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Self-assembled glycol chitosan nanoparticles for disease-specific theranostics

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ABSTRACT

Hydrophobically modified glycol chitosan (hGC) conjugates spontaneously form self-assembled nanoparticles (NPs) in aqueous conditions, and glycol chitosan NPs (CNPs) have been extensively studied for the past few decades. For disease-specific theranostics, CNPs could be simply modified with imaging agents, and the hydrophobic domains of hGC are available for encapsulation of various drugs. Based on the excellent physiochemical and biological properties, CNPs have been investigated for multimodal imaging and target specific drug delivery. In particular, a recent application of CNPs has shown great potential as an efficient theranostic system because the CNPs could be utilized for a disease-specific theranostic delivery system of different imaging agents and therapeutics, simultaneously. Furthermore, various therapeutic agents including chemo-drugs, nucleotides, peptides, and photodynamic chemicals could be simply encapsulated into the CNPs through hydrophobic or charge–charge interactions. Under *in vivo* conditions, the encapsulated imaging agents and therapeutic drugs have been successfully delivered to targeted diseases. In this article, the overall research progress on CNPs is reviewed from early works. The current challenges of CNPs to overcome in theranostics are also discussed, and continuous studies would provide more opportunities for early diagnosis of diseases and personalized medicine.

1. Introduction

In the early 2000s, nanotechnology-based medical techniques emerged as a powerful tool for theranostics. The concept of theranostics, a combination of diagnostics and therapy as a single platform, emerged with progress in molecular imaging and nanomedicine. Advances in nanomedicine contributed to theranostics with targeted drug delivery strategies to reduce the systemic toxicity of drugs. A variety of nanoprobes also have been designed for molecular imaging to visualize cellular function or changes in biomarkers, which reflect the progression and therapeutic response of a disease [1]. These nanoprobes, in most cases, can chemically interact with biomarkers to alter the signals for imaging and provide biological information on pathological lesions [2]. The combination of these two advanced technologies for theranostics is expected to achieve early diagnosis and personalized medicine in the near future, and nanoparticles will play a significant role in the concept of theranostics.

In general, nano-sized particles accumulate more in pathological lesions than in normal tissues. In particular, tumor-accumulation of nanoparticles (NPs) has been extensively studied for cancer theranostics. Based on the size-dependent property, NPs can extravasate from angiogenic blood vessels which consist of coarsely connected vascular endothelial cells. The poor lymphatic systems cause retention of NPs in tumors, and the so-called enhanced permeability and retention (EPR) effect is the most well-known mechanism for tumor targeted delivery of NPs [3]. Besides tumors, NPs also can escape from ruptured or damaged blood vessels and travel to other pathological lesions including trauma, hemorrhagic stroke, and inflammation [4–6]. In inflammation, a variety of immunocytes release chemotactic factors and vasodilators including histamines and bradykinins. Under such conditions, increase in local blood flow and endothelial permeability can be observed, subsequently increasing the accumulation of NPs in the pathological lesions. In addition, NPs can be delivered within macrophages. A recent study showed that macrophage migration is dependent on the size of the treated NPs [7]. The NPs with 100 nm diameters were effectively taken up by the macrophages and showed enhanced vector migration rates compared to smaller NPs 30 and 50 nm in diameter. Indeed, nanoparticle-loaded exogenous macrophages migrated into brain lesions through the disrupted blood–brain barrier [8]. These results suggest that the NPs circulating in the blood also may be phagocytized by macrophages to be delivered to the site

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of blood vessel disruption and the foci of inflammation. These reports suggest that NPs can be applied in targeted delivery to non-tumoral lesions, such as chronic inflammation and autoimmune diseases.

A variety of theranostic NPs have been developed for biomedical applications. In particular, chitosan has been widely used for nanoparticle fabrication during recent decades. Chitosan, a deacetylated chitin, is a natural polymer that has many functional groups in its backbone structure. The abundant functional groups of chitosan allow easy chemical modification, and the inherent cationic charges are useful for developing chitosan as a gene carrier. Furthermore, chitosan and chitosan derivatives are attractive materials for their excellent biocompatibility, biodegradability, and low immunogenicity. In particular, hydrophobically modified glycol chitosan (hGC) spontaneously forms self-assemblies in aqueous conditions, and the glycol chitosan NPs (CNPs) have been extensively studied for the past few decades. The hydrophobic modifications provide glycol chitosan (GC) polymers with interesting properties to form NP structures enabling them to be delivered to pathological lesions in a targeted-manner.

The CNPs have demonstrated great potential as an innovative theranostic system. In particular, a recent application of multifunctional CNPs holds promise for efficient theranostics with low systemic toxicity. In this article, we will review all of the CNPs including the early works on the development of CNPs. Several factors affecting target specificity, physicochemical/biological properties, and practical applications of CNPs in theranostics will be described to understand the research progress of the CNPs-related studies. In addition, the current issues and challenges of CNPs to overcome in theranostics will also be discussed in the last part of this review.

2. Self-assembled glycol chitosan nanoparticles (CNPs)

Polymeric amphiphiles can form micelles or micelle-like aggregates, spontaneously. In aqueous environments, the hydrophobic moieties of the polymeric amphiphiles are facing toward the core, and the hydrophilic moieties are exposed to the surface. Although chitosan has a low solubility above its pKa (6.4) in water, GC is a hydrophilic polymer which exhibits complete solubility in water in broad pH conditions [9,10]. Based on this property, several hydrophobic moieties have been introduced to hydrophilic GC polymers to form amphiphiles for preparing self-assembled NPs since 2003 [10–14]. Among them, 5β-cholanic acid is one of the most commonly used materials for hydrophobic modification of GC. Amphiphilic GC–5β-cholanic acid conjugates have been carefully and consistently studied. As other polymeric amphiphiles, the GC–5β-cholanic acid conjugates naturally build up into nano-sized micelle-like aggregates in aqueous solution (Fig. 1A). To trace the resulting CNPs *in vitro* and *in vivo*, imaging agents would be labeled to GC polymers. For example, GC polymers were labeled with near infrared fluorescence (NIRF) dyes, such as Cy 5.5 for fluorescence optical imaging. By the EPR effects or enhanced vascular permeability, the CNPs can infiltrate into pathological lesions including tumors for enhanced contrast imaging of lesions to provide more accurate anatomical information (Fig. 1B).

2.1. Optimization of CNPs and factors affecting targeting efficiency

The characteristics of CNPs are quite variable, depending on the constituents of the hydrophilic and hydrophobic components. As the hydrophilic part, various types of GCs have been investigated to improve target specificity. The molecular weight and degree of acetylation of the chitosan may affect the properties of hGC polymers and CNPs [16,17]. Consequently, different factors affecting the targeting efficiency have been considered to optimize the CNPs for *in vivo* application, especially in tumor-bearing mice [17]. Different degrees of hydrophobic substitution and the properties of the resulting CNPs were investigated as well [11,18].

In practice, the 5β-cholanic acid conjugated GC based CNPs were prepared with different molecular weights of GC polymers (GC 20 kDa-CNP, GC-100 kDa-CNP, and GC-250 kDa-CNP) [17]. Their physicochemical properties and tumor accumulation of various CNPs were comparatively evaluated in tumor-bearing mice. When the feed mole ratio of 5β-cholanic acid to GC sugar residue was fixed as 1:20, the surface charges of the GC-20 kDa, GC-100 kDa, and GC-250 kDa-CNPs were not significantly different from each other. The average diameter of the particles varied from 231 to 310 nm (Table 1). The GC 20 kDa-CNPs were relatively small with an average diameter of 231 nm, and the average diameter of GC 250 kDa-CNPs was up to 310 nm (Fig. 2A). Despite the relatively large size of the particles, GC 250 kDa-CNPs showed the most efficient accumulation in tumors *in vivo* (Fig. 2B).

The 250 kDa-CNPs with different degrees of hydrophobic substitutions (DSs) were also prepared as in Table 2 [18]. The CNPs with different DSs were almost the same in their sizes and morphology, whereas the stability and flexibility of the CNPs were not the same in substance. In particular, a filtration test confirmed the flexibility and deformability of the CNPs. The changes in NIRF intensity of Cy5.5-labeled CNPs were compared to those of polystyrene NPs, before and after syringe filtration. The polystyrene NPs with rigid structures lost their NIRF signals after the filtration, which suggested that the polystyrene NPs could not pass through a cellulose acetate filter (0.2 μm pore size) at all (Fig. 2C). Meanwhile, despite their larger hydrodynamic sizes of over 300 nm, the CNPs maintained distinct NIRF signals after filtration with a 0.2 μm pore filter, especially for those with under 35% 5β-cholanic acid content (Fig. 2D). Based on the ideal balance of stability and deformability, the CNPs containing 23 wt.% 5β-cholanic acid (CNP-23%) showed higher tumor accumulation than that of the others (Fig. 2E and F). The CNPs with a low DS (12 wt.% 5β-cholanic acid included) showed low *in vivo* tumor targeting and low serum stability, and the CNPs with a high DS (35 wt.% 5β-cholanic acid included) could not penetrate into angiogenic blood vessels in tumors to be recognized by the reticuloendothelial system (RES) in the liver and spleen.

The preparation methods of CNPs have been gradually modified for better targeting efficiency. Recently, GCs (250 kDa) were modified to introduce 150 ± 4.5 molecules of hydrophobic 5β-cholanic acid and 4.8 ± 0.7 molecules of Cy5.5 per each molecule of GC polymer [15,19].

2.2. Physiochemical properties of CNPs

The physiochemical properties of NPs should be carefully considered to achieve target specific delivery of the particles. The primary concern should be the size of the particle, because the enhanced delivery of NPs is mainly due to the size-dependent effect. In general, NPs 100 nm or less in diameter are considered for prolonged blood-circulation and high tumor selectivity minimizing or delaying RES clearance [20,21]. Interestingly, however, the most efficient tumor-specific accumulation was achieved with relatively large CNPs (around 300 nm in diameter). Considering the particle size only, the CNPs are somewhat large to expect the EPR effect. However, as described in Section 2.1, the elastic and deformable structure of CNPs permit the extravasation of NPs to be delivered to pathological lesions, such as tumors.

The particle stability would be also an important factor to maximize the targeting characteristics of NPs. In particular, the stability of NPs in serum is essential for a prolonged blood half-life *in vivo*. The CNPs are generally stable in physiological conditions for a long period, and they maintain the size and size distribution in phosphate-buffered saline (PBS, pH = 7.4, 37 °C) for 2 weeks, regardless of the DS [18]. In blood serum, however, CNPs may differ in stability depending on the DS. The stability of CNPs in serum tends to be in direct proportion to the DS value of the CNPs, and CNPs with a low DS are dissociated by serum proteins for rapid renal excretion [18].

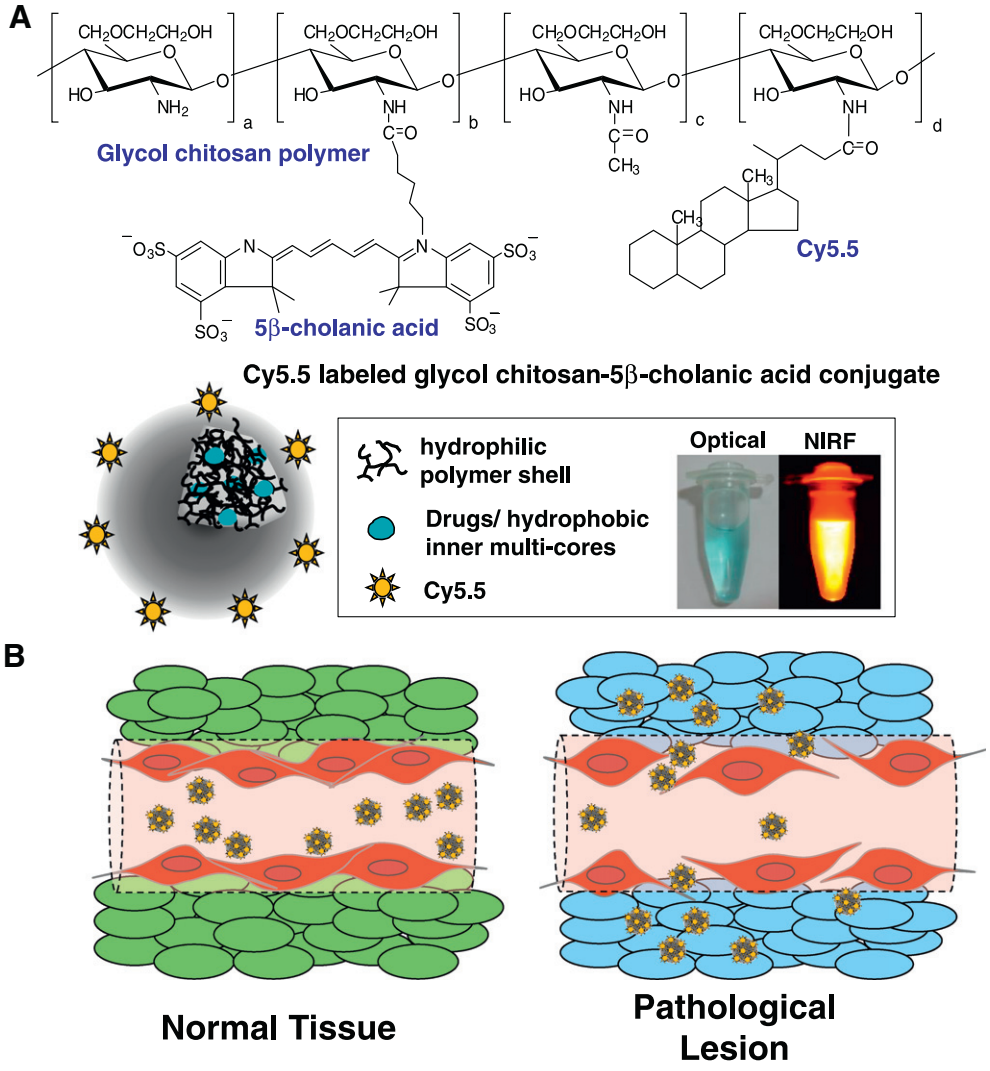

Fig. 1. Schematic diagram of theranostic glycol chitosan nanoparticles (CNPs) [15]. (A) Chemical structure of Cy5.5 labeled glycol chitosan–5β-cholanic acid conjugate and CNPs. (B) Targeted delivery of CNPs in the pathological lesions.

The surface charge of the particles can influence the cellular fate of the NPs as well [22]. A recent study showed that GC based NPs demonstrate a pH-dependent surface charge [23]. Based on this property, GC based NPs can circulate in the blood (physiological fluid, pH = 7.4) without interaction with negatively charged serum protein, and they make transition to a cationic net charge at an acidic microenvironment including tumors. Indeed, chitosan-based NPs with a positive surface charge showed a higher cellular internalization rate, compared to negatively or neutrally charged ones [24]. Moreover, the positively charged chitosan NPs showed adequate lysosomal escape after cellular uptake, whereas the negatively and neutrally charged ones tended to be entrapped in the lysosomes [24]. In the case of 5β-cholanic acid conjugated GC based CNPs, they showed rapid cellular uptake and lysosomal escape which may be attributed to the slight positive surface charge of the particles. Moreover, pH-dependent surface charge of GC based NPs would allow blood stability and cancer specific cellular uptake of CNPs.

Various CNPs could be delivered to pathological lesions including tumors, mainly due to the size-dependent effects. Although each of the characteristics of the particles such as size, shape, stability, rigidity, and surface property has influence on the biodistribution of the NPs, a single factor cannot definitely determine the *in vivo* fate of the NPs. Nonetheless, predicting the pharmacokinetics and *in vivo* behavior of NPs is sometimes uncertain, and the physiochemical properties and biological properties should be considered together.

Table 1
Characterization of CNPs consisting of different molecular of GC polymer [17].

Samples	Feed mole ratio[a]	Mn[b]	DS[c]	μ_2/Γ^2	Size[d] (nm)	ξ (mV)
GC-20 kDa-CNP	5	21,180	4.7	0.003	231	10.1
GC-100 kDa-CNP	5	109,070	4.7	0.011	271	11.4
GC-250 kDa-CNP	5	271,420	4.8	0.015	310	10.8

[a] Feed mole ratio of 5β-cholanic acid per 100 sugar residues of glycol chitosan.
[b] Number-average molecular weight, estimated from the colloidal titration result.
[c] Degree of substitution of 5β-cholanic acid per 100 sugar residues of glycol chitosan, determined by colloidal titration.
[d] Mean diameter measured by dynamic light scattering (DLS).

2.3. Biological properties of CNPs

In biomedical applications, chitosan and its derivatives have many advantages and unique biological properties, including biocompatibility, low immunogenicity, and muco-adhesiveness [25,26]. Based on the biocompatible property of chitosan, the cytotoxicity of CNPs is fundamentally low. To evaluate cytotoxicity, human breast cancer cells (MDA-MB231) were treated with various doses (0.1 mg/mL to 50 mg/mL) of two different types of CNPs [27–29]. Both the hydrotropic oligomer-conjugated GC (HO–GC NPs) and 5β-cholanic acid conjugated

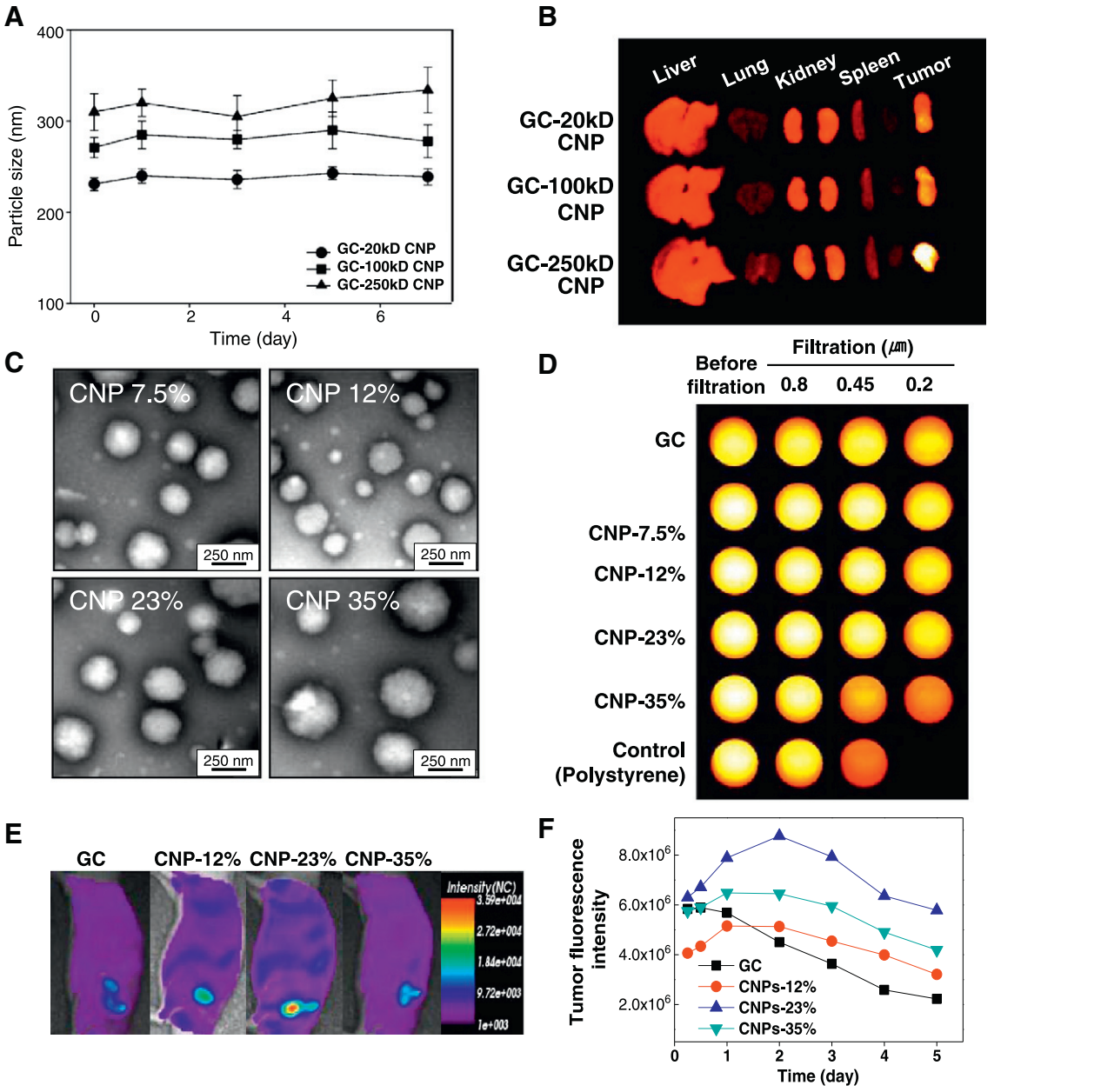

Fig. 2. Optimization of CNPs for tumor-specific delivery [17,18]. (A) Time-course of changes in particle sizes for various GC based CNPs. (B) Representative NIRF images of excised tumors and organs of SCC tumor-bearing mice, after intravenous injection of various GC based CNPs at 72 h. (C) Transmission electron microscopy (TEM) images of CNPs. (D) Deformability of CNPs measuring by filtration test. (E) Biodistribution of CNPs in SCC tumor-bearing mice at 48 h post-injection. (F) Time-course of tumor accumulation of CNPs.

Table 2
Physicochemical characteristics of GC-250 kDa-CNP with different ratio of 5β-cholanic acid conjugation [18].

Samples[a]	Feed ratio[b]	Mn	DS[c]	μ_2/Γ^2	Size (nm)	ξ (mV)
CNP-7.5%	60	268,750	52	0.013	366	15.9
CNP-12%	100	280,000	83	0.011	349	19.5
CNP-23%	200	307,500	159	0.009	359	22.1
CNP-35%	300	337,500	243	0.009	340	22.8

[a] Weight ratio of conjugated 5β-cholanic acid per one glycol chitosan polymer was determined by a colloidal titration method.
[b] Feed ratio of the number of 5β-cholanic acid molecules per one glycol chitosan polymer.
[c] Degree of substitution of the number of 5β-cholanic acid per one glycol chitosan polymer, determined by colloidal titration.

GC based CNPs showed excellent cell viability (>90%) at a concentration of 50 mg/mL. The bare CNPs, without drug loading, showed low cytotoxicity, and these CNPs could be utilized as safe carrier systems for theranostics.

The typical intracellular fate of CNPs can be explained by the muco-adhesive property of chitosan and the cellular uptake mechanisms of 5β-cholanic acid conjugated GC based CNPs [30–32]. To identify the impact of specific endocytic pathways on the internalization of CNPs, Cy5.5-labeled CNPs were traced in cultured HeLa cells after treatment with several endocytic inhibitors [31]. Chlorpromazine, filipin III, and amiloride were used to inhibit clathrin-mediated, caveolae-mediated endocytosis, and macropinocytosis, respectively. Cellular uptake of the CNPs was interrupted to a certain degree with each of the three inhibitors, which suggests that multiple endocytosis mechanisms are involved in the cellular uptake of CNPs. In addition, the macropinocytosis-dependent pathway is thought to be the most

critical in the internalization of CNPs. After cellular uptake, lysosomal escape of the CNPs were observed by fluorescence tracking and TEM imaging suggesting that they can be valuable carriers to release loaded macromolecules in cytosols [32].

3. Application of CNPs for *in vivo* diagnostic imaging

To provide anatomical information on diseases, CNPs can be labeled using simple contrast agents for medical imaging. The EPR effects, enhanced vascular permeability, or vascular leakage induce the accumulation of CNPs in pathological lesions, and the diseased region generally has an intense image signal. Based on this property, various contrast agents and relevant imaging modules for CNPs have been studied. As shown in Section 2, NIRF dyes and whole body NIRF scanning devices are the most common used together to trace CNPs for *in vivo* diagnostic imaging. From the early days of molecular imaging, fluorescence imaging for diagnosis has been widely used because of its advantages, such as high sensitivity, multi-color imaging, and changeability of the signal by fluorescence resonance energy transfer (FRET) [33–35].

Over the course of time, constant attempts to use CNPs for diagnostic imaging resulted in the use of other imaging modules including magnetic resonance (MR), positron emission tomography (PET), computerized tomography (CT), and ultrasound (US) devices (Fig. 3) [36–42]. For MR imaging, superparamagnetic iron oxides (SPIOs) or gadolinium (Gd(III)) ions were encapsulated in CNPs. In addition, radio-isotopes, gold, and gas-generating polymer encapsulated CNPs allow for PET, CT, and US contrast imaging, respectively. Each imaging module has its own advantages and disadvantages, including sensitivity, imaging resolution, tissue penetration, biological risk, and cost.

3.1. Experimental application of CNPs in various imaging modules

Multi-modal imaging can provide more credible and accurate imaging of diseases. Because each imaging module has its own strengths and limitations, researchers have attempted to develop multi-probe labeled NPs. In particular, dual labeling of NIRF dye and MR contrast agent is the most popular method to compensate for the weak points of single-modal imaging [43,44]. As described above, NIRF optical imaging has many advantages which allow for rapid screening of diseases with a

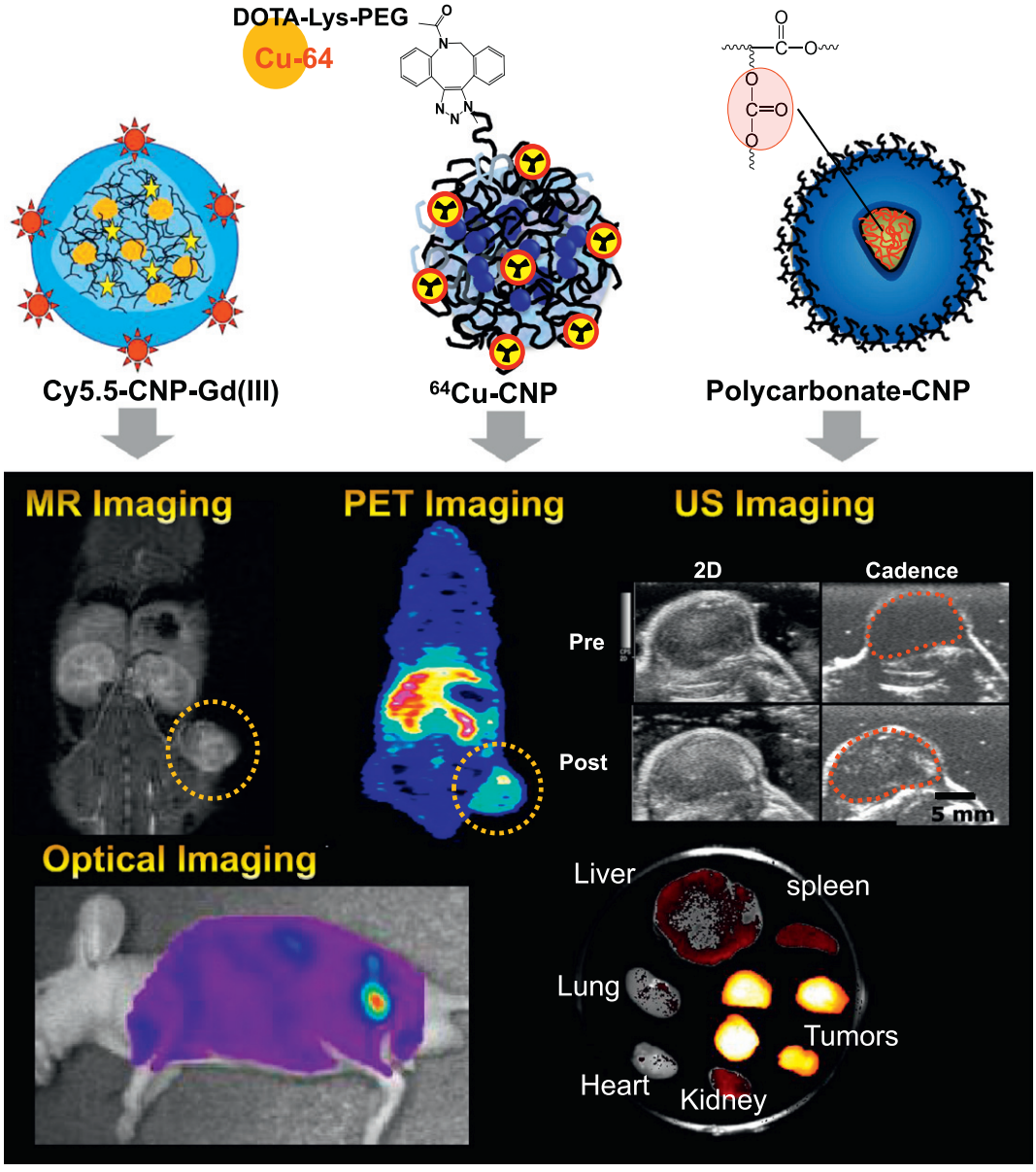

Fig. 3. Diagnostic imaging of tumors using CNPs in various imaging modules [36–38].

low dose of NPs. However, it often has the limitation of a low spatial resolution and a tissue penetration depth such that pathological lesions deep in the tissues cannot be visualized. By contrast, MR imaging offers high spatial resolution and deep tissue penetration, but it takes a long time and high dose for effective imaging. Consequently, MR and optical combined imaging is expected to complement each other. In addition to MR/optical dual labeling, a third imaging probe also can be applied for tri-modal imaging using NPs [44,45]. These multi-modal images have several attractive attributes for development and application to molecular imaging strategies.

As with other theranostic NPs, CNPs have been developed for multi-modal imaging to compensate for the weakness of single-modal imaging [36,41,42]. Cy5.5 labeled and Gd(III) encapsulated CNPs (Cy5.5-CNP-Gd(III)), for instance, provide tumor diagnostic images with both optical and MR devices [36]. The Cy5.5-CNP-Gd(III) included up to 6.28 wt.% Gd(III), and it successfully showed a tumor in the T1 weighted MR image as in the NIRF image. The excellent spatial resolution of T1 MR images enables reconstruction of tumor lesions in 3 dimensions. In addition, the Cy5.5-CNP-Gd(III) still retains the favorable properties of typical CNPs, including long blood circulation and excellent tumor targeting ability. SPIO loaded CNPs have also shown highly selective accumulation at tumors, and they were effective in displaying tumor regions in T2 weighted images [42]. The dual optical/MR imaging using CNPs could provide complete information on tumors, based on their highly tumor-selective accumulation.

Besides MR/optical dual imaging, different types of CNPs for multi-modal imaging are expanding for application in diagnostic imaging of pathological lesions. In practice, the development of optical/PET, optical/CT, optical/US, or optical/MR based trimodal imaging has been investigated for further applications in theranostic CNPs. Each combination would provide advanced information on pathological lesions, including the expression of biological molecules and changes in the microenvironment. Consequently, multimodal imaging platforms using CNPs could passively provide more opportunities for early diagnosis of diseases, based on the high quality information collected through the advantages of CNPs.

3.2. Molecular imaging using proteinase-sensitive probe coated CNPs

CNPs themselves are useful for imaging pathological lesions. CNPs may provide anatomical information on lesions, such as the size and localization of tumors. To detect molecular changes, however, surface modifications of CNPs would be required. Several modifications of CNPs, including antibodies, targeting peptides, or proteinase-sensitive probes, have facilitated the imaging of molecular changes in pathological lesions [46–48]. Antibody/affibody or targeting peptide grafted CNPs can visualize the expression of receptor proteins, based on the differences in affinity of cells with CNPs.

The other important modification is 'smart' nanoprobes, which are particularly useful to monitor the molecular changes in pathological lesions. 'Smart' nanoprobes are usually designed with dark-quenched NIRF dyes, which are linked with enzyme-sensitive peptide substrates. The chemical structures of the 'smart' nanoprobes, for example, can be simply explained as shown in Fig. 4A. The black hole quencher-3 (BHQ-3) is a dark quencher that has no native fluorescence but absorbs the fluorescence in the range from 620 to 730 nm. When FPR-675 (a NIRF dye with an excitation and emission maxima from 675 to 695 nm, respectively) and BHQ-3 are linked with a short amino acid sequence from the matrix metalloproteinase (MMP) specific peptide, the fluorophores are efficiently quenched showing no fluorescent signals. However, the probes can recover the NIRF after exposure to MMPs, because of de-quenching of the dye by the cleavage of MMP-sensitive peptide sequences. Because the exclusive use of proteinase-sensitive peptide based nanoprobes generally demonstrates rapid renal excretion *in vivo*, the nanoprobes are usually coated or conjugated to NPs for longer blood circulation and target-specific delivery (Fig. 4B). The probes definitely retain their activatable properties after binding to CNPs, and the polymeric probe-CNPs generated intense signals *in vivo* for a longer period of time [47,48]. The MMP sensitive probe (MMP-probe) coated CNPs showed substantial NIRF with 1.9 nM of a low dose MMP-3, based on the high sensitivity of the MMP probe. Moreover, the MMP probe-CNPs recovered fluorescent signals in direct proportion to the dose of the relevant enzymes (Fig. 4C). They also showed high specificity to MMPs (Fig. 4D), and the enzyme activities could be quantitatively analyzed by measuring the NIRF signal after the treatment of the probes-CNPs.

Similarly, a variety of 'smart' nanoprobes have been developed to visualize the specific enzyme activity. Depending on the substitution of amino acid sequences, the 'smart' probes can monitor the activity of specific enzymes, including MMPs, cathepsin B, and caspases-3 and -7 [46–52]. Each probe conjugated CNP has been used for monitoring the activity of relevant enzymes to provide biological information on diseases. The MMP probe-CNPs have been widely applied for chronic inflammation models, and they could indicate the degeneration and progression of diseases, such as osteoarthritis and rheumatoid arthritis. The CNPs with caspase-sensitive probes can visualize the extent of apoptosis, which is useful to evaluate the therapeutic efficiency of anti-cancer drugs. In addition, the differentiation potency of stem cells or precursor cells could be evaluated with the cathepsin B-sensitive probe coated CNPs because cathepsin B is usually involved in cell differentiation and over-expressed in differentiating cells [53–55]. Likewise, based on target specific delivery and high sensitivity, the enzyme-sensitive probe-coated CNPs offer great potential as molecular imaging probe systems for theranostics.

3.3. Diagnostic application of CNPs

Theranostic NPs have been most extensively studied in the field of cancer research, and CNPs have also been commonly applied to cancer theranostics [26,56,57]. In particular, 5β-cholanic acid conjugated GC based CNPs have attracted considerable attention for their excellent tumor-specific distribution and biocompatibility, and they have been investigated in various tumor models. Liver and brain tumor models were useful in understanding more about the *in vivo* tumor targeting ability [58]. In a liver tumor model, the CNPs showed selective accumulation in tumor tissues (Fig. 5A). The adjacent normal liver tissues presented low NIRF intensity, suggesting that the CNPs can avoid RES recognition in the liver. The brain tumor was also clearly delineated on the whole body NIRF image (Fig. 5B), and the CNPs were still highly distributed in the brain tumor. It could be due to the partial disruption of the blood–brain barrier (BBB) in tumor lesions [59], and all the results suggest a possibility for the wide application of CNPs in cancer diagnosis.

Because of their clinical significance, metastatic tumors should be considered in cancer theranostics. To examine whether CNPs could be delivered to tiny metastasized tumors, the biodistribution of 5β-cholanic acid conjugated GC based CNPs were evaluated in metastatic cancer models. Red fluorescence protein-expressing melanoma cells (RFP-B16F10) were injected *via* tail vein to establish a lung-metastasis cancer model, and the CNPs accumulated in the tumors of the lungs as well as in subcutaneous tumors [58]. Moreover, an unintended second metastatic tumor (2.6 mm in diameter) in the axilla was distinguished from the surrounded tissues in whole body NIRF scanning. Taken together, CNPs could be a promising system for cancer diagnostic imaging regardless of the type and location of the tumors.

Although CNPs have been mainly investigated in cancer research, they also have been widely applied in theranostics for other various diseases. In practice, CNP-based 'smart' nanoprobes have provided valuable information on the progression of arthritis. Rheumatoid arthritis (RA) and osteoarthritis (OA) are a chronic inflammatory disease in joints, which is characterized by the destruction and functional loss of joints [60,61]. Conventional diagnostic methods including X-ray and micro-CT often fail in the early diagnosis of RA and OA

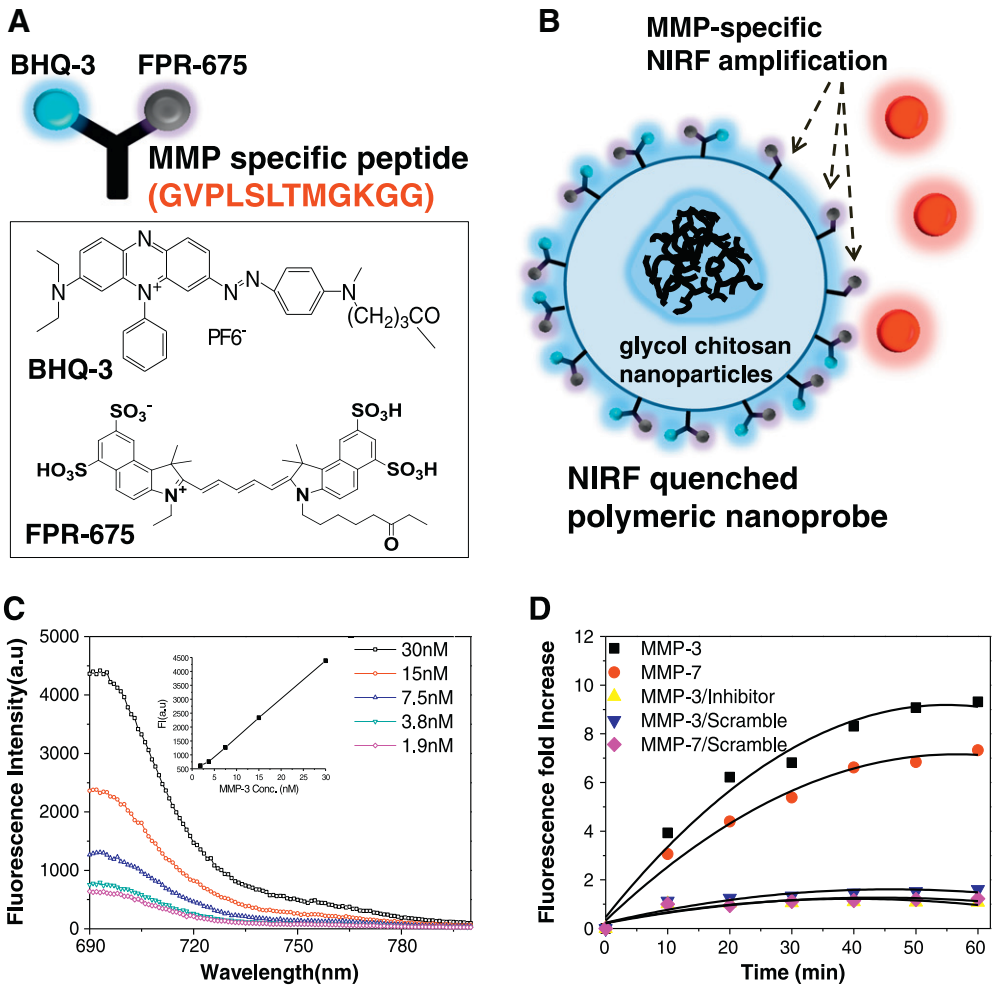

Fig. 4. Matrix metalloproteinase 3 (MMP-3)-specific polymeric probe [48]. (A) Chemical structure of the MMP-3-specific nanoprobe. (B) MMP probe coated CNPs. (C) Fluorescence recovery of the MMP-3-specific polymeric probe in the presence of MMP-3. (D) Fluorescence recovery of MMP-3-specific nanoprobe in the presence of various types of MMPs (MMP-3, MMP-7, and MMP-3 plus inhibitor).

because the initial stage of the diseases does not include distinct radiographic or molecular changes in the inflammatory lesions. However, CNP-based MMP probes can measure the MMP activities in the articular lesions from the early stage of the diseases, based on the high sensitivity of the probes. Furthermore, progression and therapy of the diseases could be followed up non-invasively and repeatedly through the monitoring of the MMP activity (Fig. 5C and D) [48]. The MMP probes were also developed as a diagnostic kit for synovial fluid from human OA patients [62].

Surface modification of the CNPs actualizes the non-invasive imaging of atherosclerotic lesions. A specific sequence of atherosclerotic plaque targeting peptide (CRKRLDRNC; termed AP peptide) was tagged to a hGC conjugate [63,64], and it formed self-assembled AP peptide-CNPs. Because the AP peptides show affinity to atherosclerotic plaques, the AP peptide-CNPs selectively bound to TNF-α-activated aortic endothelial cells *in vitro*. Moreover, the atherosclerotic plaques in a low-density lipoprotein receptor-deficient (Ldlr −/−) mouse were successfully imaged using the AP peptide-CNPs [64]. The AP peptide-CNPs could serve as effective imaging probe of atherosclerosis, and surface modifications with a targeting peptide may offer a clinical use for CNPs in a more wide variety of diseases.

4. Targeted drug delivery using theranostic CNPs

Nano-formulations of polymeric micelles and micelle-like aggregates have been traditionally considered as promising drug carrier systems [65–67]. Their hydrophobic domains in the core are available for encapsulation of hydrophobic drugs, and the hydrophilic outer shell can protect the drugs before they reach the target site. CNPs have not only been designed for carrying chemo-drugs, but also for the delivery of other therapeutic agents, including therapeutic genes, peptides, and photodynamic chemicals.

4.1. Delivery of chemical drugs

As with other micelle-like aggregates, CNPs can be utilized to carry a cargo of hydrophobic chemo-drugs. CNPs showed potential as theranostic NPs because of their superb tumor targeting ability and low cytotoxicity. Although the CNPs basically have a tumor-homing property, the tumor targeting efficiency of bare NPs is not always correlated with the loaded drug efficacy [14]. Moreover, the loaded drug contents can also influence the physiochemical properties and tumor targeting ability of the CNPs. Consequently, each formulation combining hGC conjugates and a particular drug should be individually and carefully optimized to achieve the best results for an effective therapy.

A variety of CNPs, such as N-acetyl histidine (NAcHis)–GC NPs, HO–GC NPs, and 5β-cholanic acid–GC NPs have been developed for hydrophobic chemo-drugs delivery [14,15,19,27–29,68–70]. For cancer theranostics, anticancer drugs including docetaxel (DTX), camptothecin (CPT), paclitaxel (PTX), and doxorubicin (DOX) are commonly loaded in the CNPs with a simple dialysis method. The HO–GC NPs, for example,

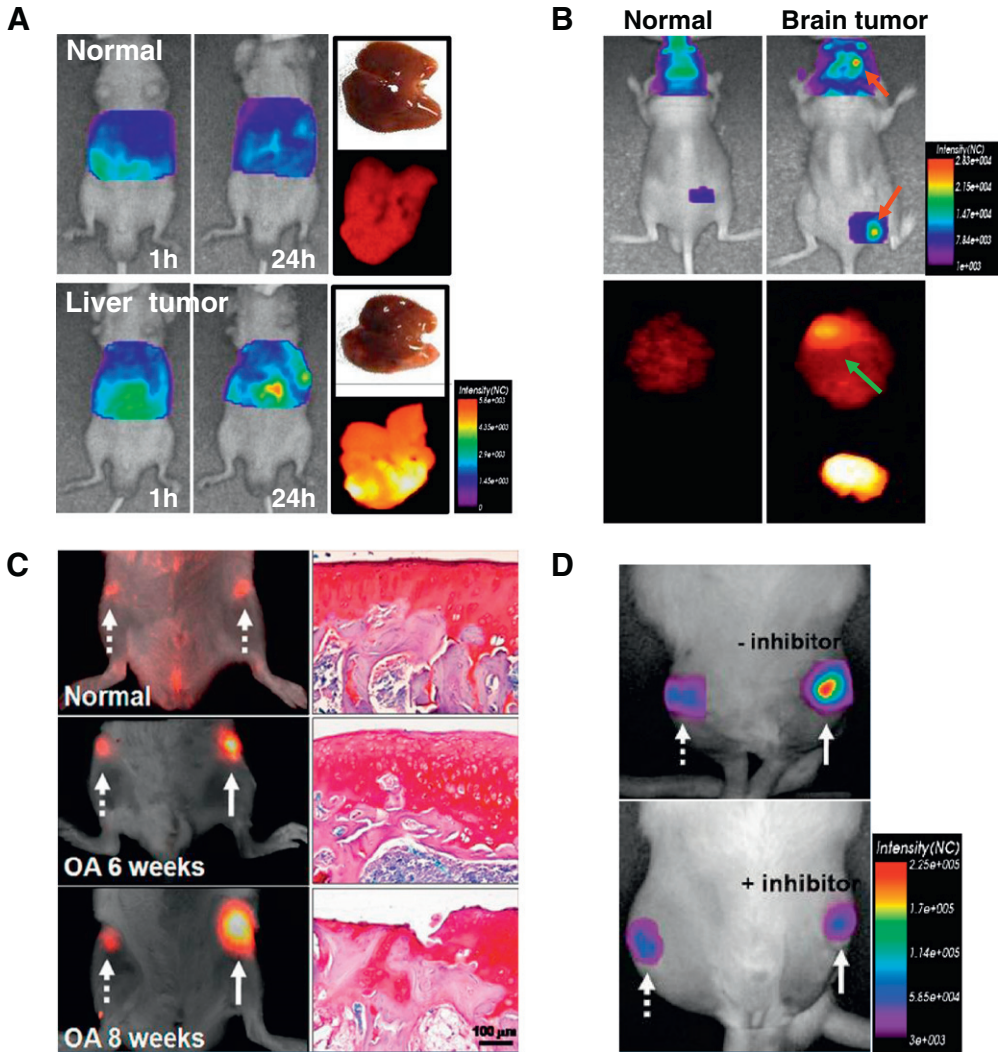

Fig. 5. Diagnostic application of CNPs in various disease models [49,58]. (A) Tumor diagnostic imaging of CNPs in liver tumor model. (B) Tumor diagnostic imaging of CNPs in brain tumor model. (C) Diagnostic application of CNPs in osteoarthritis (OA) model. (left: normal cartilage; right: OA induced cartilage) (D) *In vivo* NIRF tomographic images of OA cartilage with or without the addition of the MMP-13 inhibitor. (Top: without MMP-13 inhibitor; Bottom: with MMP-13 inhibitor in OA-induced cartilage).

could hold a large amount of PTX inside [68]. Generally, it was reported that the drug-loading amount of PTX was about 10 wt.% in nanoparticles [25], but the HO–GC NPs showed a 95.1% loading efficiency with a 20 wt.% PTX feed ratio. The HO–GC-PTX NPs also showed longer blood circulation and better therapeutic effects than that of the commercial nano-formulation of the anticancer drug, Abraxane® (Fig. 6A). Considering the low cytotoxicity and high loading efficiency of HO–GC NPs, HO–GC NPs could be a competitive carrier for PTX, and the resulting HO–GC-PTX NPs are expected to be a new treatment strategy for effective cancer therapy.

4.2. Gene delivery

Therapeutic genes consisting of oligonucleotides, such as plasmid DNA (pDNA) and small interfering RNAs (siRNAs) have great significance in correcting genetic disorders. However, overcoming transport barriers to gene delivery has been a major hurdle of therapeutic applications. In general, cationic lipids and polymers have been widely used for effective gene delivery [72,73]. Chitosan could be an attractive material for a gene carrier because this natural polymer includes many of the primary amine groups which are positively charged. The positive charge of the amine groups can interact with anionic phosphate backbones of DNA molecules to form complexes. Accordingly, various chitosan-based NPs including trimethylated chitosan/DNA complexes, alkylated chitosan-based NPs, and 5β-cholanic acid–GC NPs, have been investigated for gene delivery [74–77].

Chitosan naturally shows positive charges, but the charge density of chitosan is relatively lower than that of other cationic synthetic polymers. The low charge density of chitosan results in loose structures of chitosan/pDNA complexes, and the loosely bound complexes are unfavorable for the transfection of cells due to their low stability. To build up compact complexes for a more stable and pDNA-protective formulation, DNA molecules were encapsulated in CNPs by interactions between hydrophobic moieties and hydrophobized DNA [74]. Cetyltrimethylammonium bromide (CTAB) was used to modify pDNA molecules, and the CTAB/DNA complexes were successfully encapsulated in CNPs to transfect simian kidney (COS-1) cells. Under the optimized condition, the CNPs demonstrated a higher transfection efficiency than that of Superfect®, a commercialized transfection agent. It could be attributed to the unique characteristics of typical CNPs, and the results confirmed that CNPs can be utilized for the delivery of genes as well as hydrophobic drugs.

4.3. Delivery of other drugs

In a recent trend in nanomedicine, stimuli-responsive nano-formulation has attracted much attention for more accurate targeting of treatment. Among the stimuli-responsive ones, photodynamic

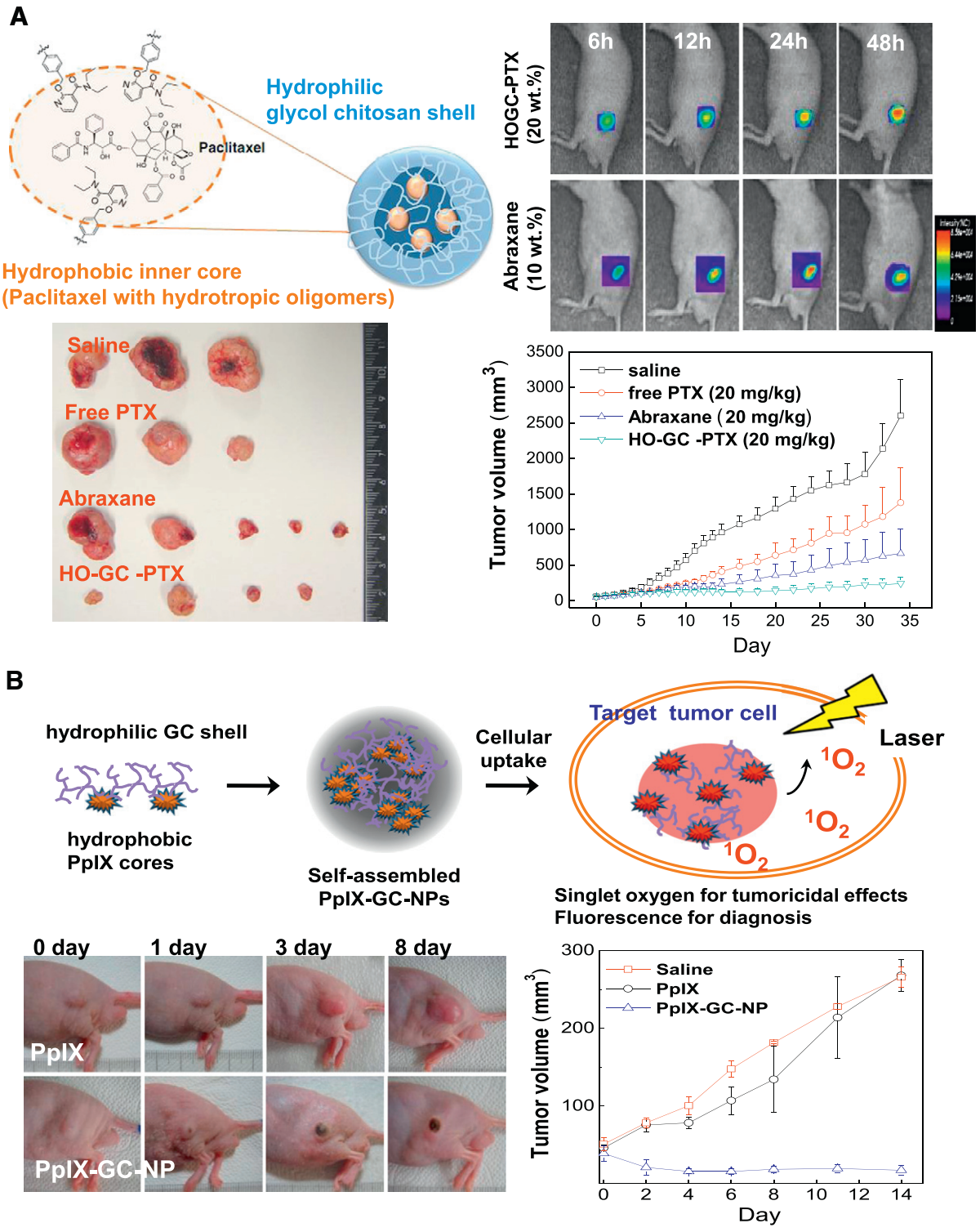

Fig. 6. Theranostic application of CNPs [68,71]. (A) Paclitaxel loaded hydrotropic oligomer-conjugated glycol chitosan nanoparticles (HOGC-PTX) and therapeutic effects compared to the Abraxane®. (B) Photodynamic therapy of PpIX-GC-NP *in vivo*.

therapy (PDT) using a combination of photodynamic chemicals and photoradiation is a new emerging strategy to reduce the systemic toxicity of anti-cancer therapy [78]. Photosensitizers emit a strong NIRF, and it enables the easy tracking of the photosensitizers. Moreover, cytotoxic free radicals are generated when photosensitizers are exposed to light, and the free radicals can induce a wide range of cell death in tumors. Primary targeting of tumors using NPs and secondary targeting with local light emission can maximize tumor selectivity and reduce systemic toxicity. The two-step targeting strategy of PDT shows excellent therapeutic effects. Therefore, CNP-based photodynamic agent delivery has been considered for the synchronous imaging and therapy of cancer [79,80].

Diverse photodynamic chemicals, such as pheophorbide a (PheoA), chlorin e6 (Ce6), and protoporphyrin IX (PpIX), have been incorporated into CNPs [71,81–83]. For instance, chemical conjugates of hydrophilic GC and hydrophobic PpIX spontaneously formed self-assembled CNPs,

and the PpIX constitutes a dense core of the particle with no fluorescence by self-quenching [71]. However, by decreasing the integrity in the cytosol, the fluorescence of PpIX was recovered after cellular uptake. In addition, cytotoxic singlet oxygen can be released when the cells are exposed to light, leading to wide necrosis of cancer cells (Fig. 6B). Photodynamic chemical carrying CNPs have demonstrated perfect therapeutic effects *in vivo*, and concurrent photodynamic imaging without further modification to the CNPs.

Peptides are biologically important for regulation of most physiological processes in the body, but most peptides are easily degradable by *in vivo* enzymes. Over the past decade, peptide drug research has been expanded, and CNPs have also been designed to protect peptide drugs before reaching the target site. The Arg–Gly–Asp (RGD) peptide, which can inhibit angiogenesis by binding to $\alpha v\beta_3$ integrin, was encapsulated into CNPs with a high loading efficiency [84]. With the solvent evaporation method, fluorescein isothiocyanate (FITC) labeled RGD peptide molecules were successfully incorporated into the CNPs despite their hydrophilic property. The CNPs showed prolonged and sustained release of RGD, indicating the suppressed migration of human umbilical vein endothelial cells (HUVECs) *in vitro*. The intra-tumoral administration of RGD peptide-loaded CNPs showed retardation of tumor growth and a decreased micro-vessel density in tumors [63]. We expect that the combination of imaging probes and adequate drugs in CNPs will present a new strategy in theranostics for simultaneous imaging and therapy.

5. Perspectives

During recent decades, nanoparticle-based drug delivery systems have made tremendous progress in theranostics. Current biomedical research on theranostic NPs provides real-time non-invasive molecular imaging of pathological lesions with target specific drug delivery. The significant advances in theranostic NPs also have enabled the controlled release of drugs, leading to improvement in therapeutic effects with convenient protocols. Riding on this success, CNPs have been steadily studied in theranostics for various diseases from the early 2000s.

Despite the technological progress, nano-formulation including CNPs faces challenges in clinical applications. Although NPs are novel and perfectly useful, current approaches to theranostics are somewhat focused on the concept of the mechanics and engineering of NPs alone. Although the optimization and improvement of NP formulations are certainly important tasks for efficient delivery, perhaps we should place more emphasis on the biological factors that affect the delivery of NPs. To overcome present limitations, it might be better to have interests in disease conditions. For instance, local blood flow and blood pressure may have an influence on the targeting ability of NPs, and controlled local blood flow may maximize the EPR effects. In addition, degradation of the extracellular matrix (ECM) around pathological lesions also may enhance targeted drug delivery because the complexities of the ECM may disturb the drug diffusion as a mechanical barrier.

In practice, many research groups have become more concerned about the biological conditions of target diseases. Today, cancer microenvironments and intra-tumoral cancer heterogeneity are typical discussion topics in the development of theranostic NPs. Despite the remaining hurdles, continuous efforts in medical science and biology will overcome the present limitations of drug delivery systems. We expect that theranostic CNPs will play important roles in the future because research on CNPs will continue contributing to the future clinical application of overall nano-formulations, and early diagnosis and personalized medicine become reality.

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