection from macrophages and preferentially accumulate in tumor tissues via extravasation[134–136]. When incorporated with targeting moieties, the nanomaterials can be even more accurately delivered to the tumor site.

These phenomena are used for tumor-specific imaging (e.g., iron oxide for MR imaging and gold for highlighting tumor borders during brain surgery). As a method for enhancing diagnostic accuracy, multi-modal imaging (e.g., PET-CT and PET/SPECT-MRI) using different complementary modalities has been widely studied[133,137]. For example, nanoparticles functionalized with radioisotopes, known as multi-modal nanoparticles, have the potential to enhance diagnostic accuracy by increasing sensitivity of detection and adding the precision of anatomical localization[138]. Recently, magnetic particle imaging (MPI)-MRI demonstrates the potential for real-time visualization of tumor and cancer-related events (e.g., angiogenesis) with nano-molar sensitivity and anatomical details[139,140].

For therapy, the most promising and common application of these phenomena is the transportation of drug molecules. One example is BIND®, a targeted therapeutic nanoparticle, which in clinical trials has effectively reduced tumor sizes at lower doses than traditional chemotherapy[141]. The nanoparticles hold the chemodrugs without leakage during circulation and release them only upon reaching the targeted tumor. Some types of nanomaterials have additional therapeutic capabilities, such as the transformation of external energy to heat (e.g., iron oxide for magnetic fields and gold for light). These heat-generating therapies are known as photothermal ablation and magnetic hyperthermia, and they have been effectively used in cancer treatments[137,142]. The hyperthermia-based therapy has regulatory approval in 27 European countries[143].

Following treatment, nanomaterials can also be utilized to assess treatment efficacy and aid in making a prognosis (e.g., complete removal, regrowth, or metastasis of tumor). Nanosystems that can provide real-time diagnosis, in tandem with therapy and/or prognosis using multi-functional nanomaterials, are called *theranostics*. Research to combine the diagnostic and therapeutic characteristics of nanomaterials within a single platform, is being actively pursued. Currently, a wealth of research is being conducted in this area to improve cancer diagnosis and therapy. However, it is still only at the initial stages of the developmental pipeline.

Clinical Significance

From a diagnostic point of view, real-time monitoring of cancer-indicative markers (e.g., from genes and/or proteins) would allow for the administration of preemptive medicines at the moment pre-cancerous symptoms are found. A nanoparticle pill that Google is currently developing is a representative example of real-time monitoring[144]. When patients swallow a pill containing magnetic nanoparticles decorated with biomolecules for the identification of cancer or heart disease, the nanoparticle can detect and report signs of targeted disease through a wearable device. This proactive monitoring concept can switch the treatment

paradigm from the curative to the preventive. Even in cases where prevention fails, there is still a large benefit to early cancer detection. It keeps more effective treatment options available, which offers the best opportunity to be cured.

From a therapeutic point of view, the targeted delivery of therapeutic molecules to a tumor using nanomaterials can potentially enhance the efficacy of therapy and significantly reduce systemic toxicity, such as that experienced with Abraxane®, the FDA-approved paclitaxel albumin-stabilized nano-formulation[145]. When combined with the imaging capabilities of nanomaterials, the therapy can be monitored for maximum accumulation time, effective release of the drug, and the patient's response to treatment. This in turn allows for more informed decision-making on timing, quantity, type of drugs, and choice of treatment procedure, as well as an evaluation of an individual's response to treatment. This could be the basis for the future of personalized cancer treatment.

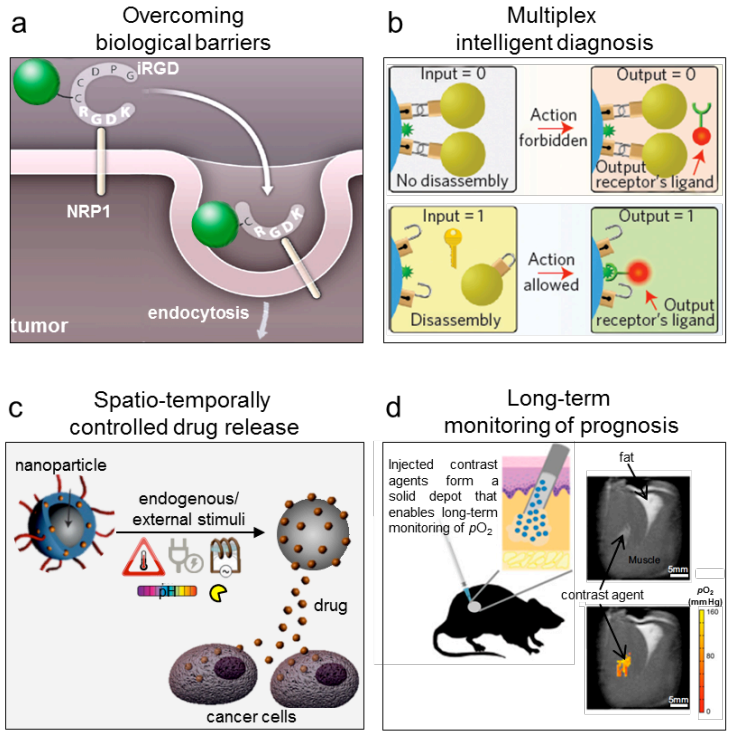

Figure 9. Challenges for future theranostic nanomaterials. (a) Nanomaterials should possess capabilities to overcome hurdles in tumor-specific delivery. One possible approach can be iRGD which allows nanomaterials to access a tumor by penetrating endothelial and tumor tissues. (b) Nanomaterials delivered to tumors should provide comprehensive information about tumor microenvironments. Logic-performing nanomaterials enable smart diagnostics by detecting and processing multiplexed molecular signatures. (c) Based on diagnostic information, nanomaterials should initiate spatio-temporally controlled therapy in response to external or endogenous stimuli. (d) After completing therapy, the non-toxic nanomaterials can be left inside the body and continuously give prognostic information (e.g., oxygen level). (*(a) Reprinted with permission from Feron, 2010; (b) from Nikitin et al., 2014; (c) from Mura et al., 2013; and (d) from Liu et al., 2014*).

Future Challenges

Although current theranostic nanomaterials have great potential, next-generation design concepts and their effective implementation strategies are required (**Figure 9**). Future nanosystems should be able to pass through biological barriers (e.g., BBB, hypoxic tumor regions, stroma, etc.) to reach any tumor sites of the body. One possible approach can be integrating nanomaterials with functional

peptides (i.e., tumor-penetrating peptides) which allow the nanomaterials to reach deep inside an extravascular tumor[146,147]. Magnetic targeting might be another potential solution if the magnetic force exerted on the nanomaterials can be made strong enough to overcome the drag force of blood flow[148,149]. This requires precise control of the direction and intensity of the applied external magnetic field.

When the theranostic nanomaterials arrive at the target site, they should provide quantitative and comprehensive information on the multiple molecular signatures of cancer cells. Current single target-specific imaging and qualitative sensing are not adequate for accurate diagnosis because tumorous environments are complex and heterogeneous[150]. Therefore, nanomaterials should be developed to have multiplexing and logic capability that detects numerous molecular signatures and intelligently reports them to us for accurate diagnostic results[151]. Considering the expression level of those signatures, such diagnostic nanomaterials should possess high sensitivity (e.g., at least pico-molar) for cancer-related biomolecule detection[126].

The nanomaterials have to be designed to sensitively and precisely respond to the corresponding stimuli.

After the diagnosis, spatio-temporally controlled therapeutic action should only start upon reaching the target region in order to lessen collateral damage. The remote trigger of the action can be either multiple and logical combinations of endogenous tumor microenvironments (e.g., pH and enzymes), or exogenously controlled physical stimuli (e.g., light and electromagnetic field)[152,153]. The nanomaterials have to be designed to sensitively and precisely respond to the corresponding stimuli. Simultaneous or sequential execution of therapeutic methods from one nanomaterial also needs to be pursued to overcome cancer resistance (e.g., multidrug resistance)[154]. Finally, when the therapy is complete, the remaining nanomaterials need to be able to assess the treatment's efficacy and aid in making a prognosis[155]. They should of course be fully biodegradable or clearable over time, and in order to meet regulatory requirements, their safety should be ensured for prolonged use through investigation of their clearance (e.g., renal and biliary routes, etc.).

Milestones to address these critical areas that researchers should be able to be achieve over the next 5-15 year time frame include many aspects. In the next 5 years, researchers will establish new sets of design principles to control physical, chemical, structural, and biological properties of nanomaterials for improved sensitivity and specificity in tumor microenvironment monitoring, cancer detection, and therapeutic effect; understand

sub-cellular level interactions between nanomaterials and cancer cells for effective tumor targeting; and evaluate the diagnostic and therapeutic effectiveness of developed nanomaterials by employing *in vitro/in vivo* models. Looking further ahead over the next 10 years, researchers will devise nanomaterials that overcome the biological barriers that limit accessibility to tumors; create nanomaterials with optimal circulation time for enhanced tumor accumulation with minimal off-target effects; endow a multiplexing capability to nanomaterials to identify multiple targets for diagnostic imaging/therapy in real-time; verify the ability to reproducibly initiate therapeutic activity only at tumor/cancer cell sites *in vivo*; and determine nanomaterial safety by characterizing biodistribution, PK/PD depending on size, shape, surface chemistry, etc. In 15 years, researchers will have optimized the theranostic properties of nanomaterials, specifically for prevention/early-detection of cancer, monitoring of cancer heterogeneity, and significant increment in therapeutic index; establish nano-regulatory with industries and the FDA; and make several highly effective nanotechnology based imaging and/or therapeutic agents in the late stage of clinical trials or in the market.

Theranostics: Targeted Theranostics in Cancer

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Introduction

The major challenges in the effective treatment of cancer patients are low efficiency in drug delivery and intrinsic drug resistance in highly heterogeneous human tumors[156,157]. Chemotherapy drugs have short blood half-lives and limited amounts of drugs can be delivered into tumors despite high doses of drugs being administrated to patients that cause severe systemic toxicity. Therefore, improvement of drug delivery into tumor cells should be one of the most important strategies for enhancing therapeutic responses in human cancer.

At present, nanoparticle formulated chemotherapy drugs, such as Doxil (liposome encapsulated doxorubicin) and Abraxane (paclitaxel-albumin protein complex), are FDA-approved nanotherapeutic agents for drug delivery into tumors, which utilize the enhanced permeability and retention (EPR) effect mediated by leaking tumor vessels[158–160]. Various non-targeted or targeted liposome and polymeric nanoparticle drug carriers are in preclinical developments and clinical trials[75,161]. Although those nanotherapeutics have shown promising anti-tumor effects and reduction in systemic toxicity in animal tumor models and in cancer patients, lack of novel approaches for timely assessment of efficiency of intratumoral drug delivery and response remains an issue. It is well known that human tumors are heterogeneous in vasculatures, tumor stromal components, and abnormalities of tumor cells, which contribute to significant differences in physical barriers for drug delivery and intrinsic barriers in drug sensitivity. Therefore, effective cancer therapy not only requires new drug delivery approaches, but also personalized evaluation of drug delivery and the subsequent early tumor response, in individual patients, using noninvasive tumor imaging. This 'precision' version of oncology would make it possible to maximize effectiveness of therapeutic agents by selecting the most efficient drug delivery approach while simultaneously minimizing systemic toxicity through timely replacement of ineffective therapeutic agents.

Current advances in the development of multifunctional nanoparticles with the abilities of targeted drug delivery and imaging intratumoral drug accumulation and distribution, i.e., *theranostics*, offer a unique opportunity for the integration of targeted and image-guided cancer therapy using a single nanoparticle platform[162,163]. First, imaging properties allow for

determining whether a cellular target is expressed by tumors and if this targeted approach is able to deliver sufficient nanoparticles into a specific tumor by non-invasive imaging (**Figure 10A**). In so doing, the cancer patients with the highest likelihood of a clinical response to the targeted theranostic nanoparticle can be selected. This is particularly important for patients with tumors, which are not easily accessible for biopsy. To overcome drug resistance, two or more therapeutic agents can be loaded to a single nanoparticle for targeted delivery into tumor cells, simultaneously, to enhance the synergistic effect of the drugs. This approach has clear advantage over conventional combination chemotherapy since drug molecules with different chemical properties vary in their pharmacokinetics, bioavailability, and stability. Encapsulation or conjugation of drugs to theranostic nanoparticles will significantly improve the blood half-lives of drugs, and protect drug molecules from binding to serum proteins and becoming inactivated by enzymes, leading to targeted delivery of large amounts of active drug molecules into tumor cells.

Following systemic delivery, non-invasive imaging modalities, such as MRI, PET, ultrasonic, photoacoustic, and optical imaging, can be used for determining nanoparticle-drug delivery efficiency (**Figure 10B**). Using an imaging modality with high resolution and anatomic information, it is feasible to monitor early tumor responses following targeted therapy to identify imaging signatures that predicate a good or poor response such that ineffective drugs will be replaced with more potent therapeutics in a timely manner (**Figure 10C and D**). Finally, targeted delivery of multimodal imaging theranostic nanoparticles enables intraoperative detection and removal of drug resistant tumors using image-guided surgery (**Figure 10E**).

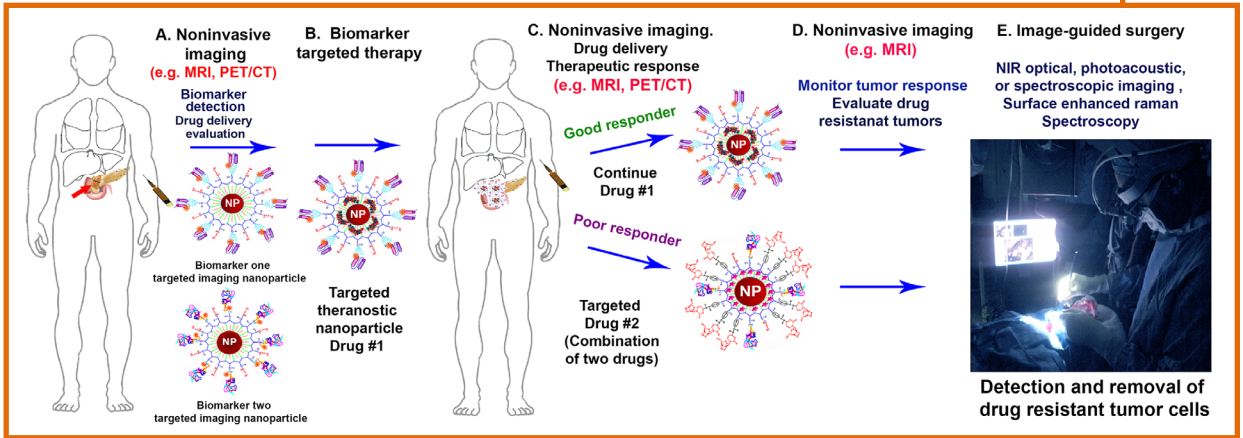

Figure 10. Clinical paradigm for theranostic nanoparticles. An outline of steps [A-E] along the clinical path of which theranostic nanosystems would display their inherent importance in oncology.

The development and translation of image-guided and targeted therapy using theranostic nanoparticles have clinical significance in the treatment of several aggressive cancer types, such as triple negative breast, pancreatic, ovarian, lung, colon, and liver cancers. For example, neoadjuvant chemotherapy has been given to triple negative breast cancer (TNBC) patients before surgery. About 22% of TNBC patients showed a good therapeutic response (pathologic complete response) and an excellent prognosis[164]. TNBC patients with drug resistant tumors following neoadjuvant therapy have a high incidence of tumor recurrence and a poorer survival. Image-guided neoadjuvant therapy using theranostic nanoparticles will allow for the selection of more potent therapeutics for individual patients while reducing systemic toxicity. Additionally, the integration of image-guided and targeted therapy using theranostic nanoparticles offers the possibility of reduction of tumor burdens of un-resectable pancreatic cancers, including over 50% of pancreatic cancer patients with locally advanced diseases[165], for potentially curative surgery. Optical image-guided surgery enables for complete removal of drug resistant tumors in those patients. Therefore, success in the development of targeted theranostic nanoparticles and innovative imaging approaches has the potential to change the paradigm of future clinical management of cancer patients.

The importance of theranostics in cancer therapy has promoted rapid advances in the development of various types of theranostic nanoparticles.

Current State of the Art

The importance of theranostics in cancer therapy has promoted rapid advances in the development of various types of theranostic nanoparticles. However, challenges in the development of such a class of multifunctional nanoparticles are well recognized. As a drug carrier, it is necessary to select nanomaterials that are biodegradable with low toxicity even after repeated administrations at high doses. It requires high drug loading and conditional drug release in tumor cells. Production of strong and lasting imaging signals is also required. Active targeting to cell surface receptors highly expressed in tumor cells is critical for increasing not only drug delivery into tumor tissues, but also into tumor cells by endocytosis. Theranostic nanoparticles targeting multiple cell types in the tumor, such as tumor endothelial cells, stromal fibroblasts and macrophages, and tumor cells have been shown to enhance intratumoral delivery of targeted nanoparticles[166]. Examples of the cellular receptors that are highly expressed in tumor stromal and tumor cells are uPAR, IGF-1R, folate receptor, and integrin αvβ3. Several examples of cellular receptors that are highly expressed in tumor cells include EGFR, HER2, MUC1, and CEA.

Theranostic nanoparticles have been produced by conjugation and encapsulation of radiotracers to nanoparticles for PET imaging or gadolinium for MRI[167]. Those approaches are used for converting liposomal, polymeric, silica, and dendrimer nanoparticles into theranostic agents. PET/CT detects targeted delivery of radioisotope labeled nanoparticles with high sensitivity. However, repeated administrations of large amounts of radioactive agents and exposure to high doses of ionizing radiation in combination with CT imaging are the major concerns. Relatively short half-lives of radioisotopes require the theranostic nanoparticles to be administrated into the patients in a short time after labeling with radiotracers. This also makes it difficult to monitor therapeutic responses, which often take days or weeks.

Near infrared (NIR) fluorescent dye conjugated or encapsulated nanoparticles are promising optical imaging probes for image-guided surgery, which represents another theranostic application. The effect of pH-sensitive or protease-activated polymeric nanoparticles carrying NIR dyes on identification of tumor margins for surgical resection has been demonstrated in animal tumor models[168,169]. Results from a recent clinical trial using RGD peptide conjugated ultra-small fluorescent silica nanoparticles labeled with a radiotracer (iodine) showed that it is safe for systemic administration in human melanoma patients and the nanoparticles were cleared through renal excretion[28].

Metallic magnetic iron oxide and gold nanoparticles are commonly used theranostic nanoparticle platforms in preclinical studies. Biodegradable magnetic iron oxide nanoparticle (IONP) with MRI contrast is one of the most promising theranostic nanoparticles for clinical translation. Therapeutic agents are conjugated to or encapsulated in the surface coating of the nanoparticles. Targeted theranostic IONPs have been developed and their effects on tumor growth and MRI of nanoparticle-drug delivery have been demonstrated in preclinical studies[170–172]. In comparison with other imaging modalities, MRI has imaging depth and high-resolution 3D-imaging capability for interrogation of heterogeneous intratumoral drug distribution. IONPs can serve as both T_1 and T_2 contrast agents depending on the core sizes and MRI scan methods[173–175]. IONPs are relatively stable in the tumor for an appropriate length of time for monitoring tumor responses to therapy by MRI. In combination with clinical contrast enhanced MRI imaging signatures of the early tumor response may be identified. A drawback of MRI is relatively high costs. Further

...MRI has imaging depth and high-resolution 3D-imaging capability for interrogation of heterogeneous intratumoral drug distribution.

improvements of T_1-contrast imaging approaches should increase sensitivity and specificity of detecting small tumor lesions in organs with a low MRI contrast, such as the liver and lung. Targeted IONPs conjugated with NIR dyes can be used for intraoperative detection of drug resistant tumors[166,176].

Theranostic applications of gold nanoparticles have been developed[77,177]. Targeted delivery of gold nanoparticles generates plasmonic photothermal bubbles that promote drug release from nanoparticle drug carriers in the endosome of cells[178]. Although gold-based theranostic nanoparticles have been produced and tested in animal tumor models, there is a concern about its low biodegradability and lack of a well-defined mechanism of clearance following systemic delivery in large therapeutic doses.

A multi-spectral imaging approach using a Raman endoscopic imaging device and tumor targeted surface-enhanced Raman scattering (SERS) gold nanoparticles has been developed for cancer detection and image-guided resection. Feasibility of multiplexed tumor imaging using SERS has been demonstrated in animal tumor models and in excised human colon tissues[179]. Image-guided hyperthermia treatment using NIR signals produced by photosensitizing agents conjugated to metallic nanoparticles has also been tested in animal tumor models[180]. Accumulation of the nanoparticles in tumors allows for image-guided therapy by precisely applying a laser to the tumor sites.

Future Science and Clinical Development

Clinical development of theranostic nanoparticles has to address challenges that are common for all cancer therapeutics and nanoparticle drug delivery systems as well as unique requirements for its dual therapeutic and imaging applications. Research areas that may have the most impact on clinical translations includes: (1) Development of ultra-small and biodegradable nanomaterials with high imaging signal strengths, high drug loading capacity, and conditional drug release ability; (2) Innovative targeting approaches and nanoparticle designs that significantly enhance passive and active targeting for intratumoral drug delivery, avoid non-specific uptake by macrophages, and have the ability of overcoming tumor stromal barrier for improving drug delivery into tumor cells; (3) Combined delivery of potent therapeutic agents for the treatment of drug resistant tumors; and (4) understanding mechanisms of nanoparticle-drug delivery and interactions of targeted theranostic nanoparticles with tumor cells and tumor microenvironment in animal tumor models that are highly relevant to human cancers, such as human patient tissue derived xenograft (PDX) tumor models and transgenic mouse tumor models. Finally, large-scale production of Good Manufacturing Practices grade theranostic nanoparticles for human use will be the major challenge. It requires the production of consistent nanoparticle core and coating, efficiency

in drug loading, and conjugation of large amounts of endotoxin-free and bioactive targeting ligands to the nanoparticles.

With the joint efforts of the NCI Alliance of Nanotechnology for Cancer and investigators at academic institutes and within industry, several advances should come to fruition over the upcoming 5-15 year time frame. In the next 5 years, researchers will complete preclinical studies for 5 to 6 targeted theranostic nanoparticle platforms; File IND applications for 3 to 4 of the above nanoparticles for Phase I clinical trials; and begin 2 phase I clinical trials for image-guided surgery using targeted imaging nanoprobes. Looking further ahead over the next 10 years, researchers will generate 3 to 4 new theranostic nanoparticles and image-guided cancer therapy protocols in Phase 1 clinical trials; 1 to 2 Phase II/III clinical trials using an integrated image-guided and targeted therapeutic clinical protocol for personalized cancer treatment; and receive FDA approval of 1 targeted imaging nanoparticle for image-guided surgery. Even further out over the next 15 years, researchers will complete 1 to 2 Phase 11/III trials; gain FDA approval of 1 theranostic nanoparticle and associated image-guided therapy protocol; and initiate 5 to 6 new clinical trials using theranostic nanoparticles and image-guided treatment protocols.

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Section IV: *In Vitro* Empirical Models to Understand *In Vivo* Response

Nanostructured Materials as Models for Cell Motility and Metastasis

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Introduction

Metastasis, i.e. cancer cells migrating from the primary tumor to a distant site in the body, where secondary tumors develop, is a major contributor to mortality[1]. Despite progress, many questions remain unresolved regarding the mechanisms involved. It is now clear that it is not just the cells, but also their environment - and in particular the dynamic interplay between them - that dictates whether metastasis is likely to occur. Thus, there is a need for well-defined model systems that enable determinants of metastasis to be studied systematically. We summarize recent breakthroughs and future opportunities for nanostructured materials to contribute to this area.

Metastasis, adhesion and migration

Stages of the development of metastases (**Figure 1**) can be summarized as follows: (1) detachment of cancer cells form the primary tumor by reduced adhesion to neighboring cells; (2) invasion through surrounding tissues by clearing the path to allow cell migration; (3) intravasation of cells through the vasculature to enter the bloodstream and remaining in circulation under flow; (4) attachment to endothelial tissue and subsequent extravasation to the secondary site; (5) proliferation and establishment of secondary tumor[2]. Changes in interactions of cells with their environment, typically adhesion and migration, are critical at every step. Adhesion in this context can refer to cell-cell and/or cell-matrix (ECM) interactions. Migration for our purpose can be either adhesion-dependent or -independent, and may involve active matrix degradation by cell-secreted or cell-surface expressed enzymes- typically matrix metalloproteases (MMPs). Interestingly, there is a substantial body of literature focused on the use of model systems to show how biochemical, mechanical and topographical signals in the cell's environment (typically focusing on stem cells[3]) influence cell fate. The development of exactly such in vitro model systems is now gaining pace for cancer metastasis research.

Designed 3D matrices as model systems to study metastasis

Designed nanostructured materials with precisely tunable properties that mimic aspects of the extracellular environment have the potential to lead us to a better understanding of the role that the tumor microenvironment plays in triggering metastasis[4]. It is now well established that 3D models are more relevant to mimic the tumor/metastasis microenvironment *in vivo*[5]. Commonly used matrices are naturally derived, including commercially available 3D culture systems such as Matrigel™, collagen gels or fibroblast-derived matrices. These materials can be informative as model systems- for example, collagen scaffolds were used to study and identify MMP independent migration pathways relevant to metastatic invasion[6]. Recognizing that natural ECM possesses a highly complex 3D organization that dictates function (which is currently impossible to mimic), matrices have been prepared by decellularizing of various tissues in order to preserve the native integrity of ECM and explore its ability to influence metastasis[7]. While effective in certain contexts, these naturally derived materials are unlikely to reveal molecular level understanding of cell-matrix interactions, as natural systems are not fully defined, have variable compositions, cannot be easily tailored and often contain biologically active materials (e.g. growth factors).

A range of synthetic materials have therefore been developed that can serve as a 'blank canvas' upon which bioactive groups can be rationally introduced. Typically, 'base' materials are selected which have seen previous use in biomedical context, such as poly-ethylene glycol (PEG), poly(lactic-co-glycolic acid) (PLGA) and poly-ε-capronolactone. Synthetic peptide-based materials such as commercially available Puramatrix™ are simplistic mimics of the ECM, which allow for cell culture under well-defined conditions. A number of designs of such self-assembling systems have been developed over the years, typically involving building blocks of 8-20 amino acid residues that can be easily functionalized with bioactive peptides. More specifically for the three primary components necessary to study metastatic disease, we discuss the current state-of-the-art for each.

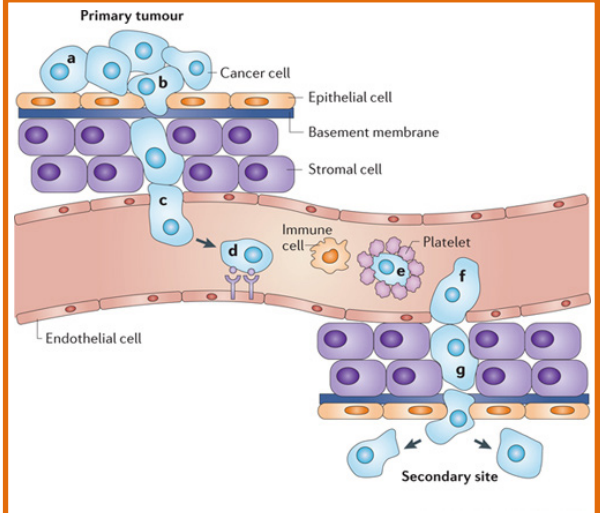

Figure 1. (*Reprinted with permission from Schroeder et al., 2012*)[2].

Adhesion

Adhesion typically involves integrins, the trans-membrane portion of focal adhesions that connect the cytoskeleton inside the cell to the extracellular matrix on the exterior. They bind to bioactive ligands in the surrounding matrix, such as the tri-peptide

RGD (arginine-glycine-aspartic acid). Introduction of RGD ligands into synthetic polymers is now straightforward using well-established polymerization techniques. There is much scope here for the inclusion of different ligands beyond RGD. For example, when using PEG-based hydrogels functionalized with adhesion peptides RGD and YIGSR (the integrin-adhesive regions of fibronectin and laminin, respectively) it was found that cancerous and non-cancerous mammary epithelial cells responded differentially to the adhesion cues[8]. Methods are now also available to introduce bioactive ligands and even entire proteins in precisely defined rations in self-assembled peptide materials[9].

In addition to the concentration of bioactive ligands, their presentation (spatial orientation, clustering) is critical. Questions about spatial organization can be addressed using precisely patterned ligands on surfaces, which may be achieved utilizing block copolymer micellar nanolithography. This approach has been used to demonstrate adhesion dependence with varying distance between RGD ligands, which in turn influenced melanoma cell fate[10]. While this is a 2D approach, the information that is obtained may be used to inform spacing of ligands in 3D constructs. In addition to static presentation of RGD ligands, a number of approaches are now available to dynamically regulate adhesion using switchable RGD ligands (by photolytic uncapping of protected precursors)[11]. These approaches have not yet been used in the context of metastasis and hold great promise in controlling temporal presentation of bioligands.

Migration

Cancer cell migration makes use of a combination of adhesion and enzymatic degradation, involving MMPs and hyaluronases (although non-enzymatic migration is also known[6]). The first designed PEG based gels crosslinked by MMP cleavable peptides were described over a decade ago[12]. Introduction of MMP cleavable linkers in PEG gels was recently used in a metastasis model. A PEG-heparin hydrogel was described that mimics the tumor angiogenesis microenvironment by incorporating RGD (adhesive), MMP-9 responsive (matrix degradation) and glycosaminoglycan (bioactive building block) motifs to take into account different metastasis characteristics[13].

Stiffness

Matrix stiffness is a known determinant of cell fate[3]. Methods are now available to tune this parameter precisely in PEG based materials as well as synthetic self-assembled peptide structures. An example is the use of collagen coated polyacrylamide hydrogel systems with tunable stiffness to study the metastatic potential through matrix stiffness induced epithelial to mesanchymal transition (indication of cancer cell invasiveness)[14]. The effects of

bio-adhesion and matrix mechanics could be investigated separately by varying either the cross-link density or ligand concentration in a gel that also included MMP degradable linkers. Results were shown to be similar to that observed in matrigel, demonstrating that key cell behaviors can be accurately mimicked in fully synthetic gels[15].

Future aspects and conclusions

We note that designed nanomaterials could be used in conjugation with microfluidics, providing access to confined environments while under flow[16]. This would enable (i) mimicry of extravasation[17]; (ii) development of structures for the efficient capture of circulating tumor cells (CTCs)[18] or (iii) study of the interactions of CTCs with endothelial barriers[19].

Tumors contain a variety of cell types (stromal, immune, in addition to tissue specific cells) so accurate mimicry of the microenvironment would require the presence of mixtures of cells. Key to fully understanding migration and invasion will be the development of microscopy techniques. This could include visualization of the invasive protrusions associated with metastasis e.g. using super-resolution (STED) microscopy. This could be combined with FRET approaches to monitor MMP activity and cell migration in real time.

Looking out 10 years, it is highly likely that researchers will be able to use this information in the clinical translation of nanomaterial based models to new materials based therapies.

Clearly, a wide range of synthetic and natural materials, processing and functionalization methods is currently available to create *ex vivo* models to study aspects of metastasis. What is missing, are fully designed model systems, that could mimic all critical aspects of the tumor microenvironment in a more controlled way, opening up opportunities to rationally and systematically vary environmental factors and discover which ones dominate. Not only are designed nanomaterials likely to provide new insights, they can also inform new therapies. There are tremendous opportunities for nanoscience to design artificial (synthetic) cell-compatible hydrogels as models to study metastatic cancer.

Milestones to address these critical areas that researchers should be able to be achieve over the next 3-10 year time frame include many aspects. In the next 3 years, researchers will be able to develop tunable scaffolds (stiffness, ligand incorporation, degradability) based on self-assembled structures as models to study each step of metastasis; biological findings

will inform materials design and, by close collaboration between cancer experts, chemists, materials scientists and engineers, new models should be developed to investigate specific aspects of metastatic disease; and superresolution fluorescence microscopy to visualize invasion. Looking further ahead over the next 5 years, researchers will be able to deliver specific, optimized matrices for establishment of secondary tumors; and a quantitative comparison of new *in vitro* models with current animal models. Looking out 10 years, it is highly likely that researchers will be able to use this information in the clinical translation of nanomaterial based models to new materials based therapies.

Microfluidic Models to Study Cell Extravasation and Metastasis

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Introduction

Metastatic cancer remains the leading cause of mortality. While there have been considerable advances in the development of new approaches to the treatment of cancer, the control of metastasis is still one of the major challenges[20,21]. Despite its tremendous importance, a fundamental understanding of the processes that constitute the metastatic cascade remains elusive. As a result, there are few therapeutic approaches available to block the various steps of metastasis. Two factors contribute significantly to this glaring deficiency. First, modern animal models of metastatic disease[22,23,24], although responsible for much of what we have learned, provide inadequate insight into the disease process for lack of the ability to image the details of cancer progression, and because of the limited ability to control and monitor the local chemical and mechanical environments. In addition, there the inevitable questions regarding differences in behavior between cells from humans and those from test animals still exist. Second, the existing *in vitro* models using traditional cell culture methods such as well-plate systems and transwell assays[25], are unable to capture many of the key features that regulate the various stages of metastasis. The gap between *in vitro* and *in vivo* models is considerable, and both have severe limitations.

Further contributing to this knowledge gap is the enormous complexity of the metastatic cascade, which consists of multiple steps: local invasion of cells from the primary tumor into the surrounding tissue, entry into the circulation by intravasation, survival and transport via circulation to a remote site, extravasation into the metastatic site, and finally, recolonization (**Figure 2**)[26]. The challenges to producing a realistic *in vitro* model of any of these steps are enormous, yet recent progress in the development of microfluidic assays capable of 3D culture of multiple cell types, some with an intact endothelial monolayer, has given rise to optimism.

In the past several years, considerable progress has been made. This is largely due to projects funded through the new emphasis by the NCI on assay development and the physical aspects of cancer growth and invasion. And, although we are still at the early stages, advances have been impressive.

Current capabilities

Recent progress has resulted from new capabilities in several strategic areas, and advances in microfluidic technologies have enabled many of these. New approaches and models have appeared within the past decade, both in the context of primary tumor and metastasis[25], although for this chapter, we focus attention exclusively on the latter, with an emphasis on extravasation. Microfluidic assays typically consist of multiple channels or regions containing hydrogels with spatial arrangement and dimensions that facilitate chemical and mechanical signaling among various cell types seeded within the interconnected compartments. The goal of these devices is in creating a local microenvironment among the cellular compoents that replicates many aspects of *in vivo* interaction[25]. For some time, it has been possible to culture cells in 3D microenvironments, simulating the extracellular matrix of tissues[27]. Progress in 3D culture subsequently led to numerous studies in cell migration[28] and the culture of tumor spheroids with microvessels[29]. Studies have examined the role of various cytokines, including spatial concentration gradients, on the initiation of dispersion from a tumor, in some cases documenting the cells' transition from an epithelial to mesenchymal state (EMT)[30]. The capability to suspend cells in 3D and to generate gradients of either chemoattractants or hydrostatic pressure across matrix-containing regions has facilitated new studies on 3D migration[31], and the effects of matrix properties[32], other interacting cell types within the matrix[33], and interstitial flows such as exist at the tumor margin or in the vicinity of blood or lymphatic vessels[34].

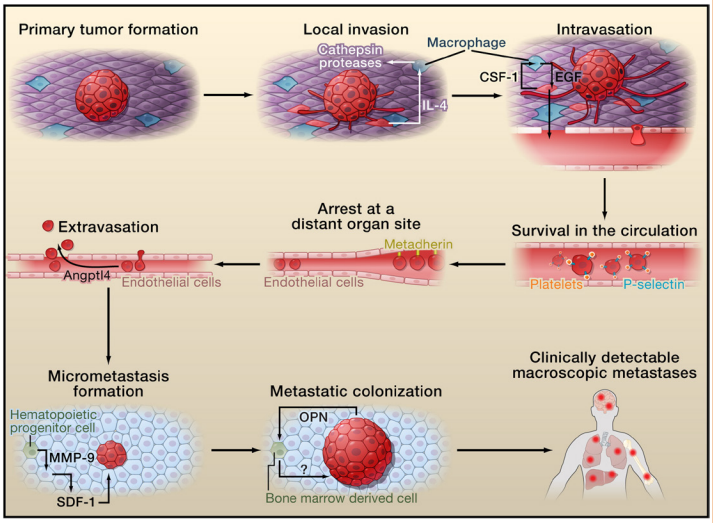

Figure 2. The metastatic cascade. From primary tumor to clinically observable metastases (*Reprinted with permission from Valastyan and Weinberg, 2011*)[26].

When one or more of the channels is lined with an endothelial monolayer, a model for intravasation can be produced by inducing cells seeded into the adjacent matrix to transmigrate into the channel[33]. Similarly, tumor cells introduced into the channel can adhere to the endothelium and transmigrate into the adjacent gel region, mimicking the process of extravasation into the remote host tissue[35]. In some cases, a microvascular network has been established within the gel region that can be perfused with a tumor cell-containing medium, leading to even greater realism in that the tumor cells can then either adhere to or become lodged in the smaller vessels, as they

would in the capillaries of the target organ[36]. Recent studies have also begun to introduce certain organ-specific cells into the matrix, demonstrating that the different rates of extravasation of a particular type of cancer can be replicated within relatively simple *in vitro* systems[37,38].

Future challenges

The use of microfluidics to model metastasis has been rapidly accelerating, but many barriers remain. One of the greatest challenges is to progressively improve the realism of the model while at the same time, keeping it sufficiently simple to use so that these methods remain accessible to the broader cancer research community. In the case of the primary tumor microenvironment, the introduction of cancer associated fibroblasts and tumor associated macrophages, along with the cells of the local microvessels will further enhance the realism of the models. Similarly, the addition of organ-specific stromal cells to models of the remote, metastatic organ will be an important step. Aside from the cellular environment, the matrix properties also need to be carefully considered, since the current choice of type 1 collagen, fibrin or even Matrigel has a significant influence on behavior. Most researchers currently use cell lines, but these should eventually give way to patient-derived tumor cells, and even to the potential for patient-derived induced pluripotent stem (iPS) cells for the creation of more realistic models.

Development of patient-specific models holds the potential for direct clinical application of microfluidics.

One of the greatest current limitations of microfluidics is that the cell numbers and volumes are small, thus making it difficult to employ many of the traditional biochemical or genetic analyses to probe cell function. Methods need to be developed for improved interrogation of the systems (e.g., protein analysis, RNA-seq) including the capability of real-time monitoring of signaling factors or cell function, beyond what can currently be accomplished by imaging.

As researchers expand to model other tissue types, new challenges will emerge. The difficulties in generating a realistic model of the blood-brain barrier are well recognized. Creating models of other organs such as those with high cell densities and intricate internal structural organization – liver, kidney, pancreas – will remain one of the most difficult problems to overcome.

Clinical potential

Development of patient-specific models holds the potential for direct clinical application of microfluidics. Use of iPS cell based systems, patient-derived explants, circulating tumor cells extracted from patient blood, or other similar models will eventually lead to the ability to screen for a therapeutic protocol that is optimized for each patient. In the context of metastasis, this implies an approach that would reduce the tendencies for the primary cancer to spread and recolonize. In addition, improvements in usability and increases in throughput will ultimately facilitate the transition into the clinic, and enable moderate to high throughput screening for combination therapies.

Milestones to address these critical areas that researchers should be able to be achieve over the next 3-10 year time frame include many aspects. In the next 3 years, researchers will have been able to develop many more organ-specific models of metastasis; and patient-specific assays for drug selection based on surgical or biopsy specimens. Looking further ahead over the next 5 years, researchers will be able to deliver multiple organ models on a single chip; high-throughput drug screening platforms; and potentially metastatic cancer-on-a-chip. Looking out 10 years, it is highly likely that researchers will be able to deliver iPS cell based models for patient specific drug screening in the clinic as well as, the really important milestone of, point-of-care assays for diagnosis and treatment planning.

In Vitro Models of the Blood-Brain Barrier

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Introduction

The blood-brain barrier (BBB), or neurovascular unit, is a complex dynamic system responsible for providing nutrients and essential molecules to power the brain while at the same time ensuring that signaling in the brain is not disrupted by fluctuations in chemistry, inflammation, or the entry of toxins or pathogens[39,40]. The blood-brain barrier maintains homeostasis by transducing signals from the vascular system and the brain, and comprises the brain microvascular endothelial cells (BMECs) that form the 600 km of capillaries, the basement membrane, and surrounding pericytes, astrocytes, and neurons. For example, the brain regulates oxygen supply by signaling via astrocytes, which have end-feet that completely surround the capillaries.

The highly specialized endothelial cells that form the lumen of microvessels and capillaries in the brain are characterized by high transendothelial electrical resistance (TEER > 1000 Ω cm^2), low permeability, expression of tight junction proteins (e.g. claudin-5 and occludin), transporters (e.g. LAT-1), and broad spectrum efflux pumps (e.g. P-gp). The two main components of the blood-brain barrier security system are the tight junctions and the efflux pumps. The formation of tight junctions at the boundaries between endothelial cells almost completely prevents paracellular transport into the brain. The array of broad-spectrum efflux pumps, primarily on the luminal surface, returns almost all non-essential small molecules back into circulation. Notable exceptions are caffeine, alcohol, and anesthetics. A consequence of this security system is that it is extremely difficult to deliver drugs to the brain following oral or intravenous administration. More than 98% of small molecule drugs and 100% of large molecule drugs do not cross the blood-brain barrier[41]. As a result, there are many diseases of the brain for which there are no drug treatments. Treatable brain disorders are limited to depression, schizophrenia, chronic pain, and epilepsy.

Recently it has become recognized that many diseases of the brain are associated with disruption of the blood-brain barrier[40]. While the details of these disruptions are not well understood, they most likely result in local increases in permeability that can lead to the disruption of signaling.

Current state of In Vitro BBB Models for Translational Development.

In the pharmaceutical industry and in academic research, the initial screening of drugs for treatment of central nervous system (CNS) diseases is performed using the transwell assay where the permeability of a drug is determined from the amount that crosses a monolayer of type II Madin-Darby Canine Kidney cells (MDCK.II)[42]. These are dog kidney epithelial cells and not human brain endothelial cells although this represents state-of-the-art in the field of pharmaceutical development for CNS drug therapies. MDCK cells transfected to express different efflux pumps can be used to assess whether molecules are substrates for these pumps. In many cases permeability coefficients obtained from the transwell assay are in reasonable agreement with brain perfusion studies in animal models, although the correlation to humans is not well understood. The transendothelial resistance and hence paracellular transport can be decreased by seeding astrocytes and pericytes, or astrocyte extract, in the basolateral compartment of the transwell chamber, highlighting the importance of these cells in the neurovascular unit[43].

A fundamental problem in BBB research is that animal-derived cell lines and immortalized human BMECs do not fully recapitulate the characteristics of human BMECs. For example,

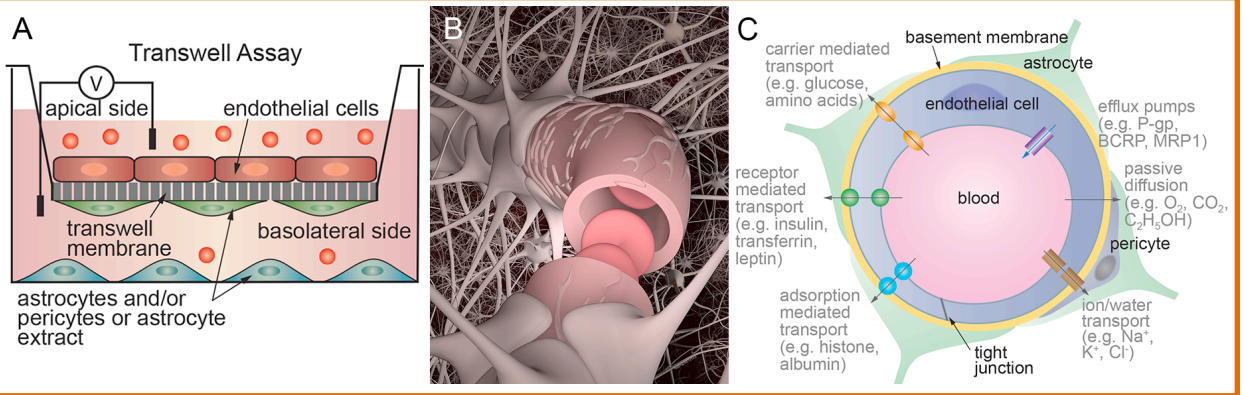

Figure 3. (A) The transwell assay is the standard in vitro tool for determining the permeability of a solute across the blood brain barrier. MDCK cells are widely used since they express tight junction proteins. Paracellular transport can be minimized by seeding astrocytes and/or pericytes, or astrocyte extract in the basolateral chamber. (B) The blood-brain barrier is modulated by functional interactions between brain microvascular endothelial cells, astrocytes, pericytes, and neurons, mediated by the 3D extracellular matrix and basement membrane. Shear flow in the microvessels and the high curvature also play a role in upregulating the blood-brain barrier phenotype. (C) The highly specialized endothelial cells in the brain are characterized by tight junctions that effectively limit paracellular transport, transporters that supply nutrients and other essential molecules, and an array of efflux pumps that return most solutes that cross the luminal membrane back into circulation.

the TEER values of MDCK monolayers are typically around 200 Ω cm^2, almost an order of magnitude lower than physiological values for the brain microvasculature (≈ 2,000 Ω cm^2). The disadvantages of primary hBMECs are that they are not readily available and lose some of their characteristics when cultured *in vitro*. Similarly, the distribution of efflux pump expression varies across species resulting in very different concentrations in the brain. Therefore the lack of physiologically relevant cell lines is a major limitation to advancing the field[44].

The traditional *in vitro* approach to screening drugs for cancer therapy is to assess efficacy by incubating the drug with the relevant cancer cells in culture, and then to assess permeability and brain penetration using the transwell assay (**Figure 3**). In recent work, the transwell assay has been modified to screen drugs for cancer therapy by seeding patient-derived glioma cells in the basolateral compartment and using a live/dead assay to assess efficacy. This approach mimics the pharmacokinetics by exposing the glioma cells to a concentration of the drug that is modulated by blood-brain barrier transport[45].

Recent developments suggest that stem cell engineering may be a solution to the lack of physiological endothelial cells for blood-brain barrier research.

Recent developments suggest that stem cell engineering may be a solution to the lack of physiological endothelial cells for blood-brain barrier research. Human brain microvascular endothelial cells have been derived from induced pluripotent stem cells[46,47]. The derived cells express relevant tight junction proteins, transporters, and efflux pumps, and treatment with retinoic acid results in TEER values in excess of 2,000 Ω cm^2. While more extensive characterization of these derived cells remains to be accomplished, these results could revolutionize the field.

Future of In Vitro BBB Models in Research and Development

The transwell assay provides a relatively high throughput assessment of blood-brain barrier transport, but does not capture the 3D cylindrical geometry of microvessels, the shear stress on the endothelium resulting from blood flow, or the local microenvironment. Engineered microvessel platforms using human cell lines that recapitulate the physiological blood-brain barrier have the potential to rapidly accelerate scientific discovery and the development of new therapies for diseases such as malignant brain cancer[48].

Further advances in stem cell engineering are likely to provide readily available human cell lines for blood-brain barrier research. Methods to harvest patient-derived cells will also be key in developing patient-specific therapies.

The blood-brain barrier remains a major roadblock in delivering drugs to the brain. New strategies for delivering drugs to the brain may include cell penetrating peptides, highjacking transporters (so-called Trojan horse approaches), or transiently increasing the permeability of the blood-brain barrier (e.g. vasomodulators, focused ultrasound, etc.).

The nature of disease-associated disruptions in modulating the local permeability of the blood-brain barrier and their role in disease remain important challenges that will be crucial to developing therapies for many diseases of the central nervous system.

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Section V: Tools and Resources to Accelerate Clinical Translation

Pre-Clinical Characterization of Nanomaterials

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The biggest challenge in preclinical characterization of nanomaterials is the diverse array of skills and knowledge required for a complete understanding of the formulation (**Figure 1**). A multidisciplinary team of experts including chemistry, immunology, toxicology, pharmacokinetics, pathology, and more is often required for an advanced evaluation of a nanomedicine, even and especially at the preclinical stage. Every data analysis and result depends on knowing exactly what the test material comprises. There have been numerous reported cases where toxicity was incorrectly assigned to a nanomaterial when in fact the toxicity stemmed from residual excipients, synthetic byproducts, biological impurities, undetected particle instability, or other anomaly[1–6].

The Nanotechnology Characterization Lab (NCL) was set up in 2004 as part of the NCI's Alliance for Nanotechnology in Cancer program to provide preclinical characterization services to oncology nanomedicine developers around the globe. The NCL staffs experts in a variety of fields who provide critical insight to organizations pursuing nanomedicine translation, but may not have the wide-ranging expertise or resources required for translational advancement. Having characterized more than 650 nanomaterial samples from nearly 100 different organizations, the NCL has had a unique opportunity to observe nanomaterial characterization challenges, including how the field has progressed over the years and insight into what lies ahead.

Challenges in Chemistry

It has been widely established that a nanomaterial's physical and chemical properties directly influence a variety of biological performances, including biodistribution, clearance, and immunotoxicity[7–10]. Therefore, a thorough characterization of these parameters is paramount to ensuring safe *in vivo* administration of the material. With this realization, the depth of routine physicochemical characterization performed on nanomaterials has increased dramatically. The recognition of the unequivocal importance of characterization and consistency is arguably the most significant advancement in this field.

The challenges associated with nanomaterial physicochemical characterization have shifted over the last decade. Initially, researchers grappled with proper ways to assess size, charge, or composition, including which measurement technique was most suited and what the most appropriate measurement conditions were. Now it is well accepted that materials should be analyzed by multiple orthogonal analytical techniques and under the appropriate biologically relevant conditions. However, with the evolution of more advanced nanotechnologies, new challenges in characterization are arising. One challenge at the forefront of physicochemical characterization of nanomaterials is surface analysis. It is imperative to know whether the surface ligands are covalently attached or simply physisorbed, which would allow their premature dissociation from the formulation. Furthermore, the density / coverage of the surface and the orientation and accessibility of the ligand(s) can also be important biological factors. As the number of surface modifications increases, so will the complexity in characterization. This is a particularly challenging area because techniques developed for one type of nanomaterial (e.g., liposomes) will not necessarily work for others (e.g., metals). Having realized the importance of surface properties for biological performance, there will be considerable advancements in tools to evaluate surface properties over the next few years[11]. Our laboratories and others have already begun to invest significant resources into this area.

Resources for scale-up and GMP manufacture of nanomedicines remain as another critical area of need for future development. The NCL is continually asked for advice on where to go for scale-up and / or GMP production services. There are limited establishments with the capabilities to meet this increasing demand for late-stage preclinical synthesis of complex nanomedicines. National efforts are underway now to address this critical gap in translation.

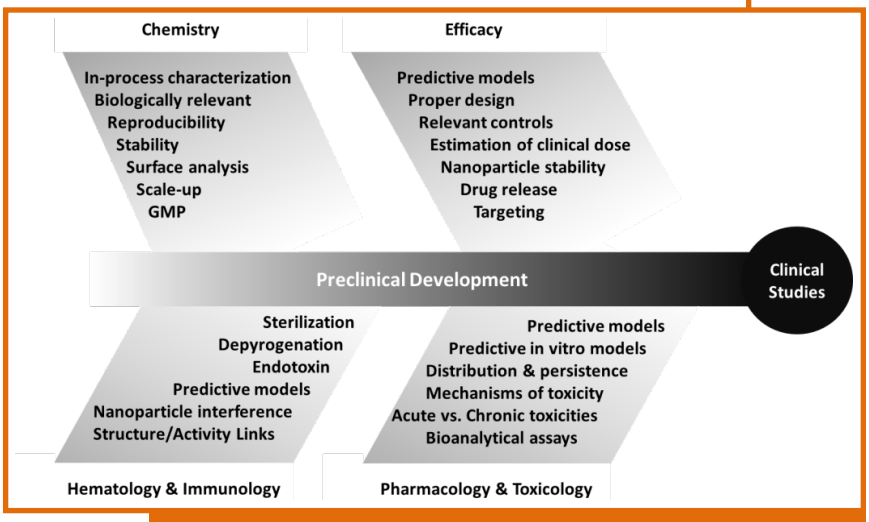

Figure 1. Challenges in Preclinical Characterization of Nanomedicines. Preclinical characterization of nanomedicines requires analysis in a variety of fields, each of which has their own set of challenges. Some of the most significant challenges associated with chemistry, immunology, efficacy and pharmacology/toxicology are noted.

Challenges in Immunology

Although, there has been increasingly more effort put into the early immunological evaluation of nanomaterials, immunology continues to be an underappreciated area during the preclinical stage. Structure-activity relationship studies have been an integral part of the early understanding of nanoparticle immunological influences. The association of nanoparticle physicochemical traits to immunotoxicities has afforded a significant knowledgebase to which the field needs to continue to build upon. However, many challenges associated with immunological evaluation of nanomaterials still remain, including sterility, sterilization, depyrogenation, biological contaminants (e.g., endotoxin and b-glucan), and accuracy and predictability of *in vitro* and *in vivo* methods.

Endotoxin detection and quantification is an area many researchers continue to struggle with. Nanoparticles are notorious for interfering with many of the traditional immunology assays, especially endotoxin quantification assays. A significant amount of research has been published on identifying and circumventing this interference, particularly as related to endotoxin, but educational efforts in this area need to continue[12–18]. Many researchers often avoid endotoxin evaluation until late in their preclinical development. This can be a costly oversight. Not only can the identification and elimination of the contamination source be expensive and time consuming, high endotoxin levels could adversely affect data interpretation.

Predictive *in vitro* and *in vivo* models for evaluating immunotoxicology continue to be one of the most important aspects of nanoparticle immunological characterization. Common immunological and hematological reactions to nanoparticles include hemolysis, complement activation, thrombogenicity, and cytokine storm. Many of these toxicities can be detected using *in vitro* assays, some of which are known to be predictive of corresponding *in vivo* toxicities. For example, a 5% hemolysis rate *in vitro* has been shown to correlate to hematocrit and hemoglobin changes *in vivo*[19]. Other hematotoxic effects, (e.g., myelosuppression) can also be studied *in vitro*, but knowledge of the *in vivo* nanoparticle biodistribution is needed for accurate data interpretation. In such situations, a systematic approach combining both *in vitro* and *in vivo* data is proven to be the most reliable characterization approach.

Future work in the immunological evaluation of nanomaterials will require monitoring the long-term effects of nanoparticles on the immune system. Delayed type reactions are triggered by nanoparticle influences of immune cell function and are often very complex, frequently involving many different cell types. Although specialized *in vitro* immune function tests have been developed and shown to be predictive of *in vivo* toxicities for small

molecules, applicability of these to nanoparticles is challenged by a distinct biodistribution profile and mode of transport across biological barriers. Many of these challenges have been reviewed in detail[20].

Challenges in Efficacy

Without question, the biggest challenge in preclinical assessment of efficacy is the availability of appropriate and predictive animal models. Most efficacy studies are conducted using human cancer cell lines in immune-deficient mouse strains that compromise the plausible interaction between immune cells and nanomaterials *in vivo*. Additionally, these xenograft models are unable to adequately recapitulate the tumor stroma, which plays an important role in tumor progression and can impede drug delivery.

There has been significant progress in the development of more suitable *in vivo* cancer models with the sequencing of cancer genomes and improved molecular biology tools. Several genetically engineered mouse models (GEMMs) have been generated to evaluate tumor growth and progression by utilizing noninvasive imaging modalities. Histopathological analysis of genetically engineered mouse tumors at different stages of disease progression has shown reasonable similarities to human disease. In addition to GEMMs, another focus has been on patient derived xenografts (PDX). PDX models implant human tumor cells in a mouse, providing a more relevant tumor microenvironment and genetic complexity that can better predict clinical outcomes. Future progress in this area will require further refinement of existing tumor models using improved understanding of cancer initiation and progression (e.g., most common genetic predictors of disease progression, signaling pathways, role of tumor stroma).

Experimental design issues also often plague *in vivo* efficacy analysis. Because of the cost of *in vivo* animal studies, it is not uncommon for researchers to forego some needed controls or preliminary analyses. For example, it may be necessary to run several small scale preliminary experiments to gain a better understanding of the maximum tolerated dose (MTD), nanoparticle stability, or drug release *in vivo*. Lack of the adequate controls is another common omission. A good efficacy evaluation should test materials at their respective MTDs and include controls of the platform, current standard of care, and the non-targeted particle where applicable.

Challenges in Pharmacology & Toxicology

Similar challenges exist for preclinical pharmacology and toxicology testing as with preclinical efficacy studies—the availability of appropriate models and proper experimental design. Development of predictive *in vitro* and *in vivo* models of toxicity would be big advancements

in the pharmacological and toxicological understanding of nanomaterials. There are differences in the mononuclear phagocytic system (MPS) between the animal species utilized that could affect accurate prediction of pharmacology and toxicology in humans. There have already been significant improvements in the development of bioanalytical assays in this area. For example, novel methods for analysis of drug release in biological matrix have allowed for a better understanding of nanoparticle stability, tendency for aggregation, drug release, and quantification of encapsulated and unencapsulated drug fractions.

Acute toxicities of nanomaterials are being well studied now; however, long-term chronic toxicities associated with nanomaterials should be further explored and will be an area of future development for this field. A better understanding of the mechanisms of nanomaterial toxicity (e.g., oxidative-stress, lysosomal dysfunction, inflammation) will aid these efforts, and research is ongoing now towards this goal. Additionally, bioanalytical challenges such as determination of dose linearity; estimation of clinical dose; and distribution and persistence of nanoparticles in tissues will be critical for the translation nanomedicine.

Preclinical characterization of nanomaterials has shown considerable advancement over the last decade.

Conclusion

Preclinical characterization of nanomaterials has shown considerable advancement over the last decade. Methods are being continually developed and optimized to meet the needs of the evolving complexity of nanomedicines. Detailed nanoparticle surface characterization, predictive immunotoxicity assays, and quantitative evaluation of the encapsulated vs. free drug fractions highlight the growth of this field. Continuing to pursue new methods development as well as conducting research directed at understanding the nano-bio interface will uncover additional relationships between nanoparticle structure and biological activity. This information will be invaluable for devising new strategies for using nanotechnology to improve upon existing pharmaceuticals and deliver novel therapies in the future.

Pharmacokinetics and Pharmacodynamics Characterization of Nanotherapeutics

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Introduction: Complex Pharmacology of Nanoparticles

Major advances in nanoparticles (NPs) have revolutionized drug delivery capabilities over the past decade. They provide numerous advantages, such as greater solubility, duration of exposure, less toxicity and delivery to the site of action over their small molecule counterparts, nevertheless NPs display substantial variability in systemic clearance and distribution, tumor delivery, and pharmacologic effects (efficacy and toxicity)[21]. NP research has historically focused on the development of NP formulations with less emphasis on evaluating the complex pharmacology and biology of NPs, which significantly influences the successful translation of these agents. This report is an overview of factors that affect the pharmacokinetics (PK) and pharmacodynamics (PD) of NPs in preclinical models and patients.

Major advances in nanoparticles have revolutionized drug delivery capabilities over the past decade.

The disposition of NPs is dependent upon the carrier, not the therapeutic entity, until the drug gets released from the carrier[22]. The nomenclature used to describe PK of NPs includes: encapsulated (the drug within or bound to the carrier), released (active drug that gets released from the carrier), and sum total (encapsulated drug plus released drug). After the drug is released from its carrier it is pharmacologically active (unless the released form is a prodrug) and subject to the same routes of metabolism and clearance as the non-carrier form of the drug. The pharmacology of NPs is complex and thus comprehensive PK studies must be performed in order to assess the disposition of encapsulated or released forms of the drug in plasma, tumor and tissues[23]. Considerable inter-patient variability exists in the PK/PD of NPs and appears to be associated with variability in the function of the mononuclear phagocyte system (MPS), which is the primary clearance pathway for NPs[24]. It is difficult to evaluate the factors that affect the PK and PD of NPs in animals and human patients, due to the fact that they are different and thus animal models may not be predictive of the effects displayed in patients[25].

NPs may be taken up by a wide variety of cells in the blood and in tissues; however, it has been discovered that NPs are primarily taken up by circulating monocytes and dendritic cells (DC) in blood, Kupffer cells in the liver, DC in the lymph nodes, and macrophages in the spleen all of which are components of the MPS[26,27]. Uptake mechanisms may occur through different pathways and are often facilitated by the adsorption of opsonins to the NP surface and subsequent phagocytosis by MPS cells. Although, the uptake of NPs by the MPS does appear to be the predominant factor that affects the clearance of NPs from the blood as well as the distribution of NPs to tissue and possibly even the tumor itself. Yet, it is currently unclear if the distribution of NPs from the blood and into tumor and/or tissues occurs by capture (i.e., the NP enters the tissue and then is taken up by the MPS cell) or hijacking (i.e., the MPS cell takes up the NP in the blood and carries it to the tissue)[28]. This complex issue complicates the optimal design of NPs and, moreover, the evaluation of the primary factors that alter NP delivery to solid tumors. **Figure 2** illustrates the complex interaction between NPs and the MPS. The following two sections will discuss, in more detail, these factors with respect to NP PK/PD and subsequent delivery to solid tumors.

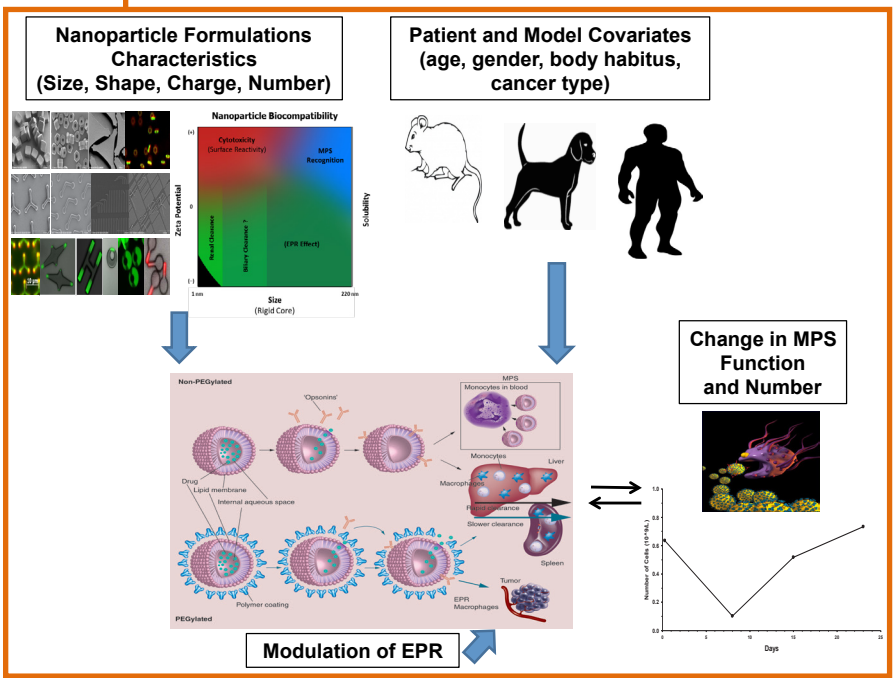

Figure 2. Summary of the complex bi-directional interaction between NPs and MPS. The factors affecting the PK and PD of NPs consist of the interactions between the characteristics of the NP carrier and host related factors. The NP characteristics consist of the size, shape, surface modifications, surface charge, and number of NPs administered. Several mediators (e.g., chemokines) and factors (e.g., age, gender, body habitus, tumor type and location, other drugs) have been reported to alter the PK and PD of NPs in animal models and in patients. The uptake of NPs by the MPS cells may also alter the function and number of MPS cells.

Factors Affecting the PK and PD of Nanoparticles

The factors affecting the PK and PD of NPs consist of the interactions between the characteristics of the NP carrier and host related factors. The NP characteristics consist of the size, shape, surface modifications, surface

charge, and number of NPs administered[22]. In an attempt to minimize opsonization and the subsequent uptake by the MPS, a commonly used strategy, although this is dependent upon the NP material type used, is to conjugate polyethylene glycol (PEG) onto the surface of the NPs. However, the optimal length, amount, and configuration of PEG or other surface coatings is unclear and is unique to each NP carrier[29,30]. There also may be hidden complications of PEGylating NPs. While PEGylation does prolong the circulation of NPs in blood compared to non-PEGylated NPs, the addition of PEG may increase the interpatient variability in the clearance of NPs[31]. Moreover, the number of NPs administered per dose significantly affects the clearance and distribution of NPs[32]. This affect is most likely due the non-linear or saturable uptake of NPs by the MPS.

Several mediators (e.g., chemokines) and factors (e.g., age, gender, body habitus, tumor type and location, other drugs) have been reported to alter the PK and PD of NPs in animal models and in patients[22]. One of the more clinically relevant issues to consider is that the type and location of the tumor may alter the PK of NPs and thus it may not be optimal to administer the same dose of a nanotherapeutic to patients with different types of tumors. The mechanisms of these interactions appear to all involve the MPS. MPS is highly promiscuous and thus takes up all types of particles (e.g., drug carriers, virus, antibodies, bacteria), but appears to have only a limited capacity to take up these particles. Thus, the presence of other natural or man-made particles in the body may alter the PK and PD of NPs. There also appears to be significant differences in the MPS function and PK of NPs across species and across different strains within a species[25,33]. Moreover, the PK and interaction of NPs with the MPS after repeated doses of NPs is opposite in some animal models compared to that of human patients[34,35].

Factors Affecting the Delivery of Nanoparticles to Solid Tumors

While conventional drugs encounter numerous obstacles *en route* to their target, in theory NPs can take advantage of tumor's leaky vasculature to extravasate into tissue via the enhanced permeability and retention effect (EPR)[36]. Furthermore, the poor lymphatic drainage in tumors leads to accumulation of the NPs for prolonged duration, allowing them to release the drug in tumor cells over time. Passive NP targeting exploits the classic features of tumor biology in order to increase exposure of NPs in the tumor.

In theory, EPR is the primary route of NP delivery to tumors (even for active, targeted nanotherapies), but heterogeneity of EPR between tumor types, location of the tumor (e.g., primary versus metastatic, organ, intracranial versus extracranial) and the inability to ensure homogeneous delivery to all regions of the tumor is forcing the need to understand the more fundamental aspects of EPR[37]. Variations in the distribution of blood flow, in vessel

permeability, in microenvironment density, and specific interactions of MPS cells within the tumor may all play an important role in the distribution and penetration of NPs to tumor[38]. It has been reported that the EPR effect is directly influenced by physiologic contributions such as vascular pore dimensions, vascular structure, surrounding stroma[36]. In addition, there appear to be interactions between macrophages and others immune system cells that influence tumor microenvironment factors[28].

In theory, active targeting of NPs may further improve tumor delivery and activity by allowing the NPs to bind to specific cells in tumors using surface-attached ligands capable of recognizing and binding to cells of interest[21]. Targeting strategies have consisted of the use of antibodies, nucleic acids, carbohydrates, peptides, aptamers, and vitamins. It is currently unclear if active targeting of NPs to factors on tumor cells can overcome the inherent barriers associated with the tumor matrix. With the notable exception in the treatment of hematological malignancies, whose use of active targeting strategies would, of course, avoid these issues and barriers[39].

While NPs are able to deliver more drug to solid tumors compared to small molecule drugs, the efficiency (e.g., % of drug) of NPs to penetrate from blood and into the tumor matrix is significantly less than small molecule drugs[38]. Thus, better and more effective NPs that exploit EPR are needed as well as employing methods to evaluate and address the structural and functional hindrances in the tumor microenvironment[40]. However, a major limitation to addressing these issues remains the lack of detailed studies comparing the EPR effect and NP delivery to tumors in preclinical tumor models and human patients.

Future Directions for Understanding PK/PD in Nanotherapeutics

The pharmacology of NPs is highly complex and the factors that alter the PK and PD of NPs, especially the clearance and delivery to solid tumors are highly variable and multifaceted. Future studies need to develop novel *in vivo* and high-throughput screening methods as well as experimental designs that can successfully evaluate how NP PK and PD are affected by the variable nanotherapy schemes, the MPS, and other immunologic factors and conditions. In addition, studies are needed to evaluate the factors influencing and inhibiting the efficient delivery of NPs to tumors as well as how these factors can be overcome[40]. However, before any of these issues can be addressed, we first need to identify and profile these factors in animal models and in patients to identify which preclinical model(s) optimally predict these effects in patients.

Preclinical Animal Models for NP PK and PD

It is currently unclear which animal model most accurately predicts the PK and PD (efficacy and toxicity) of NPs, especially after repeated dosing, in patients. For example, after repeated dosing of some NPs in animal models (e.g., dogs) there is higher clearance of NP after subsequent doses (accelerated blood clearance (ABC)); whereas, in patients the clearance of NPs is reduced after repeated dosing which results in accumulation of drug[34,35]. These differences may be due to differences in MPS function of animal models versus humans. However, the disconnect between ABC in animals and reduced clearance of NPs in human patients does not occur for all NP agents. The lack of consistent changes in clearance after repeated dosing of NPs in animal models and patients further complicates the determination of the optimal models and study design for all NPs. As the type and location of the tumor may also influence the PK and PD of NPs, studies in non-tumor bearing animals may not be as predictive as needed.

Nanoparticle Formulation Characteristics

Theoretical changes made to formulations to enhance or alter the PK and PD of NPs may not readily translate to changes *in vivo* and thus comprehensive *in vivo* studies are needed to evaluate these effects. The optimal size, shape and number of NPs dosed are currently unclear[21,22]. Studies suggest that smaller NPs may be better than larger NPs as a means to overcome potential barriers in solid tumors. However, the specifics of this parameter needs to be defined. Information from other carrier-mediated agents (polymer conjugates; antibody drug conjugates (ADC)) may be used to better define the size parameter of NPs. As the number of NPs dosed appears to be a critical parameter affecting NP PK this suggests that the dose of NPs should be based on the number of NPs administered instead of the mg of drug inside of the NP. It is also unclear if the optimal NP characteristics for the treatment of one type of cancer will be the same for other types of cancers.

Analytical and Biodistribution Studies

Based on the complexity and high variability in the PK of NPs, detailed methods and studies are needed to evaluate the PK of NPs in blood, tumor and tissues[22]. It is critically important to evaluate the PK of the NP encapsulated and released form of NP drugs. This has been evaluated for some NPs in plasma; however, these studies need to be extended to evaluate encapsulated and released drug in tumor and tissues in order to be of any relevance within acute and long-term PK studies. In addition, it may be important to distinguish the exposure of NPs in various cell types within tumor and tissues. It is also becoming apparent that circulating cells in the blood (e.g., MPS cells) act as a depot site for NP agents and thus

NPs may be detectable in circulating MPS cells for a longer period of time than in plasma. Understanding how the uptake of NPs by circulating cells in the blood influences the distribution of NPs to the tumor, liver and spleen, is also important. The ability to measure intracellular exposures (e.g., lysosome or nucleus) of the NP carrier and active-anticancer agent is also critically important for all NPs, but especially important for actively-targeted NPs[41]. In parallel to analytical PK studies, we also need to evaluate the biodistribution of NPs using imaging technologies, as this will be critical to comparing EPR and tumor delivery in animal models and in patients[40].

Interaction Between NPs and the MPS

Studies suggest that there is a bi-directional interaction between the immune system, especially the MPS, and NPs[28]. MPS cells are the primary pathway responsible for the uptake and removal of NPs from blood or plasma. In addition, the interaction or uptake of NPs by the MPS may alter the function of MPS cells and even be cytotoxic to the MPS. However, this bi-directional interaction is highly variable and is dependent upon the characteristics of the NPs and factors that affect MPS function in animal models and in patients[26,27]. The type of tumor, tumor burden and location of the tumor may alter MPS function and the PK and PD of NPs and thus the appropriate dose of NP may not be the same for all malignancies. As a result studies need to be performed to profile the sequence of events and interaction between NPs and the MPS (e.g., subject covariates, opsonization, complement activation, MPS recognition, phagocytic uptake by MPS, NP PK and PD, change in MPS function, cytotoxicity to MPS) after administration of single and repeated doses of NPs in animal models and in patients.

Tumor Delivery of NPs

There is a fundamental need for preclinical tumor models to accurately represent the types of tumors seen in patients in order to conduct informative profiling and developmental studies of NPs. It is thought that metastatic, orthotopic, and GEMM are better options for NP studies than flank tumor xenografts. However, systematic studies of several types of NPs in each tumor model have not been reported and are desperately needed to advance the field of NPs in the treatment of solid tumors. In addition, studies suggest that primary and metastatic intracranial tumors have enhanced delivery of NPs compared with small molecule anticancer agents. It is unclear if the mechanism(s) of the enhanced delivery NPs to intracranial tumors is the same as non-intracranial tumors. Studies of NPs should use valid preclinical tumor models of intracranial and non-intracranial solid tumors in patients to address these issues[22,36].

Historically, investigators have predominantly tried to improve the tumor delivery of NPs by altering the characteristics of the NP carrier. One potential NP factor that needs to be further evaluated is the potential for smaller NPs to achieve greater delivery and distribution throughout the tumor matrix[42,43]. However, changes to the NP carrier may only achieve incremental improvements in the delivery of NPs to tumors due to the inherent barriers within the tumor matrix. Thus, there is a need to develop treatment strategies, regimens, methods and devices to overcome or alter the tumor barriers. These plans could include pharmacological agents or non-invasive treatment modalities. For example, recent approaches to normalize both tumor vasculature and physical forces surrounding vessels have been explored[44]. Co-medications that effect stroma and blood pressure are also known to influence EPR effect. The use of non-invasive methods that apply external beams that alter tumor barriers also holds significant potential benefits[45]. Another fundamental problem with NPs is that, even when they are able to penetrate into tumors, the release of drug from the carrier is relatively low and highly variable[23]. Thus, there is a need to develop treatment strategies to increase the release of drug from the NP and into the tumor matrix.

...researchers could individualize treatment with NPs based on selection of tumors with high EPR, tumor targets and patient specific doses.

Milestones to address these critical areas that researchers should be able to be achieve over the next 5-15 year time frame include many aspects. In the next 5 years, researchers will identify animal models that predict the PK and PD (toxicity and efficacy) of NP agents; identify the factors affecting the tumor delivery and distribution of NPs in intracranial and non-intracranial models; and develop novel analytical methods and platforms to characteristic the pharmacology of NPs as part of high throughput screens, *in vivo* models and in patients. Looking further ahead over the next 10 years, researchers will define the bi-directional interaction between NPs and the MPS, as well as other parts of the immune system, in preclinical models and in patients; optimize NP carrier characteristics to avoid delivery to normal tissues and enhance delivery to intracranial and non-intracranial tumors; and develop treatment strategies, regimens, methods and devices to overcome or alter the tumor barriers to enhance the delivery of NPs to tumors. Looking further ahead over the next 15 years, researchers could individualize treatment with NPs based on selection of tumors with high EPR, tumor targets and patient specific doses.

Informative Assessment on Novel Oncology Therapeutics in Preclinical Cancer Models

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Introduction

It was not until the most recent decade that the tremendous complexity and diversity of molecular mechanisms, which underlie malignant transformation and cancer growth, became recognized. This new found knowledge fueling advanced efforts to dissect the cancerous pathways, pinpoint predictive biomarkers and promising drug targets and propose novel more efficacious therapeutic strategies to rein in the cancer disease[46]. As a significant component of the '*bench-to-bedside*' translational research arsenal, animal models of cancer occupy a capstone position and have become a broadly recognized mainstay in support of the preclinical phase for drug development's critical path[47,48]. In particular, mouse models have been constructed – either entirely surgically, by engrafting tumor cells/fragments into a judiciously chosen type of rodent recipients, or by using more 'cutting-edge' technologies via molecular engineering to edit the mouse genome in order to program selected sets of endogenous murine cells for oncogenic transformation (e.g., for the purpose of developing cancerous lesions of specific nature in pre-determined organs or anatomic locations). Presently, these models, which are reviewed in further details below, are broadly employed within a variety of experimental paradigms. The bulk, of which, are aimed at interrogating candidate therapeutics relative to their bioavailability, toxicity, mechanisms of systemic distribution, excretion and therapeutic action, as well as to their anti-tumor efficacy prior to moving these compounds into costly clinical testing workflows[49–51]. Such step-wise strategy has proven itself advantageous in preserving strained resources available to drug developers, while increasing scale and throughput of therapeutic testing; avoiding costly mistakes while mitigating the emotional burden of treating cancer patients; and, ultimately, accruing invaluable data to informatively guide clinical decisions in cancer disease management.

Patient-Derived Xenograft Models

Recognizing the heterogeneity and cellular complexity of cancer and the concomitant ability to reproduce the individual aspects of diverse malignancies in animal models is of critical importance for directing an informative preclinical assessment. This is of particular importance for evaluation of targeted and pathway-specific therapeutics, which display

efficacy only within a limited subset of the cancer patient population (e.g., that feature the appropriate molecular signature(s) of disease). Furthermore, individual (and not infrequently highly similar histo-morphologically) tumors may display acquired drug resistance to standard-of-care and first-line therapeutics; which mandates further evaluation of molecular content of the resistant disease's portion, followed by application of advanced next generation cancer therapeutics and/or combinatorial treatment regimens. With the purpose of attacking multiple components of the pro-oncogenic environment, which triggered the acquired resistance to mono-therapeutic intervention, in the first place. Last, many particularly aggressive tumor types reveal the notorious intra-tumoral heterogeneity, as evidenced by the presence in the same tumor mass of distinct sub-populations of transformed cells, all driven by divergent combinations of oncogenic drivers. This heterogeneity represents yet another tremendous challenge for selection of the most efficacious and durable therapeutic treatment available.

As such, patient-derived xenograft (PDX) models are constructed by grafting freshly dissected cancerous tissue (e.g., gained during tumor de-bulking surgeries or via diagnostic biopsies) either subcutaneously or orthotopically into carefully selected immunocompromised recipient mice. These can be reliably generated with a high take rate from a variety of tumor types[52–54]. Moreover, recent advances in the PDX modeling field have afforded preclinical drug developers the ability to derive models from metastatic or relapsed cancerous lesions as well as cancerous cells that have been deposited via tumor exfoliation or invasive growth into either ascitic fluid or blood circulation (e.g., circulating tumor cells)[55,56].

Among the myriad of substantial benefits PDX models' offer for preclinical

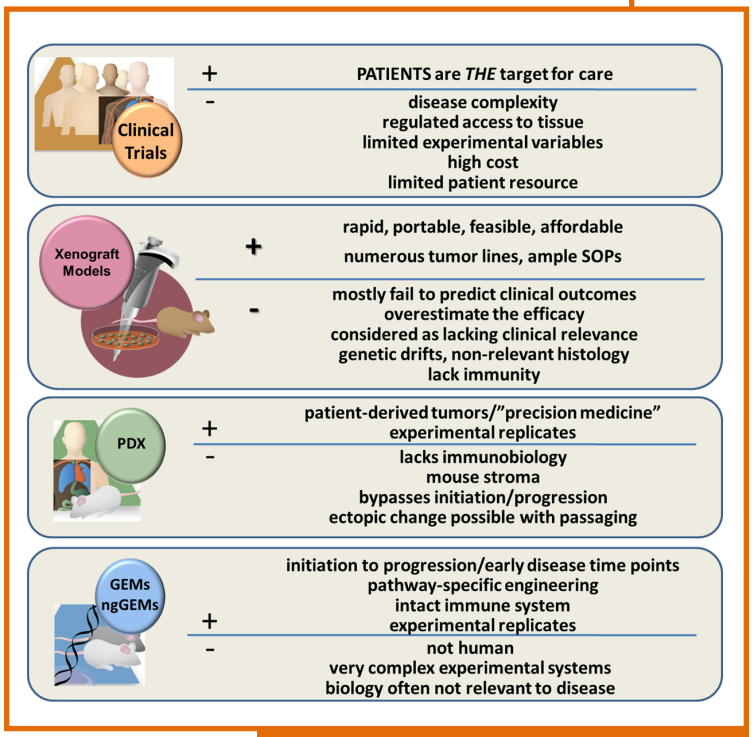

Figure 3. Comparative summary of cancer model types currently employed in preclinical evaluation vs. the clinical trials framework for oncology drug assessment. Various human-in-mouse grafted, mouse-in-mouse grafted and autochthonous/*de novo* models offer benefits for translational experimentation. All the while, featuring drawbacks limiting their applications and justifying integrated options of preclinical assessment in multiple relevant models.

assessment that should be highlighted when compared to the conventional established cell line-derived xenografts include, better preservation of original tumors' mutagenomes; the ability to mimic minimal residual and metastatic disease phases; and a faithful resemblance of therapeutic responses *vis-a-vis* those observed in parental tumors. Furthermore, the PDX models reveal histopathologic patterns and biomarker expression signatures closely approximating those of donor tumors. Also, they allow interactions between stroma or other tumor microenvironment components and the transformed tumor cells to be observed. Despite these advantages in employing PDX models for preclinical evaluation, several shortcomings should be mentioned limiting application of these models for broader use as a uniform testing platform. Mice bearing primary grafts of clinically obtained tissue specimens are immunocompromised – albeit efforts are underway in multiple organizations to reconstitute PDX recipient mice with a functional human immune system – thus largely excluding applications of PDX animals in the assessment of therapeutic strategies pursuing anti-tumor vaccination or activation of tumor immune surveillance mechanisms (e.g., immunomodulatory therapies). Furthermore, gradual passaging of PDX tumors, required to expand the pool of graft-bearing animals available for preclinical experimentation, is prone to substantial genetic and epigenetic drift, which is documented for several types of clinical malignancies. This is due to the fact that, although initially abundant at early passages, human stroma undergoes gradual replacement by its murine counterpart. This has the effect of disrupting the physiologic integrity of the tumor-stroma interaction and/or attenuating the signaling mechanisms required for sustained proliferation. The end result for the model is a misinterpretation of drug efficacy. Despite these challenges, as evidenced by rapidly growing interest and investments from multiple drug development organizations, PDX models have proven themselves as a superior predictive preclinical testing resource and are expected to gain further attention within the community of preclinical oncology experts.

Genetically Engineered Mouse Models

Genetically engineered mouse models (GEMMs), in the context of testing scientific hypotheses, have been extensively vetted as a strategy to elucidate a variety of biological mysteries, which range from developmental biology to mechanistic foundation of clinically challenging ailments. Albeit, it was not until recently when the GEMMs of oncogenic maladies started earning a widespread recognition as a predictive platform for assessment of cancer treatment options and discovery of novel diagnostic signatures, disease biomarkers, and promising drug targets. This could perhaps be best justified by the inherent complexity of cancer GEMMs, not infrequently requiring management of multi-allelic mouse inter-crosses and/or entailing implementation of tedious technologically complex workflows (e.g., inducing carcinogenesis by surgical application of infectious agents, monitoring tumor progression *in situ* via sophisticated imaging techniques, or statistically assessing

the whole gamut of disease histo-pathologic, cellular and molecular outcomes). However, once characterized and validated, the advantages of employing cancer-specific GEMMs for preclinical assessment are numerous. GEMMs provide virtually the only available experimental setting for cancer modeling that affords the cancer biologist and oncologists to monitor dynamics of autochthonous tumors from initiation through to late stage progression and metastatic spread. All the while, simultaneously capturing the disease's stochastic nature, molecular heterogeneity, and tumor-microenvironment interactions. Pending successful humanization of PDX models, the GEMM is, so far, the only experimental system featuring the presence of the fully intact immune system, an indispensable prerequisite for testing immunomodulatory therapies and anti-cancer vaccination strategies. Such models can be precisely engineered to activate a selected set of oncogenic drivers in a predefined cell sub-population or type, in the desired anatomic location. Finally, GEMMs could mimic important facets of cancer such as acquired drug resistance, incidence of minimal residual or metastatic disease, genomic instability, and heterogeneity. Although serving as a platform for numerous variables and multiple preclinical testing paradigms, genetically engineered mice remain undoubtedly the most laborious and expertise demanding preclinical asset. Of which, the application of GEMMs can be further limited by inconsistency in disease appearance, replicability, penetrance and latency, availability of robust colony management infrastructure, and the particular high-throughput options for genotyping and *in vivo* imaging. As a result, several dedicated and integrated Centers have been established. These Centers are tasked with developing optimized tractable strategies for preclinical assessment in GEMMs aimed at addressing these and other challenges impeding the broad application of GEMMs for preclinical drug development in oncology and other fields (e.g., autoimmune and neurodegenerative disorders). Such organizations are, not only expected to act as pivotal points of preclinical expertise, but are structured to offer contractual or partnership support to third parties as well as to be the hubs that disseminate best practices, optimized SOP's, and other resources. With the end goal of facilitating the application of cancer GEMMs for basic and translational purposes.

Non-Germline GEM and Syngeneic GEM-Derived Allograft Models

Despite the undeniable advantages GEMMs present for the preclinical drug evaluation arena; reaching the experimental throughput to match demand of drug developers and cancer translational biologists remains a formidable challenge. This is further amplified, today, by an almost exponential expansion of drug discovery pipelines propelling the demand for more robust preclinical assessment. This is particularly true for multiple promising and physiologically relevant models that display prolonged latency (e.g., in excess of one year from cancer disease initiation to detectable tumor), low penetrance, or significant attrition due to inconsistent or ectopic cancer incidence. A collection of

novel experimental approaches to model cancer disease in a more expedient, practical, flexible, standardized and ultimately cost-conscious way, designated non-germline GEMMs (ngGEMMs), has recently emerged and is gaining rapid adoption in both reputable academic labs and drug development organizations[57]. For example in one of the ngGEMM techniques, conventional GEMMs are bred to obtain preimplantation embryos that are converted into pluripotent embryonic stem (ES) cells, *ex vivo*, which contain the complete combination of desired oncogenic alleles (usually engineered as inducible mutations)[58]. The resultant ES cells undergo extensive genetic and karyotypic characterization prior to being employed for the production of chimeric animals according to well-established embryologic procedures. Such strategies afford the scalable, low cost maintenance of very broad portfolios of GEMMs to enable large synchronized experimental cohorts while simultaneously eliminating the need for costly step-wise interbreeding of multiple alleles and concomitant high volume genotyping. The end result is the models' improved clinical relevance[59]. Furthermore, in chimeric – but not in conventionally bred – models, a progeny of ES cells, genetically programmed for cancerous transformation, are intercalated into the hosts' embryo-derived tissue that lacks genetic alteration. Accordingly, this develops into non-pathogenic surrounding anatomic structures. This is to the contrary of oncogenic processes happening in tissues of conventionally bred animals, by which broad activation of oncogenic events in the entire target cellular subset or even whole tissue (e.g., the genetic field effect) result in either multiple "coalescing" lesions, not amenable to consistent longitudinal monitoring, or gives rise to overly aggressive tumors, limiting the therapeutic window beyond practicality. Some recently employed strategies utilizing modified ES-based chimeric ngGEMMs, have been used to rapidly assess systemically (i.e., in the context of the actual cancer disease) the biologic impact(s) of potential disease modifiers or putative drug target genes via targeted alteration of its expression in ES cells (e.g., using RNAi or CRISPR/Cas9 technologies) and subsequent tests of carcinogenicity *in vivo*[60]. The chimeric ngGEMM production technique carries only a few potential pitfalls that stem from intrinsic epigenetic instability of the pluripotent stem cells, risks of acquiring additional ectopic mutagenesis events, or undergoing loss of pluripotency in the course of ES passaging.

Yet another type of ngGEMM preclinical resource is referred to as mouse-in-mouse transplantation, or GEM-derived allograft (GDA), models. Construction of GDA animals entails dissection of cancerous tissues (either primary tumor or metastatic lesions, or even isolation of bloodborne CTC cells from murine circulation) and subsequent re-introduction of these cells – either as a dissociated single cell suspension, or as subcutaneously or orthotopically tissue fragments, – into a recipient mouse of identical genetic background[61,62]. Such syngeneic host animals, similar to conventional genetically engineered mice, harbor a fully intact immune system and thus are applicable for both investigation of

the immuno-oncology interface in cancer as well as testing of relevant IMT therapeutics. These GDA mice are generally characterized by a higher consistency and associated reproducibility in tumor appearance and histology, as well as shortened timeframe from implantation to development of enrollment-grade tumors ready for preclinical experimentation[63,64]. The dissociated cells derived from primary lesions can furthermore be genetically manipulated *ex vivo*, by established transfection or transduction techniques to, for example, visualize the grafted tumor or its derivative secondary metastatic lesions via expression of tracer markers such as fluorescent GFP/RFP proteins. Similar elegant approaches could be further extended to rapidly interrogate the functional implications of a suspected tumor modifier or candidate drugs' target genes with respect to their carcinogenic potential and/or sensitivity vs. resistance to pharmacologic challenges. This would be simply achieved via manipulating their expression level in tumor cells that will be subsequently tested in the GDA mice *in vivo*. **Figure 3** summarizes several of the aforementioned model types, also comparing them to conventional cell line-based xenograft models in a "strengths-weaknesses" format.

...efforts are underway in several organizations to assemble the proficient resources to advance the preclinical arena towards consolidated expertise in cancer disease modeling.

Conclusions and Future Directions: Integrated Strategies for Informative Preclinical Assessment in Predictive Animal Models

A common belief shared by a majority of the mouse modeling experts suggests that there is no "ideal" or "perfect", one-size-fits-all cancer model type. Or more specifically, that no single strategy of engineering the oncologic disease in mice will allow unambiguous and adequately granular recapitulation of all aspects of clinical malignances to facilitate straightforward predictions of disease progression path or deduction of unequivocally failure-proof treatment plans. To the contrary, an integrated multidisciplinary approach enabling simultaneous assessment of multi-dimensional data sets gathered from different cancer models that are subject to a battery of experimental assays presents itself as the most promising avenue in guiding clinical development and is strongly advocated for by preclinical science professionals. Although challenges still persist in identifying the best-fit robust, while sufficiently reproducible and portable, experimental frameworks. And more importantly, frameworks satisfying the unmet need criteria of the oncology field and attuned to current rigorous trends in precision medicine. Luckily, efforts are underway in several

organizations to assemble the proficient resources to advance the preclinical arena towards consolidated expertise in cancer disease modeling. The ultimate package of deliverables from such coordinated activities (e.g., pursued at the NCI Center for Advanced Preclinical Research, see https://ccr.cancer.gov/capr-about for further information) is anticipated to include collections of best practices and standard operating procedures; information on optimized materials, reagents, instrumental base, partnership business models and intellectual property mechanisms; and access to integrated enterprise quality information systems designed to accumulate, warehouse, evaluate, share and disseminate the full spectrum of preclinical data from multiple sources. But above all, such initiatives will offer tutelage and access (and whenever applicable or justified, sponsorship) to experimentally validated portfolios of preclinical modeling resources. Resources, of which, have been carefully selected to support flexible testing for the variety of novel diagnostic approaches, disease outcome monitoring and assessment methodologies, or improved oncology therapeutics. It is also both reasonable and enticing to argue that the current and projected progress in application of translational cancer models for preclinical drug development will galvanize and pave the way for collinear efforts in other clinical arenas – such as neurodegenerative or cardiovascular diseases, inflammation, and autoimmunity – to produce a similar toolkit of methodologies that explore relevant preclinical murine models for devising better treatment options.

...such initiatives will offer tutelage and access to experimentally validated portfolios of preclinical modeling resources.

Multiscale Modeling and Simulation to Guide Rational Nanomaterials Design

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Over the last decade, new nanomaterials, devices and systems have been developed for the diagnosis, imaging and treatment of multiple malignancies[21,65,66]. Nanoparticles with different geometrical and physico-chemical properties have been engineered, loaded with multiple agents, and systemically administered for the detection and treatment of primary and metastatic tumors[67,68]; nano/micro-fluidic chips have been presented for the rapid screening of potential medications and for the identification of cancer biomarkers[69,70]; and miniaturized devices have been designed for molecular imaging on patient-derived histological samples[71]. Although most of these nano-systems are developed following rather empirical approaches, mathematical modeling and computer simulation, over multiple biophysical scales, are crucial in understanding their *in vivo* behavior and optimizing their performance for clinical translation. As computational sciences have already had a profound impact across multiple disciplines of science and technology development, 'Computational Nanomedicine' could have an equally pervasive impact in our ability to rationally engineer novel and more efficient nanostructures, nanodevices, and nanomaterials for biomedical applications. Current efforts and future perspective in this field are discussed briefly below and in order of biophysical scale, from large to small.

Whole-animal scale modeling.

Multi-compartment mathematical models are now extensively used to understand, predict and compare, the *in vivo* pharmacokinetics (PK) of therapeutic and imaging agents[72]. In particular, based on anatomical and biological information, these models divide the whole-body in multiple compartments, which are interconnected via specific transport and adsorption parameters. Since PK models have been successfully applied for estimating the organ-specific absorption, distribution, and excretion of systemically injected small molecules; similar approaches are now being established for the biodistribution of nanoparticles (NPs). However, the predictive power of these PK models is still quite limited by empiricism and the lack of mechanistic information on the organ-specific deposition and sequestration of NPs.

Most recently, compartment-based models have been adopted for predicting the blood concentration of cancer biomarkers[73]. These models are extremely relevant to early cancer detection and aim at elucidating the correlation between blood biomarker concentration

and tumor size. Unfortunately, clinical data are not generally available to address such a question, thus this is an area where mathematical modeling can be helpful. Specifically, using a one-compartment model integrated with a conventional tumor growth law, it was possible to estimate the blood concentration of tumor biomarkers over time (**Figure 4**). Based on published data on ovarian carcinoma and considering CA125 as a tumor biomarker, the model computed that 8 years are required in order to detect a continuously growing malignant mass with the currently available clinical tools. These computational models clearly emphasize the need for developing more sensitive detection techniques, but also imply that increases to the blood concentration of biomarkers for facilitating earlier detection are necessitated[74].

Tumor and single-organ scale modeling.

Sophisticated multi-scale and multi-physics computational models have been developed for predicting the response of malignant masses to different treatments, including molecular and nano-based therapies as well as radiation and thermal ablation interventions[75]. These models have similarly been used for understanding and optimizing the vascular transport and tumor accumulation of NPs[76,77]. In particular, using an immersed finite element method, the vascular distribution of NPs was studied in whole blood (**Figure 5**). These computer simulations, supported by experimental intravital microscopy data, demonstrated that small NPs (≤ 100 nm) tend to distribute quite randomly within capillaries without interacting with red blood cells. Inversely, large NPs (> 500 nm) preferentially accumulate next to the vessel walls, in a size-dependent manner. This data suggests that sub-micron particles could be more efficiently employed for targeting the diseased vasculature as compared to conventional 100 nm NPs, whose tumor accumulation is primarily driven by the Enhanced Permeability and Retention (EPR) effect. Still focusing on the vascular deposition of NPs, computational models have been developed to predict the accumulation of systemically injected NPs in the tumor neovasculature[77]. By combining a mesoscale model for the vascular adhesion of NPs with a multi-dimensional tumor growth model, it was predicted that the fraction of NPs accumulating in the malignant tissue depends only on the vascularity. Additionally, it was observed that a moderate NP affinity for the tumor endothelium provided the optimal balance between spatial distribution and absolute tumoritropic accumulation. Clearly, this is another example where multi-scale

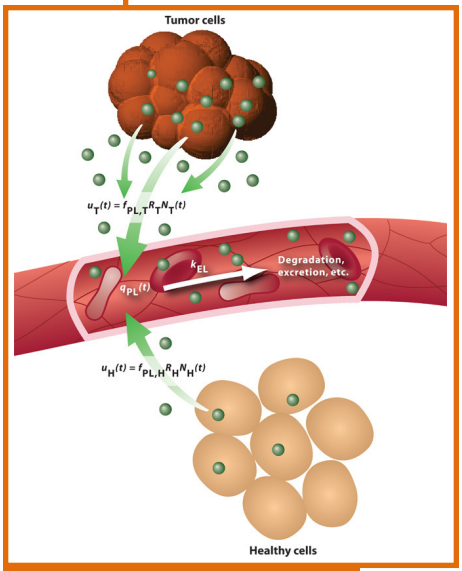

Figure 4. One-compartment model for plasma biomarker kinetics (*Reprinted with permission from Hori and Gambhir, 2011*)[73].

and multi-physics mathematical modeling provides input for rationally engineering NPs with enhanced tumoritropic accumulation.

Computational models can also be used to directly compare the therapeutic efficacy of a single bolus injection of drug molecules with an equivalent dose administered via NPs[78]. By modeling the interplay between mass transport in the microvasculature and blood perfusion in the extravascular volume, computer simulation allowed prediction of interstitial drug concentrations, rates of metabolization, and fractions of cell killing over time. These studies concluded that, for an equivalent injected dose, nano-based treatments ensure higher intratumor drug accumulation and longer exposure times as compared to single bolus injections, thus resulting in higher apoptotic indexes.

Cell and single nanoparticle scale models

Mathematical modeling has been fundamental in elucidating the biophysical mechanisms regulating NP transport dynamics within the vasculature and via internalization into cells[80]. For instance in vascular adhesion, numerical simulations suggested that oblate spheroidal particles would more avidly adhere to the vessel walls as compared to spherical particles of identical volume[81]. Also, mathematical models demonstrated that NP size and shape play a crucial role in modulating cellular endocytosis[82,83]. More recently, computational models for NP cell uptake and drug release were developed to characterize the multi-drug resistance in cancer cells[84]. Supported by experimental evidence, these models revealed that NP-mediated delivery increases both the total concentration and temporal exposure of chemotherapeutic molecules to the target cells. As a consequence, the respective IC_{50} values were improved upon as compared to free drug molecules.

Mathematical models can also be directly used to improve the performance of nanomaterials. For instance, by using molecular dynamics simulation, the diffusion of molecules within nanoporous structures, around nanoparticles, and proteins can be studied (**Figure 6**). Following this approach, the magnetic resonance imaging performance of mesoporous particles loaded with iron oxide NPs and Gd-macromolecules was predicted and optimized for future clinical use[79].

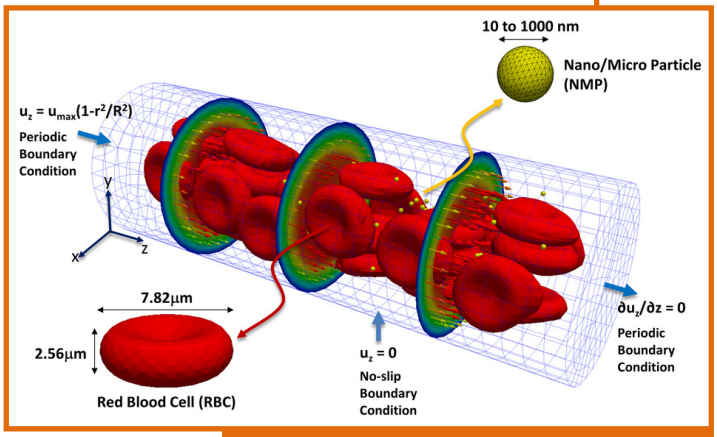

Figure 5. Modeling the transport of NPs into whole blood (*Reprinted with permission from Lee et al, 2013*)[76].

Future perspectives

'*Computational Nanomedicine*' could play a major role in facilitating and accelerating the clinical translation of nanotechnologies and in enabling what is often referred to as precision medicine. At the individual NP level, molecular dynamics simulation can be used to engineer NPs with new architectures enhancing the loading efficiency of drug molecules and contrast agents. This will allow us to reduce the injected doses and limit potential side effects; to improve upon imaging contrast agents for early disease detection; and enable combination therapies (i.e., polypharmacy) to be more rapidly correlated to efficacy. At the cell scale, mathematical models are needed to elucidate the role of thermal ablation therapies and mechanical stresses on cell proliferation and drug resistance. At the organ level, more sophisticated models of tumor growth. Those which account for the spatio-temporal heterogeneity of malignancies, occurrence of *de novo* and acquired drug resistance, presence of tumor initiating cells, and tissue deformability, known to modulate cell growth and migration, will have to be developed. The integration of cell scale and tumor growth models will help us designing new intervention strategies, where diseased cells and tumor microenvironment are coupled for synergistic and efficient targeting. Finally, more efforts should be devoted in developing truly multi-physics and multi-scale computational PK models for predicting patient-specific biodistribution of NPs. These mechanistic PK models should be derived by the hierarchical integration of cell/organ level mesoscopic models with conventional schemes for pharmacokinetic analyses. In this effort, the contribution of multi-modal imaging data will be crucial in the validation phase as well as in the actual clinical utilization for acquiring patient-specific information to be fed back into the computational models. In a near future, mechanistic PK models will help doctors to identify *a priori* the optimal *4S* – size, shape, surface properties and mechanical stiffness – NP properties for maximizing tumor accumulation; and the proper combination of therapeutic agents for eradicating the disease in each individual patient, allowing for eventual realization of 'precision medicine.'

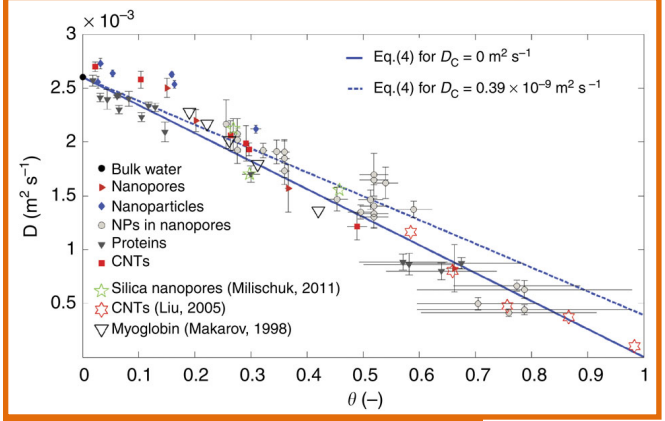

Figure 6. Molecular dynamics representation of a silicon nanopore containing iron oxide nanoparticles, a single walled carbon nanotube, a green fluorescence protein (top). Correlation between the diffusion coefficient of water molecules *D* and a geometrical parameter *Θ* (*Reprinted with permission from Chiavazzo et al, 2014*)[79].

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Section VI: Commercialization of Nano-Products for Cancer

Commercialization of Cancer Nanomedicines: Opportunity and Challenges

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Chemotherapeutics in Cancer Therapy

The treatment of cancer remains an ever-growing problem. In developed countries, the most common approach to treating solid tumors, in particular, starts with surgical resection followed by chemotherapy and/or radiotherapy. Such a clinical treatment strategy, requiring sophisticated hospitals with sophisticated staff, equipment and supplies, which are quite costly. For the developing nations of the world, this approach may be an insurmountable economic challenge. And, the efficacy of this approach has not resulted in a dramatic improvement in overall survival rates for most cancers[1].

Using nanoparticles to deliver potent anti-cancer agents to solid tumors, which represent 85% of all cancers reported annually, has the potential to change this paradigm, and potentially change patient outcomes. As solid tumors grow, whether primary or metastatic cancer, new blood vessels grow to support that growth. These new blood vessels are leaky with fenestrations ranging in size from 0.2-1.2 μm^2. This unique biology provides an ideal opportunity for systemically administered nanoparticle-based medicines (nanomedicines), ranging in size from 10-100 nm, to target tumors by exiting the circulation through these fenestrations, potentially resulting in improved biodistribution, bioavailability, safety and efficacy. In effect, the leaky tumor neovasculature argues that solid tumors should only be treated, prior to surgery, *in situ* with nanomedicines, taking advantage of this unique biology and potentially improving the therapeutic index of potent anti-cancer drugs. Recognizing this therapeutic opportunity is the clinical rationale for changing the current cancer treatment paradigm for the vast majority of solid tumors from a surgery first protocol, to medical treatment first.

If nanomedicines are effective in significantly reducing or eliminating cancers, making subsequent surgeries less complex or unnecessary, then this treatment regimen is a clear opportunity for the pharmaceutical industry to help reduce healthcare costs worldwide. Such a public health strategy might effectively improve patient outcomes for the largest number of cancer patients. And, the potential role nanomedicines might play in this paradigm shift, worldwide, represents a major motivating factor for biotechnology

and pharmaceutical companies to seriously explore the clinical development of cancer nanomedicines.

Since the tumor neovasculature is inherently leaky, irrespective of cancer type or disease stage, this biology may be used again and again in its treatment. So, from the perspective of biotechnology and pharmaceutical companies, treating cancer as a chronic medical disease that requires periodic nanomedicine treatments to control/suppress recurrent disease is an added economic incentive to develop nanoparticle-based cancer medicines.

Design of Cancer Nanomedicines

However, the leaky tumor neovasculature is both an opportunity and a challenge for nanoparticle-based medicines. As noted above, the opportunity exists for nanomedicines smaller than 100 nm to passively exit the circulation and remain in the tumor interstitial space, the "enhanced permeability and retention" (EPR) effect. But, is the EPR effect sufficient for the delivery of cancer killing drugs? Comparative data have shown that inclusion of a tumor targeting ligand that binds to a cell surface receptor reduces the time for a nanomedicine to reach a solid tumor from hours to minutes[3]. Consequently, in the design of new nanomedicines for commercialization having a tumor-targeting ligand needs to be considered.

...the opportunity exists for nanomedicines smaller than 100 nm to passively exit the circulation and remain in the tumor interstitial space...

Conversely, a challenge that the leaky tumor neovasculature creates for systemically administered cancer therapeutics, including nanomedicines, is that other similar or smaller-sized blood components also leak into the tumor interstitial space, creating an interstitial pressure gradient in tumors, where the fluid pressure inside the tumor is greater than it is outside the tumor[4]. This high interstitial fluid pressure (IFP) creates a physical barrier, preventing systemic cancer treatments, such as nanomedicines, from reaching their target, the cancer cells.

Clinically, the effect of destroying the high tumor IFP has been most dramatically seen in patients with in-transit melanoma or sarcoma[5]. Using hyperthermic limb perfusion to locally treat these patients first with a vascular disrupting agent, which destroys the high tumor IFP, followed by chemotherapy, has, on average, been reported to result in an 85% complete local response. In effect, this regional limb perfusion protocol eliminates this physical barrier, enabling follow-on chemotherapy to reach its target and kill the cancer cells.

By design, if a nanomedicine is able to destroy tumor blood vessels, then, using the tumor targeting mechanisms noted above, the systemic administration of a nanomedicine to a cancer patient prior to surgery could eliminate the high tumor IFP. With this added mechanism of action, such nanomedicines might have the greatest potential of achieving the high response rates seen with regional limb perfusion. Consequently, incorporating an agent capable of destroying the high tumor IFP should also be considered when creating cancer nanomedicines for systemic treatment of solid tumors.

Looking to the future of creating commercializable cancer nanomedicines, some critical first steps in design and manufacture need to be considered. For example, translation of a nanotechnology-based research concept into a commercial nanomedicine product requires that thought be given to the biocompatibility of the material comprising the nanomedicine platform, the therapeutic payload (ideally a new drug entity), the immunogenicity of the resultant nanomedicine, the ability to actively target tumors and attack cancer cells, the metabolism and elimination of the material comprising the nanomedicine platform, and the ability to scale-up the nanomedicine manufacturing process to commercial lot sizes in a current good manufacturing process (cGMP) facility. And, the resultant product must be stable, with a two-year shelf life at a minimum. Without a clear understanding of these issues, as well as patent protection of the accompanying intellectual property, the translation of a nanotechnology-based drug concept into a nanomedicine product might never be achieved.

Regulatory and Financial Hurdles to Commercialization

Many of the issues noted above must be satisfactorily addressed in the *Investigative New Drug (IND) application* that is required by the Food and Drug Administration (FDA) to initiate human clinical testing. And for nanomedicines specifically, the Chemistry, Manufacturing and Controls (CMC) section of the IND is quite critical in that the Sponsor must fully explain the composition of the new drug, how the nanomedicine is formulated, its stability under various conditions that might approximate its use, and the analytical tests used to interrogate the final drug product and its components. Providing this critical data is a challenge for new nanomedicines, and being sure that the data meet the requirements of the FDA for new product registration and sale is not guaranteed. And, such uncertainty is often perceived as a risk for pharmaceutical companies and for investors, such as venture capital companies that oftentimes provide the necessary capital to develop new technologies.

Such uncertainty stems in part from the fact that the FDA has not issued specific guidance or analytical benchmarks that all nanomedicines must achieve. In fact, the FDA has

maintained that the current procedures for new drug testing and evaluation sufficiently cover the development of nanomedicines[6]. In addition, current FDA policy states that each nanomedicine should be reviewed and evaluated on a case-by-case basis, similar to other drugs in clinical development.

Herein lies the conundrum for the development of new nanomedicines. Developers of nanomedicines typically want as few regulatory hurdles as possible to allow for maximum creativity and flexibility, while large pharmaceutical companies, who usually have the expertise and resources for later stage drug development and commercialization, want as much specificity as possible about the regulatory requirements for final drug product approval to better estimate their financial commitment/exposure in bringing a new nanomedicine to market.

Each element needs to be optimized to create a new nanomedicine product formulation that can be commercialized.

To help overcome this obstacle, nanomedicine stakeholders need to create a nanomedicine development matrix to streamline optimization of the final drug product. For example, to create the ideal ratio of each nanomedicine component to insure that the new formulation has all the functionality needed for optimal safety and efficacy may require that each new nanomedicine formulation be tested directly *in vivo* for pharmacokinetics and biodistribution, looking for longer half-life of the therapeutic payload and specific organ/tissue targeting, respectively, initially skipping over both *in vitro* and *ex vivo* testing. By going from new formulation to in vivo testing, back and forth, might provide the quickest, most cost-effective strategy to define a successful nanomedicine formulation.

The Opportunity

Therefore, to truly improve the outcome of patients with solid tumors, as an example, the ideal cancer nanomedicine needs to: avoid immediate immune detection by the mononuclear phagocyte system (MPS); carry a novel active pharmaceutical ingredient (API), not re-package an already approved drug; target tumors by both passive (EPR) and active (receptor binding) mechanisms; disrupt the high IFP in tumors; and be manufactured using a scalable, robust, reproducible, and cost-effective process. Each element needs to be optimized to create a new nanomedicine product formulation that can be commercialized. And, commercialization most likely requires that patents be issued domestically and internationally to protect the composition of the final drug product, its method of production and its use.

Academia and industry need to seize the opportunity that nanotechnology-based medicines present for changing the cancer treatment paradigm and the outcome for patients with solid tumors; not focusing on perceived challenges and risks, but on the potential to dramatically impact cancer care for the world's population by treating cancer patients with safe and effective cancer nanomedicines prior to surgery, even for resectable tumors.

Manufacturing Challenges of Nano-Products

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Why Bother with a Nanoparticle?

This brief chapter will survey the field of Nano-product manufacturing. First, the term "nano-product" implies that there is some similarity between all things "nano". Outside of the obvious shared dimensional quality, nano-products are actually widely divergent. For this review we will limit ourselves to discussing oncology related nano-particulates and not consider devices fabricated at the nano-scale. Such particles range from simple nano-particulates of pure drug to highly structured multicomponent particles and delivery systems. The term includes solid structures, liquid phases and systems that incorporate small and/or large molecules. Further, "nano" is really nothing new and, on a commercial level, we have been manipulating nanostructures for a very long time. The difference is that now we are more conscious of it and have a much greater ability to measure both what we are doing and its impact.

Because of the many possible nanoparticle structures, they can serve a host of roles in oncology therapeutics and vaccines. On a mechanical level, nano-structures can be biomimetic and engineered to be site selective. Chemically, behaviors such as solubility, reactivity and affinity can be manipulated. Further, nanoparticles can be co-formulated with other technologies imparting even greater flexibility. Ultimately, nanoparticle drug constructs can provide a variety of performance benefits that increase effectiveness: improved pharmacokinetics, improved safety profiles, improved stability, and targeted delivery.

As an indication of the activity in this space, in a Jan 17, 2013 article[7] on nanomedicine products that are approved or in various stages of clinical study by the European Medicines Evaluation Agency were summarized. Of the 247 products noted, there were a total of 33 approved drugs at the time of the study. In the oncology space, **Table 1** gives a list of approved nanotechnology-based oncology products from a publication on cancer nanomedicines[8].

Table 1: Nanotechnology Oncology Products Approved as of 2014				
Product	***Nanoplatform/ agent***	***Indication***	***Status***	***Company***
Doxil	PEGylated liposome/ doxorubicin HCl	Ovarian cancer	Approved 11/17/1995 FDA50718	Ortho Biotech (acquired by JNJ)
Myocet	Non-PEGylated liposome/ doxorubicin HCl	Metastatic breast cancer	Approved in Europe and Canada, in combination with cyclophosphamide	Teva Pharma B.V.
DaunoXome	Lipid encapsulation of daunorubicin citrate	First-line treatment for advanced HIV-associated Kaposi's sarcoma	Approved in USA	Galen Ltd
ThermoDox	Heat activated liposomal encapsulation of doxorubicin	Breast cancer, primary liver cancer	In Phase III in USA	Celsion
Abraxane	Nanoparticulate albumin/paclitaxel	Various cancers	Approved 1/7/2005 FDA21660	Celgene
Rexin-G	Targeting protein tagged phospholipid/ microRNA122	Sarcoma, osteosarcoma, pancreatic cancer, and other solid tumors	Fully approved in Philippines in 2007, Phase III Fast Track Designation, Orphan Drug Status Acquired in USA	Epeius Biotechnologies Corp
Oncaspar	PEGylated asparaginase	Acute lymphoblastic leukemia	Approved 6/24/2006	Sigma-Tau Pharmaceuticals
Resovist	Iron oxide nanoparticles coated with carboxydextran	Liver/spleen lesion imaging	Approved 2001 for European market	Bayer Schering Pharma AG
Feridex	Iron oxide nanoparticles coated with dextran	Liver/spleen lesion imaging	Approved in 1996 by FDA	Berlex Laboratories
Endorem	Iron Oxide nanoparticles coated with dextran	Liver/spleen lesion imaging	Approved in Europe	Guerbet
DepoCyt	Liposome/ cytarabine	Lymphomatous meningitis	Approved in USA	Sigma-Tau Pharmaceuticals

Scale Up Principles

The progression of a formulation manufacturing process from the benchtop to GMP is a critical step for all pharmaceuticals – it is also often very challenging. It involves the simultaneous increase in scale and the maturation of the various unit operations. Even if a formulation is very effective biologically, if it can't be reproducibly scaled to commercially relevant quantities, it is of questionable value. Therefore, from the beginning of the product

development process one needs to keep in mind eventual commercialization, i.e., using off-the-shelf manufacturing equipment if possible, using excipients that are available in the appropriate grade and generally recognized as safe (GRAS), and using processes that have a high probability of being scaled. Deviations from these are of course possible and are, in fact, quite common but their impact needs to be evaluated in real-time. In addition to safety, efficacy and quality, cost needs to be considered. Clearly, the lower the cost the greater number of people that can be potentially helped although subsidies of one kind or another can mitigate even truly expensive therapies. Also one needs to keep in mind that the infrastructure to handle highly potent compounds, as are typically required for oncology agents, is relatively scarce and that this, coupled with the need for GMP and special expertise around nanoparticles, limits the number of available commercial resources. So, early identification and involvement of a scaling partner is key. For academic groups this typically means partnering with a commercial CDMO. For commercial developers, recruitment of internal resources or an appropriate sub-contractor is needed. Either way, early transfer of the product production function will speed development and greatly enhance later chances of success.

The QBD[9] (quality by design) approach is the organizing framework under which the pharmaceutical industry now operates. A review of QBD is not appropriate here but, in brief, it is a proactive scientific approach to pharmaceutical development that pivots around the desired product attributes and provides for the establishment of well-defined processes that result in a reproducible product. During the QBD process, CQA's (critical quality attributes) are defined. CQA's are product properties that are key to safe and effective performance - the amount of drug per dose, the rate of dissolution or the sterility of an injectable are typical examples. Operating by QBD principles and using tools such as DOE (design of experiments), a well-run scale up program will progress in scale generally by increments of 10 fold. Going from mg to grams for instance or 100 mL to the liter scale. Scale up not only considers drug product production, but material acquisition, training, filling, packaging, storage, and administration. As one progresses in scale, greater attention should be paid to the equipment and processes and each weighed against their respective commercial viability.

The QBD approach is the organizing framework under which the pharmaceutical industry now operates.

Production methods and product attributes are intimately linked. Two methods of particle size reduction can yield similar size distributions but different polymorphs as a simple example. All data generated in a drug product development effort is potentially part of

the regulatory submission. This includes details on both active pharmaceutical ingredient (API) and drug product production. Some of the performance data, mainly toxicological, is required and is performed under GLP's (Good Laboratory Procedures). The purpose of this requirement is of course to insure, or at least to be able to assess the risk to, the safety of the clinical trial participants. Thus, the product used in that testing absolutely needs to be identical, in all of its CQAs, to the clinical trial materials. For a product composed purely of API, the manufacturing process used for that API is less important since equivalency of the API from one process to another can be established with some certainty. For complex nanoparticles, the situation is less clear-cut. CQA's are sometimes difficult to define early in development and thus the impact of a manufacturing variation likewise becomes difficult to quantify. For this reason, optimally, by the time legally mandated testing is being performed the manufacturing process should be essentially the same as that which will be used for clinical trial material production. In practical terms, generally speaking, this means that the process should be scaled to a clinically relevant degree no less than 12 months from the estimated first-in-human trial. To accomplish this, process rationalization should start, as a rule of thumb, at least two years prior to the first-in-human target date and, ideally, as early as possible. The more complex the product, the earlier rationalization should begin.

While each product will present its own set of challenges, there are some recurring themes. Perhaps the most frequent shortcoming manufacturers encounter in the advancement of therapeutic nanoparticles is a lack of thorough characterization of the product and the identification, to the extent possible, of the CQA's. This requires, among other things, an early emphasis on the appropriate analytical methods, which is something that is frequently neglected. Other common errors include advancing very low yield processes, failure to identify GMP sources of materials, advancing products based on single batch results, using non-scalable production methods, failure to involve regulatory expertise early on, and inadequate consideration of intellectual property constraints.

Characterization

After a therapeutic nanoparticle is identified, the qualities that enable its benefits should be well understood. Scaling a poorly characterized product is a waste of time. Basic properties should all be well documented and can include, among others, particle size, zeta potential, pH, viscosity, encapsulation efficiency, API assay and related substances, dissolution, solid state, binding efficiency and batch-to-batch variability (i.e., reproducibility). As a rule, one should have a basic idea of stability and use different lots of raw materials, if available, to test potential impact, if any. Raw materials that are themselves variable should be evaluated to establish if that variation impacts product success.

Yield

While many if not most newly developed products will have low yields, a commercially viable product must at least have the promise of adequate yields. At first this can be a paper exercise but should become a focus early on.

Sourcing

All materials used in production of products for human use will be required to be made under cGMPs or, in rare instances where GMP materials are not available and the need is compelling, be controlled to a degree that simulates GMP quality. In development, when possible, all materials used should be from GMP suppliers. This does not mean that the materials need be of GMP quality only that equivalent GMP supplies are available. By their nature however, nano-therapeutics will often incorporate unique excipients that are not available under GMP's. While not inherently bad, and potentially necessary, any such material adds a very significant cost, time and regulatory burden to the drug product development path. Educated assumptions as to their impact should be incorporated into the plan so that rational decisions as to their relative value can be made.

...efficacy should ideally be demonstrated multiple times using multiple batches of the therapeutic with proper controls.

Proof-of-Concept

While not actually a scale up issue, advancing thinly documented therapies wastes finite resources. Great scientific advances don't always make great drug products. Prior to dedicating resources to scale up, efficacy should ideally be demonstrated multiple times using multiple batches of the therapeutic with proper controls. As above, characterization is key.

Processes

After initial proof-of-concept, efforts towards using commercially viable processes should be made whenever possible. At the nano-scale, changes in process invariably result in product changes and these may or may not impact performance in a predictable way. In addition to process driven attribute changes, production methods are evaluated as to practicality. As an example, using a precipitation process at 0.1% solids would mean that for every kg of product one would produce 1,000 kg of waste. For a nanoparticle that might only contain

5% of API that translates to 1 kg of API generating 20,000 kg of waste. While potentially possible, this is certainly less than attractive. Early efforts at practical processes are vital.

Regulatory

This encompasses many aspects including, among others, toxicology and manufacturing conditions. Early developers will benefit from having access to regulatory advice to provide an understanding of the regulatory path for the various kinds of products. As an example, for a sterile product, knowledge of the relative overhead of a terminally sterilized product vs. one aseptically produced will greatly aid the developer in their process choices.

Intellectual Property

As of this writing, the US Patent Office is issuing patents with numbers approaching 9 million. Assessing one's own invention against this pool is hard enough but when one also needs to consider API patents, method of use claims and various manufacturing techniques as part of the intellectual property pool to be considered, the job becomes truly daunting. As a practical matter, developers need to be current at least in their field's literature. When approaching advanced preclinical development, involving an IP professional is advisable if the developer is financially capable of doing so.

Manufacturing

As above, nanoparticles encompass a wide variety of structures so there is no one manufacturing system to review. In general, the caveats for manufacturing include those under scale up with the addition of the necessary Quality and cGMP overhead. Independent of the nuances of a specific nano-product, the steps common to all manufacturing efforts include: technology transfer, analytic method validation and process validation. Each of these involve literally dozens of steps themselves and are intimately linked to each other. Listing them as separate efforts is purely for organizational purposes.

...developers need to be current at least in their field's literature.

Technology transfer involves moving the process from the innovators' lab to the manufacturing site. In this author's experience, this is best done during preclinical development. This allows the manufacturer to gain experience with the process and help it mature along a commercially viable path. Usual practice is that decisions around process improvement, packaging, specifications, labeling and final sourcing have not been made at the time of transfer. In the scheme presented in this chapter much of the process development effort is

effectively shifted to the CDMO making that partnering choice even more important. When possible, it is most efficient to have the same partner do both scale up and manufacturing. This saves time and a great deal of money as transferring methods is costly. A good manufacturer will also help insure that the background information needed in regulatory filings is properly assembled and ready for presentation.

Analytical methods evolve from basic-to-advanced following along with the product itself. The term "phase appropriate" is often used to describe this maturation process. The analytical methods insure the quality of the drug product, its consistent behavior, and ultimately its safety. For in-human studies the analytical methods need to be robust and, most developers will state, validated. Certain methods, sterile filtering, do not vary by development stage and needed to be fully validated even for a Phase I. This is for obvious safety reasons: a microbial contaminate in an injection could have catastrophic results. Clarity on analytical method, stage and purpose is critical. As an example, "stability" has a specific meaning from a regulatory perspective: the product has the same physicochemical properties, within predetermined limits, at some time post-manufacture as it did at the time of manufacture. On the other hand, an innovator often views stability as meaning that the product still works (i.e., has the desired biological activity, after some period of time). Both definitions are valuable and awareness of each is needed for an efficient development process.

Pharmaceutical manufacturing is a unique discipline but should not be separated from the development process.

Once the manufacturing process is locked, each unit operation needs to be refined to the point that the manufacturer has confidence in its repeatability. Ideally there is some way to monitor each unit-op to assess its function in real-time although this, referred to Process Analytic Technology (PAT) in QBD terms, is often not feasible in early stage clinical manufacturing. At a minimum, the process as a whole is demonstrated through engineering runs to produce the desired product, meeting the predetermined specifications. Invariably, because deep product production experience is lacking by definition, early clinical production relies heavily on post-production quality testing. Again, this points to the importance of the proper development of analytical methods. For certain types of products various unit operations are actually validated. This is most evident in sterile processes where the product is either produced under aseptic conditions or terminally sterilized. For aseptic production media fills are required. A media fill is a dry run of the entire process in the clean room with thorough microbial sampling of staff, product and facility to demonstrate the processes ability to

produce a sterile product. For terminally sterilized products, as above, the sterilizing process itself is fully validated.

Future Direction for Manufacturing

Pharmaceutical manufacturing is a unique discipline but should not be separated from the development process. Rather, discovery-to-commercialization should be viewed as a continuum with the handoff from one group to another taking place in phases. The basics of nano-based manufacturing are here and established today. The next 5 to 10 years will see incremental improvement in processing capabilities mostly, we believe, in the areas of aseptic handling and throughput. Why? Simply because that is where the acute need is. Along with this will come standardization and dissemination of procedural operations, again driven by regulatory mandates, not the result of any real innovation. The innovation opportunity lies in the emergence of a disruptive change, not to the nano-products themselves but to the method of manufacture. Among other properties, such a manufacturing advance will be …"cheaper, simpler, smaller and …… more convenient to use"[10] and, if history is any indication, it will be the smaller more nimble companies that champion this change and its adoption.

Regulatory Evaluation of Nanotechnology in Diagnostics for Human Use*

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Background

Nanotechnology is a rapidly evolving field that has tremendous potential to advance human health and medicine. Nanomaterials have already been integrated into medical products designed to treat and diagnose serious and life threatening disease[11]. However, as often is the case, people assume that new is better; or what works well in the laboratory will work well, without modification, in a clinical setting. The zealousness to bring the latest and greatest to market, or be the first to publish on a particular topic can be at the expense of generating a high quality, well characterized, final product, which in the case of medical applications risks injury to the end user, i.e., the patient. It is the role of medical product regulation and regulatory agencies worldwide to both protect and promote the public health. United States Law, in the form of the **Federal Food, Drug, and Cosmetic Act of 1938** (the Act) and the **Public Health Service Act of 1944** (the PHS Act) give primary authority to regulate medical products to FDA.

Introduction to Diagnostic Device Regulation

FDA protects the public health by insuring that medical products are safe and effective for their Intended Use. They promote the public health by guaranteeing that the best and most innovative medical products are available to the public.

Products intended to diagnose a disease or condition, whether implantable (such a heart monitor within a pace maker), *in vivo* (such as an electroencephalogram used on a living person) or *in vitro* (using materials collected from a living person such as blood and urine tests) are considered medical devices. Devices are regulated by FDA's Center for Devices and Radiologic Health (CDRH), with a few exceptions[12]. *In Vitro* diagnostic devices (IVDs) are a special category of device with specific labeling requirements[13]. Whether a product is safe and effective is determined partially by risk classification. Depending upon the classification, an appropriate level of review of the scientific, clinical and manufacturing data for the product is applied[14,15].

While exceptions to each rule exist, generally: *Class I* devices are considered low risk and are therefore exempt from FDA review prior to being placed on the market. Manufacturers of these devices are still required to follow several procedures, referred to as General Controls. These include registration of the company with FDA; listing of all medical products the company sells; following current Good Manufacturing Practices (cGMP, known as the Quality System Regulations for devices); establishing a system for handling customer complaints, establishing a system for preventative actions, corrections and corrective actions (CAPA); performing corrections and removals as necessary (recalls); and providing labeling that is complete, truthful and accurate.

Such nanotechnology-containing devices may still be determined to be substantially equivalent to legally marketed devices or exempted from future premarket notifications and FDA review.

Manufacturers of *Class II* (moderate risk) devices are subject to the same General Control procedures as a Class I product, as well as additional Special Control procedures. The Special Controls are procedures designed to mitigate the moderate risks identified with the device. Special Controls include a submission of pre-market notification for FDA review. This procedure is described in FDA guidance documents and under section 510(k) of the Act. *Such applications are referred to by FDA and industry as, a 510(k) submission*. Review is based on a demonstration of substantial equivalence to another legally marketed Class II device, referred to as the predicate. The idea being that if the clinical value of the predicate is established, the manufacturer of a similar device only needs to show that their device is analytically and technically the same as the predicate. Clinical data is generally not required. If the new is found to be substantially equivalent to the predicate device, the 510(k) device is "cleared" for marketing. Manufacturing facilities are inspected after the device has been cleared.

Class III devices are considered the highest risk. Manufacturers of these devices are required to obtain pre-market approval (PMA). Approval of a PMA application generally requires a clinical study and inspection of both the clinical study sites and the site of manufacturing prior to the device coming on the market. Companies are also required to report all changes to device design or manufacturing[14].

Regulation of New Technologies - Nanotechnology

The Agency does not recognize a formal definition for nanotechnology[16,17], but we ask the same question of any new technology that comes into the Agency: Does it affect the safety or effectiveness of the device for its intended use? In general, the presence of a material that has not previously been used in a medical product may raise additional questions/ concerns from regulators. That said, simply adding nanotechnology to a medical device does not necessarily cause it to fall into a different classification than similar marketed Class I or II devices. Such nanotechnology-containing devices may still be determined to be substantially equivalent to legally marketed devices or exempted from future premarket notifications and FDA review.

FDA regulation has evolved over the years and will continue to do so to accommodate new emerging technologies...

If the nanotechnology enables a device to function through different principals than the predicate device, it likely would not be considered substantially equivalent, but the risk of using the new device may still not be considered high. When any new technological characteristic creates a unique device, FDA's *de novo* classification process provides a pathway for a device to be put into Class I or Class II for which general controls or general and special controls provide a reasonable assurance of safety and effectiveness, but for which there is no legally marketed predicate device. For example, special controls for a nanotechnology may reasonably include requirements for well-done physical and physiological characterizations of the new material. Once the nanotechnology-enabled device is classified as Class I or II through the de novo process, similar devices could come to market as exempt devices or by use of the 510(k) pathway, rather than premarket approval.

Combination Products

It has long been a goal of visionaries in the field of nanotechnology to generate a nanomachine that could diagnose, treat and ultimately cure a patient on the cellular level[18,19]. Moving towards such goals, nanotechnology has enabled medical products to develop beyond single mode of action devices into multifunctional platforms performing several functions – such as nanotheranostics that combines therapeutics with diagnostics. Medical products are regulated according to their primary mode of action (PMOA). In the case of products with multiple modes of action, so called combination products, it falls to the FDA's Office of Combination Products to determine whether a product achieves its primary therapeutic benefit from its action as a drug, a biologic product, or a medical device.

Once this determination is made, the regulation of the product will be assigned to the appropriate Center, either CDRH, the Centers for Drug Evaluation and Research (CDER) or Biologics Evaluation and Research (CBER). The Center(s) who have expertise in the additional parts of the combination product are consulted in the review process to insure consistency. For example, contrast agents for MRI are regulated as drugs by CDER while IVD's intended to screen the blood supply are regulated as biologics by CBER. Review of these products may reasonably include consults to MRI and IVD specialists, respectively, and hence involve CDRH. If we envision a potential nanotheranostics product for ex vivo therapy, where tissue may be removed from a patient, manipulated outside of the body, and the re-introduced to the patient, the regulatory framework would likely be related to both the ex vivo biology (regulated by CBER) and the diagnostic device (regulated by CDRH) and potentially CDER depending on the nature of the therapy.

Future Scientific and Clinical Developments

The current regulations, as they stand, provide a sound framework upon which to develop medical products that incorporate nanotechnology. That said, two major factors are found to influence future regulations:

1. The introduction of new technologies in to the medical products realm. FDA has had to deal with smartphones, genetic engineering, personalized medicine and other paradigm shifts in medicine that were precipitated by new scientific discoveries.

2. The behavior of entities marketing medical products. Major shifts in Food and Drug law have occurred because of findings of fraud, corruption, poor quality, false or off-label advertising. These findings, unfortunately, do not usually come to light until after tragedy has struck.

FDA regulation has evolved over the years and will continue to do so to accommodate new emerging technologies, such as nanotechnology, that have the potential to significantly benefit human health and medicine.

Regulatory Evaluation of Nanotechnology in Drug Products*

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In recent years, there has been an increased focus on developing novel drug delivery systems, targeted therapies, and medical devices, including *in vitro* diagnostics, through the use of nanotechnology and nanomaterials. Such focus is translating to an increasing number of submissions for drug products and medical devices to the United States Food and Drug Administration (FDA). Although subject to the same regulatory standards and pathways as any drug or device, unique properties that arise from the small size and large surface area of nanomaterials may lead to additional scientific considerations when following current FDA guidelines and practices.

FDA has not defined the term "nanotechnology" or related terms, given the wide diversity the Agency has seen with these products. FDA has, however, published general guidance on products involving the use of nanotechnology[20]. According to this guidance, when considering whether an FDA-regulated product involves the application of nanotechnology, FDA will ask:

1. Whether a material or end product is engineered to have at least one external dimension, or an internal or surface structure, in the nanoscale range (approximately 1 nm to 100 nm), and

2. Whether a material or end product is engineered to exhibit properties or phenomena, including physical or chemical properties or biological effects, that are attributable to its dimension(s), even if these dimensions fall outside the nanoscale range, up to one micrometer (1,000 nm).

History of Nanotechnology in Drugs and Devices

The Center for Drug Evaluation and Research (CDER) is responsible for reviewing applications for new and generic drugs, new indications for already approved products, and active ingredients and labeling for over-the-counter drugs. CDER reviews each drug product application on its merits, regardless of the presence (or absence) of nanomaterials. CDER has a long history of approving drug products that contain nanomaterials (**Table 2**)[21]. In

recent years, the number of applications to CDER has increased, with over 350 individual applications submitted to date.

Table 2: Representative drug products involving the application of nanotechnology

Platform/Type	*Example*		
	Name	*NDA Approval Year*	*Indication*
Liposome	DOXIL® (Doxorubicin)	1995[a]	Ovarian cancer; AIDS-related Kaposi's Sarcoma; Multiple Myeloma
Inorganic nanoparticle	FERRLECIT® (Sodium ferric gluconate complex)	1999[b]	Iron deficiency anemia in patients with chronic kidney disease (CKD).
Protein nanoparticle	ABRAXANE® (Paclitaxel)	2005	Metastatic breast cancer; Locally advanced or metastatic non-small cell lung cancer (NSCLC); Metastatic adenocarcinoma of the pancreas
Polymer nanoparticle	MACUGEN® (Pegaptanib sodium)	2004	Neovascular (wet) age-related macular degeneration.
Emulsion	RESTASIS® (Cyclosporine)	2002	To increase tear production
Lipid complex	AMPHOTEC® (Amphotericin B)	1996	Invasive aspergillosis
Nanotube	SOMATULINE DEPOT® (Lanreotide acetate)	2007	Acromegalic patients who have had an inadequate response to or cannot be treated with surgery and/or radiotherapy
Nanocrystal	TRICOR® (Fenofibrate) 48mg/145mg tabs	2004[c]	Primary hypercholesterolemia or mixed dyslipidemia; Severe hypertriglyceridemia.
Micelle	TAXOTERE® (Docetaxel)	1996	Breast Cancer; Non-Small Cell Lung Cancer; Hormone Refractory Prostate Cancer; Gastric Adenocarcinoma; Squamous Cell Carcinoma of the Head and Neck Cancer

[a] First ANDA approval in 2013.
[b] First ANDA approval in 2011.
[c] First ANDA approval in 2012.

Nanotechnology was first exploited in "first generation" products of nanocrystals or liposomes, where the drug products were typically reformulations of previously known, often poorly water soluble, drug substances. Nanotechnology was used to increase bioavailability, alter biodistribution, or both. In recent years, a "second generation" of products has begun to emerge, which incorporates more complex structures and functions into the drug formulation (example: drug delivery systems with targeting capabilities).

Medical devices are regulated by FDA's Center for Devices and Radiologic Health (CDRH). Products intended to diagnose a disease or condition, whether implantable *in vivo* (such as

a heart monitor within a pace maker), external *in vivo* (such as an electroencephalogram used on a living person) or *in vitro* (using materials collected from a living person such as blood and urine tests) are considered medical devices. CDRH reviews each medical device application, regardless of the presence (or absence) of nanomaterials, by asking the same question: Is this product safe and effective for its Intended Use. Under the Federal Food, Drug and Cosmetic Act, Code of Federal Regulations (CFR) title 21, 860.3, medical devices are classified into three categories based on risk: class I, class II and class III, often referred to as low, moderate and high risk, respectively. Device classification determines the regulatory pathway and the types of controls to which a medical device may be subject. Although CDRH does not have a long history of clearing/approving medical products that contain nanotechnology, there are a limited number of *in vitro* diagnostics that have been cleared/approved and the current regulations, as they stand, provide a sound framework upon which to regulate such devices.

Review Considerations for Drug Products and Devices Containing Nanomaterials

FDA has multiple guidance's for products involving the application of nanotechnology. These guidance's may be Agency-wide, Center-specific, or even product-specific. **Table 3** lists several of the relevant FDA guidance's involving nanotechnology.

Nanotechnology was used to increase bioavailability, alter biodistribution, or both.

In general, drug product applications contain the following information:

- Description and composition
- Physicochemical characterization
- Description of the manufacturing process and packaging
- Specifications needed for product release
- Analytical methods and validation of these methods used to characterize the drug product
- Stability studies to support an expiration date, or shelf life, and in-use conditions.

Table 3: FDA Guidance on Nanotechnology		
Guidance Category	***Name***	***Weblink***
NANOTECHNOLOGY		
General and cross-cutting topics	Considering Whether an FDA-Regulated Product Involves the Application of Nanotechnology	http://www.fda.gov/regulatoryinformation/guidances/ucm257698.htm
Food	Assessing the Effects of Significant Manufacturing Process Changes, Including Emerging Technologies, on the Safety and Regulatory Status of Food Ingredients and Food Contact Substances, Including Food Ingredients that are Color Additives	http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm300661.htm
Cosmetics	Safety of Nanomaterials in Cosmetic Products	http://www.fda.gov/Cosmetics/GuidanceRegulation/GuidanceDocuments/ucm300886.htm
Animal & Veterinary	Draft Guidance for Industry: Use of Nanomaterials in Food for Animals	http://www.fda.gov/Cosmetics/GuidanceRegulation/GuidanceDocuments/ucm300886.htm
Chemistry, Manufacturing, and Controls (CMC)	Draft Guidance for Industry: Liposome Drug Products Chemistry, Manufacturing and Controls; Human Pharmacokinetics and Bioavailability; and Labelling Documentation	http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070570.pdf
GENERIC DRUG PRODUCTS		
Bioequivalence Recommendations	Draft Guidance on Doxorubicin Hydrochloride	http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM199635.pdf
Bioequivalence Recommendations	Draft Guidance on Amphotericin B	http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM384094.pdf
Bioequivalence Recommendations	Draft Guidance on Verteporfin	http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM384173.pdf
Bioequivalence Recommendations	Draft Guidance on Paclitaxel	http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM320015.pdf
Bioequivalence Recommendations	Draft Guidance on Sodium Ferric Gluconate Complex	http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM358142.pdf
Bioequivalence Recommendations	Draft Guidance on Ferumoxytol	http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM333051.pdf
Bioequivalence Recommendations	Draft Guidance on Iron Sucrose	http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM297630.pdf
Bioequivalence Recommendations	Draft Guidance on Sirolimus	http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM089640.pdf
Bioequivalence Recommendations	Draft Guidance on Paliperidone Palmitate	http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM270384.pdf

The presence of nanomaterials, due to their unique properties, may warrant emphasis on different portions of the review of the drug product. There is a great diversity in drug products containing nanomaterials, ranging from metal colloids to polymeric micelles. Such diversity can make it difficult to apply generalities to all drug products containing nanomaterials. Despite the diversity, some common attributes exist when considering the quality of drug products containing nanomaterials. These include:

- Size and size distribution
- Nanomaterial composition
- Three dimensional structure
- API to nanomaterial ratio
- State of API (e.g., encapsulated, bound, etc.)
- Surface functionalization and state of the surface ligands (if any)
- Surface coating quantitation, density and polydispersity
- Zeta potential or surface charge

In addition, how the characterization of these quality attributes is conducted may vary greatly from one application to another, and is generally more involved than technologies or methods that have been traditionally used for other drug products. Finally, it is generally recognized that orthogonal or complementary methods are needed for key quality attributes of drug products containing nanomaterials due to the high impact of these critical physicochemical properties on the ultimate product performance.

Nanotechnology in medical diagnostics and devices

In general, the presence of a material that has not previously been used in a diagnostic medical device may raise additional questions or concerns from regulators. However, simply adding nanotechnology to a medical device does not necessarily cause it to fall into a different classification than similar marketed Class I or II devices that do not incorporate nanotechnology. Such nanotechnology-containing devices may still be determined to be substantially equivalent to legally marketed devices (called a predicate device) or exempted from future premarket notifications and FDA review.

Nanotechnology may enable medical products to develop beyond a single mode of action into multi-functional platforms performing several functions...

If the nanotechnology enables a device to function through a different principle than the predicate device, it likely would not be considered substantially equivalent to a predicate, but the risk of using the new device may still not be considered high. In such cases, FDA's *de novo* classification process provides a pathway for the device to be put into Class I or Class II. For devices, for which there is no legally marketed predicate device, general controls or general and special controls provide a reasonable assurance of safety and effectiveness. For example, special controls for a nanotechnology may reasonably include requirements for well-done physical and physiological characterizations of the new material. Once the nanotechnology-enabled device is classified as Class I or II through the *de novo* process, it can be used as a predicate for similar devices and these could come to market as exempt devices or by use of the 510(k) pathway, rather than premarket approval (PMA).

Nanotechnology may enable medical products to develop beyond a single mode of action into multi-functional platforms performing several functions – such as nanotheranostics that combines therapeutics with diagnostics. In the case of products with multiple modes of action, so called combination products, it falls to the FDA's Office of Combination Products to determine the primary mode of action (PMOA) of a product. Once this determination is made, the regulation of the product will be assigned to the appropriate Center, either CDRH, CDER or Biologics Evaluation and Research (CBER). The Center(s) who have expertise in the additional parts of the combination product are consulted in the review process to ensure consistency.

Future Regulatory Outlook

The number and complexity of submissions of drug and medical device products containing nanomaterials is expected to increase in the next 5-10 years as the potential of nanotechnology within the medical field is fully realized. Although not treated differently within the regulatory pathway, these drug and medical device products often have different emphasis on parts of the review process due to the specialized properties of the nanomaterials and the product's intended performance (drugs) or use (devices). In either case, an understanding of the scientific basis of the functioning of the nanomaterial within the product, as well as the instrumentation used to characterize it, will assist both applicants and reviewers alike in speeding these products to market.

*Disclaimer: The views presented in these articles do not necessarily reflect those of the Food and Drug Administration.

Section VI: References

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