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Review

Chitosan and Its Derivatives for Gene Delivery

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Abstract: Non-viral vectors, including lipid- or polymer-based systems, have attracted much attention to date as a gene delivery vehicle, due to safety issues with viral vectors. Chitosan, a naturally existing cationic polymer, has shown great potential as a gene delivery carrier, as it has low immunogenicity and toxicity, excellent transcellular transport ability, and is relatively easy to chemically modify. This review summarizes and discusses the general features of chitosan and its applications as a delivery carrier of DNA and RNA.

Keywords: chitosan, nanoparticle, gene delivery, targeting, siRNA.

Introduction

Gene delivery is used to introduce genetic material into cells in order to alleviate symptoms or prevent the occurrence of a particular disease.[1] Appropriate carriers, typically viral or non-viral vectors, are essential for gene delivery, as it is difficult to move naked DNA, a negatively charged macromolecule, through negatively charged cell membranes. Several viral vectors such as retroviruses and adenoviruses have been frequently employed. However, critical safety issues have been raised in clinical uses.[2,3] Therefore, non-viral vectors have been extensively investigated as alternatives. Several features required for non-viral gene delivery systems include (1) commercial availability and stability, (2) ease of bulk synthesis, (3) high binding efficiency, (4) ability to transfect most cells, and (5) lack of immunogenicity or biohazardous activity.[4] Generally speaking, when cells are treated with DNA/vector complexes, the complexes adhere to the cell surface via either electrostatic interaction or receptor-mediated uptake. Endocytosis is the dominant mechanism for entry of the complexes, and the released DNA from the endosome enters into the cytosol in a partially de-condensed form. DNA finally reaches the

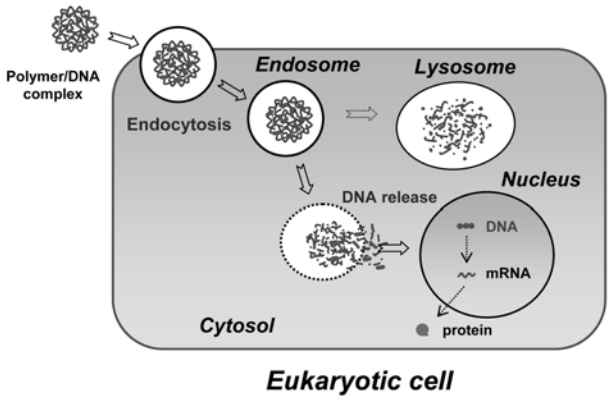

Figure 1. Schematic description for transfection of eukaryotic cells with polymer/DNA complexes.

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nucleus via passive diffusion and nuclear membrane crossing, and the DNA is transcribed into mRNA, resulting in gene expression.[5,6] A schematic description for transfection of eukaryotic cells with polymer/DNA complexes is shown in Figure 1.

Liposomes, self-closed colloidal particles in which bilayered membranes encapsulate a fraction of the medium, have been frequently used as a non-viral gene delivery system.[7] Cationic liposomes are used as a gene carrier to reduce the net negative surface charge of DNA in an attempt to reduce charge-charge repulsion at the surface of cell membranes.[8,9] However, the application of liposomes *in vivo* is limited due to poor biocompatibility and rapid degradation.[10] An alternative approach to the development of non-viral vectors is the use of cationic polymers designed to complex with DNA. Polymer-based non-viral gene carriers have been frequently used due to the avoidance of potential immunogenicity and toxicity, the possibility of repeated administration, and the ease of establishing a good manufacturing practice (GMP).[11] Simple mixing of DNA with poly(L-lysine) or DEAE-dextran resulted in the formation of polyelectrolyte complexes for transferring DNA into cells. But, these systems still showed a low transfection efficiency, cytotoxicity, and limited usefulness for systemic administration due to the rapid clearance following intravenous injection.[12,13] New types of polymers such as poly(ethylene glycol)-*b*-poly(L-lysine),[14] poly(L-lysine)-*g*-poly(D,L-lactic-*co*-glycolic acid),[15] poly-*N*-(2-hydroxypropyl) methylacrylamide-*b*-poly(trimethylammonioethyl methacrylate chloride),[16] polyethylenimine-*g*-poly(vinyl pyrrolidone),[17] and dendrimers[18] have been synthesized and developed for gene delivery.

Although non-viral gene delivery systems have been dominated by synthetic polymer- or lipid-based gene carriers, natural polymers may provide a useful means of developing non-viral gene carriers due to their distinctive characteristics.[19] For example, chitosan, a positively charged natural polysaccharide, has shown great potential as a gene delivery carrier. In this review, we discuss the general features of chitosan and its use as a delivery carrier of DNA and RNA.

General Properties of Chitosan

Chitin, composed of (1,4) linked *N*-acetyl-β-D-glucosamine, is the second most abundant natural polymer in the world, and is primarily obtained from shrimp and crab. When the degree of deacetylation (DD) of chitin reaches about 50%, it becomes soluble in aqueous acidic media and is called chitosan. Chitosan has a repeated structure of (1,4) linked β-D-glucosamine, and has an apparent pK of 6.5. Traditionally, commercial products are composed of 80% β-D-glucosamine and 20% *N*-acetyl-β-D-glucosamine (Figure 2).[20] Chitosan is generally soluble at pH below 6, and its solubility is usually tested by dissolving it in 1% or

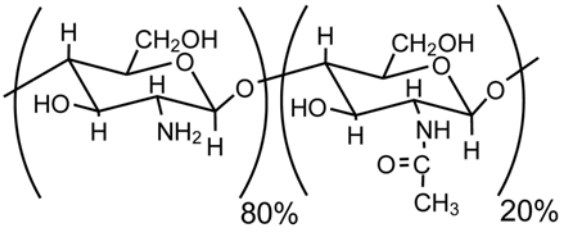

Figure 2. Chemical structure of chitosan.

0.1 M acetic acid. The solution properties of chitosan depend on its molecular weight, degree of deacetylation, and distribution of acetyl groups in the main chain, as the deacetylation is usually carried out in the solid state and generates an irregular structure due to the semicrystalline feature of chitosan.[21,22] ^{1}H-NMR is the most convenient technique to measure the content and distribution of acetyl groups in chitosan.[23] ^{13}C and ^{15}N solid state NMR are also frequently used to investigate the distribution of acetyl groups in the chain (e.g., random or blockwise), which may influence the solubility and inter-chain interactions.[24,25]

Chitosan is a cationic polymer and has been widely used in the areas of food, cosmetics, biomedical and pharmaceutical applications, etc.[26-29] The extensive biological properties of chitosan enable various biomedical applications and include (1) biocompatibility and biodegradability, (2) binding capability to cells, (3) acceleration of wound healing, (4) haemostatic properties, (5) anti-bacterial and anti-fungal properties, and (6) anti-tumor properties. A large variety of useful forms such as beads, films, sponges, tubes, powders, and fibers, can be obtained from chitosan.[30] Chitosan is degradable by enzymes such as lysozyme and chitosanase, and the degradation rate is also dependent on the temperature, ionic strength, and pH of the medium *in vitro*.[31] In general the lower the degree of deacetylation of chitosan, the faster the degradation. Further, chitosan has been proven to be biodegradable when implanted into animals.[32]

Chitosan/DNA Polyelectrolyte Complexes

Simple and direct mixing of chitosan and DNA generated nanoparticles by a coacervation process, which was useful for oral allergen-gene immunization. Oral administration of nanoparticles containing a dominant peanut allergen gene (pCMVArah2) substantially reduced allergen-induced anaphylaxis, indicating a prophylactic use of the nanoparticles in treating food allergies.[33] The molecular weight (MW) and degree of deacetylation (DD) are critical factors when using chitosan as a gene delivery carrier, because they affect DNA binding and release, and ultimately the *in vitro* and *in vivo* gene transfection efficiency. A549 cellular uptake of chitosan/pEGFP-C2 complexes was significantly reduced by decreasing the MW or DD of chitosan.[34] The decreased DD also resulted in a decrease of overall luciferase expression

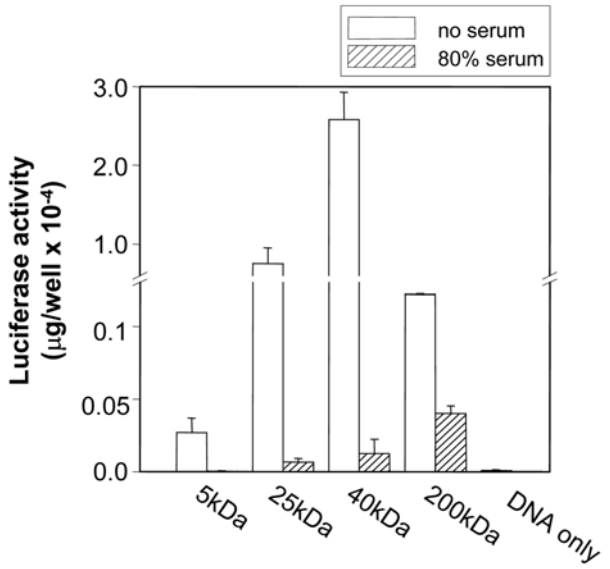

Figure 3. *In vitro* transfection activity of COS-1 cells using chitosan with different molecular weights (reprinted with permission from ref. 37, Copyright 2001 American Chemical Society).

levels in HEK293, HeLa, and SW756 cells. In contrast, intramuscular luciferase expression levels increased with decreasing DD.[35,36] In the absence of serum, it was evident that the use of chitosan 40 kDa was the most efficient way to deliver DNA to COS-1 cells, whereas chitosan 5 kDa showed the lowest transfection efficiency, likely due to the low stability of the complexes. The transfection efficiency in the presence of serum, however, increased with increasing the MW of chitosan (Figure 3).[37] Despite extensive studies, it is still unclear how the MW and DD of chitosan influence the transfection efficiency and several contradictory results have been reported.

When chitosan was mixed with poly(acrylic acid), nanoparticles with diverse microstructures (e.g., solid sphere, core-shell structure, etc.) were formed, depending on the molecular weight, ratio of amino groups to carboxyl groups, and incubation temperature.[38] Chitosan also formed well-defined toroidal and rod-like structures when complexed with DNA, depending on the molecular weight and degree of deacetylation.[39] An inclusion of alginate improved the transfection efficiency of chitosan-based nanoparticles while maintaining biocompatibility and low toxicity. Competition binding assays showed that the presence of alginate reduced the strength of interaction between chitosan and DNA, which eventually contributed to an improved transfection efficiency.[40]

Chitosan Derivatives for Gene Delivery

Various chitosan derivatives have been synthesized and used for gene delivery, as chitosan is a linear polyamine whose reactive amino groups, as well as primary and secondary hydroxyl groups, can be chemically modified.[41-43] Chitosan oligomers can be chemically modified with hydrophobic cholesterol groups, and form nano-sized self-aggregates in aqueous environments. The physicochemical properties of the nanoparticles are closely related to the molecular weight of chitosan and the number of hydrophobic groups per chitosan chain. Nanoparticle/DNA complexes enhanced the *in vitro* transfection efficiency on human embryonic kidney 293 cells.[44,45] Hydrophobically modified chitosans containing deoxycholic acid groups were also synthesized to prepare self-aggregated nanoparticles with a mean diameter of 160 nm in aqueous media (Figure 4), and these were used to form complexes with plasmid DNA.

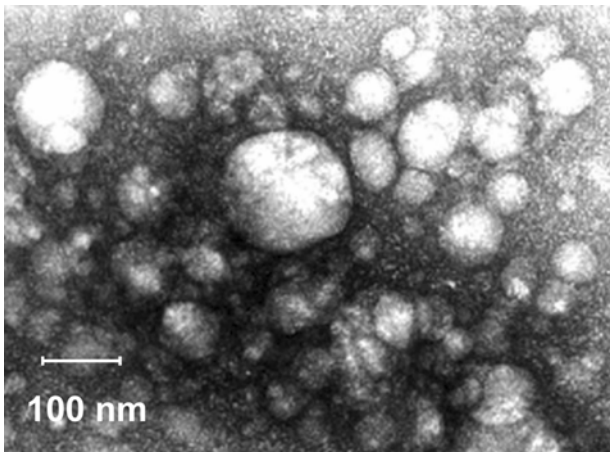

Figure 4. Transmission electron microscopic picture of deoxycholic acid-modified chitosan self-aggregates negatively stained with an aqueous solution of uranyl acetate.

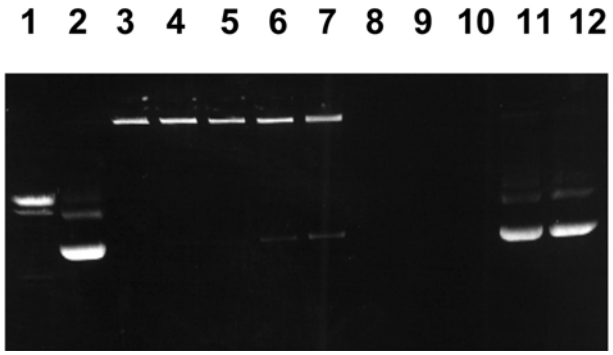

Figure 5. Migration of chitosan/DNA complexes in the presence of excess amounts of heparin incubated for 2 h at room temperature. DNA was mixed with chitosan self-aggregates (lanes 3-7) or with Lipofectamine® (lanes 8-12) at a fixed charge ratio (+/-) of 4/1. Lane 1, DNA molecular weight marker II; lane 2, DNA only; lanes 3 and 8, complex only; lanes 4 and 9, 5; lanes 5 and 10, 10; lanes 6 and 11, 50; lanes 7 and 12, 100 equivalent of heparin to plasmid DNA.[47]

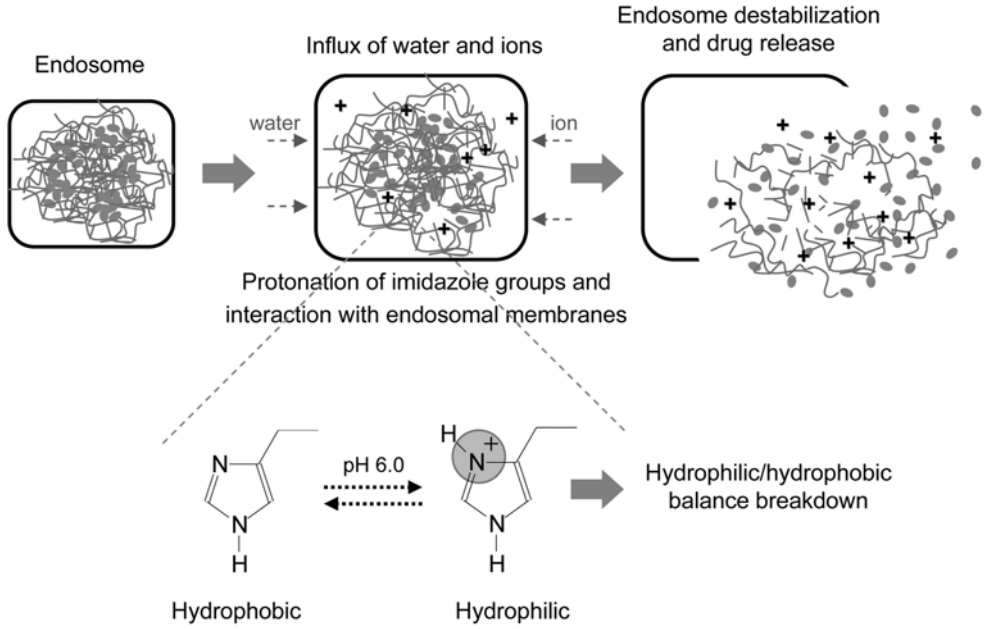

Figure 6. Schematic representation of endosomal escape and drug release of histidylated chitosan nanoparticles (reprinted from ref. 49, Copyright 2006, with permission from Elsevier).

Complex formation between DNA and chitosan self-aggregates, as well as the complex stability were strongly dependent on the charge ratio, pH, and incubation time (Figure 5).[46,47] Alkylated chitosans were prepared by modifying chitosan with alkyl bromide and used to deliver a plasmid-encoding CAT into C2C12 cells. The transfection efficiency increased with an increase of the number of carbons in the alkyl side chain, due to the enhanced entry into cells facilitated by hydrophobic interactions and easy unpacking of DNA from the chitosan/DNA complexes.[48] *N*-Acetyl histidine-conjugated glycol chitosan was synthesized and used to form self-assembled nanoparticles with mean diameters of 150-250 nm for the intracytoplasmic delivery of drugs. In slightly acidic environments, the nanoparticles were disassembled and drugs were released into the cytosol (Figure 6).[49] Thiolated chitosan was prepared by the reaction of thioglycolic acid with chitosan in order to enhance the cell penetration properties. Nanocomplexes of thiolated chitosan with plasmid DNA encoding green fluorescent protein (GFP) were formed and their mean diameter was in the ranges of 75-120 nm, depending on the weight ratio of chitosan to DNA. Thiolated chitosan/DNA nanocomplexes induced significantly higher GFP expression in HEK293, MDCK, and HepG2 cells than the unmodified chitosan. Gene expression from disulfide-cross-linked nanocomplexes that were intranasally administrated to mice lasted for at least 14 days.[50]

Chitosan was trimethylated to introduce quaternization on the backbone, and the toxicity and transfection efficiency of this derivative were tested. The derivative was significantly less toxic to COS-7 and MCF-7 cells than linear polyethylenimine (PEI), and was able to transfect MCF-7 cells with greater efficiency than PEI.[51] PEGylated trimethyl chitosan copolymers were synthesized in order to enhance the solubility of chitosan in water, as well as to improve the biocompatibility of trimethyl chitosan. PEGylation with PEG 5 kDa decreased the cytotoxicity of trimethyl chitosan to a great extent.[52] PEGylation of chitosan (e.g., chitosan-*g*-PEG) enabled higher gene expression than chitosan/DNA complexes in the liver, and transgene expression by chitosan-*g*-PEG/DNA complexes in other organs after portal vein infusion increased with increasing the degree of PEG grafting in chitosan.[53]

Targeted Delivery

Lactose-modified chitosan was synthesized to make chitosan/DNA complexes specific to hepatocytes, which enhanced delivery of the complexes to the late endosome of the cells.[54] Lactobionic acid bearing a galactose group was also conjugated to water-soluble chitosan to enhance the transfection efficiency into HepG2 cells, due to the existence of asialoglycoprotein receptors in the cells.[55] A branch-type of galactosylated chitosan was prepared by introducing an L-lysine spacer to chitosan, followed by covalent conjugation of lactobionic acid to the lysine spacer. The glycoconjugated chitosan showed an especially higher binding efficiency toward galectin-1, a galactose-binding lectin, due to its multivalent galactose units. Varying the length of the spacer led to a significant change in the attachment and pro-

liferation of HepG2 cells.[56] Chitosan oligomers were modified with a trisaccharide containing an *N*-acetylglucosamine residue at the free end, aimed at targeting cell-surface lectins and improving cellular uptake in HEK293 cells. Interestingly, *in vitro* gene expression levels in human liver cells and human bronchial epithelial cells were enhanced when using the trisaccharide-conjugated chitosan. Luciferase gene expression was also enhanced after lung administration to mice using the chitosan derivative.[57] Mannosylated chitosan was prepared to induce mannose receptor-mediated endocytosis of the IL-12 gene directly into dendritic cells that reside within the tumor. Mannosylated chitosan suppressed tumor growth and angiogenesis in mice bearing CT-26 carcinoma cells and significantly induced cell cycle arrest and apoptosis.[58] Mannose-modified chitosan was also used to deliver DNA into macrophages expressing a mannose receptor.[59]

Chitosan/DNA nanoparticles (100-250 nm in diameter with a narrow distribution) were prepared using a complex coacervation process and modified with either transferrin or KNOB protein. The transferrin-conjugated nanoparticles yielded a maximum of a 4-fold increase in the transfection efficiency in HEK293 cells and HeLa cells, whereas KNOB-conjugated nanoparticles improved the gene expression level in HeLa cells by 130-fold. The clearance rate of PEGylated chitosan/DNA nanoparticles in mice following intravenous administration was slower than that of unmodified nanoparticles.[60] Folic acid-modified chitosan was prepared using reductive amidation and used to form nanoparticles through a complex coacervation process, in order to promote targeting and internalization of chitosan/DNA complexes and to improve the transfection efficiency.[61] Water-soluble chitosan was chemically modified with urocanic acid bearing an imidazole ring, which can play a critical role in endosomal rupture by the proton sponge effect. The transfection efficiency of urocanic acid-modified chitosan/DNA complexes into 293T cells was enhanced in parallel with an increase of urocanic acid contents in the complexes.[62]

Chitosan for siRNA Delivery

Recently, small interfering RNA (siRNA) has been extensively investigated and proven to be much more effective and potent for controlling gene expression in mammalian cells than conventional antisense strategies.[63] siRNA consisting of 21-23 nucleotides can regulate gene expression in mammalian cells through RNA interfering (RNAi), and has been used in blocking genes expressed in various infectious disease and cancer. However, due to the rapid degradation and poor cellular uptake of siRNAs *in vitro* and *in vivo*, the appropriate vectors are necessary for efficient delivery.[64]

Although chitosan-based carriers have been found to be useful for the safe and cost-effective delivery of DNA, few research groups have reported on the efficacy of chitosan as a delivery carrier of siRNA. Chitosan and siRNA duplexes (21-mers) formed polyelectrolyte complex nanoparticles, ranging from 40 to 600 nm in diameter, and rapid uptake of Cy5-labeled nanoparticles into NIH 3T3 cells was observed. Effective *in vivo* RNA interference was achieved in bronchiole epithelial cells of mice after nasal administration of the nanoparticles, indicating the potential application of chitosan-based nanoparticles in RNA-mediated therapeutics.[65] Chitosan/siRNA nanoparticles prepared from chitosan with high MW (65-170 kDa) and DD (80%) formed stable nanoparticles (200 nm in diameter), and showed a great gene silencing efficiency.[66] Chitosan nanoparticles were also prepared by ionic cross-linking using sodium tripolyphosphate (TPP), and chitosan/TPP nanoparticles with entrapped siRNA showed great potential as a vector for safe and cost-effective siRNA delivery.[67] Chitosan-coated poly(isohexylcyanoacrylate) nanoparticles encapsulating anti-RhoA siRNA were intravenously administered in xenografted aggressive breast cancers and significantly inhibited the tumor growth while reducing toxicity, indicating the usefulness of chitosan/siRNA systems for cancer treatment.[68]

Conclusions

In this article, we summarized the general features of chitosan and its application as a gene carrier. Naked DNA or RNA does not efficiently transfect most cells, and often requires repeated treatments. The combination with delivery vehicles, however, can protect DNA or RNA from degradation, induce a slow release effect, and significantly enhance the transfection efficiency. Even though non-viral carriers may offer a wide range of possibilities for gene delivery, an appropriate method should be selected and adapted according to the types of molecules and cells. Most of non-viral gene carriers do not allow for a permanent effect and do not reach the efficacy of viral vectors. Although chitosan has been widely used for the delivery of DNA and RNA, chitosan still encounters limitations to its use, such as low transfection efficiency.

The transfection efficiency can be enhanced by controlling the molecular weight, degree of deacetylation, size and stoichiometry of chitosan/DNA complexes, pH of media, serum concentration, administration route, receptor-mediated cellular uptake, unpacking and release of DNA from chitosan/DNA complexes, etc. Optimization of these controlling parameters and overcoming current limitations will accelerate future clinical applications of chitosan in the area of gene delivery as a successful therapeutic.

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