Chitosan Nanoparticles in Gene Delivery

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22.1 Introduction

Gene therapy is a remarkable approach for the treatment of a wide range of diseases and is a method to prevent, treat, and modulate genetic and acquired diseases by changing the expression of genes that are responsible for these diseases. It has gained more attention over the past decade. However, the major challenge of this kind of therapy is the development of safe and effective gene carrier systems for delivering DNA to the target tissue followed by internalization since nucleic acids are sensitive to enzymatic attack of nucleases and show poor cellular uptake (Mahato et al. 1999).

At present, there are two classes of gene delivery systems: viral and non-viral systems. Viral gene delivery systems have been largely employed in clinical trials, because of their high transfection efficiency. They efficiently deliver exogenes to host cells by taking advantage of intracellular trafficking. However, several serious drawbacks, including the lack of specificity to target cells; safety concerns, such as risk of potential immunogenicity; and chromosomal insertion of viral genome, and other drawbacks like restricted plasmid size and complicated

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production, limit the practical use of viral vectors. The death of a patient in a gene therapy trial using viral vectors has focused research on non-viral vectors (Glover et al. 2005). Non-viral vectors have attracted much attention as safer alternatives to viral vectors and also due to several advantages, such as ease of production, lower immunogenicity against the repeated administration, well-defined physicochemical properties, unlimited DNA packing capacity, and low cost. Non-viral vectors have a high degree of molecular diversity for making extensive modifications to overcome extra- and intracellular obstacles of DNA delivery (Jeong et al. 2007; Leong et al. 1998). Cationic lipids, liposomes, cationic polymers, and physical methods are used for DNA internalization. Although cationic lipids are easily transferred into the cell and have high transfection efficiency, they are rapidly cleared from the blood, which limits their application (Midoux et al. 2009). They have also some drawbacks such as toxicity and instability (Leong et al. 1998; Borchard 2001). A range of natural and synthetic cationic polymers have also been used, including chitosan, poly(L-lysine) (PLL), poly(ethylenimine) (PEI), protamine, spermine, modified gelatin, poly (β-amino ester), poly (lactide-co-glycolide), and poly (ε-caprolactone), to prepare polymer-DNA nanoparticles for gene delivery (Bhavsar and Amiji 2007). Cationic polymers are more stable than cationic lipids, and they can be condensed with DNA and deliver it to the cells (Hirano and Noishiki 1985; De Smedt et al. 2000).

Among cationic polymers, chitosan is an attractive gene delivery carrier, since it is biocompatible, biodegradable, nontoxic, cheap and has low immunogenicity (Hirano and Noishiki 1985). Chitosan, α-(1–4)2-amino-2-deoxy-β-D-glucan, is a linear cationic polysaccharide prepared by partial alkaline deacetylation of chitin (Skaugrud 1989). Chitin is mostly obtained from exoskeleton of crustacea (shell of shrimp and other crustaceans). Chitosan is a weak base with pKa value of the D-glucosamine residue of about 6.2–7.0 and is therefore insoluble at alkaline and neutral pH value (Fig. 22.1) (Hejazi and Amiji 2003).

The primary amine groups provide special properties to chitosan and make it very useful in pharmaceutical application and gene therapy.

From the technological point, it is very important that chitosan is hydrosoluble and positively charged as these properties are responsible for its interaction with negative charged molecules such as DNA, upon their contact in an aqueous solution.

Chitosan can effectively bind DNA and protect it from the degradation effect of nucleases. Chitosans have different degree of N-acetylation (40–98 %) and molecular weight (50–2000 kDa) (Hejazi and Amiji 2003).

Because of these good properties of chitosan, chitosan nanoparticles have been appealing as a gene delivery carrier in recent years. Chitosan has been used for the first time as a delivery system for DNA by Mumper et al. (1995), and since then chitosan is successfully used as a non-viral gene delivery system both *in vitro* and *in vivo* (Erbacher et al. 1998; MacLaughlin et al. 1998; Roy et al. 1999; Aral et al. 2000; Koping-Hoggard et al. 2001; Ozbas-Turan et al. 2003; Chen et al. 2004).

In gene therapy, transfection is hampered by several problems, including targeting the gene delivery system to the target cell, transport through the membrane of the cells, degradation in endolysosomes, and intracellular trafficking of DNA to the nucleus (Borchard 2001). Chitosan-based gene delivery systems appeared to have the ability to overcome these major obstacles for transfection (Ishii et al. 2001).

In earlier studies, chitosan-DNA was used in many pharmaceutical forms including complexes, self-aggregates, emulsions, microspheres, and nanoparticles (Erbacher et al. 1998; Ozbas-Turan et al. 2003; Lee et al. 1998, 2005a, b; Mao et al. 2001) as a promising gene carrier. On the other hand, gene therapy is based on two basic strategies: (1) gene replacement or correction for the purpose of production of a protein appropriate to a cell function and (2) inactivation of a gene causing cell dysfunction.

In this chapter, transfection efficiency, factors influencing transfection and cellular uptake, and

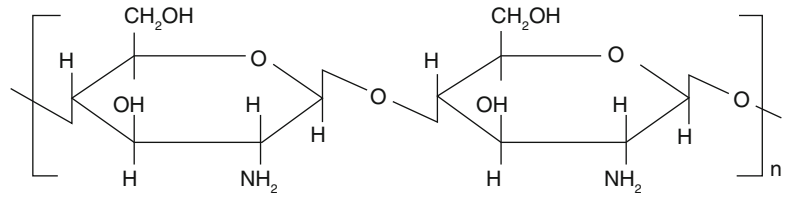

Fig. 22.1 Structure of chitosan

administration routes of chitosan-nucleic acid nanoparticles are discussed.

22.2 Factors Affecting Transfection Efficiency

Transfection is the process of introducing nucleic acids into cells. Different factors may affect the transfection properties of chitosan-based systems. Therefore, optimization of the transfection efficiency of chitosan-DNA nanoparticles is needed for clinical trials. Useful reviews about this topic are available in literature (Kim et al. 2007; Mao et al. 2010) (Fig. 22.2). Some of the factors mentioned below (such as presence of serum and others) are mostly related to parenteral administration, but will be discussed in the chapter as general important data about the gene delivery with chitosan.

22.2.1 Molecular Weight of Chitosan

Molecular weight of chitosan used in gene delivery is an important formulation variable. The effect of molecular weight of chitosan in chitosan-DNA particles has been attributed to chain entanglement effect. Longer polymer chains in high molecular weight chitosan more easily entangle free DNA after initial electrostatic interaction has occurred. Low molecular weight chitosans having shorter polymer chains may not be energetically favorable for chitosan-DNA particle formation (Kiang et al. 2004b). Conflicting results are found in the literature about the influence of chitosan molecular weight on the transfection efficiency. MacLaughlin et al. (1998) studied the transfection efficiency of commercial and prepared depolymerized chitosan (32 and 120 kDa)-DNA complexes on *Cercopithecus aethiops* SV40-transformed kidney cells (Cos-1) and noted that molecular weight of chitosan had limited effect on plasmid expression *in vitro*. Similar observations were reported by several other groups (Koping-Hogard et al. 2003, 2004; Romoren et al. 2003).

However, the group of Sato et al. (2001) investigated factors influencing the transfection efficiency as well as cell transfection mechanisms of chitosan-DNA complexes; the results indicated that chitosans of 10–50 kDa are excellent for gene transfer (Ishii et al. 2001; Sato et al. 2001). According to their suggestion, molecular weight of chitosan may affect the stability, cellular uptake, and the dissociation of DNA from chitosan-DNA complex after endocytosis. A certain combination of deacetylation degree and molecular weight of chitosan is suggested to be an important factor in transfection efficiency by affecting particle stability (Lavertu et al. 2006). Csaba et al. (2009) encapsulated plasmid DNA into chitosan nanoparticles prepared with high and low molecular weight polymers and found that nanoparticles based on low molecular weight chitosan mediated higher plasmid DNA expression than nanoparticles prepared with high molecular weight chitosans.

A charge ratio as high as 60:1 (+/-) was required for the formation of physically stable polyplex when using low molecular weight of chitosan. The degree of polymerization (DPn) of chitosan is also important, chitosan having degree of polymerization around 18 complexed with DNA and transfected *in vitro* and *in vivo* (for lung administration) successfully (Koping-Hoggard

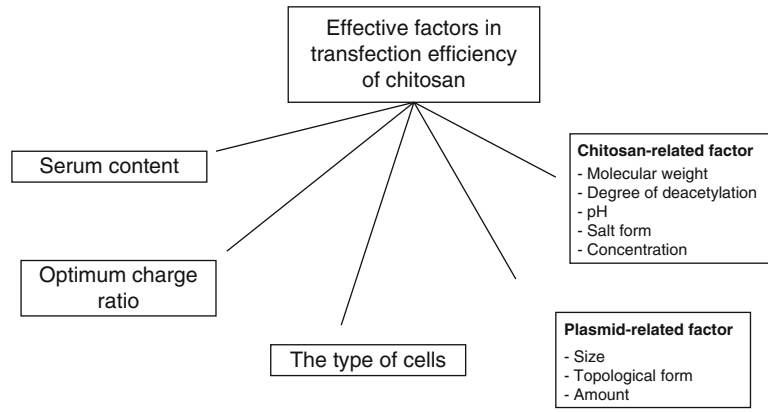

Fig. 22.2 Factors affecting transfection efficiency of chitosan-based gene delivery system

et al. 2004). These polyplexes more easily dissociated and indicated *in vitro* higher transfection than high molecular weight chitosan. *In vitro* transfection requires relatively more stable polyplexes for successful gene transfection than *in vivo* transfection. However, *in vivo* transfection efficiency of chitosan may be dependent on factors other than physical stability and is directly related to the ability of chitosan to protect DNA against the deoxyribonuclease (DNase) enzymatic attack. DNA degradation by DNase is shown to be a major barrier to an efficient *in vivo* transgene expression (in the lung). Therefore, a fine balance must be provided between DNA protection (higher with high molecular weight chitosans) and efficient intracellular unpacking (better with low molecular weight of chitosan) for obtaining high transfection efficiency with chitosans (Koping-Hoggard et al. 2004; Kim et al. 2007).

Akbuga et al. (2004) evaluated interleukin-2 encoding plasmid DNA (pDNA)-loaded chitosan microparticles using low and medium molecular weight chitosan and the effect of molecular weight of chitosan on *in vitro* transfection. The molecular weight of chitosan used and the amount of pDNA influenced the *in vitro* IL-2 production in the cell.

22.2.2 Degree of Deacetylation (DD)

There have been few papers concerning the influence of chitosan properties (other than molecular weight) on gene transfection. In general the degree of chitosan deacetylation greatly affects the characteristics of the chitosan such as charge density (the number of available primary amines for binding), crystallinity, solubility, and degradation rate (Tomihata and Ikada 1997).

Highly deacetylated chitosan (above 80 %) has been generally used to prepare chitosan-DNA complexes (Erbacher et al. 1998; Koping-Hoggard et al. 2001; Mao et al. 2001). In another study Kiang et al. prepared chitosans with various degrees of deacetylation and used in nanoparticle formation. They investigated DNA-binding efficacy, morphology, and gene transfer efficiency of these chitosans (Kiang et al. 2004b). The decreased degree of deacetylation of chitosan (90, 70, and 62 %) resulted in a decrease in luciferase expression levels in human embryonic kidney (HEK 293), human epithelial cervix adenocarcinoma (HeLa), and human squamous cell carcinoma (SW 756) cells. DNA-binding efficacy decreased with decreasing deacetylation degree.

In another study, similar results were reported by Huang et al. (2005). Various degrees of deacetylation (DD 46–88 %) relating chitosan were used in forming chitosan-pEGFP-C2 (Clontech) nanoparticles and cellular uptake and transfection were evaluated in the human lung carcinoma (A549) cell model. Cellular uptake of nanoparticles was significantly reduced by decreasing degree of deacetylation of chitosan. Lavertu et al. (2006) have tried to optimize the effective parameters (mol. wt, DD, and N/P ratio)

related to chitosan. They obtained maximum expression level by using a certain combination of deacetylation degree and molecular weight of chitosans. High expression of DNA could be achieved by simultaneously lowering molecular weight and increasing DD or lowering DD and increasing molecular weight. In other words, the stability of chitosan-DNA systems may be central to describe the effect of parameters (mol. wt, DD, N/P ratio, and transfection medium) on the transgene expression, as complexes that are not sufficiently stable will dissociate early and will show low or no expression. Contrary, the highly stable complexes might not release DNA once inside the cells resulting in low expression. Particle (complex) stability is strongly dependent on electrostatic binding (Lavertu et al. 2006).

22.2.3 Charge Ratio

Electrostatic interaction between chitosan and nucleic acids leads to the spontaneous formation of nanoparticles of different sizes and shapes (Mao et al. 2010). The ability of chitosan nanoparticles to transfect cells efficiently depends on several factors such as the N/P charge ratio represented by the amine group of chitosan-to-phosphate group of DNA or RNA (+/-) ratio used to form nanoparticles. Because only ~90 % of the amino groups in chitosan were protonated at pH 5.5–5.7, an N/P ratio, instead of charge ratio, was used. Large aggregates formed at N/P ratio around 1 but below 0.75 and above 2 yielded submicron size particles. Higher thermal dynamic stability was obtained for nanoparticles prepared with an N/P ratio between 3 and 8 (Mao et al. 2001).

Ishii et al. (2001) noted that the transfection efficiency of the complexes increased at charge ratios of 3 and 5 and decreased at higher charge ratios, due to the relationship between cell uptake of DNA and transfection efficiency.

Similar results were reported by Lavertu et al. (2006). They found that an optimal N/P ratio is needed, because using a low N/P ratio yields physically unstable complexes and poor transfection, while stable complexes prepared at too high N/P ratio can also show decreased transfection as reported previously (Koping-Hoggard et al. 2001, 2003).

22.2.4 pH

The transfection efficiency of chitosan nanoplexes[1] is dependent on the pH of the culture medium because the charge density of chitosan changes with the pH of the buffer. At pH 5.5–5.7, about 90 % of the amino groups of chitosan are protonated (Mao et al. 2001). Binding capacity of chitosan with negative charged DNA increases as pH of medium decreases (Sato et al. 2001). At neutral pH, the degree of protonation decreases; this means that actual charge of the chitosan polyplex[2] is different from the charge of chitosan-DNA complexes of the same charge value made at pH 5.5 (Romoren et al. 2003).

Sato et al. (2001) compared the transfection efficiency of luciferase reporter vector (pGL3)/chitosan complexes in tumor cells between pH 6.9 and 7.6, and the results showed that transfection efficiency at pH 6.9 was higher than that at pH 7.6 because complexes at pH 6.9 are positively charged and easily bind with the negatively charged cells through electrostatic interaction. Similar data were reported by Koping-Hogard et al. (2003), who investigated the influences of pH (2.5–6.5) on the shape of complexes and observed coil-globular-shaped complexes at pH 6.5.

The fraction of soluble globules (globular-shaped complexes) increased as the pH decreased from 6.5 to 3.5, and further acidification decreased the solubility of globules which eventually precipitated at pH 2.5. Different pH values of transfection media, in the range of 6.5–7.4, were tested by Lavertu et al. (2006). Comparable numbers of transfected cells (HEK 293) were found for pH 6.5 and 7.1, while drastically low-

[1] Particle-based systems can be defined on their main groups: involving the combination of DNA with cationic polymers (polyplex), submicron colloidal systems (nanoplex), or lipids (lipoplex).

[2] See Footnote 1.

ered transfection was observed at pH 7.4. They reported that the decrease of medium pH value increases the surface charge of the chitosan-DNA nanoparticles and increases chitosan binding affinity to DNA; thus, complexes are more stable and more efficient in slightly acidic medium. Similar results were reported by different groups (Zhaou et al. 2006; Liu et al. 2005). Chitosan-DNA polyplexes are shown to be very stable under acidic and low ionic strength conditions; however, after changing to physiological conditions, they tend to form aggregates and indicate a decreased binding affinity for DNA with neutral surface charge of polyplexes (Strand et al. 2005).

22.2.5 Presence of Serum

One of the problems for *in vivo* gene delivery mediated by cationic liposomes and lipids is that gene expression is inhibited by serum (Goldman et al. 1997). The development of gene delivery systems that are stable in serum is very important for the improvement of gene therapy by non-viral vector. Sato et al. (2001) investigated the effect of serum on the transfection efficiency of chitosan complexes. The results indicated that the presence of serum enhanced the gene transfer efficiency about 2–3 times than without serum. This effect may be caused by increasing cell function (viability, membrane permeability) in the presence of serum. However, the addition of 50 % fetal bovine serum (FBS) to transfection medium resulted in the decrease of transfection efficiency of chitosan complexes because of cell damage induced by the addition of high amount of serum.

Erbacher et al. (1998) reported that chitosan-DNA complexes more efficiently transfected human epithelial cervix adenocarcinoma (HeLa) cells in the presence of 10 % serum than in its absence.

22.2.6 Plasmid Concentration

The amount of plasmid DNA incorporated within the particles (complexes) plays an important role in the efficiency of transfection process. The transfection efficiency increases with plasmid DNA concentration up to a critical point; thereafter, the transfection keeps constant or decreases significantly (Mao et al. 2010; Romoren et al. 2003).

MacLauglin et al. (1998) used complexes containing 25–400 μg of plasmid, and a plasmid concentration of 100 μg was selected and formed adequate complexes. The results indicated that as the plasmid concentration increased, the diameter of complexes increased. A higher increase in size was observed by increasing DNA concentration and using a higher molecular weight chitosan (102 kDa) than a lower molecular weight chitosan (32 kDa). Thus, formulating complexes of a specific diameter is possible by adjusting plasmid concentration.

Romeron et al. (2003) reported that the concentration of DNA is an important factor for the magnitude of expressed luciferase in the epithelioma papulosum cyprini cells (EPC).

Increasing the DNA concentration from 0.5 to 2.5 μg per well leads to an increase in gene expression (normal dose-response phenomenon). Saturation in the expression levels was observed by a further increase in plasmid concentration to 5 μg per well (Romoren et al. 2003). Similarly, Zhaou et al. (2006) showed that transfection level increased as the plasmid dose increased (0–8 μg/well) in chondrocytes; however, at higher plasmid dose (16 and 32 μg/well), transfection efficiency greatly decreased. This decrease is attributed to the aggregation of chitosan nanoparticles, resulting in lower cellular uptake.

In chitosan-tripolyphosphate nanoparticles, the effect of plasmid DNA loading (5, 10, and 20 %) on the transfection efficiency of nanoparticles was investigated, and 0.5, 1, and 2 μg pDNA doses were used. Based on the results obtained, a dose of 1 μg was chosen for *in vitro* studies (Csaba et al. 2009).

Plasmid encoding interleukin-4 (pIL-4) gene-loaded chitosan-TPP nanoparticles were prepared using 3 different concentrations of plasmid. Encapsulation efficiency, particle size, and transfection efficiency were influenced by the amount of DNA. Nanoparticles formulated with high

amount of DNA showed the highest IL-4 production (Ozdemir et al. 2011).

22.2.7 Chitosan Salt Form

Chitosan is only soluble in acidic solutions, but its salt forms are water soluble and have improved transfection efficiency. Weecharangsan et al. (2008) studied polyplexes formulated with different chitosan salts such as hydrochloride, acetate, lactate, aspartate, and glutamate and found that the transfection efficiency was dependent on the salt form.

22.2.8 Chitosan Concentration

In addition to abovementioned factors, concentration of chitosan was investigated as a parameter influencing the transfection efficiency. Stable and uniform nanoparticles were formed with chitosan concentration in the range from 50 to 400 μg/mL of chitosan. After i.m. injection of pDNA-loaded chitosan microspheres in rats, a clear effect of chitosan concentration on gene expression was not seen (Aral et al. 2000).

Plasmid IL-4 was successfully encapsulated into chitosan-TPP nanoparticles prepared with different chitosan concentrations (0.25, 0.125 %). Size of nanoparticles changed with the concentration of chitosan. Transfection levels of pIL-4 nanoparticles were also affected by the chitosan concentration, i.e., they were higher when lower concentration of chitosan and higher concentration of DNA were used (Ozdemir et al. 2011).

22.2.9 Cell Type

Chitosan-mediated DNA transfection depends on the cell type; therefore, it is needed to test a gene carrier on different cell lines especially cells that resemble those that will be targeted (Leong et al. 1998; Mao et al. 2001; Corsi et al. 2003). Mao et al. (2001) reported preparation of chitosan-DNA nanoparticles and cell dependency of the transfection efficiency of these particles. Higher gene expression levels were found in human embryonic kidney (HEK 293) cells and bronchial epithelia cells (IB-3-1) compared with that in human tracheal epithelial cell line (9HTEo) and HeLa cells (Mao et al. 2001). Cellular membrane composition varies among the cell types and can facilitate or hinder the binding of the particles and internalization.

In another study, Corsi et al. (2003) evaluated the transfection potential of chitosan-DNA nanoparticles using three different cell lines such as human mesenchymal stem cells (MSCs), HEK 293, and human osteosarcoma cells (MG63) and compared them with Lipofectamine® 2000 (commercial cationic lipid) (Life Technologies). The transfection of HEK 293 cells is superior to that seen with MG63 cells and MSCs. Their results suggested that transfection efficiency of chitosan nanoparticles is depended on the cell type.

22.2.10 Plasmid-Related Factors

Although the role of different factors in transfection was investigated largely, the effect of plasmid-related properties on gene transfection was not studied detailed. The group of Akbuga et al. (2003) investigated the effect of different sized (small and large) plasmids on transfection, and similar transfection data were obtained with two different sizes of plasmids after *in vivo* application to rats. They injected chitosan microspheres containing different forms of plasmid (pMK3) into the muscles of the rats and monitored the transfection profile over 12 weeks. Higher protein production was obtained with microspheres containing a mixture of super coiled and open circular forms (60:40), while the linear form induced lower protein production. Transfectivity of relaxed (a topological form of DNA) or super coiled forms of this reporter plasmid was nearly equal (Akbuga et al. 2003). After co-encapsulation of two plasmids into the same microsphere structure, *in vivo* transfection efficiency was investigated. Plasmid DNAs were continuously released from chitosan microspheres

after their i.m injection to rats, and high β-galactosidase and luciferase productions were determined after a long post-transfectional period (Ozbas-Turan et al. 2003).

22.3 Preparation Techniques of Chitosan Nanoparticles

Chitosan nanoparticles are prepared by coacervation and ionic gelation using sodium tripolyphosphate. DNA is encapsulated into the chitosan nanoparticles or is adsorbed onto the surface of nanoparticles. Another preparation method is the direct formation of nanoparticles from DNA and chitosan by complexation (Fig. 22.3).

22.3.1 Coacervation

Chitosan-DNA nanoparticles were prepared by mixing equal volume of chitosan solution (pH 5.5) and sodium sulfate solution containing DNA (Mao et al. 2001).

Preparation of chitosan microspheres by this method was first described by Berthold et al. (1996). Interleukin-2 (IL-2) expression plasmid (pCXWN-hIL-2)-loaded chitosan microspheres were evaluated using a coacervation preparation method that reported previously by Akbuga et al. (2004) and Aral et al. (2000). IL-2 gene encapsulation was found high (82–92 %). High level of IL-2 expression was measured in MAT- LyLu, the rat prostate adenocarcinoma cell line (Akbuga et al. 2004). Kiang et al. (2004b) prepared chitosan-DNA nanoparticles using coacervation method and reported that degree of chitosan deacetylation is an important factor in gene transfection efficiency *in vitro* and *in vivo*.

22.3.2 Ionic Gelation

This method relies on the interaction between positively charged chitosan and negatively charged polyanion. Nanoparticle formation occurs spontaneously due to the molecular linkages between positively and negatively charged agents. First time Bodmeier et al. (1989) reported ionotropic gelation of chitosan with tripolyphosphate (TPP) for drug encapsulation; however, their approach was to design bead rather than nanoparticle. Then Calvo et al. (1997) have developed chitosan particles based on the same principles.

Chitosan particles with different characteristics can be obtained by changing concentrations of chitosan, TPP, and relative volumes of phases.

Particle size of nanoparticles prepared by this method changed between 93 and 336 nm dependent on molecular weight of chitosan. Nanoparticles showed high DNA encapsulation (almost 100 %) independent of molecular weight and a very well-defined spherical shape (Csaba et al. 2009).

22.3.3 Simple Complexation

Nanoplexes were prepared by mixing a solution of chitosan with nucleic acid solutions. Particle sizes change between 150 and 500 nm. This technique is simple and mild. Particle size depends on molecular weight of chitosan used. The zeta potential of nanoparticle is affected by changing pH and deacetylation degree of chitosan (Lavertu et al. 2006; Huang et al. 2004).

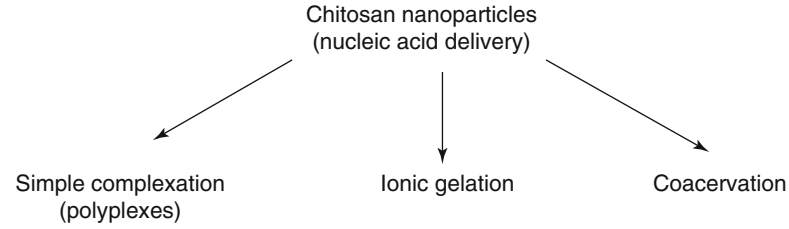

Fig. 22.3 Schematic representation of the preparation method of chitosan-DNA nanoparticles

22.3.4 Nucleic Acid Loading into Nanoparticles

DNA loading in nanoparticles can be done by two techniques, during the preparation of nanoparticles (incorporation) or adsorption after the formation of particles (incubation). In these systems, DNA is physically encapsulated into the matrix or adsorbed onto the surface.

22.4 Modified Chitosans

Despite the several advantages of chitosan as a gene delivery carrier, the application of chitosan/nucleic acid systems is restricted by low transfection efficiency originating from its low solubility and low stability at physiological pH and its slow endosomal release (Mao et al. 2010; Wang et al. 2011). For that reason, several strategies are taken to address these challenges, and hydrophobic and hydrophilic modifications were made to obtain proper chitosan derivatives. Mentioned below modifications of chitosan affecting skin delivery also will be discussed as general important data about other *in vitro* and *in vivo* studies.

22.4.1 Hydrophilic Modification

Positively charged chitosan-DNA complexes can form aggregates following interaction with blood components such as negatively charged serum albumin and other opsonins, resulting in rapid clearance and short circulation time (Dash et al. 1999). In a series of hydrophilic modifications, such as quaternization (Thanou et al. 2002) was applied to enhance transfection efficiency leading to increased water solubility of chitosan at physiological pH, reduced opsonization of the chitosan polyplex, and improved intracellular plasmid release (Mao et al. 2010).

As being hydrophilic, flexible polymer polyethylene glycol (PEG) has long circulating properties, which make it an attractive polymer for modifying the carriers to increase their hydrophilicity and serum half-life (Veronese and Pasut 2005).

22.4.2 Hydrophobic Modification

Hydrophobic modification could enhance cell binding, alleviate serum inhibition, protect from enzymatic degradation, and facilitate intracellular plasmid DNA association, which have been proven to mediate favorable gene transfection (Liu et al. 2010). Hydrophobic modifications of chitosan such as deoxycholic acid (Chae et al. 2005), N-alkylation (Liu et al. 2003), thiolation (Lee et al. 2007), stearic acid (Hu et al. 2006), and uronic acid (Kim et al. 2003) were studied.

N-alkylation is an example of functional group modification of chitosan. The first proposed alkylated chitosan (ACS) was prepared from dodecyl bromide and chitosan (Liu et al. 2001).

Chitosan was hydrophobically modified by deoxycholic acid to yield self-aggregates in aqueous media. These chitosan-deoxycholic acid self-aggregates were used as a delivery carrier for plasmid DNA in mammalian cells (COS-1 cell line). Self-aggregates have a small size with unimodal size distribution (Lee et al. 1998).

Yoo et al. (2005) developed self-assembled nanoparticles using a hydrophobically modified glycol chitosan for gene delivery.

The derivation of the primary amino groups of chitosan with coupling reagents bearing thiol functions leads to the formation of thiolated chitosans. Three types of thiolated chitosans have been developed: chitosan-cysteine conjugates, chitosan-thioglycolic acid conjugates, and chitosan-4-thio-butyl-amidine conjugates. Various properties of chitosan are improved by thiolation: (1) permeation through intestinal mucosa can be enhanced, (2) display *in situ* gelling property, and (3) a prolonged controlled release of embedded molecules (Bernkop-Schnurch et al. 2004). Lee et al. (2007) prepared a highly effective gene delivery system using a 33 kDa thiol-modified chitosan.

22.4.3 Amphiphilic Derivatives

To obtain favorable characteristics for gene delivery, hydrophilic and hydrophobic modifications have been applied to chitosan (CS).

Amphiphilic linoleic acid (LA) and poly (β-malic acid) (PMLA)-double grafted chitosan (LMC)/pDNA nanocomplexes were prepared and characterized. Hydrophobic LA and hydrophilic PMLA substitutions suppressed nonspecific adsorption and enhanced pDNA dissociation. However, enzymatic stability and cellular uptake were promoted by hydrophobic LA grafting. *In vitro* transfection increased 8.0-fold. Higher i.m. gene expression in mice compared to chitosan was reported (Wang et al. 2011).

Transfection activities of N-imidazolys-O-carboxymethyl chitosan/pDNA complexes were studied in HEK 293 cells. High transfection efficiency which is dependent on the degree of imidazoyls substitution was obtained. This result may be due to its high solubility, high DNA-binding capability, and low cytotoxicity (Shi et al. 2012).

22.4.4 Specific Ligand Modification (Active Targeting)

Successful therapy cannot be guarantee if the active molecule does not reach the target site of cell. In spite of the advantages of chitosan as a gene delivery vector, the application and transfection efficiency are limited by low cell specificity, and cellular uptake of nanoparticles mostly occurs via nonspecific adsorptive endocytosis depending on surface properties of chitosan nanoparticles (Mao et al. 2010; Park et al. 2010). Therefore, for improving cellular uptake efficiency and specificity to the target cells, chitosan-based systems are modified by conjugating a cell-specific ligand that specifically recognizes and binds to membrane-bound proteins of target cells. Particularly, cancer cells often overexpress some specific antigens or receptors on their surfaces, which can be used as targets in drug development. The specific ligand-receptor interaction leads to cellular uptake of the chitosan/nucleic acid systems via receptor-mediated endocytosis.

Several targeting molecules and ligands (such as transferrin, folate, galactose, and mannose) have been used for receptor-mediated chitosan modifications.

22.4.5 Other Modification Approaches

One of the important causes of low transfection efficiency of chitosan-based system in the intracellular surrounding is the inefficient release of the nucleic acid of chitosan-DNA complexes from endosomes into the cytoplasm. Therefore, better transfection strategies are needed. For improvement in the endosomal escape capability of the chitosan, imidazole moieties – as a pH-sensitive group – are introduced into the chitosan backbone to mimic the action of PEI (Moreira et al. 2009). For enhancement of endosomal escape, histidine-modified chitosan was reported by Chang et al. (2010). This effect may be due to high buffering capacity of histidine even if introducing very small amount (3–4 %).

Various studies were made on grafting low MW PEI to chitosan in order to enhance the buffering capacity of chitosan, also lowering the cytotoxicity of PEI. PEIs with low molecular weight are nontoxic but have poor transfection efficiency. Branched PEIs with high molecular weight have better DNA transfer ability but high toxicity.

Kiang et al. (2004a) added poly (propyl acrylic acid) (PPAA) in the chitosan-DNA complexes for enhancing the transfection efficiency, because PPAA, a highly pH-sensitive polymer, exhibits maximum membrane disruption capability at below pH 6.0 and results in the disruption of the endosomal membrane to release the vesicle contents into the cytosol. Addition of PPAA to chitosan-DNA complexes enhanced gene expression in both HEK 293 and HeLa cells.

22.4.6 Use of Cell-Penetrating Peptides

The cellular plasma membrane constitutes are effective barrier for many macromolecules

(Bolhassani 2011). Cell-penetrating peptides are short amphipathic and cationic peptides that are rapidly internalized across cell membranes. Some peptide sequences known as protein transduction domains (PTD) or membrane translocalization signals (MTS) were used for the delivery of plasmid DNA (Tung and Weissleder 2003).

With the addition of nuclear localization signal peptide to chitosan-DNA complexes, high gene expression was obtained with the negligible cytotoxicity (Opanasopit et al. 2009).

22.5 Skin Delivery

The skin is an attractive target for the therapeutic and prophylactic gene medicines (containing nucleic acids such as DNA, siRNA, shRNA, and carrier). It is not only the largest human organ but also a good biological barrier to the absorption of drugs and foreign compounds. However, different penetration pathways including hair follicles, sweat ducts, sebaceous glands, and two stratum corneum penetration pathways, the inter cell clusters and the intercorneocyte clusters (being better sealed and more transport resistant), are known, which allow the penetration of exogenous substances into the skin (Cevc and Ulrich 2010). Controllable and reliable molecule delivery across the skin barrier can be provided with stable and deformable nano-sized carriers. The skin has been recently investigated for plasmid DNA delivery as an alternative to parenteral administration of DNA. However, the medical use of gun technology or the needle-free devices are very limited (Cui and Mumper 2001). For achieving local and systemic effects of drugs, the skin is a potential route for drug delivery by nanoparticles. Cui and Mumper (2001) developed chitosan-based nanoparticles (200–300 nm) for topical immunization. They prepared two types of nanoparticles, pDNA-condensed chitosan nanoparticles and pDNA coated on chitosan- carboxymethylcellulose (CMC) nanoparticles, and showed that both chitosan and depolymerized chitosan oligomer can complex CMC to form stable nanoparticles. Plasmid DNA was coated on these pre-formed nanoparticles. Several different chitosan-based nanoparticles containing pDNA were applied topically to the skin of mice. These nanoparticles showed an enhanced luciferase expression in the skin 24 h after topical application. Beside this, for immune response, significant antigen-specific IgG titer was measured as a determinant for expressed β-galactosidase after 28 days of application. Ozbas-Turan and Akbuga (2011) investigated *in vitro* and *in vivo* skin gene transfer of DNA-loaded chitosan/TPP nanoparticles using plasmid that has SV 40 promoter encoding β-galactosidase (pSV-β-galactosidase) (Promega, Madison, WI) as a reporter gene. *In vitro* transfection studies [*Mus musculus* embryo fibroblast cell line (NIH/3 T3) and human dermal fibroblast (HDF)] have revealed that chitosan/TPP nanoparticles are suitable delivery systems for DNA. Lower β-galactosidase level was measured in HDF than in 3 T3 cell lines. In animal studies, usability of nanoparticles was tested and compared in baby and adult rats because of the differences. In baby rats, the hair follicles are virtually in the anagen phase so that the size of the anagen follicles is enlarged and the cells grow in a synchronous manner (Raghavachari and Fahl 2002). Therefore, nanoparticles were tested in both adult and baby rats. Higher gene expression was measured in baby rats than in adult rats. On the other hand, contradictory results were obtained concerning the charge of nanoparticles applied to the skin (Cui and Mumper 2001; Shi et al. 1999). While Cui and Mumper (2001) reported that negative charged nanoparticles achieved higher gene expression, Shi et al. (1999) and Fan et al. (1999) demonstrated the feasibility of cationic particles to be used for topical gene immunization. The results of Ozbas-Turan and Akbuga (2011) were in accordance with the results of Shi et al. (1999). According to histological data of Ozbas-Turan and Akbuga (2011), β-gal expression was mainly localized in the dermis and hypodermis particularly close to the hair follicles. Badea

et al. (2007) used cationic nanoparticles as a topical formulation for the skin delivery of interferon gamma.

Gene guns have been used for the delivery of nucleic acid-coated gold particles through the stratum corneum to the epidermis by helium. Using the gene gun, coated DNA can be inserted into the cytoplasm and nuclei of cells enhancing expression of the encoded protein. However, non-biodegradable gold particles may cause adverse side effects when accumulated (Lin et al. 2006, 2008). Instead of gold particles, biodegradable nanoparticles composed of chitosan and poly-γ-glutamic acid were prepared by an ionic-gelation method for transdermal DNA delivery and used with a low-pressure gene gun. Chitosan-DNA nanoparticles are compared with nanoparticles containing CS/γ-PGA (poly-γ-glutamic acid)/DNA, provided an enhanced penetration depth of DNA into the mice skin and increased gene expression. Lee et al. suggested that this enhancing effect may be due to the fact that chitosan-poly-γ-glutamic acid/DNA nanoparticles were more densely arranged (showing very close packing) than chitosan nanoparticles, thus having a larger mobility for penetration nanoparticles into the skin barrier (Lee et al. 2008).

Chitosan exhibits outstanding properties, such as having a protonable amino group for complexation with nucleic acids, mucoadhesive properties, and permeation-enhancing ability. These remarkable properties of chitosan offered opportunities for its biomedical application (Croisier and Jerome 2013). In burn treatment different types of skin substitutes, i.e., epidermal equivalents, dermal equivalents, and composite equivalents, have been used (Pereira et al. 2007). However, the major limitation of dermal equivalents for treatment of deep burn is slow vascularization, which may result in graft necrosis. To enhance angiogenesis plasmid DNA encoding vascular endothelial growth factor-165 (VEGF-165) was complexed with N,N,N-trimethyl chitosan chloride (TMC) and loaded into bilayer porous collagen-chitosan/silicon membrane dermal equivalents (BDE). These skin substitutes were applied for the treatment of full-thickness burn wounds in the skin. Different BDEs were then transplanted in porcine full-thickness burn wounds. After treatment the TMC/pDNA group had a higher number of newly formed mature blood vessels and faster regeneration of the dermis compared to control groups. After 14 days, a further ultrathin skin grafting was observed on the regenerated dermis, leading to complete regeneration of the skin of the burn wound (Guo et al. 2010, 2011).

Skin is a highly immune-reactive organ containing antigen-presenting cells such as Langerhans cells (LCs), particularly in the epidermis, and it provides a favorable site for DNA vaccines (Lee et al. 2010). Multifunctional core-shell polymeric nanoparticles composed of PLGA-core and glycol chitosan-shell were prepared and applied into the epidermis via a gene gun. Nanoparticles transfected DNA directly into LCs present in the epidermis. Transfected LCs then migrated to lymph nodes and expressed the encoded gene products in the skin draining lymph nodes (Lee et al. 2010). Here, LCs may be activated by antigens in the periphery and move to the lymphoid organs to stimulate an immune response.

Salva et al. (2011) evaluated chitosan/pGM-CSF (plasmid encoding granulocyte macrophage colony-stimulating factor) complexes *in vitro* and suggested that this system may be useful for wound healing.

Conclusions

The skin represents a site for the treatment of cutaneous diseases as well as systemic diseases. Methods for gene delivery via skin have been developed as a therapeutic strategy for the treatment of different skin disorders. Both viral and non-viral methods have been studied. However, due to safety concerns, the use of viral methods is being questioned and non-viral alternatives are gaining major interest. Chitosan-based gene delivery by topical DNA application onto the skin has great potential.

Although important development was achieved in chitosan-based systems for gene delivery, the therapeutic effectiveness still requires to be improved for clinical administration. Standardization of chitosan for

pharmaceutical use, regarding solubility, *in vivo* stability, cell uptake, and unpacking, is an essential problem that must be solved. Also more *in vivo* studies are needed to be carried out in order to overcome the hurdles related to skin delivery of chitosan particles.

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