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Multifunctional Chitosan Nanoparticles for Tumor Imaging and Therapy

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Abstract Chitosan and its derivatives have been widely used for various biomedical applications because of their unique chemical and biological characters. The amine groups in the backbone of chitosan allow chemical modification to change the physical properties of chitosan. Based on hydrophobic or charge interactions with this chitosan polymer backbone, stable self-assembled nanoparticles can be fabricated in aqueous condition. These nanosized structures enable intravenous injection and show large accumulation in tumor tissue, indicating great potential in imaging and drug delivery. The main objective of this review is to introduce various chitosan nanoparticles and their recent applications for tumor diagnosis and therapy.

Keywords Chitosan · Molecular imaging · Nanoparticle · Tumor

Contents

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1 Introduction

For cancer diagnosis, many noninvasive imaging methods have been developed and applied to living subjects, and they are feasible for assessing the biological or biochemical processes related to cancer metabolism. Various nanoparticles have been developed especially for this purpose, and they have allowed fine tumor imaging in living systems at different time points, providing valuable information. These nanoparticles are also notable tools for drug and gene delivery and could increase the efficacy of therapy. Furthermore, they enable the "theragnosis," the combined diagnosis and therapy, of various diseases, including tumors, whereby imaging agent and drug are included in one nanoparticle. The building blocks of nanoparticles may consist of natural and synthetic polymers that are biocompatible and biodegradable [1–3]. Among them, natural polymers include nucleotides, proteins, and polysaccharides. Polysaccharides are especially privileged materials in nanoparticle fabrication because of their physical and biological properties, such as stability or the sufficiency of functional groups [4, 5].

Among the natural polysaccharides, chitin and chitosan are very widely used in biomedical fields. Chitin is a biodegradable polymer of high molecular weight, and is one of the most common polysaccharides found in nature. It originates from the abundant exoskeletons of crustaceans. Like cellulose, chitin is a fiber, and it presents unique chemical and biological qualities that can be used in various medical applications.

Chitin and chitosan have similar chemical structures, and they are made up of cationic polysaccharide linear chain of D-glucosamine and *N*-acetyl-D-glucosamine linked by glycosidic bonds. Chitosan is obtained by removing enough acetyl groups (CH_3–CO) for the molecule to be soluble in most diluted acids. This process, called deacetylation, releases amine groups (–NH) and gives the chitosan a cationic characteristic. This is especially interesting in an acid environment where the majority of polysaccharides are usually neutral or negatively charged, and these cationic charges of the amine groups enable chitosan molecules to be soluble in acidic aqueous condition. These primary amine groups in a deacetylated subunit of chitosan have a pK_a value of about 6.5. Therefore, chitosan is soluble in acidic solution such as acetic acid, citric acid, aspartic acid, glutamic acid, hydrochloric acid, and lactic acid. However, at neutral or alkaline pH it loses some of the surface charge and becomes insoluble, forming aggregates. In addition to pH, the solubility of chitosan is greatly influenced by its degree of deacetylation and molecular weight, and by the ionic strength of the solution.

This incomplete solubility of chitosan in aqueous condition could be a obstacle in handling for many uses, and therefore various chitosan derivatives were developed

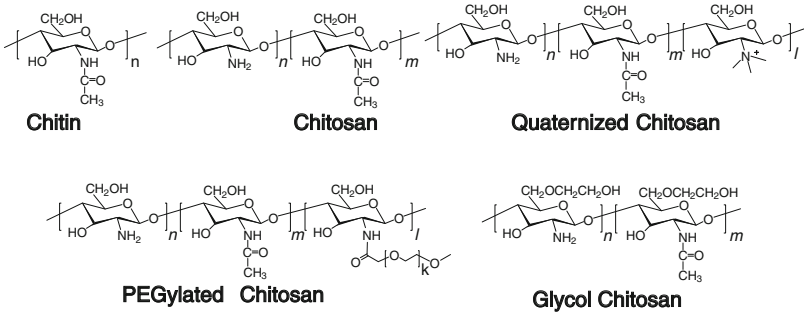

Fig. 1 Chemical structure of chitin, chitosan, and its derivatives

for improved solubility and other purpose (Fig. 1). Quaternized chitosan (*N*,*N*, *N*-trimethyl chitosan; TMC) and polyethelene-glycol-conjugated (PEGylated) chitosan are more soluble in water than the original form. The amine groups of quaternized chitosan are mainly protonated at neutral pH, therefore the overall charge of quaternized chitosan is positive. This positive charge has advantages for good solubility, enhanced cellular uptake, and condensing ability with gene drugs, but chitosan aggregates with anionic serum protein.

PEGylated chitosan is very stable even in serum because of its bioinert PEG group, but it also has lower efficiency of cellular uptake than natural chitosan. Glycol chitosan (GC) is a chitosan derivative containing short ethylene glycol groups in its backbone, and these glycol groups inhibit aggregation with anionic serum protein and provide good solubility, but they are not long enough to inhibit cellular uptake. These chitosan derivatives were developed to improve the properties of original chitosan, and have shown their usefulness in biomedical fields such as imaging and therapy [6–11].

In this article, we will review the application of chitosan-based nanoparticles in tumor imaging and therapy. The first part of the review is focused on the mechanism of efficient tumor accumulation using chitosan nanoparticle. The second part considers the recent applications of chitosan nanoparticles for tumor imaging, and the last part is concerned with antitumor therapy using drug- or gene-containing chitosan nanoparticles.

2 Tumor-Targeted Delivery of Chitosan Nanoparticles

Nanosized particles, with diameters of <100 nm to 1 μm, have been utilized for targeted delivery of imaging agents, drugs, or therapeutic genes. Nanoparticle delivery systems enable the prolonged circulation time of imaging agents or drugs, and can be controlled by targeting tactics [12]. Intravenously administered nanoparticles need to slip through the tumoral blood vessels to reach their target tumor site. Although targeting moieties can improve tumor-homing efficiency,

some nanoparticles show high accumulation at a tumor site without a targeting ligand. Nanosized particles show a tendency to extravasate from leaky blood vessels and to be easily retained in the tumor site. This effect is known as passive targeting for enhanced permeability and retention (EPR) [13]. However, chemically modified or targeted ligands conjugated to chitosan nanoparticles showed better performance for imaging or therapy in many studies. Therefore, localization of nanoparticles at tumor sites depends on a combination of the effects of various factors such as charge and size of particles, and the presence of a targeting ligand.

2.1 Passive Targeting (EPR Effects)

The origin of the EPR concept dates back to the late 1970s, when the selective accumulation of macromolecular drugs in tumor tissues was discovered [13]. The specific passive accumulation of macromolecules was attributed to defective tumor blood vessels with poorly organized endothelium at the tumor site and a poor lymphatic drainage system. Since then, researchers have capitalized on this concept for the delivery of various drugs by conjugating them with polymers or encapsulating them within nanoparticles. Nowadays, it is evident that long-circulating macromolecular polymer–drug conjugates and nanosized particulates such as micelles or liposomes accumulate passively in the tumor tissue due to the EPR effect. To maximize the EPR effect, the size, surface charge, and deformability of the nanoparticles should be considered carefully. Therefore, some chemical modifications have been developed to improve the EPR effect by changing the chemical or physical properties of chitosan nanoparticles.

The EPR effect has been confirmed in various kinds of biocompatible macromolecules, and chitosan-based nanoparticles are also able to passively accumulate in the tumor through this effect. Chitosan has a free amine group in a unit of hexosaminide residue. With this functional group, hydrophobic groups can be conjugated to hydrophilic chitosan or GC polymer to refine the self-assembling capabilities. These polymeric amphiphiles form self-assembled nanoparticles in an aqueous environment via hydrophobic interactions between the hydrophobic parts, primarily to minimize interfacial free energy. Therefore, chitosan-based nanoparticles can be readily obtained by chemically attaching the hydrophobic moiety to the backbone of chitosan and its derivatives. Various hydrophobic groups like oleic acid, cholesterol, stearic acid, deoxycholic acid, 5β-cholanic acid, and some hydrophobic drugs have been conjugated to chitosan and its derivatives. These chitosan conjugates have improved physicochemical and functional properties compared to native chitosan. Furthermore, these self-assembled hydrophobically modified chitosan nanoparticles can encapsulate a quantity of drugs or imaging agents inside the particles or present them on the surface by conjugation. Many studies using self-assembled nanoparticles have been carried out for imaging and drug delivery purposes.

These nanoparticles can circulate in the bloodstream for a relatively long time without recognition by phagocytes and can easily accumulate at the leaky vasculature throughout the EPR effect. In general, the results have demonstrated that nanoparticles based on chitosan and its derivatives are stable in a physiological solution for a long period of time without significant change in the particle size. When these nanoparticles are systemically administrated into tumor-bearing mice, they can circulate in the bloodstream for at least 1 day, thereby increasing the probability of the nanoparticles reaching the target site. It should be emphasized that a significant amount of chitosan-based nanoparticles have been reported to be selectively accumulated into the tumor site, primarily owing to the EPR effect (Fig. 2).

Chitosan conjugated with ethylene glycol is totally water soluble, regardless of pH values, and the GC retains a positive charge [14]. GC has been additionally modified with polymers or hydrophobic analogs to further improve its ability as a drug carrier [15–17]. Previously, Park et al. evaluated three different molecular weights (20, 100, and 250 kDa) of GC nanoparticles [16] to evaluate their effect on the EPR mechanism. The mean diameters, in vitro colloidal stability, and surface charges of the GC nanoparticles were not significantly different. However, fluorescence imaging data revealed that the GC with higher molecular weight (250 kDa) showed longer blood circulation time and higher tumor selectivity. In previous studies, GC nanoparticles modified with deoxycholic acid or bile acid were also characterized [6], and these GC nanoparticles, loaded with drugs such as paclitaxel, cisplatin, or doxorubicin, were applied for cancer therapy (reviewed in Sect. 4.1) [7–9].

PEG and PEGylated nanoparticles have many advantages for biomedical applications, and PEGylation is another representative method for enhanced passive targeting. PEGylation of chitosan nanoparticles also improves their physical stability and prolongs blood circulation time [16]. Paclitaxel- or camptothecin-loaded PEGylated chitosan nanoparticles were investigated and found to be phagocytized less than unconjugated nanoparticles [10, 11].

2.2 *Active Targeting*

The EPR effect is the basis of the tumor targeting and is generally accepted for selective delivery of many macromolecular anticancer drugs. However, drug accumulation in tumor tissue does not always mean a successful therapeutic effect,

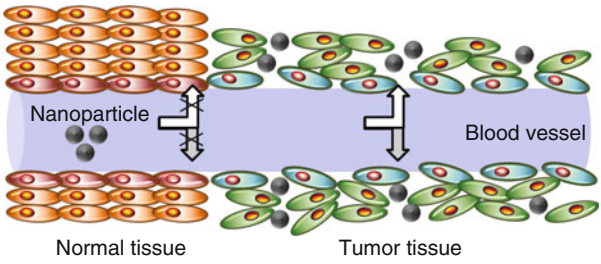

Fig. 2 Illustration of enhanced permeation and retention (EPR) effect and accumulation of nanoparticles in tumor tissue

e.g., for drugs or genes that cannot reach the target cells or inside organelles in their original form. Active targeting is a tactic that enhances the efficiency of a nanoparticle delivery system by using conjugation with targeting moieties, and receptor-mediated endocytosis is the major cellular mechanism used in active targeting. Consequently, overexpression of particular antigens or receptor proteins on the surface of tumor cells is often utilized in these targeting tactics, and the expression of specific receptors on the tumor cells may be helpful for successful active targeting. Ligand-targeted nanoparticles can recognize tumor cells with high efficiency and showed lower toxicity in nontargeted tissues in cases of drug delivery [18, 19]. Several targeting moieties, including specific ligands or antibodies, have been studied and utilized for chitosan-based nanoparticle systems.

Folic acid is a vitamin with a low molecular weight (441 Da), and the folate receptor is known to be overexpressed in various tumor cells, including lung epithelia and ovarian cancer [20–22]. The high binding affinity of folate to its receptors has been applied to modification of chitosan nanoparticles for receptor-mediated endocytosis, and folate is one of the most common molecules in use for active targeting of nanoparticles in cancer research. The improvement of transfection efficiency of delivered gene involves not only targeting ability or endocytosis, but also the nuclear localization ability of the arginine residue of the delivered gene [23]. Folate-conjugated chitosan nanoparticles loading paclitaxe, aminolevulinic acid (5-ALA), pyrrolidinedithiocarbamate, or doxorubicin were applied for cancer therapy [24–27]. Various studies on the active targeting of chitosan nanoparticles using folate and other moieties are summarized in Table 1.

Transferrin is a 80-kDa blood plasma protein that transports iron ions to cells carrying transferrin receptors. Transferin-conjugated chitosans are also used as tumor-targeting nanoparticles, because transferrin receptors are overexpressed in malignant tumor cells. Transferrin, as a targeting ligand, has the advantage that it can be internalized into the cells without the effects of the drug efflux system associated with p-glycoproteins [28]. Moreover, overexpression of the transferrin receptor is closely related to drug resistance in tumor cells [29]. Dufes et al. reported that nanoparticles based on transferrin-conjugated chitosan showed a significantly higher cellular uptake than those with unconjugated chitosan [30].

Glycyrrhetinic acid is the bioactive compound of licorice species, which are perennial herbs [31]. Glycyrrhetinic acid-modified chitosan nanoparticles have affinity to human hepatocellular carcinoma cells (QGY-7703 cells) and demonstrated a 19.0-fold increased uptake in vitro. Doxorubicin (DOX)-loaded glycyrrhetinic acid-modified chitosan nanoparticles could inhibited tumor growth successfully in H22 cell-bearing mice [32].

Short peptides are also favorable targeting moieties for many researchers. In previous studies, some peptides were conjugated to chitosan-based nanoparticles as targeting moieties. Arg-Gly-Asp (RGD) peptide showed increased selectivity for small interfering RNA (siRNA) delivery when linked to chitosan nanoparticles [33]. RGD-linked chitosan nanoparticles showed a high therapeutic efficacy against ovarian carcinoma in vivo. Moreover, bombesin, a 14-amino acid peptide binding to the bombesin receptor, has been studied for therapy of gastric-releasing peptide

Table 1 Cancer-targeted chitosan-based nanoparticles and their active targeting moieties

Targeting moiety	Drug or gene	Remarks	References
Folate	Paclitaxel	Cytotoxicity of paclitaxel-loaded folate-conjugated chitosan nanoparticles was evaluated in vitro	[24]
	δ-ALA	Folic acid–chitosan conjugates were used as a vector for colorectal-specific delivery of 5-ALA for fluorescent endoscopic detection	[25]
	PDTC, doxorubicin	Doxorubicin released from pH-sensitive folate–chitosan micellar nanoparticle. The particle showed the ability for co-delivery of PDTC and doxorubicin in vitro	[26]
	Paclitaxel	Folate-conjugated stearic acid-grafted chitosan oligosaccharides increased cellular uptake in cells expressing abundant folic acid receptors	[27]
Transferrin	Doxorubicin	Transferrin-conjugated chitosan nanoparticles showed an increased uptake efficiency in vitro and tumoricidal activity in vivo compared to the nontargeted chitosan	[30]
Glycyrrhetinic acid	–	Glycyrrhetinic acid-conjugated chitosan nanoparticles showed affinity to human hepatocellular carcinoma cells and could inhibit H22 tumor cells in vivo	[32]
Arg-Gly-Asp (RGD) peptide	siRNA specific for POSTN, FAK, and PLXDC1	RGD–chitosan conjugates, as an αvβ3 integrin-targeted delivery system, was used for targeted siRNA delivery. Enhanced antitumor therapeutic efficacy was confirmed	[33]
Bombesin peptide	–	Bombesin was conjugated to *N*-acetyl histidine-modified GC for targeting gastric-releasing peptide receptors overexpressed in prostate cancer cells. Iron oxide was loaded to be tested as a probe for MRI	[34]
Mannose	Murine IL-12 gene plasmid	Mannosylated chitosan/IL-12 plasmid complexes delivered the plasmid to the dendritic cells around tumor cells, and could inhibit tumor growth in vivo with higher efficiency	[35]

ALA Aminolevulinic acid, *GC* glycolchitosan, *FAK* focal adhesion kinase, *IL-12* interleukin-12, *PDTC* pyrrolidinedithiocarbamate, *PLXDC1* plexin domain-containing protein 1, *POSTN* periostin, *RGD* Arg-Gly-Asp peptide, *siRNA* short interfering RNA

receptor (GRPR)-positive tumors. Bombesin conjugated to *N*-acetyl histidine-modified GC nanoparticles were able to bind to prostate cancer cells (PC-3) over-expressing GRPR, and iron oxide-loaded nanoparticles could be applied as a probe for magnetic resonance imaging (MRI) [34].

Because immature dendritic cells display abundant mannose receptors for endocytosis or phagocytosis, mannose can be applied to active targeting of chitosan nanoparticles. For interleukin-12 (IL-12) gene delivery to dendritic cells around the tumor cells, mannose ligand was considered for modification of the chitosan nanoparticles [35]. The study showed that mannosylated chitosan and IL-12 plasmid complexes could inhibit tumor growth, and that biocompatible mannosylated chitosan nanoparticles were effective as a gene carrier for cancer immunotherapy.

3 In Vivo Tumor Imaging with Chitosan Nanoparticles

Nanoparticles have been studied for biomedical imaging with various imaging agents, which enables noninvasive in vivo live imaging. Molecular imaging modalities for this purpose include optical imaging, positron emission tomography (PET) or single photon emission computed tomography (SPECT), and MRI. Although conventional imaging modalities have been used for molecular imaging of biological targets, these modalities lack the target site specificity required for precise diagnosis of disease. Therefore, targeted delivery of imaging agent using nanoparticles could play an important role in molecular imaging. In addition, each modality has a different character with respect to sensitivity, resolution, time, and cost. Therefore, each imaging modality has its own advantages and limitations; consequently, appropriate choice or combination of these modalities is highly required according to the type and degree of disease.

3.1 Optical Imaging

Light is the representative property that can be applied to imaging, and the emission photons can be produced by bioluminescent proteins, fluorescent proteins, or fluorescent dyes coupled to nanoparticles. The most important advantage of optical imaging is the high sensitivity of visualizing target molecules such as peptides, drugs, DNA, or siRNA. For in vivo optical imaging, probes emitting in the near-infrared fluorescence (NIRF) spectrum are favored because NIRF has emission wavelengths of approximately 600–1,000 nm, which are not well absorbed in biological tissues. Moreover, autofluorescence from connective tissue and other biological fluorophores does not interfere strongly in the NIR [36]. Although some new NIRF probes with high photostability and low cytotoxicity have been developed, polymethines such as Cy5.5 are still the most common NIR fluorophores used for fluorescence imaging in vivo.

Whole-body NIR fluorescence intensity monitoring has been performed in tumor-bearing nude mice after administration of probe containing nanoparticles. Previous studies showed tissue distribution and tumor accumulation of chitosan nanoparticles in vivo. To assess biodistribution of nanoparticles, fluorescent images

are acquired at various time points up to 72 h post-injection using various photon counting scanners such as eXplore Optix system [9, 16], the IVIS Spectrum small-animal in vivo imaging system [34], or Kodak Image Station 4000 MM [37]. Although the tumor-homing efficiency of the nanoparticles was somewhat differentiated according to the different characteristics of each nanoparticle, many studies demonstrated that chitosan-based nanoparticles were accumulated in tumors. Tumor-bearing mice at the peak tumor-accumulation time point can be sacrificed to estimate the tissue distribution of nanoparticles ex vivo. NIRF images of dissected organs and tumors are obtained using cameras with a Cy5.5 filter set (680–720 nm) [9]. The chemical structure, noninvasive fluorescent optical imaging, and biodistribution of Cy 5.5-conjugated chitosan nanoparticles are shown in Fig. 3 [34].

Quantum-dots (QDs) are colloidal semiconductors. They exhibit unique optical and electronic properties that are closely related to the size and shape of each crystal. QDs are in the limelight for optical applications due to their high sensitivity and the stability towards photobleaching. However, they were not originally biocompatible because traditional QDs are made with toxic metal ions, such as cadmium. To increase the stability or the efficacy of delivery, chitosan derivatives have been utilized for coating or encapsulating QDs. Tan et al. developed chitosan

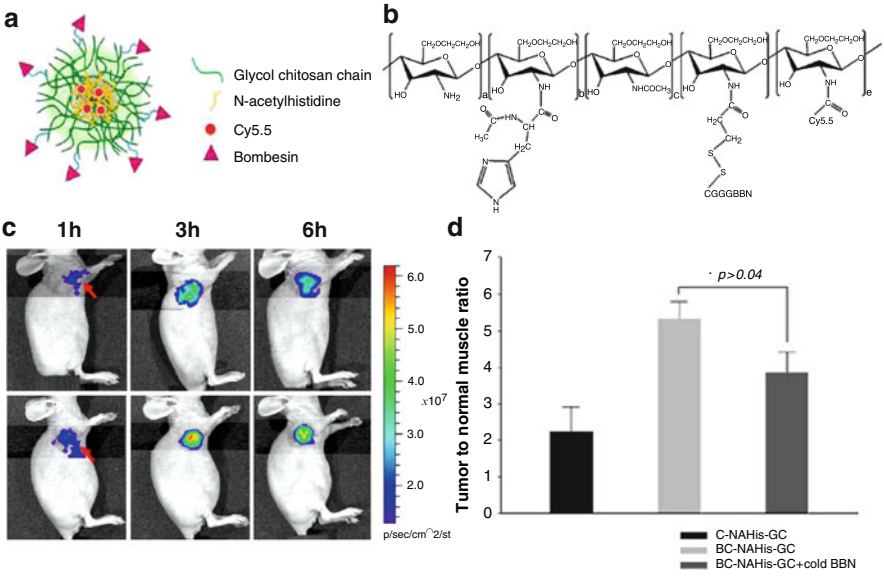

Fig. 3 Fluorescent optical imaging of tumor with bombesin–Cy 5.5-*N*-acetylhistidine–glycol chitosan nanoparticles (BC-NAHIS-GC). (**a**) BC-NAHIS-GC nanoparticle. (**b**) Chemical structure of BC-NAHIS-GC. (**c**) In vivo noninvasive fluorescent imaging of mice with PC3 tumors: C-NAHIS-GC (*upper*) and BC-NAHIS-GC (*lower*). (**d**) Ex vivo quantification of fluorescence in tumor tissue (tumor to normal ratio). From Lee et al. [34]

nanoparticles doped with QDs for delivery of HER-2-specific siRNA, and the results suggested that they are easily internalized into cells [38]. In another study, cadmium–selenium hybrid QDs were crosslinked to chitosan–poly(methacrylic acid) nanogels [39]. The covalently crosslinked hybrid nanogels had good structural stability and changed physical property in response to a pH change to release the anticancer drug temozolomide. In in vitro cytotoxicity tests, there were no signs of morphological damage to B16F10 cells, and free hybrid nanogels were low-cytotoxic in concentrations up to 520 mg/mL for 2 h. These studies only contain in vitro data, but we expect that chitosan nanoparticles have potential for efficient in vivo optical imaging using QDs.

3.2 MR Imaging

MRI has been one of the most powerful noninvasive diagnostic imaging modalities and provides detailed anatomical information for tumor diagnosis [40]. In clinical diagnosis, contrast agents such as iron oxide nanoparticles and gadolinium (Gd)-based molecules are necessary to improve enhancement of signals in the disease tissue surrounding the normal tissue. There are two major subgroups of MR contrast agents that are currently being developed. The first type are positive contrast agents like Gd-based chelates, which cause a reduction in the T1 relaxation time and appear extremely bright on the T1-weighted images [40, 41]. The second type are negative contrast agents such as superparamagnetic iron oxide nanoparticles (SPIONs), which produce a predominantly spin–spin relaxation effect, resulting in shorter T1 and T2 relaxation times [40]. This enables the enhancement of T2-weighted images and gives dark MR images. The functional modification of these MR contrast agents with nanoparticles for efficient delivery, tumor targeting, and molecular imaging has been attempted by many researchers [40, 42, 43].

Among contrast agents, SPIONs have attracted the interest of many researchers over the last 10 years in an effort to achieve an efficient MRI for more accurate tumor diagnosis [40, 44]. Although considerable efforts have been made to develop magnetic nanostructures, further modification is required to improve the physiological character of SPIONs. SPIONs can be easily prepared in nanosizes and show promising magnetic properties. However, it has been shown that bare SPIONs tend to aggregate in suspension, resulting in limitations for clinical applications [40, 42]. Furthermore, for in vivo application when injected intravenously, SPIONs need to be suspended in aqueous solution with good stability. They can be easily functionalized with biological substrate for efficient tumor targeting without rapid elimination of them by the reticulo-endothelial system (RES). Therefore, for in vivo application of SPIONs as MRI contrast agents, stabilization of SPIONs is highly desirable. Surface modification of SPIONs with biocompatible polymers (mostly coated with dextran) such as PEG, polylactic acid, albumin, or hyaluronic acid has been explored to achieve enhanced colloidal stability, prolonged blood circulation,

and improved specificity to target site [42, 45–47]. Surface-modified SPIONs showed enhanced blood circulation and sufficient stability for in vivo applications, such as diagnosis of disease, MR-guided drug delivery, and imaging of cell trafficking and migration [40, 48–50]. There are also several studies on the modification of SPIONs with chitosan-derived polymers, which can be used to coat and stabilize the SPIONS due to their biocompatibility. Shi et al. prepared SPIONs coated with carboxymethyl chitosan (CMCS) for imaging human mesenchymal stem cells (hMSCs) with MRI [51]. In this preparation, CMCS was used for increasing the stability of SPION, resulting in good dispersion in aqueous media and facilitating the uptake of the CMCS-coated SPIONs by stem cells.

Nanoparticles have been introduced into a multidisciplinary research field for various biomedical applications such as diagnosis and therapy. Particularly, the use of polymeric nanoparticles is attractive for the development of multimodality imaging platforms due to their biodegradable and biocompatible character [52–54]. Polymeric nanoparticles can encapsulate SPIONs, providing enhanced MR relaxivity and tumor targeting. Cheong et al. have recently developed SPION-loaded chitosan-based nanoparticles for hepatocyte-targeted gene delivery and imaging [55]. The encapsulation of SPION has been performed using water-soluble chitosan (WSC)–linoleic acid (LA), which formed self-assembled nanoparticles (SCLNs) through conjugation of the hydrophobic moiety, LA. In this study, encapsulated SPIONs in SCLNs with pEGFP (plasmid DNA encoding enhanced green fluorescent protein) are useful for monitoring gene delivery into hepatocytes by MR imaging. Oleic acid-coated SPIONs were prepared with a diameter of 11.7 nm and loaded into WSC-LA nanoparticles by a solvent-evaporation method, resulting in SPION clusters inside the hydrophobic core, with a content of 37.68% (w/w).

As described above, Gd-based chelates were considered as a T1 MRI contrast enhancement agent. Saha et al. reported chitosan-based Gd-DTPA microspheres for Gd neutron-capture therapy and as an MRI agent [56]. However, the size distribution of 3–12 μm makes it difficult to apply in vivo. As an optical/T1 dual imaging agent, Tan et al. prepared chitosan nanoparticles that encapsulated both QDs and Gd-DTPA [57]. They demonstrated that Gd-DTPA within the chitosan nanobeads had a high MR relaxivity, which may be useful for a MR contrast agent, but they did not carry out in vivo experiments. In a similar fashion, we recently reported that Cy5.5-conjugated self-assembled chitosan nanoparticles (CNPs) grafting Gd-DOTA [Cy5.5-CNP-Gd(III)] have been used as an efficient MR and optical imaging probe (Fig. 4) [58]. The highest weight ratio of Gd(III) in the Cy5.5-CNP-Gd (III) nanoparticles was 6.28 wt%, with a diameter of 350 nm. CNPs have proved their potential for in vivo molecular imaging and drug delivery systems due to their efficient accumulation in solid tumor tissues through the EPR effect and their ideal in vivo characteristics, such as biocompatibility and biodegradability, and prolonged blood circulation time. Therefore, Cy5.5-CNP-Gd(III), in which MR and NIRF dual modalities are introduced to the chitosan nanoparticles, can be successfully used for in vivo tumor imaging by optical and MR imaging systems.

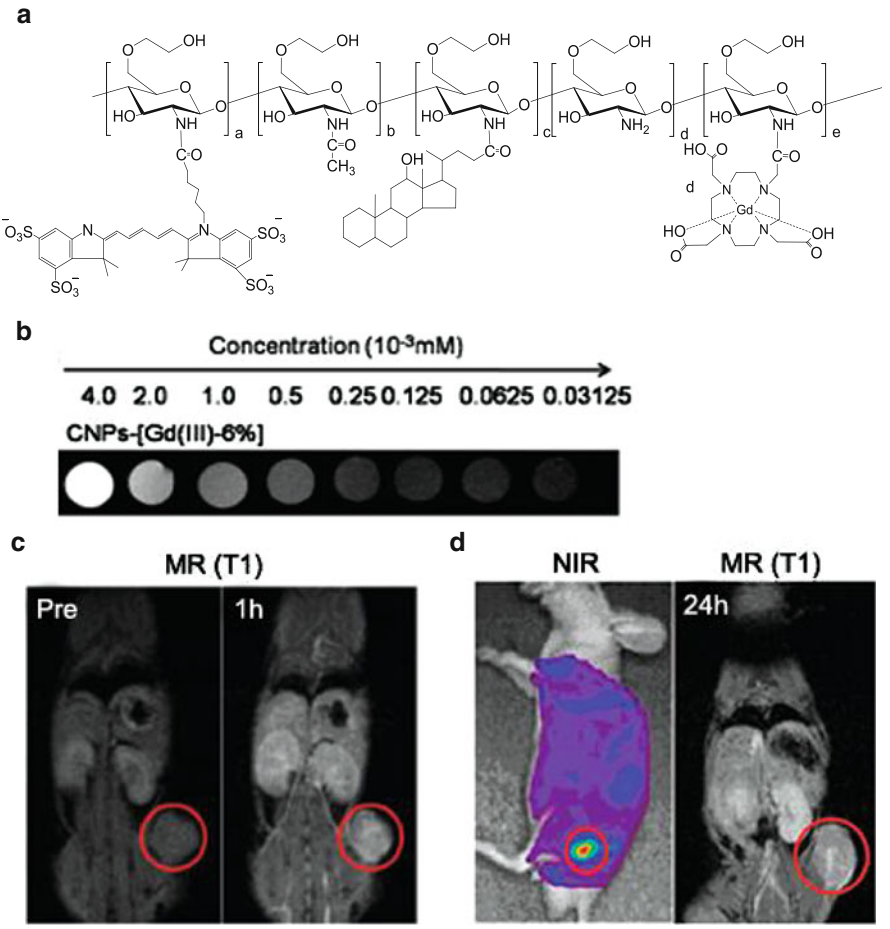

Fig. 4 MR/optical dual imaging with Gd(III) encapsulated in Cy5.5-conjugated glycol chitosan nanoparticles [Cy5.5-CNP-Gd(III)]. (**a**) Chemical structure of Cy5.5-CNP-Gd(III). (**b**) T1-weighted spin-echo MR image (1.5T) of Cy5.5-CNP-Gd(III) in phosphate-buffered saline, showing bright MR signals. (**c**) Coronal slices of T1-weighted MR images of Cy5.5-CNP-Gd(III) in SCC7 tumor-bearing mice. *Circles* indicate SCC-7 tumor tissue. (**d**) NIRF and T1-weighted MR dual images at 1 day post-injection of Cy5.5-CNP-Gd(III) (5 mg/kg). From Nam et al. [58]

4 Application for Tumor Therapy

Various chemical or biological drugs for clinical uses have been incorporated into chitosan-based nanoparticles. Many studies have described the characteristics, efficiency, and safety of these chitosan-based nanoparticle systems containing chemical drug or therapeutic agents, such as antitumor genes or siRNA. Many amphiphilic chitosan derivatives constitute a hydrophobic core of self-assembled

nanoparticles, and these nanoparticles can load hydrophobic drugs inside themselves because of the affinity of drug and core-forming molecules. Therapeutic genes usually require different methods to build up nanoparticles. The cationic chitosan provides electrostatic interaction with negatively charged therapeutic genes, and this charge–charge interaction can be utilized for producing nanosized complexes. These therapeutic nanoparticles can reach tumor sites through the EPR effect and targeting moieties, and they need to overcome intracellular barriers and release the therapeutics into the cytosol for achievement of desired goals [59].

4.1 Anticancer Drug Delivery

Doxorubicin (DOX) is the one of the most widely used anticancer drugs and inhibits the nucleic acid synthesis of cancer cells. DOX has many advantages as an anticancer drug, but it shows a number of side-effects on heart and bone marrow, resulting in a narrow therapeutic index [60]. Since targeted DOX delivery to cancer cells can reduce the side effects, researchers have attempted to load DOX to tumor-homing nanoparticles. DOX has a primary amino group and becomes positively charged at pH 7.4. DOX in its natural form is hydrophobic, and it is commonly used in a hydrochloric acid salt formulation [61]. In a previous study, DOX-GC conjugates containing up to 5 wt% DOX formed self-assembled nanoparticles in aqueous solution [62]. The final DOX loading content in the nanoparticles was 38.9%, and the DOX-GC conjugates precipitate at DOX loadings above 5.5 wt% because of increased hydrophobicity. The physicochemical properties and the cytotoxicity of DOX-modified and loaded GC nanoparticles was evaluated [7]. DOX-GC nanoparticle showed lower cytotoxicity than the free form of DOX. In another study, DOX and dextran conjugates were encapsulated in 100-nm chitosan nanoparticles [63]. When encapsulated in the chitosan nanoparticles, DOX-dextran enabled faster in vivo tumor regression. In addition, several active targeting chitosan nanoparticles using transferrin or folate could also deliver DOX to tumor cells with high efficiency [26, 30].

As a mitotic inhibitor, paclitaxel (PTX) is another drug actively used in cancer chemotherapy. PTX is soluble in diverse organic solvents, but poorly soluble in aqueous solutions. Originally, it was dissolved in Cremophor EL (polyethoxylated castor oil) and ethanol, but it bound to albumin and showed unintended toxicity. Previous studies using chitosan-based nanoparticles loaded with PTX presented relatively low cytotoxicity compared to PTX in Cremophor EL. Hydrophobically modified GC nanoparticles, 200–400 nm in size, had a similar antitumor effect to PTX formulated in Cremophor EL without severe toxicity [8]. Qu et al. demonstrated that PEGylated chitosan micelles also showed decreased plasma protein adhesion and increased circulation time, thus evading the elimination of PTX in the late stage of intravenous injection [10]. Docetaxel is a semisynthetic analog of PTX, and was loaded into hydrophobically modified GC nanoparticles in an attempt to reduce the side effects of free docetaxel by using tumor targeting [64].

Camptothecin (CPT) is a hydrophobic quinoline alkaloid that inhibits the enzyme topoisomerase I. FDA-approved CPT-based anticancer drugs are used in first therapy of cancer with 5-fluorouracil [60], but insolubility and rapid hydrolysis have prevented wide clinical applications of CPT in cancer therapy. CPT has an anticancer active lactone form in acidic conditions (pH < 5), which becomes an inactive carboxylate form at basic pH [65]. CPT was incorporated into polymeric micelles using *N*-phthaloylchitosan-grafted polyethylene glycol methyl ether (mPEG), and *N*-trimethyl chitosan (TMC) or hydrophobically modified GC nanoparticles [11, 66, 67]. Stability tests investigating the preservation of CPT within the nanoparticles under physiological condition (pH 7.4, 37°C) and optimal controlled release of the active lactone form supported the idea that modified chitosan-derived nanoparticles have considerable potential as CPT carriers. The biodistribution, tumor targeting ability, time-dependent excretion profile, and anticancer efficacy of CPT-GC nanoparticles were also confirmed in vivo by noninvasive animal imaging systems [67]. Liu et al. evaluated the high therapeutic effects of TMC nanoparticles with the recorded tumor growth and histopathological changes (Fig. 5) [66]. In addition, quantitative analysis of expression of a specific protein like proliferating cell nuclear antigen (PCNA) supported the possible therapeutic value of the nanoparticles. The tumor volume in the group treated with CPT-loaded nanoparticles was significantly smaller, and PCNA expression was also reduced compared with other groups. As shown, chitosan-based nanoparticle systems can be practically applied for delivery of hydrophobic anticancer drugs.

4.2 Photodynamic Therapy

Photodynamic therapy (PDT) is one of the therapeutic methods especially used for the treatment of various cancers [68]. In this method, a photosensitive drug called the photosensitizer is injected locally, intravenously or intraperitoneally, and should accumulate in target tissue with high concentration. Because of the fluorescence of photosensitizers and their high concentration in the target tumor site compared with surrounding normal tissue, the visualization and imaging of tumors are enabled by fluorescence [69]. To achieve efficient tumor therapy, the tumor should be exposed to laser light, and the laser-excited photosensitizer produces reactive oxygen species like singlet oxygen ($^1O_2$). These can kill tumor cells by phototoxic damage to major cellular organelles such as mitochondria [70]. Especially for in vivo therapy, laser of the red or near-infrared wavelength is more widely used than blue wavelengths, because of the differences in absorbance by photosensitizers. This optimal absorption wavelength of the photosensitizer is a crucial factor in PDT because the light of a longer wavelength can penetrate through the tissue deeply and enable maximum therapeutic results for large tumors. PDT has been applied clinically for the treatment of a variety of cancer types. In the last decade, many promising results have been obtained for lung cancer, head and neck cancer, skin cancer, locoregional breast cancer recurrences, and prostate

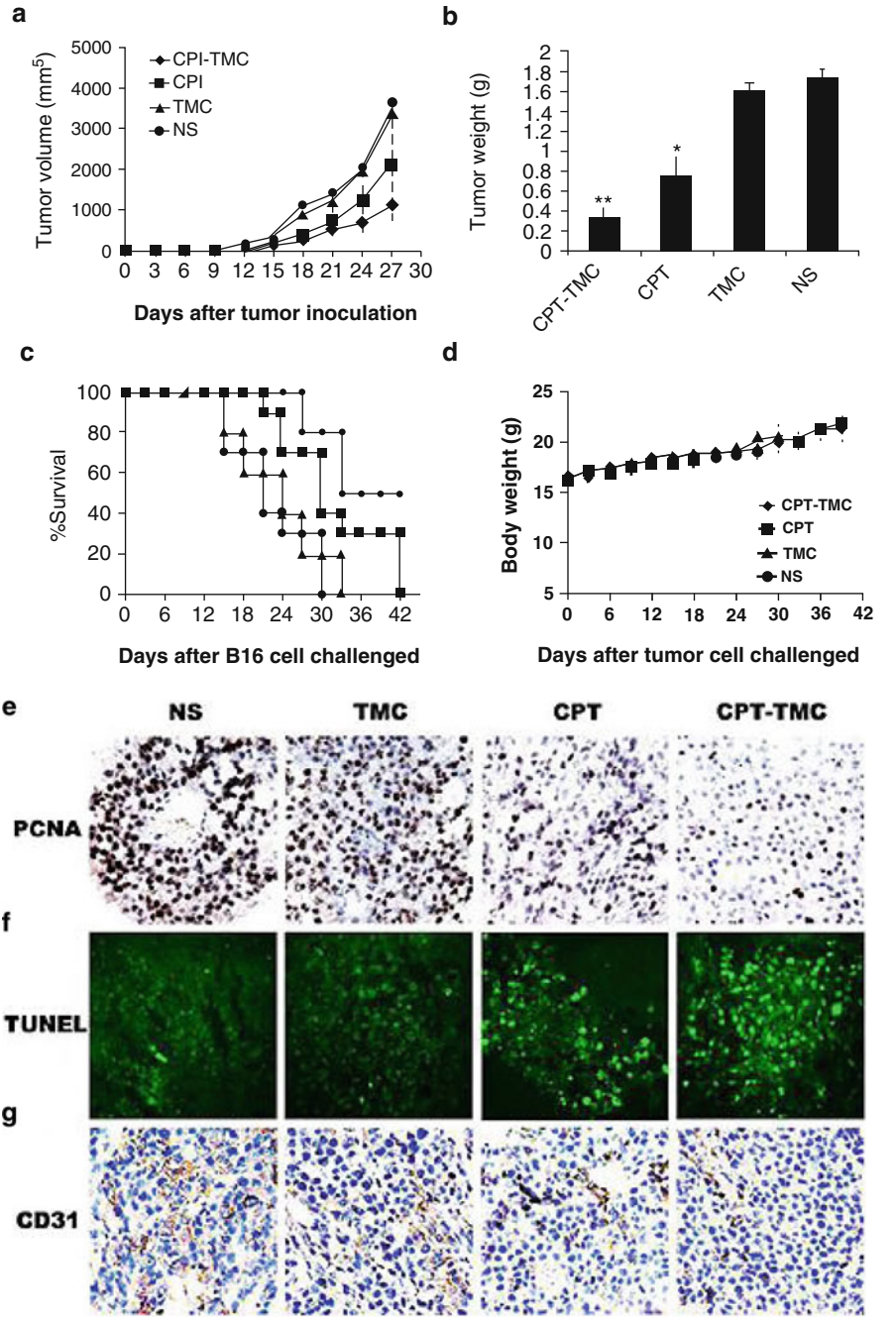

Fig. 5 Tumor therapy with camptothecin encapsulated in *N*-trimethyl chitosan nanoparticles (CPT-TMC). (**a**) Tumor volume growth curve of B16-F10 tumor-bearing mice with CPT-TMC. Comparison of (**b**) tumor weight, (**c**) survival curves, and (**d**) body weight changes in mice treated

cancer [71, 72]. PDT is also an attractive clinical modality for several other therapeutic applications like inflammations [73].

In spite of these promising results, there are some problems still to be solved in current PDT. The major limitation is the nonselective accumulation of the photosensitizers, which could cause severe damage to normal tissue after laser irradiation. Moreover, patients should stay in the absence of sunlight for a long time after the administration of the photosensitizer, because photosensitizers may result in skin phototoxicity. Various delivery methods such as dendrimers, liposomes, micelles, and nanoparticles have been developed to overcome these problems [74, 75].

Chitosans and chitosan derivatives are also attractive materials in PDT. Chen et al. used GC as immunoadjuvant in photodynamic therapy with two photosensitizers, photofrin and *m*-tetrahydroxyphenylchlorin [73]. The treatment with GC could enhance antitumor immune response and the effects were more highly effective than other immunoadjuvants like complete Freud adjuvant or *Corynebacterium parvum*. The combination of immunological stimulation by GC and active tumor destruction by photosensitizers could significantly enhance the cure rate of tumor therapy.

Hu et al. encapsulated chlorine e6 (Ce6) in stearic acid-grafted chitosan micelles [76]. The size of micelles was 270–300 nm and they could contain 5–20% Ce6. They were stable spherical nanostructure in aqueous condition and enabled sustained release of Ce6 over 12 h. Importantly, they could enhance the uptake of Ce6 to A549 (human lung cancer) and HeLa (human cervix carcinoma) cells, and showed high phototoxicity, proving their potential for cancer therapy.

In vivo PDT with chitosan was performed by Schmitt et al. [77]. They used chitosan nanogel prepared by crosslinking with tripolyphosphonate and Ce6, as photosensitizer. This chitosan nanogel was about 40–140 nm and could more efficiently deliver Ce6 to macrophage cells than fibroblast cells. Macrophages play a crucial role in inflammatory disease, and they are major targets of clinical treatments at the inflammation site. In vivo evaluation of serum amyloid A (SAA) levels showed the clinical results of a rheumatoid arthritis model. SAA levels after PDT with Ce6 encapsulated in chitosan nanogel were comparable with those obtained after intra-articular injection of methylprednisolone, the currently used corticoid for rheumatoid arthritis patients.

Recently, Lee et al. showed highly efficient PDT with an in vivo tumor model using chitosan-based nanoparticles (Fig. 6) [78]. They prepared self-assembled amphiphilic glycol chitosan–5β-cholanic acid conjugates and encapsulated the hydrophobic photosensitizer, PpIX, with a high drug-loading efficiency of over

Fig. 5 (Continued) with CPT-TMC and in mice treated with CPT or TMC alone. NS indicates treatment with normal saline. (**e**) Tumor sections immunostained with an antibody against PCNA showing the differences in positive nuclei in tumor tissues. (**f**) Apoptosis of tumor tissues examined by TUNEL assays. (**g**) Tumor sections immunostained with anti-CD31 antibody (*brown*) for angiogenesis assay. From Liu et al. [66]

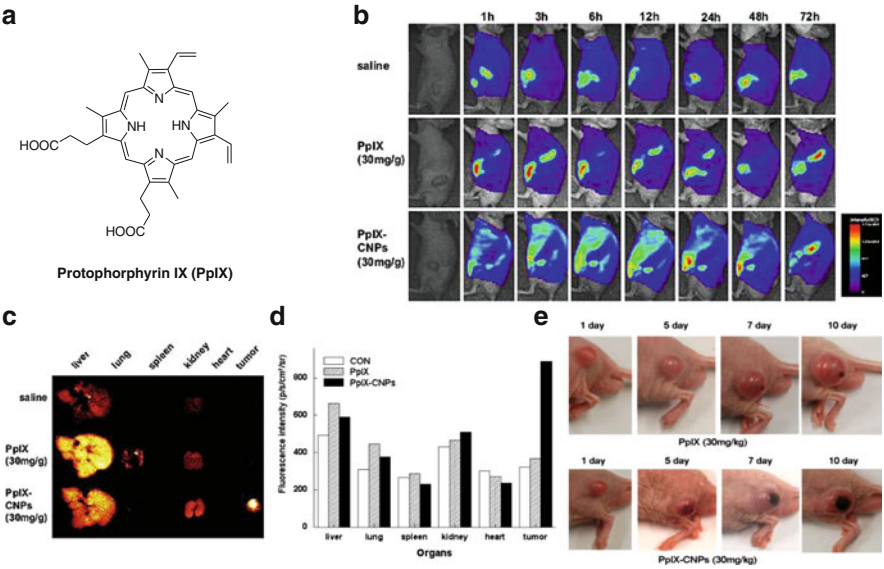

Fig. 6 Photodynamic therapy with protophorphyrin IX encapsulated in glycol chitosan nanoparticles (PpIX-CNP). (**a**) Chemical structure of the photosensitizer PpIX. (**b**) In vivo time-dependant whole-body imaging of athymic nude mice bearing SCC7 tumors after i.v. injection of PpIX-CNPs. (**c**) Ex vivo images of normal organs (liver, lung, spleen, kidney and heart) and tumors excised at 3 days post-injection of PpIX-CNP (**d**) Quantification of in vivo biodistribution of free PpIX and PpIX-CNPs recorded as total photon counts per second per centimeter squared per steradian (p/s/cm^2/sr) for each excised organ. (**e**) Photodynamic therapeutic efficacy of free PpIX (30 mg PpIX/kg body weight) and PpIX-CNPs (30 mg/kg of PpIX) in SCC7 tumor-bearing mice. From Lee et al. [78]

90%. These PpIX-loaded chitosan-based nanoparticles (PpIX-CNPs) showed a sustained release profile of PpIX and were biocompatible to tumor cells without irradiation. With SCC7 murine cell carcinoma cells, we observed fast cellular uptake of the PpIX-CNPs and the released PpIX exhibited high phototoxicity after laser irradiation. In vivo imaging and therapy with SCC7 tumor-bearing mice showed enhanced tumor specificity and increased therapeutic efficacy of PpIX-CNPs compared to free PpIX. These results indicated that these chitosan-based nanoparticle systems have great potential as fine delivery system for photodynamic therapy.

4.3 Gene Delivery

Research on gene delivery has expanded during the past 20 years because gene therapy is a potential candidate for therapies to overcome many obstinate diseases. Gene materials include plasmid DNA (pDNA), oligonucleotides, and small RNA

molecules. Recently, the use of siRNA for gene silencing has been paid more attention because of its highly specific silencing ability. The siRNAs targeting overexpressed oncogenes or mutated tumor suppressor genes are being vigorously investigated in cancer studies. In spite of the specific gene suppression effect, therapeutic genes are not yet widely used in clinical trials because naked therapeutic genes are easily degraded by nucleases in plasma and are rapidly cleared through the kidneys. Moreover, they show poor cellular uptake and low transfection efficiency in their original form due to the strong negative charge of phosphate backbone [79, 80]. Therefore, many kinds of viral vectors or nonviral vectors are being developed for gene therapy. The transfection efficiency of nonviral vectors is still lower than that of viral vectors, but they have advantages of safety and cost.

Chitosan-based carriers are being used as one of the typical nonviral vectors. The primary amine groups in the chitosan backbone present a positive charge at acidic pH, and this cationic property is utilized for application as a gene delivery carrier. The cationic chitosan backbone can interact with anionic pDNA or siRNA to form stable nanosized particles in an aqueous condition. At this moment, it is crucial to consider the proper ratio of chitosan derivatives to therapeutic genes for obtaining particles with desirable physicochemical properties. The physicochemical properties such as size and electrical ζ-potential are important because the stability of the particles and the ability for gene delivery are entirely dependent on them. The particle size of the chitosan–gene complex is also dependent on the molecular weight of the chitosan polymer. The degree of chitosan deacetylation can affect DNA binding, release, and transfection efficiency [81, 82]. Previous studies reported that chitosan–siRNA nanoparticles formed using chitosan of high molecular weight (114 and 170 kDa) and high degree of deacetylation (84%) formed the most stable structure and exhibited the highest gene knockdown in vitro [81, 83].

Numerous researchers have studied chitosan nanoparticles as carriers of pDNA, and showed that PEGylation or thiolation of chitosan prolongs the plasma circulation time and enhances the transfection efficiency of pDNA [84, 85]. In a previous study, combination of PEGylated chitosan and low molecular weight polyethylenimine (PEI, 1.2 kDa) demonstrated effective gene delivery and transfection in mice bearing C6 xenograft tumors [47]. Another study implied the use of chlorotoxin–chitosan conjugates to enhance uptake into glioma cells in vivo with the same animal model and gene (pEGFP-CS2) [86]. Nanocarrier uptake into the cancer cells and the biodistribution including tumor accumulation were confirmed in the paper. In another paper, gene therapy using the p53 gene and urocanic acid-modified chitosan was described [87]. Urocanic acid-modified chitosan nanoparticles mediated p53 transfection in HepG2 cells and induced high levels of p53 mRNA and protein. Consequently, apoptosis and growth inhibition of HepG2 cells were observed in vitro and in vivo (Fig. 7).

siRNA is the small RNA molecule that is typically used for the technique of RNA interference. The siRNA, when introduced into cells, forms RNA-induced silencing complexes and induces degradation of homologous mRNA and knockdown of the relevant protein [88]. Previous studies introduced a chitosan-based siRNA nanoparticle delivery system, which demonstrated 77.9 and 89.3%

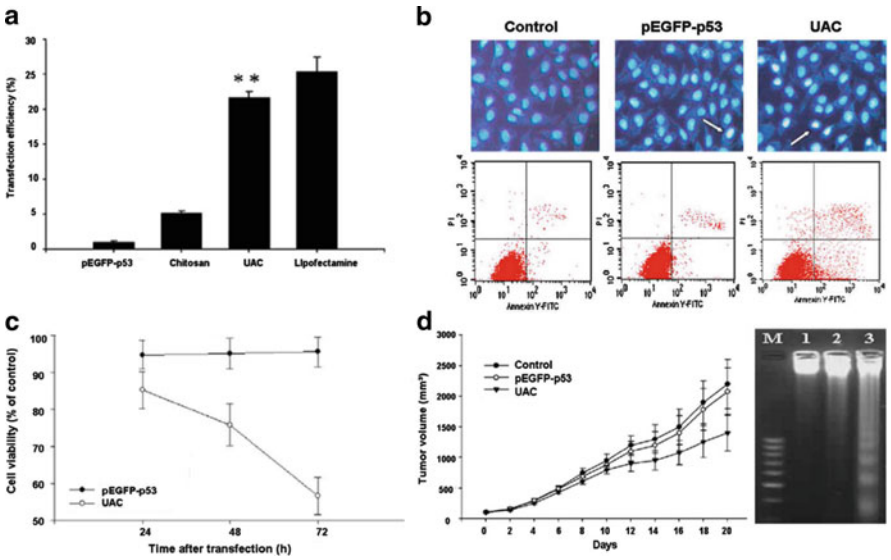

Fig. 7 Gene therapy with p53 gene (PEGFP-p53) and urocanic acid-modified chitosan nanoparticle (UAC). (**a**) In vitro transfection efficiency examined with pEGFP gene. (**P < 0.01 compared with untreated control). (**b**) DAPI staining for apoptosis imaging and FACS analysis for Annexin V. *Arrows* indicate typical apoptotic cells. (**c**) Inhibitory effects of UAC-mediated p53 transfection on the proliferation of HepG2 cells. (**d**) Tumor suppression of HepG2 tumor-bearing mice after intratumoral injection of naked DNA and UAC/pEGFPp53 complexes (*left*). DNA fragmentation assay on HepG2 nude mice xenografts after treatments (*right*). *Lane M* marker, *Lane 1* control, *Lane 2* pEGFP-p53 alone, *Lane 3* UAC/pEGFP-p53 complexes. From Wang et al. [87]

reduction of endogenous EGFP in H1299 human lung carcinoma cells and murine peritoneal macrophages in vitro [89]. Subsequently, siRNA delivery with tumor-homing chitosan-based nanoparticles was evaluated in vivo. Huh et al. introduced strongly positively charged PEI polymer to GC polymer–siRNA complexes [90]. GC-PEI nanoparticles carried red fluorescent protein (RFP)-matched siRNA to the tumor site of RFP-expressing B16F10 tumor-bearing mice, and RFP suppression was confirmed in vivo. Although low transfection efficiency of the polymer carriers has not been fully overcome, chitosan-based nanoparticles are still strong candidates for gene delivery systems in cancer treatment.

5 Conclusion

In this review, recent applications of chitosan nanoparticles for tumor imaging and therapy have been discussed. Recently, chitosan has been applied to molecular imaging based on many imaging agents, which enables in vivo live imaging. It also has profitable chemical and biological characteristics as a drug carrier, and hence

chitosan-based nanoparticles have been widely investigated for tumor therapy. Different groups of researchers have studied various sizes and characteristics of chitosan nanoparticles using chitosan polymers of different molecular weight and deacetylation degree [91]. In addition, diverse chitosan nanoparticles can be further modified by chemical conjugation with amine groups in the chitosan polymers. Hydrophobically modified, PEGylated, glycolated, or ligand-conjugated chitosan nanoparticles can improve their solubility, stability, passive targeting abilities, and cellular uptake efficiency.

Use of chitosan-based nanoparticles with diverse probes and imaging modalities could be used to trace biological and biochemical processes in living subjects. In previous studies, real-time in vivo imaging and treatment monitoring were carried out effectively. In addition, active targeting and controlled drug release enables enhanced tumor targeting and therapy. Although some chitosan-based nanoparticles that include drugs or other therapeutic agents displayed insufficient drug delivery efficiency, others showed large tumor accumulation and outstanding therapeutic effects with rational design and optimization. With the advantages of chitosan and its derivatives, such as biocompatibility or the convenience of availability, this kind of review on tumor targeting chitosan-based nanoparticles should be helpful to many biomedical researchers.

The current chitosan-based nanoparticles can be further modified to have better properties for an ideal carrier of imaging agents or drugs. Therefore, we expect that these chitosan nanoparticles will continue to provide attractive and advanced tools as nanocarriers, especially for cancer research and treatment.

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