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Review

Chitosan conjugated DNA nanoparticles in gene therapy

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ABSTRACT

Conventional gene delivery with viral or lipid carriers-are plagued by disadvantages such as low transfection efficiency, cytotoxicity and immunogenicity. Numerous techniques are being probed to help improve efficiency, including the development of biodegradable polymers with reduced toxicity, incorporation of cell targeting moieties, improved chemistry for syntheses of polymers with uniform size and topology etc. Chitosan, a naturally occurring cationic polysaccharide, is emerging as a potential candidate for gene delivery applications with its reasonable transfection efficiency combined with a minimal level of cytotoxicity. The chitosan and their nanoparticles have potential to form polyelectrolyte complex with DNA and it is useful for non-viral vectors for gene therapy applications. Hence, the objective of this review is to summarize the recent advances in gene therapy giving emphasis to the applications of chitosan nanoparticles as gene carriers in enhancing cellular uptake and transfection efficiency.

1. Introduction

Gene therapy is the treatment of human disorders by the introduction of genetic material into specific target cells of a patient, where production of the encoded protein will occur (Corsi et al., 2003). A wide variety of vectors to deliver therapeutic genes into the desired target cells have been studied (Nishikawa & Hashida, 2002). Gene therapy is currently being applied in many different health problems such as cancer, AIDS, and cardiovascular diseases (Turan, Aral, Kabasakal, & Uysal, 2003). Several trials employing gene therapy protocols have already been successfully completed in patients with cystic fibrosis (Gill et al., 1997; Porteous et al., 1997), and adenosine deaminase deficiency (Bordignon et al., 1995). Many researches are working in the field of gene delivery to develop ideal gene delivery carriers. There are several systems that can be used to transfer foreign genetic material into the human body. Gene carriers should possess certain ideal properties. The DNA to be transferred must escape the processes that affect the disposal of macromolecules. These processes include the interaction with blood components, vascular endothelial cells and uptake by the reticuloendothelial system. Furthermore, the degradation of therapeutic DNA by serum nucleases is also a potential obstacle for functional delivery to the target cell (Quong & Neufeld, 1998). Thus, an ideal gene-delivering carrier should transport genetic materials without any toxicity and immune responses (Smith, Zhang, & Niven, 1997). It must be capable of protecting the DNA until it reaches its target. To do so, the system must be small enough to allow internalization into cells and passage to the nucleus, it must have flexible tropisms for applicability in a range of disease targets, and it must be capable of escaping endosome–lysosome processing and of following endocytosis (Mansouri et al., 2004).

Currently, transport of exogenous DNA to cells can be achieved using viral and non-viral vectors or as naked DNA. The simplest non-viral gene delivery system uses naked DNA. The overall level of expression is much lower with naked DNA than with either viral or liposomal vectors. Naked DNA is also unsuitable for systemic administration due to the presence of serum nucleases. As a result, direct injection of DNA seems to be limited to tissues that are easily accessible to direct injection such as skin and muscle (Mansouri et al., 2004). The most common viral vectors used today are retrovirus, herpes simplex virus, lentivirus, adenovirus and adeno-associated virus, each having its own characteristics (Oligino, Yao, Ghivizzani, & Robbins, 2000). Although viral vectors have obvious advantages such as high transfection rate and a rapid transcription of the foreign material inserted in the viral genome, there are some evident drawbacks limiting their clinical use, such as poor target-specificity, low capacity to incorporate foreign DNA sequences to their genome (Mansouri et al., 2004), toxic and inflammatory effects, eliciting immune responses, viral wild-type mutations, and potential oncogenic effects (Lee, Kwon, Kim, Jo, & Jeong, 1998). However, the use of viruses in gene therapy could be limited by

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various factors. First and foremost, safety issues have been raised following the death of a patient during a clinical trial that investigated the potential of gene therapy using viral vectors (Ferber, 2001; Somia & Verma, 2000). Second, gene therapy using viral vectors is limited by the fact that only small sequences of DNA can be inserted in the virus genome. Third, viruses present a variety of potential problems to the patients such as toxicity, immune responses, and inflammatory responses. Lastly, insertional mutagenesis and oncogenic effects can occur when used in vivo (Lee et al., 1998).

Non-viral vectors can be administered repeatedly with minimal host immune response, are targetable, stable in storage, and easy to produce in large quantities (Mao et al., 2001). The delivery systems of non-viral vectors include cationic molecules such as cationic lipids and synthetic or natural cationic polymers, which are widely used to condense DNA and to efficiently deliver therapeutic genes within mammalian cells (Garnett, 1999; Rolland, 1998). Because of their permanent cationic charge, these interact electrostatically with negatively charged DNA and form complexes (lipo- or poly-plexes). Although cationic lipids have low immunogenicity (Deshpande et al., 1998), they are not considered for gene therapy due to their toxicity and the relatively low transfection efficiency. DNA/polymer complexes involving cationic polymers, on the other hand, are more stable than cationic lipids (De Smedt, Demeester, & Hennink, 2000), though the efficiency of gene delivery by cationic polymers is still relatively low (Rolland, 1998). Several cationic polymers have been investigated that lead to increased transfection efficiencies (Ferber, 2001; Somia & Verma, 2000). They show structural variability and versatility including the possibility of covalent binding of targeting moieties for gene expression mediated through specific receptors (Liu & Yao, 2002; Somia & Verma, 2000). Cationic polymers are able to condense more DNA than lipids. They form complexes with DNA and protect it against nuclease degradation (Gao & Huang, 1996). The cationic polymers that has been investigated include poly(D,L-lactide-*co*-glycolide) (PLGA) (Tahara, Sakai, Yamamoto, Takeuchi, & Kawashima, 2008), poly(ethylene imine) [PEI], poly(L-lysine), polybrene, tetraminofullerene, poly(L-histidine)-graft-poly(L-lysine) (Mansouri et al., 2004), etc. Natural polymers that have been tried include chitosan and its derivatives, collagen, gelatin, etc. (Dang & Leong, 2006).

Polymeric nanospheres have been used to deliver medicines because of their advantages such as high stability, easy uptake into the cells by endocytosis, and targeting ability to specific tissues or organs by adsorption or binding with ligand at the surface of the particles (Lobenberg, Araujo, & Kreuter, 1997). In particular, biodegradable nanospheres are available for delivering drugs and degraded after passing required specific site (Belbella, Vauthier, Fessi, Devissaguet, & Puisieux, 1996). Among them poly(lactide) (PLA) and poly(D,L-lactide-*co*-glycolide) (PLGA) have been approved by the FDA for certain human clinical uses (Sahoo, Panyam, Prabha, & Labhasetwar, 2002). There are two types of nanoparticle system carrying nucleic acid, i.e., DNA or RNA entrapping system (Wang, Robinson, Kwon, & Samuel, 1999) and surface binding system (Kim et al., 2005). Surface binding systems utilize an ionic interaction between cationic polymer and the anionic nucleic acid. Nucleic acid entrapping system is a reservoir type nanosphere system that has the advantages of nucleic acid protection. The application of DNA–chitosan nanospheres has advanced in vitro DNA transfection efficiency and further research data show their usefulness for gene delivery (Erbacher et al., 1998; Corsi et al., 2003).

Of these, chitosan and its derivatives have received much attention because of their excellent biocompatibility, low immunogenicity, and reduced cytotoxicity compared to other polymers (Erbacher et al., 1998; Liu & Yao, 2002; Mao et al., 2001; Roy, Mao, Huang, & Leong, 1999). Chitosan, a cationic polysaccharide obtained by alkaline *N*-deacetylation of chitin, is one of the most widely utilized polysaccharides (Romoren, Thu, & Evensen, 2002). It is a non-toxic biodegradable polymer with low immunogenicity (Muzzarelli, 1973). These characteristics make chitosan an excellent candidate for various biomedical applications such as drug delivery, tissue engineering, and gene delivery (Hoggard et al., 2001; Illum, 1998; Ishii, Okahata, & Sato, 2001; Roy et al., 1999; Lee et al., 2001; Thanou, Verhoef, & Junginger, 2001; Turan, Akbuga, & Aral, 2002; Khor and Lim, 2003; Chen et al., 2004; Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Gupta, Mahor, Khatri, Goyal, & Vyas, 2006; Jayakumar, Nwe, Tokura, & Tamura, 2007; Jayakumar, Prabaharan, Reis, & Mano, 2005; Kai & Ochiya, 2004; Kumar, Behera, Hellermann, Lockey, & Mohapatra, 2003; T Salas, Martinez, Godinez, Arco Del Nunez, & Miranda, 2008). It is a good candidate for gene delivery system because positively charged chitosan can be complexed with negatively charged DNA (Mac Lughlin et al., 1998; Fang, Chan, Mao, & Leong, 2001; Richardson, Kolbe, & Duncan, 1999). Chitosan and its derivatives have been complexed with DNA by ionic interactions between anionic phosphate backbones of DNA and primary amine groups of chitosan. This binding protects the DNA from nuclease degradation (Cui & Mumper, 2001; Illum, Gill, Hinchcliffe, Fisher, & Davis, 2001). Also, the mucoadhesive property of chitosan potentially permits a sustained interaction between the macromolecule being "delivered" and the membrane epithelia, promoting more efficient uptake (Hejazi & Amiji, 2003; Richardson et al., 1999; Takeuchi, Yamamoto, Niwa, Hino, & Kawashima, 1996) and it has the ability to open intercellular tight junctions, facilitating its transport into the cells (Illum et al., 2001). It has advantages of not necessitating sonication and organic solvents for its preparation, therefore minimizing possible damage to DNA during complexation. Various gene delivery systems based on chitosan have been described such as self-assembling polymeric and permanent oligomeric chitosan/DNA complexes, DNA–chitosan nanospheres, etc. (Borchard, 2001).

In this review, we evaluated the preparation of chitosan conjugated DNA and small interference RNA (siRNA) nanoparticles for gene delivery applications in detail. We also discussed the recent development on synthesis of chitosan nanoparticles and their applications towards clinical applications.

2. Chitosan–DNA complex nanoparticles

Polysaccharides and other cationic polymers have recently been used in pharmaceutical research and industry for their properties to control the release of antibiotics, DNA, proteins, peptide drugs or vaccines, and they have also been extensively studied as non-viral DNA carriers for gene delivery and therapy. Among them, chitosan is the most used since it can promote long-term release of incorporated drugs. The preparation of chitosan and chitosan/DNA nanospheres by using a novel and simple osmosis-based method has been recently patented (Masotti, Bordi, Ortaggi, Marino, & Palocci, 2008). With this method, they were able to prepare chitosan/DNA particles of spherical morphology with an average diameter 38 ± 4 nm. Also, the DNA incorporation was pretty high (up to 30%) and the release process is gradual and prolonged in time. Another advantage of this method is that, varying the solvent/non-solvent couple, temperature and membrane cut-off, affording useful nanostructured systems of different size and shape to employ in several biomedical and biotechnological applications, may easily modify the process.

There are many reports suggesting the advantages of chitosan as an efficient gene carrier molecule. The first report suggesting the probable suitability of chitosan as a gene delivery agent was in the year 1998 (Lee et al., 1998). They prepared hydrophobically modified chitosan containing 5.1 deoxycholic acid groups per 100

anhydroglucose units by an EDC-mediated coupling reaction. Formation and characteristics of self-aggregates of hydrophobically modified chitosan were studied by fluorescence spectroscopy and dynamic light scattering method. Charge complex formation between self-aggregates and plasmid DNA was confirmed by electrophoresis on an agarose gel. Migration of DNA on an agarose gel was completely retarded above a charge ratio of 4/1 at pH 7.2. The free DNA dissociated from the complexes was observed by electrophoresis above pH 8.0 at a fixed charge ratio of 4/1. Chitosan self-aggregate/DNA complexes achieved an efficient transfection of COS-1 cells and the level of expression with plasmid/chitosan was observed to be no less than that with plasmid/lipofectin complexes in SOJ cells.

A few years later, Mao et al. reported preparation of chitosan–DNA nanoparticles using a complex cooperation process (Mao et al., 2001). They investigated important parameters for the nanoparticle synthesis, including the concentrations of DNA, chitosan and sodium sulfate, temperature of the solutions, pH of the buffer, and molecular weights of chitosan and DNA. At an amino group to phosphate group ratio (N/P ratio) between 3 and 8 and a chitosan concentration of 100 mg/ml, the size of particles was optimized to 100–250 nm with a narrow distribution, with a composition of 35.6% and 64.4% by weight for DNA and chitosan, respectively. The surface charge of these particles was slightly positive with a zeta potential of 112–118 mV at pH lower than 6.0, and became nearly neutral at pH 7.2. With this system, they found that the transfection efficiency of chitosan–DNA nanoparticles was cell-type dependent. They also developed three different schemes to conjugate transferrin or KNOB protein to the nanoparticle surface. The transferrin conjugation only yielded a maximum of fourfold increase in their transfection efficiency in HEK293 cells and HeLa cells, whereas KNOB conjugated nanoparticles could improve gene expression level in HeLa cells by 130-fold. Conjugation of PEG on the nanoparticles allowed lyophilization without aggregation and without loss of bioactivity for at least 1 month in storage. The clearance of the PEGylated nanoparticles in mice following intravenous administration was slower than unmodified nanoparticles at 15 min, and with higher depositions in kidney and liver. However, no difference was observed at the 1-h time point.

In an approach to study the transfection mechanism of plasmid/chitosan complexes as well as the relationship between transfection activity and cell uptake, Ishii et al., 2001 used fluorescein isothiocyanate-labeled plasmid and Texas Red-labeled chitosan. They observed that, there are several factors, which contribute to transfection activity: the molecular mass of chitosan, stoichiometry of complex, as well as serum concentration and pH of transfection medium. The level of transfection with plasmid/chitosan complexes was found to be highest when the molecular mass of chitosan was 40 or 84 kDa, ratio of chitosan nitrogen to DNA phosphate (N/P ratio) was 5, and transfection medium contained 10% serum at pH 7.0. While investigating the transfection mechanism, they found that plasmid/chitosan complexes most likely condense to form large aggregates, which absorb to the cell surface. After this, plasmid/chitosan complexes are endocytosed, and possibly released from endosomes due to swelling of lysosomes along with the swelling of plasmid/chitosan complex, causing the endosome to rupture. Finally, these complexes were observed to be accumulating in the nucleus.

Another approach of chitosan–DNA nanoparticles synthesis is by the complexation of the cationic polymer with a plasmid DNA (Corsi et al., 2003). They evaluated the transfection potential using three different cell lines such as human mesenchymal stem cells (MSCs), human osteosarcoma cells (MG63) and human embryonic kidney cells (HEK293). DNA distribution within the nanoparticle was visualized by transmission electron and atomic force microscopy. The Lipofectamine 2000 (LF) reagent was used in comparison. The transfection of HEK293 cells is superior to that seen with MG63 cells and MSCs, however, not surpassing that seen with LF. But most importantly, they found that the cytotoxicity was minimal with the chitosan–DNA nanoparticles compared to greater than 50% toxicity with LF. Their results suggested that chitosan–DNA nanoparticles have favorable characteristics for non-viral gene delivery, are cell-type dependent and not cytotoxic.

Quaternized modifications of chitosan are another technique with characteristics that might be useful in DNA condensing and efficient gene delivery (Thanou, Florea, Geldof, Junginger, & Borchard, 2002). Quaternized chitosan oligomers were synthesized and these complexes were characterized by photon correlation spectroscopy and further their ability to transfect COS-1 and Caco-2 cell lines in the presence and absence of fetal calf serum was investigated. The transfection efficiency was compared with DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium sulfate) lipoplexes. Additionally, their effect on the viability of the respective cell cultures was investigated using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Their observations suggest that quaternized chitosan oligomers were able to condense DNA and form complexes with a size ranging from 200 to 500 nm. Chitoplexes proved to transfect COS-1 cells, however, to a lesser extent than DOTAP–DNA lipoplexes. The quaternized oligomer derivatives appeared to be superior to oligomeric chitosan. The presence of fetal calf serum (FCS) did not affect the transfection efficiency of the chitoplexes, whereas the transfection efficiency of DOTAP–DNA complexes was decreased. Cells remained 100% viable in the presence of chitosan oligomers whereas viability of DOTAP treated cells decreased to 50% in both cell lines. Both DOTAP–DNA lipoplexes and chitoplexes resulted in less transfection efficiency in Caco-2 cell cultures than in COS-1 cells; however, quaternized chitosan oligomers proved to be superior to DOTAP. Effects on the viability of Caco-2 cells were similar to the effects observed in COS-1 cells. This report is also in line with the earlier one suggesting that chitosan–DNA complexes present suitable characteristics and less cytotoxic compared to lipid gene carriers and thus has the potential to be used as gene delivery vectors.

A self-assembled nanoparticle using a hydrophobically modified glycol chitosan for gene delivery has been prepared (Yoo, Lee, Chung, Kwon, & Jeong, 2005). Here a primary amine of glycol chitosan was modified with 5β-cholanic acid to prepare a hydrophobically modified glycol chitosan (HGC). The modified chitosan was found to form DNA nanoparticles spontaneously by a hydrophobic interaction between HGC and hydrophobized DNA. As the HGC content increased, the encapsulation efficiencies of DNA increased while the size of HGC nanoparticles decreased. Upon increasing HGC contents, HGC nanoparticle became less cytotoxic. The increased HGC contents also facilitated endocytic uptakes of HGC nanoparticles by COS-1 cells. The HGC nanoparticles showed increasing in vitro transfection efficiencies in the presence of serum. In vivo results also showed that the HGC nanoparticles had superior transfection efficiencies to naked DNA and a commercialized transfection agent. They concluded that the HGC nanoparticles composed of hydrophobized DNA and hydrophobically modified glycol chitosan played a significant role in enhancing transfection efficiencies in vitro as well as in vivo.

Another group used reverse microemulsion technique as a template to fabricate chitosan–alginate coreshell nanoparticles encapsulated with enhanced green fluorescent protein (EGFP)-encoded plasmids (You, Liu, & Peng, 2006). These alginate-coated chitosan nanoparticles endocytosed by NIH 3T3 cells were found to trigger swelling of transport vesicles, which render gene escape before entering digestive endolysosomal compartment, and concomitantly promote gene transfection rate. Their results indicate that DNA-encapsulated chitosan–alginate nanoparticles with average

size of 64 nm (N/P ratio of 5) could achieve the level of gene expression comparable with the one obtained by using polyethyleneimine–DNA complexes.

As a novel technique, Hui et al. studied the gene delivery by chitosan–DNA nanoparticles through retrograde intrabiliary infusion (RII) and examined the efficacy of liver specific targeting (Dai et al., 2006). The transfection efficiency of chitosan–DNA nanoparticles, as compared with PEI–DNA nanoparticles, was evaluated in Wistar rats by infusion into the common bile duct, portal vein, or tail vein. Chitosan–DNA nanoparticles administrated through the portal vein or tail vein did not produce detectable luciferase expression. In contrast, rats that received chitosan–DNA nanoparticles showed more than 500 times higher luciferase expression in the liver 3 days after RII; and transgene expression levels decreased gradually over 14 days. Luciferase expression in the kidney, lung, spleen, and heart was negligible compared with that in the liver. RII of chitosan–DNA nanoparticles did not yield significant toxicity and damage to the liver and biliary tree as evidenced by liver function analysis and histopathological examination. Luciferase expression by RII of PEI–DNA nanoparticles was 17-fold lower than that of chitosan–DNA nanoparticles on day 3, but it increased slightly over time. These results suggest that gene delivery by chitosan–DNA nanoparticles through RII is a promising routine to achieve liver-targeted gene delivery and both gene carrier characteristics and mode of administration significantly influence gene delivery efficiency.

In another study designed to investigate the in vitro and in vivo transfection efficiency of chitosan nanoparticles as vectors for gene therapy, three types of chitosan nanoparticles [quaternized chitosan−60% trimethylated chitosan oligomer (TMCO-60%), C (43–45 kDa, 87%), and C (230 kDa, 90%)] were used to encapsulate plasmid DNA (pDNA) encoding green fluorescent protein (GFP) using the complex coacervation technique (Zheng et al., 2007). The in vitro study showed the efficiency of transfection in the following descending order: TMCO-60% NC (43–45 kDa, 87%) NC (230 kDa, 90%). TMCO-60% proved to be the most efficient, the optimal chitosan/pDNA ratio was 3.2:1. Further, they examined the in vivo transfection efficiency by feeding the chitosan/pDNA nanoparticles to 12 BALB/C-nu/nu nude mice and observed that the gastric and upper intestinal mucosa showed most prominent GPF expression. GFP expression in the mucosa of the stomach and duodenum, jejunum, ileum, and large intestine were found, respectively, in 100%, 88.9%, 77.8% and 66.7% of the nude mice examined. TMCO-60%/pDNA nanoparticles exhibited better in vitro and in vivo transfection activity than the other two with minimal toxicity, which makes it a desirable non-viral vector carrier for gene therapy via oral administration.

Weecharangsan et al. (2008), explored the differences in transfection levels of chitosan/DNA complexes formulated with various chitosan salts (CS) including chitosan hydrochloride (CHy), chitosan lactate (CLa), chitosan acetate (CAc), chitosan aspartate (CAs) and chitosan glutamate (CGl). CHy, CLa, CAc, CAs and CGl, MW 45 kDa were found to form a complexes with pcDNA3-CMV-Luc at various N/P ratios. CGl/DNA complexes were formulated with various chitosan molecular weights (20, 45, 200 and 460 kDa). Gel electrophoresis illustrated that complete complexes formed at N/P ratios above 2 in all CS of MW 45 kDa. The transfection efficiency of CS/DNA complexes was found to be dependent on the salt form and MW of chitosan, and the N/P ratio of CS/DNA complexes. Of different CS, the maximum transfection efficiency varied with different N/P ratios. CHy/DNA, CLa/DNA, CAc/DNA, CAs/DNA and CGl/DNA complexes showed maximum transfection efficiencies at N/P ratios of 12, 12, 8, 6 and 6, respectively. Cytotoxicity results showed that all CS/DNA complexes had low cytotoxicity. This study suggested chitosan salts have the potential to be used as safe in gene delivery systems.

Chitosan, trimethyl chitosan or polyethylenglycol-graft-trimethyl chitosan/DNA complexes were characterized concerning physicochemical properties such as hydrodynamic diameter, condensation efficiency and DNA release (Germershaus et al., 2008). Further, they evaluated the cytotoxicity of these polymers and uptake- and transfection efficiency of polyplexes in vitro. Under conditions found in cell culture, formation of aggregates and strongly decreased DNA condensation efficiency were observed in the case of chitosan polyplexes. These characteristics resulted in only 7% cellular uptake in NIH/3T3 cells and low transfection efficiencies in four different cell lines. By contrast, quaternization of chitosan strongly reduced aggregation tendency and pH dependency of DNA complexation. Accordingly, cellular uptake was increased 8.5-fold compared to chitosan polyplexes resulting in up to 678-fold increased transfection efficiency in NIH/3T3 cells. Apart from reduction of the cytotoxicity, PEGylation led to improved colloidal stability of polyplexes and significantly increased cellular uptake compared to unmodified trimethyl chitosan. These improvements resulted in a significant, up to 10-fold increase of transfection efficiency in NIH/3T3, L929 and MeWo cells compared to trimethyl chitosan. This study not only highlights the importance of investigating polyplex stability under different pH- and ionic-strength conditions but also elucidates correlations between physicochemical characteristics and biological efficacy of the studied polyplexes.

In an approach to target pDNA/chitosan complex using cell specific receptors, mannose-modified chitosan (man-chitosan) was used to target macrophages expressing a mannose receptor (Hashimoto et al., 2006). The cellular uptake of pDNA/man-chitosan complexes through mannose recognition was then observed. The pDNA/man-chitosan complexes showed no significant cytotoxicity in mouse peritoneal macrophages, while pDNA/man-PEI complexes showed strong cytotoxicity. The pDNA/man-chitosan complexes showed much higher transfection efficiency than pDNA/chitosan complexes in mouse peritoneal macrophages. Observation with a confocal laser microscope suggested differences in the cellular uptake mechanism between pDNA/chitosan complexes and pDNA/man-chitosan complexes. Mannose receptor mediated gene transfer thus enhances the transfection efficiency of pDNA/chitosan complexes.

Using a similar technique, Taku and his co-workers modified a chitosan derivative, 6-amino-6-deoxy chitosan (6ACT) by galactosylation (Satoh et al., 2007). A series of galactose-modified 6ACTs (Gal–6ACT) with degrees of substitution (d.s.) ranging from 3% to 50% per pyranose were prepared by reductive alkylation with lactose. DNA retardation assays showed that the electrostatic interaction between Gal–6ACT and plasmid DNA was not changed by galactose modification up to 50% per pyranose of 6ACT. Gal–6ACT with a d.s. of 38% was bound to galactose-recognizing lectin, RCA120. A significant increase in transfection efficiency for HepG2 cells was observed at degree of substitutions ranging from 18% to 50% and at N/P values ranging from 1.5 to 2.5. Under optimum conditions, Gal–6ACT showed about 10 times higher efficiency than 6ACT. However, a slight uptake by the galactose receptors on hepatocytes was observed by flow cytometric analysis. Moreover, Gal–6ACT with a d.s. of 38% mediated efficient gene transfer into both A549 and HeLa cells lacking the galactose receptor. These results suggest that the enhancement of transfection efficiency of Gal–6ACT was not due to the increase of receptor-mediated cellular uptake. In addition, the enhanced gene transfer efficiency was not specific to the galactose modification because the efficiency of glucose-modified 6ACT for HepG2 cells was similar as that of Gal–6ACT.

Kim et al. (2005) explored the possibility of surface modification of poly (D,L-lactide-*co*-glycolide) (PLGA) nanosphere platform with chitosan for gene delivery by using the emulsion solvent

diffusion (ESD) method (Tahara et al., 2008). By coating the PLGA nanospheres with chitosan, the loading efficiency of nucleic acid in the modified nanospheres was found to increase significantly. The release profile of nucleic acid from PLGA nanospheres exhibited sustained release after initial burst, while coating with chitosan reduced the initial burst of nucleic acid release and prolonged the drugs releasing at later stage.

Probing for a solution to track the efficiency of DNA delivery, Lee, Ha, & Yoo, 2008, employed fluorescence resonance energy transfer (FRET) to monitor the molecular dissociation of a chitosan/DNA complex with different molecular weights of chitosan. Chitosan with different molecular weights was complexed with plasmid DNA and the complex formation was monitored using dynamic light scattering and a gel retardation assay. Plasmid DNA and chitosan were separately labeled with quantum dots and Texas Red, respectively, and the dissociation of the complex was subsequently monitored using confocal microscopy and fluorescence spectroscopy. As the chitosan molecular weight in the chitosan/DNA complex increased and the Texas Red-labeled chitosan gradually lost FRET-induced fluorescence light. This observation was noticed when HEK293 cells incubated with chitosan/DNA complex and examined with confocal microscopy. This suggested that the dissociation of the chitosan/DNA complex was more significant in the high molecular weight chitosan/DNA complex. Fluorescence spectroscopy also determined the molecular dissociation of the chitosan/DNA complex at pH 7.4 and pH 5.0 and confirmed that the dissociation occurred in acidic environments. This finding suggested that the high molecular weight chitosan/DNA complex could easily be dissociated in lysosomes compared to a low molecular weight complex. Furthermore, the high molecular weight chitosan/DNA complex showed superior transfection efficiency in relation to the low molecular weight complex. Therefore, it could be concluded that the dissociation of the chitosan/DNA complex is a critical event in obtaining the high transfection efficiency of the gene carrier/DNA complex (Lee et al., 2008).

A study on the condensation of depolymerized chitosans with DNA was carried out. High molecular weight chitosan was depolymerized by oxidative degradation with $NaNO_2$ at room temperature to get 11 samples of chitosan derivatives of varying molecular weights with a view to assessing their effective molecular weight range for gene delivery applications. The results showed that chitosans having very low molecular weights and high charge density exhibited strong binding affinity to DNA compared to high molecular weight chitosans (Morris, Emilia, & Pillai, 2006; Morris, Neethu, Abraham, Pillai, & Sharma, 2008). Song et al. (2009) recently demonstrated that the synthesis of novel galactosylated chitosan (GC) through etherization of chitosan and galactose in THF using BF3-OEt2 as promoter. They found that the GC/DNA nanoparticles were able to transfect HEK293 cells and the viability of these cells was not affected (Song et al., 2009). The interactions between phosphorylcholine-substituted chitosans (PC–CH) and calf-thymus DNA (ct-DNA) were investigated focusing on the effects of the charge ratio, the pH, and phosphorylcholine content on the size and stability of the complexes using the ethidium bromide fluorescence assay, gel electrophoresis, dynamic light scattering, and fluorescence microscope. The PC–CH nanoparticles synthesized at low ionic strength with moderate degree of substitution (DS = 20% and 40%) remained stable for weeks (Case et al., 2009). Xu et al. (2009) recently synthesized methoxy poly(ethylene glycol)–polyethylenimine–chitosan (mPEG–PEI–CS) via chitosan conjugated with polyethylenimine and methoxy poly(ethylene glycol). The transfection of HEK293 cells proved that mPEG–PEI–CS/VRfat-1 plasmid had little toxicity on the growth and gene expression of cells (Xu et al., 2009).

Alkylated chitosan was found to be more efficient gene carrier and had negative impact of the alkyl group modification on the biocompatibility of the chitosan (Liu et al., 2009). An approach for the enhancement of cellular uptake and transfection efficiency of chitosan/DNA complexes through modifying the internal structure by incorporating a negatively charged poly(γ-glutamic acid) was recently reported (Peng et al., 2009). Other modifications of chitosan including methylated *N*-acryl chitosan derivatives, O-carboxymethyl chitosan along with organosilica have been well reported for gene delivery (Sajomsang, Ruktanonchai, Gonil, Mayen, & Opanasopit, 2009; Zhang et al., 2009). Duceppe and Tabrizian described nanoparticles made of ultra low molecular weight chitosan (ULMWCh)/hyaluronic acid (HA) as novel potential carriers for gene delivery (Duceppe & Tabrizian, 2009). Lee, Kim, and Yoo (2009) prepared chitosan/pluronic hydrogels as injectable depot systems for gene therapy to enhance local transgene expression at injection sites. Transfection studies employing HEK293 cells showed that released fractions from chitosan/pluronic hydrogels showed better transfection efficiency than those from pluronic hydrogels (Lee et al., 2009). Thus, numerous studies are being carried on modifying chitosan aiming at improving its transgenic efficacy.

3. Clinical applications of chitosan–DNA systems

To test the chitosan/DNA system as a potential DNA vaccine candidate, Kumar et al. utilized a strategy involving an intranasal gene transfer, referred to as IGT, complexed with chitosan–DNA nanospheres containing a cocktail of DNA encoding nine immunogenic syncytial virus (RSV) antigens (Kumar et al., 2002). This system was tested against acute RSV infection in a BALB/c mouse model. The effectiveness and mechanism of this IGT strategy were investigated, and results demonstrated that IGT was safe and effective against RSV as well as it significantly attenuates pulmonary inflammation induced by RSV infection. A single dose of about 1 mg/kg body weight was capable of decreasing viral titres by two orders of magnitude (100-fold) on primary infection. This therapy works by induction of high levels of both serum IgG and mucosal IgA antibodies, generation of effective control response and elevated lung specific production of interferon (IFN)-γ with antiviral action. Also intranasal gene transfer (IGT) significantly decreased pulmonary inflammation and did not alter airway hyper responsiveness, making it safe for in vivo use.

Another application in using chitosan–DNA gene therapy is against Coxsackie virus B3 infections, which cause acute and chronic myocarditis (Xu et al., 2004). Intranasal delivery of chitosan–DNA complex prepared by vortexing DNA with chitosan resulted in transgenic DNA expression in mouse nasopharynx and also induce mucosal SIgA secretion. Sun et al. (2004) constructed a eukaryotic expression vector pVAX1-pZP3a as an oral ZP DNA contraceptive and successfully encapsulated in nanoparticles with chitosan to target zona pellucida (ZP), the extra cellular matrix surrounding oocytes. After 5 days of feeding to mice the transcription and expression of pZP3 was found in mouse alvine chorion. Okamoto et al. (2003) investigated the potential of chitosan in the form of inhaled powder for gene delivery purposes by preparing powders using pCMV-Luc as a reporter gene and a LM chitosan (3000–30,000) as a cationic vector with supercritical CO_2. This powder was administered to the lungs of mice and their transfection efficiency was compared to that of DNA solution and DNA powder without the cationic vector. The gene powder with the cationic vector was found to be excellent gene delivery system to the lungs (Okamoto et al., 2003).

Bivas-Benita et al. (2004) proposed chitosan–DNA nanoparticles for pulmonary delivery of a DNA vaccine for *Mycobacterium tuberculosis*. The authors used an HLA-A2 transgenic mouse model to investigate the effect of pulmonary delivery of a new pDNA

encoding eight HLA-A*0201-restricted T cell epitopes from *M. tuberculosis* formulated in chitosan nanoparticles. Pulmonary administration of these nanoparticles was shown to induce the maturation of dendritic cells as well as induce increased levels of IFN-γ secretion compared to pulmonary delivery of plasmids in solution or the intramuscular immunization route (Bivas-Benita et al., 2004).

In another study, Khatri et al. investigated the preparation and in vivo efficacy of plasmid DNA loaded chitosan nanoparticles for nasal mucosal immunization against hepatitis B. Chitosan pDNA nanoparticles were prepared using a complex coacervation process (Khatri, Goyal, Gupta, Mishra, & Vyas, 2008). Prepared nanoparticles were characterized for size, shape, surface charge, plasmid loading and ability of nanoparticles to protect DNA against nuclease digestion and for their transfection efficacy. Nasal administration of nanoparticles resulted in serum anti-HBsAg titre that was less compared to that elicited by naked DNA and alum adsorbed HBsAg, but the mice were seroprotective within 2 weeks and the immunoglobulin level was above the clinically protective level. However, intramuscular administration of naked DNA and alum adsorbed HBsAg did not elicit sIgA titre in mucosal secretions that was induced by nasal immunization with chitosan nanoparticles. Similarly, cellular responses (cytokine levels) were poor in case of alum adsorbed HBsAg. Chitosan nanoparticles thus produced humoral (both systemic and mucosal) and cellular immune responses upon nasal administration. The study signifies the potential of chitosan nanoparticles as DNA vaccine carrier and adjuvant for effective immunization through non-invasive nasal route.

Albeit the conventional high molecular chitosans have a few drawbacks such as aggregated shapes, low solubility at neutral pH, and high viscosity at concentrations used for in vivo delivery and a slow onset of action (Zhou et al., 2008), the non-viral gene delivery systems based on chitosan are still regarded as one of the most efficient system for DNA vaccine delivery. To circumvent the problems with high molecular weight and to improve the efficacy of DNA delivery, Zhou et al. (2008) examined the possibility of covalently attaching low molecular weight chitosans with an average molecular mass of 6 kDa (Chito6) to gold nanoparticles (GNPs). Further, the potency of the resulting Chito6–GNPs conjugates as vectors for the delivery of plasmid DNA was investigated both in vitro and in vivo. After delivery by intramuscular immunization in BALB/c mice, the Chito6–GNPs conjugates induced an enhanced serum antibody response 10 times more potent than naked DNA vaccine. Additionally, in contrast to naked DNA, the Chito6–GNPs conjugates induced potent cytotoxic T lymphocyte responses at a low dose (Zhou et al., 2008).

4. Chitosan–siRNA complex nanoparticles

RNA interference (RNAi) has been discovered as a conserved mechanism in higher eukaryotic cells to eliminate harmful or unwanted gene expressions, and thereby contribute to the defense mechanism from viral infection siRNAs have been proven to be versatile agents for controlling gene expression in mammalian cells. They work in more potential ways than conventional antisenses (Beale et al., 2003; Khan et al., 2004; Miyagishi, Sumimoto, Miyoshi, Kawakami, & Taira, 2004). Overall, siRNA appears to be a much more robust and efficient technology offering significant potential. siRNA consisting of 21–23 nucleotides can regulate gene expression in mammalian cells through RNA interfering (RNAi). As the administration of siRNA could bypass nonspecific inhibition of protein synthesis induced by long doublestranded RNA (Sioud & Sorensen, 2003; Sorensen, Leirdal, & Sioud, 2003), it has therefore been employed as a novel tool in blocking the expression of genes such as those expressed in infectious diseases and cancers. However, similar to hydrophilic and polyanion-mediated gene therapy, siRNA also suffers particular problems including poor cellular uptake, rapid degradation by ubiquitous nucleases as well as limited blood stability (Stein, 1996; Urban-Klein, Werth, Abuharbeid, Czubayko, & Aigner 2004). As a result of these limitations, unassisted delivery of siRNA to the cells is frustrating. Although various chemical modifications of siRNA can be used to overcome these problems, these modifications posses disadvantages such as a decreased mRNA hybridization, higher cytotoxicity and increased unspecific effects (Weyermann, Lochmann, Georgens, & Zimmer, 2005). Therefore, effective systems which can protect and transport siRNA to the cytoplasm of the target cells are needed to exploit the promising potential applications offered by successful delivery of siRNA.

Chitosan can be used as a carrier deliver siRNA due to its advantages such as low toxicity, biodegradability and biocompatibility (Illum, 1998; Thanou et al., 2001). In an approach to develop chitosan nanoparticles for siRNA delivery, Katas & Alpar, 2006, prepared chitosan nanoparticles by two methods of ionic cross-linking, simple complexation and ionic gelatin using sodium tripolyphosphate (TPP). Both methods produced nanosize particles, less than 500 nm depending on type, molecular weight as well as concentration of chitosan. In the case of ionic gelation, two further factors, namely chitosan to TPP weight ratio and pH, affected the particle size. In vitro studies in two types of cells lines, CHO K1 and HEK 293, revealed that preparation method of siRNA association to the chitosan plays an important role on the silencing effect. Chitosan–TPP nanoparticles with entrapped siRNA are shown to be better vectors as siRNA delivery vehicles compared to chitosan–siRNA complexes possibly due to their high binding capacity and loading efficiency. This report suggested that, chitosan–TPP nanoparticles show much potential as viable vector candidates for safer and cost-effective siRNA delivery.

Exploring the efficiency of chitosan/siRNA nanoparticles as a therapeutic agent, Liu et al. reports that the physicochemical properties (size, zeta potential, morphology and complex stability) and in vitro gene silencing of chitosan/siRNA nanoparticles are strongly dependent on chitosan molecular weight (MW) and degree of deacetylation (DD) (Liu et al., 2007). High MW and DD chitosan resulted in the formation of discrete stable nanoparticles of 200 nm in size. Chitosan/siRNA formulations (N/P: 50) prepared with low MW (10 kDa) showed almost no knockdown of endogenous enhanced green fluorescent protein (EGFP) in H1299 human lung carcinoma cells, whereas those prepared from higher MW (64.8–170 kDa) and DD (80%) showed greater gene silencing ranging between 45% and 65%. The highest gene silencing efficiency (80%) was achieved using chitosan/siRNA nanoparticles at N:P 150 using higher MW (114 and 170 kDa) and DD (84%) that correlated with formation of stable nanoparticles of 200 nm. From their conclusions it is evident that there is stillroom for improvement and for the optimization of gene silencing using chitosan/siRNA nanoparticles and the fine-tuning of the polymeric properties would make lots of difference.

5. Conclusions

In the field of gene therapy, the development of efficient and safe carrier systems able to transfer DNA into cells is a major goal. Different systems have been developed in recent years, and chitosan is one of the most promising candidates among them. There are an enormous number of in vitro and in vivo studies, showing that chitosan is a suitable material for efficient non-viral gene therapy. In this review, we have summarized the recent advances in gene therapy giving emphasis to the applications of chitosan

conjugated DNA nanoparticles as novel non-viral delivery systems to improve the transfection efficiency.

Drawbacks with conventional high molecular weight chitosan prompted researchers to conduct studies using a series of chitosan oligomers, ranging in molecular weight from 1.2 to 10 kDa. By varying the molecular weight of chitosan, plasmid concentration and the stoichiometry of polymer–plasmid complex, the transfection efficiency and plasmid DNA uptake can be tuned. These gene delivery systems based on chitosans can be conjugated with quantum dots for tracking and also be equipped with specific ligands for cell specific interaction. Together with gene therapy applications, chitosan nanoparticles contribute to the design of siRNA polyplexes for gene silencing and also as carriers of DNA vaccines, which will fuel clinical development pipelines in the near future.

All these studies highlighting the use of chitosan nanoparticles as biocompatible non-viral gene delivery systems present a platform for further optimization studies of chitosan-based gene delivery systems, for example, with regard to steric stabilization and cell specific targeting. Another promising area of research could be the matrix-based gene delivery using chitosan nanoparticles, which would find increasing utility and appeal for regenerative medicine. Studies with stem cells would be of particular interest as the encapsulated genes to drive the preferential differentiation of the seeded cells can further augment the micro-environmental cues at the site of implantation.

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