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Review article

Controlled-release nanotherapeutics: State of translation

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ABSTRACT

This is a review of nanotherapeutic systems, specifically those that exhibit controlled release of the encapsulated bioactive compound. The survey includes the delivery of a range of bioactive compounds, from lipophilic small molecules to hydrophilic proteins and siRNA molecules. The research into enabling sustained delivery of these compounds from nanocarriers has been prolific, but clinical success has been harder to achieve. This is partly because achieving true sustained duration of action over several days is difficult when the carrier dimensions become less than about 400 nm, due to the much shorter diffusion path length compared to micron-sized carrier systems. Other options must be sought to control the efflux of incorporated bioactives, particularly when these bioactives have moderate to high hydrophilicity. A few of these options are discussed critically in this review. We also answer the question: is controlled release needed for nanotherapies? We present the case for controlled release in specific conditions, with two examples from our own work: one for treatment of glaucoma, and the second for inhibition of fibrosis following surgery. The former is sustaining the release of a small-molecule lipophilic drug, while the latter focusses on sustained siRNA delivery.

1. Introduction

It has long been accepted that controlling transport rate of drug from a drug delivery system (DDS) enables the attainment of constant levels of drug in tissue (blood, vitreous humor), while at the same time prolonging the duration of action of the drug. The constant tissue levels of drug is important for drugs (including proteins) that have a narrow therapeutic window: if the constant level is within the therapeutic window, then side effects and drug wastage are minimized. On the other hand, if the therapeutic window is large, then controlled release is useful to simply prolong duration of action: this is sometimes referred to as “sustained delivery”.

Fig. 1 provides examples of controlled/sustained release products in various dosage forms and release durations. Most of the products that claim controlled release of action of drugs have been based on diffusion-control, and on osmotic pumping. In oral drug delivery, where duration of action is limited to 24 h, most of the approved products have been osmotic systems (named OROS® by Alza Corporation) [1]: both hydrophilic and hydrophobic drugs have been delivered to the intestinal epithelium at a constant rate. Some of the best-known products are Procardia XL for nifedipine delivery, and Glucotrol XL for once-a-day glipizide treatment for type 2 diabetics.

The concept of controlled-release was extended to transdermal systems, with the scopolamine patch in the 1970’s (Transderm-Scop®): such a patch used a reservoir/membrane design to deliver the scopolamine at a constant rate across the adhesive onto the skin surface [2]. Subsequently, the same approach was used for transdermal fentanyl (Duragesic®) and nicotine (Nicoderm®). The transdermal approach is limited to a few drugs that readily permeate across skin, below about 500 Da in molar mass, and is limited in duration to 7 days at best.

For extending duration of action beyond a few days, we need implants and microparticles: the former is inserted through a (minimally-invasive) procedure or injected through a needle, while the latter is usually injected directly into tissue (sub-cutaneous, ocular). The 5-year contraceptive system, Norplant®, was the earliest approved implant; subsequently, ocular implants such as Vitrasert (biostable, for delivering ganciclovir for 3 months into vitreous humor), Ozurdex® (bioerodible, delivering dexamethasone for 6 months into vitreous humor) have been developed and approved. For delivering proteins or peptides for long periods, but without a constant release rate, biodegradable microparticles have been used. The earliest approved product was Lupron® Depot for the treatment of endometriosis or prostate

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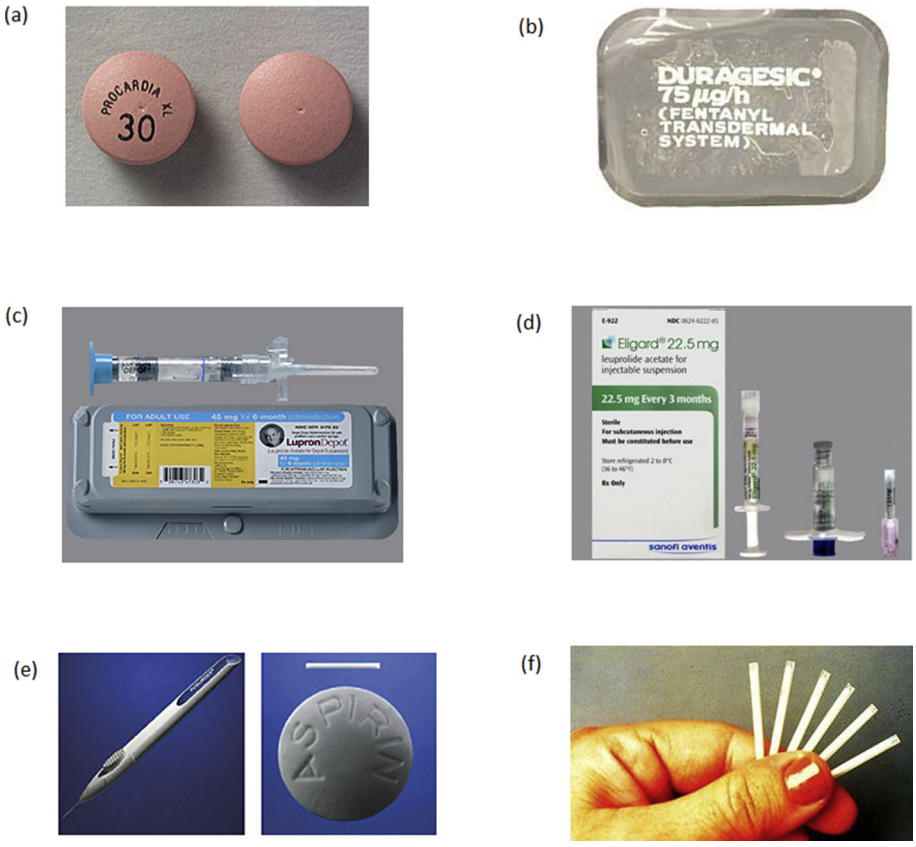

Fig. 1. Examples of controlled/sustained release products in different dosage forms and release durations: (a) Oral (osmotic) CR tablet: ProcardiaXL, releases nifedipine, up to 24 h [4], (b) Transdermal patch: Duragesic®, releases fentanyl (painkiller), up to max 7 days [5], (c) Injectable microparticles: Lupron® Depot, releases leuprolide acetate, up to 1–6 months [6], (d) Injectable (in-situ gelation) depot: Eligard®, releases leuprolide acetate, up to 3–6 months [7], (e) Injectable ocular implant: Ozurdex®, releases dexamethasone, up to 6 months [8], (f) Solid subcutaneous implant: Norplant, releases contraceptive drugs, up to 5 years [9].

cancer. Lupron® Depot delivers leuprolide acetate over periods ranging from 1 to several months, via sub-cutaneous injection. The control of release of drug is predominantly through diffusion with biodegradation contributing at a later stage.

Other concepts for controlling drug release include in situ gelling systems: the earliest approved product in this category was Eligard® which delivered leuprolide acetate for palliative treatment of advanced prostate cancer. The duration of action is about 3 to 6 months, based on dosage. In general, leuprolide acetate is a relatively stable hormone, with a broad therapeutic window. The molecule is not affected by transient exposure to organic solvents unlike other proteins.

One other related concept, similar to implants, is the approved product Gliadel®, which delivers carmustine for post-surgical management of glioblastoma, over 3 weeks. This is a fully-erodible polymer which is a copolymer of sebacic acid and carboxy phenoxy propane, and erodes over 6–8 weeks in vivo [3].

In general, for chronic conditions, controlled-release systems are beneficial in terms of patient compliance, improvement in quality of life for the patients, as well as the cost of intervention. Both small molecules and proteins have been incorporated into microparticulate injectable systems as well as in situ gelling systems. Implanted systems (biostable and bioerodible) have been developed (and approved) mostly to deliver small molecules but not proteins.

2. Controlled-release: moving from “micro” to “nano”

As shown in the previous section, the use of (degradable) microparticles for sustained/controlled drug release has conventionally centered on diffusion-controlled (and/or degradation-controlled) systems.

For diffusion-controlled systems, two different designs were often considered: (i) membrane-reservoir and (ii) matrix systems. Eqs. (1) and (2) have been widely used to estimate the release rate from membrane-reservoir and matrix systems respectively.

$$M(t) = \frac{ADKC_s}{l}t \quad (1)$$

$$M(t) = A\sqrt{2DC_sC_0}\sqrt{t} \quad (2)$$

M(t) is the amount of drug release at time t, A is the surface area of release, D is the drug diffusion coefficient through the polymer carrier, K is partition coefficient, C_s is the drug solubility in the polymer carrier, l is the thickness of the rate-controlling membrane, C_0 is the initial drug loading while t is the duration of release.

Serving as a quick guide, Table 1 presents estimated release durations that could be achieved using microsphere formulations based on the mechanism of diffusion (Eqs. (1) and (2)). Several assumptions used to generate Table 1 include: (i) initial drug loading (C_0) in every formulation is equal to twice the drug solubility limit in the carrier, (ii) where needed, partition coefficient (K) is 1, and (iii) the effect of carrier degradation on diffusivity (D) is not accounted for. The diffusion

Table 1
Estimated drug release duration from diffusion-controlled microspheres vs. nanospheres.

Types of particles	Particle diameter	Carrier design	Diffusion coefficient (cm^2/s)	Membrane thickness	Time to achieve 50% release
Microspheres	100 µm	Membrane – Reservoir	1×10^{-12}	25 µm	48 days
				10 µm	19 days
			1×10^{-10}	25 µm	12 h
				10 µm	5 h
		Matrix	1×10^{-12}	–	8 days
			1×10^{-10}		2 h
Nanospheres	100 nm	Membrane - Reservoir	1×10^{-12}	25 nm	4 s
			1×10^{-10}		0.04 s
		Matrix	1×10^{-12}	–	0.7 s
			1×10^{-10}		0.007 s

coefficients were set at 10^{-10} cm^2/s and 10^{-12} cm^2/s, to estimate their effects on slow release [10].

It is obvious from Table 1 that it is not difficult to tune the release duration from the microspheres. In fact, it is possible to extend drug release duration from a few hours to a few days and up to a few months, simply by changing the carrier design, membrane thickness, diffusivity, size of microspheres, etc. Lupron® Depot, made of PLGA microparticles, is a good example that demonstrates such versatility. Lupron® Depot has a few different formulations to deliver leuprolide acetate from 1 month up to 6 months.

Unfortunately, the same cannot be achieved easily with nano-spheres. The extremely small size of nano-spheres presents a very short diffusion path length that results in rapid release. In fact, relying on diffusion mechanism alone will lead to almost immediate drug offload from the nano-spheres. To look at it another way, sustained release over several days using diffusion control from nano-spheres mandates very low diffusivities ($< < 10^{-12}$ cm^2/s) which are difficult to achieve with materials such as PLGA or nanoliposomes where the bilayer thickness is usually of the order of 5–10 nm.

Hence, it is necessary to think of or employ other rate-controlling mechanisms if one wishes to achieve prolonged or controlled release from nanospheres. Some of these alternative strategies are discussed next.

3. Controlled-release nanotherapeutics

Even though the field of nanomedicine is still relatively young, several review papers that summarize the development of nanotherapeutics in the past 20 years are available [11–13]. These papers summarize various formulations of nanotherapeutics undergoing clinical trials at different stages and report their progress and performance. It is recognized that most of the nanotherapeutics were developed, to a larger extent, to deliver anticancer drugs and, to a lesser extent, to deliver antimicrobial/antibiotic agents or other agents (proteins and nucleic acids). It is also noted that while many formulations are currently undergoing clinical trials, successful clinical translation and regulatory approval is limited.

These reviews on nanotherapeutics provide insights on the observed clinical benefits of using nanocarriers, especially with regard to improving drug accumulation at the target site (such as solid tumors), enhancing drug solubility and decreasing total dosage administration. However, little emphasis has been placed on the presence/absence of mechanisms to sustain or control drug release from nanoformulations. In this paper, we survey various nanoformulations and analyze the mechanisms, if any, that have been employed to delay the premature release of drugs, and to thus sustain the action of the bioactive compound over several days.

How many approved products truly have controlled release in nanotherapeutics? To answer this, we refer to Table 2, which lists several approved Nanomedicine products, ranging from protein-drug conjugates (Abraxane®) through monoclonal antibodies to nano-carrier-based DDS. A partial listing of these products is given in Table 2.

Nanocrystal refers to “nanonization” of parent drug compound into nanoscopic crystals of, between 10 and 1000 nm. Several nanocrystal techniques are available, ranging from chemical precipitation, milling and (high pressure) homogenization methods. Drug nanocrystals are composed of 100% drug, with no other carrier materials present. It is one of the strategies often employed to overcome the limitations of poorly water-soluble drugs, which results in poor bioavailability. Size reduction leads to an increased surface area and hence it increases the dissolution rate. Study has also shown that below a critical size of 1–2 μm, the kinetic saturation solubility also increases with decreasing particle size below 1000 nm [14, 15].

Long-acting olanzapine nanocrystals (ZypAdhera®) are an example of commercially available products developed based on nanocrystal technology. Olanzapine is a small hydrophobic drug with limited water solubility (39.88 mg/l at 25 °C) that is indicated for the treatment of schizophrenia. ZypAdhera®, nanosuspension of the pamoate salt of olanzapine, is administered to the patients through intramuscular injection. Once injected into the gluteal muscle, the organic salt dissolves slowly, releasing olanzapine (free base) and pamoic acid. While the absorption of the dissociated free base olanzapine through muscle tissue is very fast, in-situ dissolution rate is slow; thus providing a mechanism for a sustained release of olanzapine. Depending on the formulation and doses selected, olanzapine long-acting injections can last from 2 to 4 weeks. As the absorption half-life of this product is reported to be around 30 days, it means olanzapine is still being released slowly from the depot of the first injection while the second or even the third injection has been administered. Ultimately, a steady state is achieved (roughly after ~ 3 months) when “stacking” of olanzapine concentrations from repeated injections reaches the point whereby the amount of olanzapine being absorbed systemically matches the amount of drug eliminated from the body. Therefore, to prevent long-term relapse of schizophrenia, this formulation is better as opposed to oral formulation with its associated risk of missing dose (patient's compliance) [16, 17].

It is also worth mentioning that nanocrystal approach has also been utilized to improve drug solubility of orally administered drug, which in turn enhances absorption from GI tract into the bloodstream, improving its overall bioavailability. Examples of commercially available products include Rapamune®, Emend®, Tricor®, and many more [14].

Another nanosystem approach is the use of PEGylated proteins (PEG-protein conjugates), typically 2–25 nm. It is primarily intended for parenteral administration in the treatment of viral infections and tumors. Some of the limitations faced by peptide-, protein- and antibody-based drugs include short plasma half-life and poor stability. Examples of such nanotherapeutics include pegylation of interferon α-2a (Pegasys®) and interferon α-2b (PegIntron®). Pegasys®, for example,

Table 2
Approved nanotherapeutic systems.

Nanosystem approach	Size of nano therapeutics	Sample products	Drug	Dosage form/Administration mode	Sustained-release and mechanism, if any?
Nanocrystal	50–1000 nm	ZypAdhera®	Olanzapine (Mw = 312 Da)	Reconstituted suspension for intramuscular injection	YES, controlled by slow in-situ dissolution rate in the muscle, 2–4 weeks
Pegylated proteins	2–25 nm	Pegasys®	Interferon-α2a (Mw > 19,000 Da)	Solution for subcutaneous injection	NO, but increases plasma half-life & improves uptake by liver, leading to reduced dosing interval
Nanoparticles/ Conjugates	130 nm	Abraxane®	Paclitaxel (Mw = 854 Da)	Reconstituted suspension for IV infusion over 30 mins	NO, but increases solubility of drug & employs tumor receptor-mediated pathway to target tumor site
Polymeric Micelles	20–50 nm	Genexol-PM	Paclitaxel (Mw = 854 Da)	Reconstituted solution for IV infusion over 3 h	UNLIKELY and inconclusive due to insufficient data, but increases drug solubility & maximum tolerated dose
Nanoliposomes	100 nm	Doxil®	Doxorubicin (Mw = 544 Da)	Solution for IV infusion over 1 h	YES, controlled by dissolution rate of drug crystals in the core of liposome, 3–4 days

is a covalent conjugate of recombinant interferon α-2a with PEG chain (approximate Mw = 40,000 Da). Significant improvement against viral hepatitis has been demonstrated in patients that received subcutaneous injection of pegylated interferon solution.

Even though pegylated interferons do not target the liver specifically, pegylated interferons exhibit prolonged plasma circulation time, which allows accumulated uptake by hepatocytes. This in turn enhances the therapeutic efficiency of the formulation. However, similar uptake in non-target cells cannot be prevented. In addition, it was also reported that once-weekly administration of peg-interferon α-2a provided effective hepatitis C virus suppression throughout the 3-month dosing period. This reduction in the repeat administration times, from the usual 3× weekly for non-pegylated interferon α-2a, is enabled due to the sustained plasma concentration of pegylated interferon α-2a [18,19].

Nanoparticle albumin-bound paclitaxel (nab-P), Abraxane®, is solvent-free formulation of paclitaxel that has been approved for the treatment of metastatic breast cancer and advanced non-small cell lung cancer. The albumin-bound nanoparticle, approx. 130 nm in size, encapsulates paclitaxel, a water-insoluble anti-cancer drug in its core. It is supplied as lyophilized powder for reconstitution with 0.9% sodium chloride. The reconstituted suspension is then administered intravenously as infusion for 30 min. Once injected into the circulation, the nanoparticles quickly dissolve into smaller sized (~10 nm) albumin-paclitaxel complexes. The solvent free nab-P nanoparticles offer several practical advantages over paclitaxel that requires Cremphor EL® (polyoxyethylated castor oil) to improve its solubility for parenteral administration. The advantages include (i) elimination of pre-medications for hypersensitivity to solubilizers/solvent, (ii) shorter infusion time (due to better solubility of nab-P nanoparticles) to deliver the required dose and (iii) ability to use conventional infusion equipment safely without the danger of leaching plasticizers from infusion bags/tubing [20].

Besides the practical advantages, Abraxane® formulation uses nanotechnology to combine human albumin with paclitaxel and deliver the insoluble drug in the form of nanospheres. The nanoparticles accumulate in tumors by exploiting the gp60 receptor-mediated pathway in the endothelial cell walls of tumor vasculature. Gp60 (albondin) is a glycoprotein expressed on endothelial cell surface that has high affinity for native albumin. Once the nanoparticles accumulate in the tumor, the nanoparticles then leverage on SPARC's affinity for albumin to finally penetrate the tumor cell membrane. The pharmacokinetic data of intravenous administration of Abraxane® shows that paclitaxel plasma concentration follows a biphasic elimination profile: an initial rapid decline, representing distribution to peripheral compartment and a slower second phase representing drug elimination. Higher clearance (43% faster) and larger volume of distribution (53% higher) were also reported, indicating extensive extravascular distribution. Similar terminal elimination half-life was observed for both nab-P and solvent-based paclitaxel, with mean terminal half-life ranging from 13 to 27 h [20–24].

Genexol®-PM (Samyang Biopharmaceuticals, South Korea) is another formulation of paclitaxel that is bound in the core of polymeric micelles, 20–30 nm in size. This product was launched in Korean market for breast cancer and non-small cell lung cancer in Feb 2007 and is currently undergoing clinical trials in the US. Polymeric micelles are normally formed upon the self-assembly of amphiphilic polymers, whereby the hydrophobic portion will point toward the core while the hydrophilic portion will form the outer shell. Genexol®-PM micelles were formed through the self-assembly of low molecular weight diblock copolymer, polyethylene glycol PEG-*block*-poly(D,L-lactide), PEG-*block*-PDLLA [25]. PEG is often chosen to form the outer shell as it has well-established history of use in drug products and can reduce micelle aggregation and opsonization of the nanoparticles. The core of the micelles are suitable for the entrapment of hydrophobic drugs such as paclitaxel.

Genexol®-PM micelles help to increase the water-solubility of paclitaxel and hence allows higher dose administration than paclitaxel alone. It has been shown that the maximum tolerated dose is up to 3 times higher than that of Taxol. It is suggested that saturated paclitaxel in the polymeric micelles is quickly released (95%) followed by being bound to plasma proteins within about 2.5 h [26]. This immediate offloading of the paclitaxel dose is consistent with another work on PEG-block-PLA micelles that reported micelles disruption/instability within 15 min after IV injection [27]. However, another in vitro release study reported that Genexol-PM demonstrated first-order release kinetics with ~65% drug release within 24 h and 95% drug release at 48 h [28]. Long circulating PEG-block-PDLLA micelles have previously been developed by another group who reported that the integrity of the micelles could be preserved up to 24 h [29].

Given the conflicting reports and lack of release data/published information on the release profile and mechanism of Genexol®-PM, additional studies on paclitaxel delivered by the polymeric micelles are required. The release profile could be greatly affected by the molecular weights of PEG and PLA blocks, paclitaxel content, and presence of any ligands attached on PEG. Nevertheless, even if the micelle integrity is preserved, the extremely short diffusion length in the 20-nm micelles would make it difficult to sustain the release over 48 h (see calculations above: micelles have a low "T_g"), unless different mechanisms are present (dissolution rate, drug partitioning rate, etc).

As can be seen, most of these products address tumor treatment, and hence are administered via intravenous infusion. The nanosize of these systems enables better biodistribution: the optimum size range is around 100–200 nm, with an envelope of poly(ethylene glycol) molecules added for evading clearance by elements of the RES. The PEG envelope confers the ability for these carriers to circulate long (24–48 h half-lives in blood) in the bloodstream, which enables them to accumulate at tumor sites (although the liver and spleen still take in a large fraction). In these products, the need for a certain degree of controlled release was established during the development of the nanoliposome-based product Doxil® [30]. The first-generation Doxil® formulation was simply a nanoliposome incorporating doxorubicin in the lipid bilayer, and had fast release of doxorubicin. When infused, the doxorubicin released fairly quickly and was cleared from the bloodstream in about 3–4 h [31], thus defeating the purpose of encapsulation of the drug.

Thus, in the second-generation Doxil® formulation, not only was PEG added to the liposome surface, but the drug was also loaded into the liposomal core using a pH gradient; in fact the loading was intentionally high enough for the drug to be crystallized partially. Thus, the drug efflux was retarded because although the dissolved doxorubicin diffused fairly easily across the lipid bilayer, the rate-limiting step became the rate of dissolution of the drug crystals. This allowed for control of the release for 3–4 days, sufficient to prevent premature release while circulating in the bloodstream. The clinical trials with this 2nd generation formulation were successful [30], with reduced side-effects due to the passive targeting or accumulation of the Doxil® at the tumor site.

Following the clinical success of Doxil®, several other nanotherapeutic products were approved, most of them based on nanoliposome as carrier (see Table 3).

Of these, the only one with some claim to controlled release is DepoCyt®, intrathecally (locally) administered for neoplastic meningitis; the controlled or sustained release in this case enables the product to be administered every other week, thus improving quality of life for patients. On the other hand, the DepoCyt® technology yields micro- rather than nano-particles, and this size difference may be the key reason behind the sustained release profile. In general, therefore, controlled-release nanosystems that can sustain efficacy over the long-term (several days to a month or more) have not been approved yet, although a few have made it to the clinic, as described below. It must be noted that beyond controlled release over 2–3 days, tumor-targeted products do not need any sustained release: in fact very slow release

Table 3
Some approved liposome-based nanotherapeutic products.

Product name	Drug and route of administration	Drug encapsulation site	Liposome size	Sustained release?
AmBisome®	Amphotericin B, anti-fungal; suspension (IV)	Core-loaded	100 nm	No; carrier decreases toxicity; cell-specific
DepoCyt®	Cytarabine, neoplastic meningitis, suspension (intrathecal)	Core-loaded MLV	10–20 μm	Yes; 5 days, reduces freq. of administration
Doxil®	Doxorubicin, chemotherapy, solution (IV)	Core-loaded	100 nm	Yes; 3 days, improved circulation time, passive targeting
LEP-ETU	Paclitaxel, chemotherapy, solution (IV)	Bilayer-loaded	150 nm	No; carrier used for solubilization
Visudyne®	Verteporfin, photo-activated therapy for AMD, suspension (IV)	Core-loaded	200 nm	No; carrier used to transport to back of the eye

would be detrimental once the particle reaches tumor tissue.

4. Controlled release of low molar mass drugs

The front of the eye is a good target for sustained and localized delivery of drugs, to treat various conditions including glaucoma, uveitis, corneal graft rejection and inflammation. The back of the eye also develops chronic conditions that could benefit from sustained delivery: examples include age-related macular degeneration, diabetic macular edema, cytomegalo virus infection, and some inflammatory conditions. In both areas of intervention, either via sub-conjunctival injection or via intra-vitreal injection, nano-sized carriers for the drugs would be highly beneficial, partly due to reduced scattering of light by nano-sized particles, provided aggregation can be avoided in the long term.

One example is the use of nanoliposomes for the management of glaucoma, which is characterized by high intra-ocular pressure. The condition can be alleviated by the use of drugs that either reduce production of aqueous humor, or clear up a pathway for drainage of the aqueous humor. Prostaglandin analogues such as latanoprost fall into the second class, and are administered by topical eye drops, once a day. Bioavailability is low, as a result of poor corneal penetration; the site of action is accepted to be located mainly on the ciliary body. There is also poor patient compliance to self-administration, which can lead eventually to blindness. Sub-conjunctival administration is a better option, provided the duration of action of each intervention is a month or more.

We developed a nanoliposomal formulation of latanoprost, Fig. 2, that releases the drug slowly in vitro over 45 days: rabbit studies with this formulation showed intra-ocular pressure lowering for 90 days [32]. The duration of action is dependent on the nature of the liposome, and its interactions with latanoprost [33]. The latanoprost resides in the bilayer and partitions out into the surrounding aqueous media slowly over time. A small human study validated the extended duration of action on IOP lowering [34].

5. Controlled release of protein and peptide therapeutics

As with many hydrophilic drugs, there are challenges in controlling the release of protein/peptide drugs from nanocarriers. To date, there are 13 FDA-approved nanomedicines for protein/peptide delivery [35]. Most approved protein/peptide nanotherapeutics aim to increase stability and prolong circulation time (e.g. PEGylated proteins), and one (Ontek®, a fusion protein) has been approved for active-targeting to interleukin-2 (IL-2) receptors on T-cells [35]. None of the approved nanoparticulate delivery systems offers substantial controlled release of the protein/peptide payload. The reason is that in many diffusion-controlled polymer-based systems synthesized by classical methods (nanoprecipitation, double emulsion), the drug tend to localize at the surface of the particle or become non-uniformly distributed within the matrix, resulting in uncontrollable burst release [36]. Such polymer systems are further limited by the need to use organic solvents and mechanical processes that change the 3D conformation of many protein drugs that could result in the loss of bioactivity. On the other hand, there are some disease settings whereby controlled release formulations of proteins can potentially enhance therapeutic outcomes.

5.1. Anti-VEGF proteins

Anti-angiogenesis agents are commonly used for the treatment of solid tumors and retinal neovascularization that occurs in wet age-related macular degeneration (AMD). Bevacizumab (Avastin®) was the first monoclonal anti-VEGF antibody approved for clinical use, specifically for several cancers [37]. Subsequently, another anti-VEGF antibody (Ranibizumab – Lucentis®) and a hybrid protein of VEGF receptors fused with the Fc portion of human immunoglobulin G1 (IgG1) (Aflibercept – EYLEA™) came into clinical use for wet AMD. Aflibercept is also approved for treatment of metastatic colorectal cancer under the trade name ZALTRAP®. While most of the preclinical development on cancer has been on enabling passive or active targeting of the anti-VEGF agent by the nanocarrier, administration to the vitreous humor can benefit from controlled release of the anti-VEGF molecule.

In current treatment regimens for wet AMD with anti-VEGF

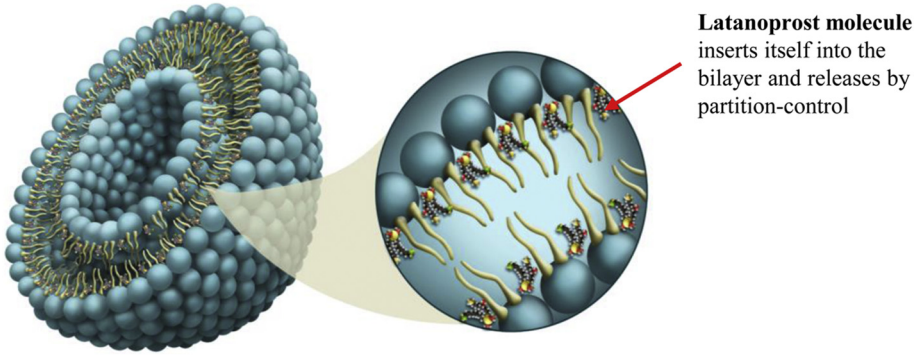

Fig. 2. Cartoon depiction of the postulated mechanism of the sustained release of latanoprost from nanoliposomal structures, based on calorimetric studies of interaction between the drug and the lipid molecules. Reprinted with permission from ref. [33].

proteins, continuous monthly intravitreal injections are invasive and can potentially lead to sight-threatening complications [38]. There is interest therefore in developing sustained delivery systems such as the refillable port delivery system (PDS) by ForSight VISION4, currently in Phase II clinical trials (www.clinicaltrials.gov ID: NCT02510794). The PDS is a non-biodegradable, depot implanted beneath the conjunctiva, and although it is less invasive than monthly intravitreal injections, it still requires invasive surgical excision for removal after 12 months of use. In light of this, hydrogels [39–41] and microparticulate [42] systems have been evaluated for sustained delivery of anti-VEGF proteins. Of these, Yu et al. has reported the sustained release of bevacizumab for at least 6 months from a modified hyaluronic acid (HA)/dextran hydrogel following intravitreal injection [41]. These controlled-release systems offer the potential of reducing the frequency of intravitreal injections needed. However, to our knowledge, none of these sustained formulations of anti-VEGF proteins are in clinical trials.

Another strategy to reduce the invasiveness of anti-VEGF administration is to deliver it via the periocular route (e.g. subconjunctival), as it is a safer alternative that avoids the risks of intraocular damage posed by intravitreal injections. If choroidal and lymphatic clearance of administered formulations can be avoided, controlled release systems delivered via this route can potentially reduce both the frequency and invasiveness of anti-VEGF administration. We have recently demonstrated the potential of nanoliposomes loaded with ranibizumab and administered them via subconjunctival route [43]. Using ex vivo porcine scleral tissues, 100 nm-sized negatively-charged liposomes were best able to penetrate into the sclera, which acts as a depot for sustained release of ranibizumab from the liposomes and subsequent trans-scleral transport [43]. This effectively avoids choroidal clearance of the nanotherapeutic. As the pore diameter of the human sclera was estimated to be 20–80 nm, it is feasible to propose the use of the sclera as a depot for nanoparticulate formulations [44]. What can be envisioned is a nanoparticulate formulation that is able to traverse the sclera following subconjunctival injection and be able to sustain the release of the anti-VEGF protein for 2–3 months.

5.2. Insulin

Insulin is the first recombinant protein approved for therapeutic use in humans. For patients with insulin-dependent diabetes mellitus, insulin is injected into a subcutaneous depot with needles, pens or insulin pump systems. A number of rapid-acting, intermediate-acting (NPH insulin), and long-lasting insulin analogues exist in the market. Rapid-acting insulin analogs (e.g. Aspart, Lyspro) have a rapid onset of action and are injected during or just before mealtimes. Due to recent advances in recombinant technology and fabrication methods in the recent decades, long-acting insulin analogs (Levermir®, Lantus®) need only be injected once or twice a day to maintain basal insulin levels. Insulin glargine (Lantus®) is a microcrystalline insulin analogs that sustains the release of insulin over 18–24 h with a 'peakless' profile [45]. This prolonged action is achieved with a modification in the amino acid sequence that changes its isoelectric point from 5.4 to 7.4, such that microcrystalline precipitates are formed in the subcutaneous space. The precipitates then dissolve slowly from the subcutaneous depot to release the insulin into the bloodstream [45]. Although this offers some rudimentary controlled release of insulin, the pharmacokinetics and safety of the formulation can be compromised if the insulin is not injected into the subcutaneous space. As it is administered in a larger dose than rapid-acting analogs, there is a risk of hypoglycemia when accidental intramuscular injection of insulin glargine occurs, due to its rapid absorption into the bloodstream [46]. Insulin degludec (Tresiba®) is the latest long-acting basal insulin that assembles into zinc-insulin multi-hexamers in the subcutaneous depot. Insulin monomers then dissociate and are released over a period of 42 h when zinc ions diffuse slowly out of the complex [47].

The controlled release of insulin has not extended beyond using long-acting insulin analogues in subcutaneous depots, but much research has also gone into the development of particulate carrier systems for insulin, and most efforts were focused on pulmonary delivery systems for insulin. Most pulmonary delivery systems do not exhibit controlled or sustained release but are instead intended for rapid absorption. After the clinical and commercial failure of several pulmonary insulin formulations, Afrezza® (using Technosphere™/insulin technology, Mannkind Corporation) eventually attained FDA approval in 2014 as a mealtime rapid-acting insulin [48]. As one important determinant of pulmonary deposition is the particle mass median aerodynamic diameter (MMAD), which predicts that nanosized particles (< 1 μm) will mostly be exhaled, nanosized powder formulations for inhaled insulin have not been the focus of translational development [49]. In addition, concerns over long-term safety, inconsistent pulmonary deposition and low cost-effectiveness have essentially excluded the pulmonary route for the development of prolonged insulin release formulations.

It is in oral delivery of insulin where nanoparticulate formulations represent a promising tool to match the long-acting analogues in subcutaneous formulations. Additionally, oral insulin is advantageous over subcutaneous insulins as it fosters patient compliance and confers physiologic benefits due to the fact that it reaches the liver directly via hepatic portal vein, enabling better hepatic regulation of glucose [50]. However, the low oral bioavailability of insulin is a huge challenge due to proteolytic degradation by digestive enzymes and low permeability [50]. In fact, an oral tablet (OI338GT) developed by Novo Nordisk using an absorption-enhancing platform (GIPET®, licensed from Merrion Pharmaceuticals) packaged with a long-acting basal insulin analog matched the efficacy of injectable insulin glargine (Lantus®) in a study involving 50 patients with Type 2 diabetes mellitus, but was discontinued due to cited low bioavailability and hence, low economic viability (American Diabetes Association 77th Scientific Sessions, 2017). Before sustained oral insulin formulations can be feasible, it is imperative that we address the challenges surrounding the uptake of insulin.

Among the materials used for insulin nanoparticles developed for oral formulations, chitosan/chitosan derivatives have been widely researched [51]. The fabrication of chitosan-based nanoparticles obviates the use of organic solvents, requiring instead ionic crosslinking by tripolyphosphate (TPP) to entrap insulin [52]. Chitosan is a known mucoadhesive, and chitosan-insulin nanoparticles has been demonstrated to prolong insulin residence time in the small intestine while enhancing the permeation of insulin across the intestinal barrier [52]. However, as is usual with proteins encapsulated into nanocarriers, a high burst release of the payload is frequently observed. Despite the anticipated electrostatic interactions between the chitosan and insulin, TPP-crosslinked chitosan nanoparticles released $> 60\%$ of insulin in the first hour, and subsequently the release profile approached a plateau at 80% by the 2nd hour [52]. In diabetic rat models, chitosan-insulin nanoparticles were observed to interact with intestinal epithelial to sustain the release of the insulin, effectively suppressing glucose levels over a period of 11 h [53]. The development of chitosan-based insulin nanoparticles for enhanced uptake and sustained delivery, alongside other nanocarrier systems (dextrans, polyesters, alginate etc.), is still in early stages of preclinical evaluation. Compared to subcutaneous insulin depots, nanoparticle systems developed for oral delivery have yet to match the sustained release of the most advanced insulin analogues (e.g. 42 h for insulin degludec). Together with the poor uptake, shifts in material choices and release strategies are necessary for oral insulin to be viable commercially. Except for a liposomal formulation currently in phase III trials (Oral HDV, Diasome Pharmaceuticals) that improves hepatic-targeting but lacks sufficient bioavailability to function as full insulin replacement therapy [54], no other oral nanocarrier-based insulin is in clinical trials yet. The design and toxicology evaluation of other novel nanocarriers can pave the way for clinical trials involving oral controlled release formulations of insulin in future.

5.3. Bone morphogenetic proteins (BMP)

The BMP proteins are known potent promoters of bone formation and several members of the family have found clinical usage in orthopedic procedures. Earlier studies have concluded the superiority of BMP-2-soaked collagen sponges over standard-of-care bone grafting in spinal fusion for degenerative disc diseases [55, 56], and over metal fixation devices for open fractures [57]. Although the efficacious delivery of BMPs has been realized in two FDA-approved biomaterial devices, such as BMP-2 in collagen sponges (INFUSE®) and BMP-7 in collagen sponges (OP-1®), the field has not been without controversy. Human studies in the early 2000s showed that bone induction is possible with BMP-2, but appropriate dosing and safety were uncertain [58]. However, starting in 2006, independent research groups started to report serious side effects that, among others, include bone overgrowth, soft tissue swelling and increased risk of cancer, with adverse event rates ranging from 20 to 70% [58]. BMP-2 was initially identified as a bone morphogenetic protein that induces osteoblast differentiation [59, 60], but is now known as a multi-functional growth factor involved in complex and integrated signaling networks, including to be found overexpressed in certain soft tissue cancers [61].

The approved BMP collagen sponges are simple drug carriers that require soaking of the sponges with recommended concentrations of BMPs in solution (e.g. 20 mg/level of fusion for INFUSE® BMP-2). Collagen sponges typically have a biphasic release profile characterized by an initial burst release (> 60% within 3 days) followed by a first order release profile over 2 weeks [62]. It is understood that the kinetics of BMP-2 release must not lie at two extremes – bolus injections will chemotactically recruit osteoprogenitor cells to the site, but do not retain sufficient residual doses to promote differentiation, while prolonged low level release do not create a sufficient chemotactic gradient to recruit osteoprogenitor cells to the site [63]. It appears that the approved collagen sponges owe their efficacy to hitting the intermediate range of release rates. However, together with the serious side effects observed in human patients, other lines of evidence point toward non-optimized dosage and release kinetics. There is disagreement over the required dosage, as a recent study has found comparable spinal fusion rates between the reduced dosage of 6–12 mg/level of BMP-2 and the recommended dosage (20 mg/level) [64]. Nevertheless, many studies used high dosages [65, 66], probably due to uncertainty over the range of effective doses. Such high doses are probably responsible for side effects such as abnormal bone formation and soft tissue swelling [67]. As a growth factor-based protein therapeutic, an optimal release profile for BMP-2 is required also to minimize serious side effects resulting from the initial spike of local concentration.

To overcome this challenge, research on electrospun nanofibrous scaffolds [66], hydroxyapatite nanoparticles [68], and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) nanoparticles [69] have been attempted to attain better controlled release of BMP-2 but without any follow-up clinical trials. One of the most promising approaches to achieve controlled release of BMP-2 may be embedding nanoparticulate BMP-2 into biodegradable scaffolds. Li et al. demonstrated the loading of bovine serum albumin/BMP-2 nanoparticles into electrospun polyester nanofibers, and subsequently attained BMP-2 release of up to 35 days [70]. The efficacy of the scaffold co-loaded with BMP-2 and dexamethasone was validated in a rat calvarial (skullcap) defect model [70].

6. Sustained gene silencing: controlled release of siRNA

Although the first report of gene silencing via the use of short interfering RNA (siRNA) was first reported in 1998 [71], its therapeutic benefits have not been translated into an approved product, although an NDA has been filed (by Alnylam Pharmaceuticals) in February 2018 for TTR-mediated amyloidosis, using a lipid nanoparticle system for delivery of the siRNA. The significant barriers to translation include its rapid clearance from blood, as well as its degradation by nucleases and the inability of naked siRNA to enter cells readily [72]. Clearly a protective and sustained-release nano-carrier system is crucial to realizing the therapeutic potential of siRNA.

The siRNA itself may be stabilized chemically by suitable modifications [72], but for protection against clearance and for cellular penetration, the nano-carriers require modification. It is no accident that most of the siRNA systems in the clinic target the liver [72] since nano-carriers naturally accumulate in the liver upon systemic administration. The numerous nano-carrier types tested so far have been well described by Kanasty et al. [73]. It is now recognized that most, if not all, of these carrier systems lack the ability to sustain the release of the siRNA over long periods: this inability has been the probable cause of some failures in the clinic.

In general, preventing premature efflux of hydrophilic entities from nano-carriers is a huge challenge that has not been fully overcome by research efforts. Several types of nano-carriers have been evaluated, including complicated cyclodextran-polymer based systems [74]; PLGA-lipid hybrid nanoparticles [75]; PEGylated liposomes with cholesterol [76]. Of these systems, only the PLGA-hybrid nanoparticle system, Fig. 3, was reported to sustain the release of the siRNA over several days, so it is worth examining the concept in more detail here. It should be noted that long blood half-lives (6–8 h) were reported for the PEGylated-liposomal systems with cholesterol in vivo, indicating some level of sustained release or controlled efflux of the siRNA.

The hybrid nanoparticles were prepared using a modified double-emulsion technique [73]. First, droplets of siRNA solution were dispersed in an organic solvent with dissolved PLGA and cationic ethyl phosphocholine lipids (EPC). In the second emulsion, a DSPE-PEG lipid was used to self-assemble into the “outer” lipid bilayer, surrounding the siRNA-cationic lipid complexed PLGA solid core.

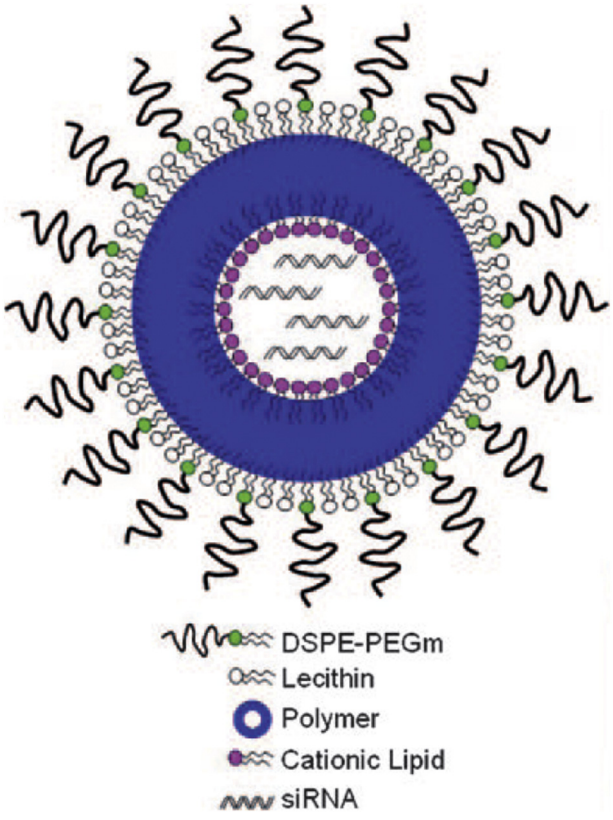

Fig. 3. Cartoon depiction of the cationic lipid-siRNA core inside a PLGA solid layer, that is then covered by a DSPE-PEG liposomal bilayer; this construct enables sustained release of the siRNA over several days. Reprinted with permission from ref [75].

The sustained release of the siRNA is presumably controlled by diffusion through the solid PLGA although it is not clear whether the diffusing species is the cationic lipid-siRNA complex, or free siRNA. Nevertheless, this appears a promising sustained-release siRNA system for further exploration.

We have long believed that the Layer by Layer (LbL) self-assembly process has the potential to yield a sustained-release nano-carrier system for siRNA along with the ability to penetrate cells by suitable manipulation of the layers [77]. The number of layers allows the researcher to fine-tune amount of drug loaded, as well as allowing for drug combinations to be delivered in a single particle. For example, Deng et al. constructed a LbL nanoparticle with both the anticancer drug doxorubicin and siRNA, resulting in an enhanced effect of reduction in target gene expression in breast cancer tumors by 80% [78]. It should be noted that this system has some level of sustained release (biphasic release for siRNA, complete release at 50 h). These nanoparticles demonstrated long blood circulation half-lives and accumulated in tumor tissue by virtue of the enhanced permeation and retention (EPR) effect. Gene silencing over a period of 15 days however, required repeated injections, suggesting that a single dose application of these nanoparticles may not suffice for sustained efficacy.

Other workers have reported on sustained efficacy of siRNA action in vitro and in vivo [79] over 2–3 weeks with a single administration. Unfortunately, clinical translation of the reported carrier is hampered by the use of gold NPs as the core and Poly (L-Lysine) as the peptide, neither of which are biocompatible. Other siRNA delivery vehicles have been constructed based on a variety of biomaterials, including cationic polymers [80], nanofibrous peptides [81], thermosensitive cationic hydrogels [82] and hybrid lipid-polymers [75]. However, there is no clear evidence from these studies that the sustained gene silencing was achieved in vivo for an endogenous target gene via a clinically relevant route of administration. Instead, these reports detailed gene silencing in experimental backdrops far from the clinical setting, including knockdown of a non-mammalian gene [79], experimentation only on cultured cells [80, 81], or investigation of xenografted tumors where tumor cells were pre-treated with the delivery vehicles carrying siRNAs before transplantation into recipient mice [75, 79, 82].

In summary, truly prolonged silencing of an endogenous gene in a target tissue by a single direct in vivo application has not been indubitably established for these nano-carriers. Our study has gone beyond previous work to prove that clinically relevant endogenous gene silencing can be achieved on a prolonged basis using a single dose application of siRNAs incorporated in LbL nanoparticles.

We have developed a system that has sustained efficacy of action against a fibroblast gene over 2 weeks, rather than a few hours. To this end, we have optimized SPARC-silencing siRNA incorporated in an LbL system based on hydroxyapatite and poly-L-arginine, as a biocompatible, effective anti-scarring agent with prolonged efficacy of up to 14 days in mouse eyes. The LbL system serves to protect the SPARC siRNA layers from degradation by nucleases, with the cationic polymer layer enabling more efficient and effective cellular uptake. The release of the siRNA is controlled by the molar mass and charge density of the polyelectrolyte layers, and in vitro studies show sustained release over several days. The release is also pH-sensitive, indicating that the "release" occurs by exfoliation of the layers. In a mouse model of fibrosis following simulated glaucoma surgery, we have shown anti-fibrotic activity for 14 days (reduced collagen deposition).

The direct injection of these nanoparticles into the conjunctiva is a method that can be easily administered in the clinical setting. Moreover, these nanocarriers can be readily monitored since the target area is localized, accessible and visualisable. By injecting the LbL nanoparticles directly into the target conjunctival tissue after experimental surgery, it has been shown that these carriers can facilitate knockdown of endogenous *SPARC* gene expression, a clear demonstration of therapeutic efficacy. Furthermore, the nanoparticles did not induce an adverse foreign body reaction which is a normal physiological response/mechanism involving inflammation and known to result in loss of functionality of nanoparticles. Most importantly, the long-term silencing effects facilitated by the nanocarriers suggested that re-injections are potentially not required and surgical success can be further improved along with a reduced surgical morbidity.

To achieve a more sustained anti-fibrotic effect, for other applications, an additional layer of siRNA was added to the previously designed NP configuration. Two different NPs were fabricated. The particle configurations were, A: HA/ARG/6FAM-siRNA/ARG and B: HA/ARG/6FAM-siRNA/ARG/6FAM-siRNA/ARG/6FAM-siRNA/ARG. NP A has only 1 layer of siRNA while B has 3 layers of siRNA. To confirm that NP B has a higher siRNA content, 1 mg of each of the two particles (triplicates conducted) were dispersed in trypsin and shaken overnight at 37 °C to disassemble the particles to release all their siRNA contents and compared (measured with Tecan plate reader). From the results, NP A fabricated with 1 mg of HA contained 0.47 nmol of siRNA while NP B fabricated with 1 mg of HA contained 1.15 nmol of siRNA. NP B has about 1.5 times more siRNA than NP A.

NPs A and B fabricated were used for siRNA release study to investigate their release profiles. NPs fabricated from 1 mg HA of NP A and B (triplicates conducted) were dispersed in 300 μl of PBS and spun at 1500 rpm at 37 °C in the thermoshaker. At specific time intervals, samples were spun down with their supernatant collected and tested for siRNA content released using the Tecan plate reader at gain 60. The results are shown in Fig. 4.

From Fig. 4, it can be seen that both NPs can sustain the release of siRNA over 120 days. The 3 layered NP released an average of 115.9 pmol of siRNA while the 7 layered NP released an average of 254.3 pmol of siRNA by day 118. The 7 layered NP released about 1.38 times more siRNA as compared to the 3 layered NP.

7. Status

Very few nanocarrier-based products with controlled release of the incorporated bioactive, have been approved. For small molecule drugs, there have been some clinical trials, including our own nanoliposomal system for glaucoma, but no approvals or NDA filings yet. For larger

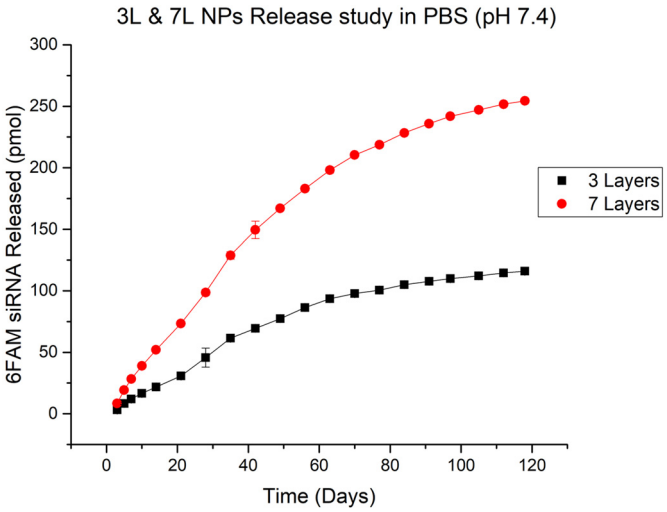

Fig. 4. Graph of cumulative siRNA release from 3 (HA/ARG/6FAM-siRNA/ARG) and 7 layered (HA/ARG/6FAM-siRNA/ARG/6FAM-siRNA/ARG/6FAM-siRNA/ARG) LbL NPs over 118 days. Each NP fabricated from 1 mg of HA were dispersed in 300 μl of PBS and constantly shaken at 37 °C over 118 days. At the stipulated time points, the particle samples were centrifuged down and the supernatant collected and measured with the Tecan plate reader to affirm release amount compared against FAM siRNA standard curve. Fresh PBS was added for subsequent time point measurements. All samples and measurements were conducted in triplicates.

bioactives, one lipid nanoparticle-based system incorporating a siRNA against a liver protein, has gone through successful clinical trials and an NDA was filed in 2018. On the other hand, sustained efficacy of action of hydrophilic bioactives, including siRNA, has been demonstrated mainly using a layer-by-layer approach to coat nanoparticle cores. At least one company has been formed to deliver sustained-efficacy solutions for medical conditions; the MIT-spin off Layer Bio is reportedly developing such systems for glaucoma and for wound healing. In this regard, our own system based on a hydroxyapatite core with multiple layers of siRNA and cationic polymer, has shown promising sustained gene silencing effects in a mouse model of glaucoma surgery.

This paper has catalogued several chronic conditions where sustained delivery of the therapeutic agent would be hugely beneficial for the patient, including retinal diseases caused by unwanted neovascularization. Additionally, for many of these conditions, a nanosized carrier is also essential, for various reasons, including the ability to penetrate target cells. However, achieving true sustained release (for days rather than hours) from nano-sized entities remains a huge challenge. The situation calls for new material systems. If successfully met, with judicious new approaches, such systems will prove immensely beneficial to patients.

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