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Chitosan-DNA/siRNA Nanoparticles for Gene Therapy

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1. Introduction

Human diseases can be treated by the transfer of therapeutic genes (transgene) into specific cells or tissues of patients to correct or supplement defective, causative genes. Gene therapy offers a solution to controlled and specific delivery of genetic materials (DNA and RNA) to targeted cells. The success of gene therapy depends on the ability to deliver these therapeutic materials to targeted site. Viral vectors (e.g. adenovirus) are very effective in term of transfection efficiency, but they have limitations *in vivo*, particularly by their safety concern and non tissue-specific transfection. Non-viral gene transfer systems are limited by their lower gene transfer efficiency, low tissue specificity and transient gene expression.

Chitosan is a polysaccharide usually obtained from deacetylation of chitin, which may be extracted from various sources, particularly from exoskeletons of arthropods such as crustaceans. The goal of this chapter is to introduce the readers to chitosan as a DNA/small interfering RNA (siRNA) delivery vector, as well as different variable strategies to improve cellular transfection and its potential clinical application. the first section is to present of chapter (section 1). The second section presents the discussion about barriers to DNA/siRNA delivery *in vitro* and *in vivo*. It is important to have a clear overview of obstacles to the *in vivo* treatment with DNA/siRNAs. Different *in vivo* administration routes will encounter different physiological barriers, and complications may be furthered by different cells in organs and tissues (section 2). The third section provides the readers with an understanding of the key steps of cellular internalization of DNA/siRNA non-viral vectors. Internalization of non-viral vector-based DNA/siRNA delivery system into cells typically occurs through endocytosis (section 3). the fourth section describes chitosan as a vector for gene therapy (section 4) followed by chitosan structure and physicochemical

behaviour (section 5), general strategies for chitosan modification (section 6), chitosan-DNA delivery system (section 7), chitosan-siRNA delivery system (section 8), and potential applications of chitosan–DNA/siRNA nanoparticles (section 9). Our current research will be summarized in the section of conclusion.

2. Barriers to gene delivery using non-viral vectors

2.1 Viral gene vectors

Gene transfer can occur through 2 delivery systems: viral or non-viral vectors. Viral gene therapy consists of using viral vectors which, given their structure and mechanisms of action, are good candidates or models to carry therapeutic genes efficiently, leading to long-term expression. Viruses are obvious first choices as gene transport. They have the natural ability to enter cells and express their own proteins. Nowadays, most viral vectors used are retroviruses, herpes virus, adenoviruses and lentiviruses. Unfortunately, certain viral vectors (for example, adenoviruses) can elicit a robust cellular immune response against viral and some transgenic proteins, so their use has been limited to studies in immune-compromised animals (Seiler et al., 2007). Adeno-associated viruses (AAV), which have been considered safe, appear to be immunogenic in several experimental settings (Vandenberghe & Wilson, 2007) and in a clinical trial (Mingozzi & High, 2007). Some serious adverse events have occurred with viral gene therapy. One patient died of fatal systemic inflammatory response syndrome after adenoviral gene transfer in 1999 (Raper et al., 2003). Two children developed leukemia-like clonal lymphocyte proliferation after recombinant retroviral gene transfer in 2000 (Hacein-Bey-Abina et al., 2003), and 1 of them died after unsuccessful chemotherapy late in 2004. Attention focused recently on the tragic death of a young female patient in a gene therapy study (intra-articular injection of AAV vectors) of severe RA in 2007 (Kaiser, 2007).

2.2 Non-viral gene vectors

Non-viral gene transfer systems offer several potential advantages over virus vectors. They are non-infectious, relatively non-immunogenic, have low acute toxicity, can accommodate large DNA plasmids or RNA, and may be produced on a large scale (Castanotto & Rossi, 2009; Gary et al., 2007). Non-viral gene therapy has been explored by physical approaches (transfer by gene gun, electroporation, ultrasound-facilitated and hydrodynamic delivery) as well as chemical approaches (cationic lipid-mediated gene delivery and cationic polymer-mediated gene transfer). Numerous chemical non-viral gene transfer systems have been proposed, including naked DNA, cationic liposomes, histones, and polymers (Gao et al., 2007; Ulrich-Vinther, 2007). The main drawback of non-viral vectors as gene carriers is their typically low transfection efficiency (Gao et al., 2007; Giannoudis et al., 2006). Furthermore, the *in vivo* delivery of non-viral liposome/plasmid DNA complex triggers an immune response (Sakurai et al., 2008). Non-viral gene therapy with cationic liposomes has already been tested in clinical trials that dealt with the treatment of inherited genetic disorders (for example, cystic fibrosis) (Hyde et al., 2000) and cancer (Ramesh et al., 2001). Synthetic and natural cationic polymers (positively-charged) have been widely used to carry DNA or siRNA (both negatively-charged) and condense it into small particles, facilitating cellular internalization via endocytosis through charge-charge interactions with anionic sites on cell surfaces. Although existing non-viral vectors have been found to enable DNA expression after *in vivo* delivery, the efficiency and duration of ensuing gene expression have proven to

be unsatisfactory. Research efforts to improve the *in vivo* DNA-delivery efficacy of non-viral vectors are ongoing.

2.3 Barriers to DNA delivery

Systemic gene delivery involves a systemic approach in which exogenous genes are delivered to cells in a certain tissues, and secreted gene products are released into the circulatory system where they could modulate disease processes throughout the body. Systemic non-viral gene delivery has become an attractive alternative to viral vectors because of their safety, versatility and ease of preparation (Li & Huang, 2006). Genes can be delivered systemically (intramuscularly, intravenously, subcutaneously or, in animals, intraperitoneally). Otherwise, hydrodynamic-based gene delivery through systemic DNA injection offers a convenient, efficient and powerful means for high-level gene expression in animals (Liu & Knapp, 2001; Suda & Liu, 2007). This method is expected to be evaluated in patients soon (Romero et al., 2004). The limitations of the systemic approach to gene therapy are essentially the advantages of local delivery: exposure of non-target tissues to the therapeutic agent may have toxic effects or may compromise the immune system of the patient. Certain proteins will likely require very high levels of synthesis to achieve therapeutic function.

Ideally, gene therapy must protect DNA against degradation by nucleases in intercellular matrices so that the availability of macromolecules is not affected. Transgenes should be brought across the plasma membrane and into the nucleus of targeted cells but should have no detrimental effects. Hence, interaction with blood components, vascular endothelial cells and uptake by the reticuloendothelial system must be avoided. For DNA-based gene therapy to succeed, small-sized systems must internalize into cells and pass to the nucleus. Also, flexible tropisms allow applicability to a range of disease targets. Last but not least, such systems should be able to escape endosome-lysosome processing for endocytosis.

2.4 Barriers to siRNA delivery

The discovery of small interfering RNAs (siRNAs) has given renewed vision to the treatment of incurable diseases and genetically-associated disorders. Short double stranded (ds) RNA of 21-23 bp was cleaved by the RNAse III-like protein Dicer and incorporated into RNA-induced silencing complexes (RISC) (Hammond et al., 2000). Chemically-synthesized siRNAs and short hairpin RNA (shRNA) expression plasmids, which are sequence-specific for mRNA targeting, are methods commonly employed to mimic Dicer cleavage (Chen et al., 2007). However, siRNAs are susceptible to nuclease destruction and cannot penetrate the cell membrane because of their highly-charged backbone. An effective delivery system would enclose siRNA in carriers for protection and transport to the cytoplasm of targeted cells but should have no detrimental effects such as specific and non-specific off-targeted effects. Off-target effects can be divided into two categories: specific and non-specific off-targeted effects. Off-targeted effects may cause inflammation including interferon response, cell toxicity, and unintended gene knockdown.

Turning siRNA into drugs is a 3-step process. The design and *in vitro* screening of target siRNAs are followed by incorporating stabilizing chemical modifications in lead siRNAs, as required, and end in the selection as well as *in vivo* evaluation of delivery technologies that are appropriate for the target cell type/organ and disease setting (Vaishnaw et al., 2010). After nearly 10 years of study and development, many problems have been resolved, such as improving the stability of siRNAs, and avoiding 2 types of off-target effects. A recent

anti-influenza study showed that the anti-viral activity of siRNA as found to be due to active siRNA. However, a different non-targeting control siRNA also had significant anti-viral activity (Mook et al., 2007). siRNA targeting vascular endothelial growth factor for patients with age-related macular degeneration (AMD) are currently in clinical trial. But further study showed that the inhibition is a siRNA classic effect, which is sequence- and target-independent (Jackson & Linsley, 2010). The off-target effect can be minimized by optimizing the rules and algorithms for siRNA design (Vaishnaw et al., 2010). However, several other factors limit the utility of siRNAs as therapeutic agents, such as competition with endogenous RNA, induction of immune responses, degradation in lysosomes after endocytosis (Dominska & Dykxhoorn, 2010; Wang et al., 2010). Unprotected, naked siRNAs are relatively unstable in blood and serum and have short half-lives *in vivo* (Gao et al., 2009). Naked siRNAs do not freely cross cellular membranes because of their large molecular weight (~13 kDa) and strong anionic charge. They are rapidly degraded by nuclease. Physiological barriers hinder siRNAs from reaching their targets, thereby reducing their therapeutic efficacy. Moreover, siRNA molecules have unfavorable physicochemical properties (negative charge, large molecular weight and instability). Therefore, they need delivery systems to overcome physiological obstacles and prolong vascular circulation by reducing renal clearance, protecting them from serum nucleases, improving their effective bio-distribution as well as targeted cellular uptake with endosomal escape and, finally, promoting trafficking to the cytoplasm and loading onto RISC. Therefore, delivery systems are required to facilitate siRNA access to intracellular sites of action.Barriers to siRNA delivery depend on the targeted organ and routes of administration. For example, intravenous (IV) administration is the most commonly used technique. The endothelial wall in the vasculature presents the primary delivery barrier to siRNAs. The endothelial barrier is often altered by inflammatory processes (e.g., RA, infection) (Moghimi et al., 2005). siRNAs leave a blood vessel to enter tissue. After reaching target cells, they undergo internalization via endocytosis, escape from endosomes, and release into the cytosol and, finally, load onto RISC. At the same time, siRNAs undergo elimination. The mononuclear phagocyte system is responsible for removing circulating foreign particles from the bloodstream by the phagocytosis of resident macrophages (Moghimi et al., 2005).

3. Cellular internalization of non-viral vector delivery system

There are seven steps should be overcome before the expression of exogenous DNA. They are (1) complexation, (2) *in vivo* administration, (3) endocytosis, (4) escape from endolysosome, (5) release of DNA, (6) trafficking through cytoplasm and (7) finally import of DNA into nucleus. (If siRNA is used as exogenous nucleotide, the last two steps can be ignored; but if vector-expressed siRNA is used, the process remains the same.) During each step, many factors may come into play, inducing toxicity, immunogenicity or affecting transfection efficiency. (1) During complexation, the non-viral vectors-DNA interaction is driven mainly by the electrostatic interaction between the polycation and the charged phosphate groups leading to reversible linear to globule transition of DNA. The ability of the non-viral vectors to condense DNA into nanoparticles is often critical for transfection efficiency since DNA must be protected from DNase degradation. (2) Different *in vivo* administration routes will meet different physiological barriers. Therefore, it is suggested that the corresponding primary cells and similar physiological barriers should be tested *in vitro* as far as possible, before *in vivo* administration is attempted. (3) The following step is to

reach its target, the cell by endocytosis. In this respect it is well accepted that the polyelectrolyte complex polycation-DNA exhibiting a net positive charge binds to negatively charged cell membrane. (4) After the internalization the following crucial step in gene delivery with cationic polymers is the escape of the polymer/DNA complexes from the endosome. (5) The inefficient release of the DNA/polymer complex from endocytic vesicles into the cytoplasm is indicated as one of the primary causes of poor gene delivery. (6) and (7) the following step, the nuclear envelope is the ultimate obstacle to the nuclear entry of plasmid DNA. This obstacle is also considered crucial and two main mechanisms were proposed to explain how plasmid DNA enters into the nucleus: (i) a passive DNA entry into the nucleus during cell division when the nuclear membrane is temporarily disintegrated or (ii) an active transport of the DNA through the nuclear pores.

4. Chitosan as a vector for gene therapy

Cationic polymers, such as chitosan, are promising candidates for DNA transport in non-viral delivery systems (Kean & Thanou, 2010; Tong et al., 2009). Chitosan, a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit), and has once been considered as an attractive gene transfer candidate for its superior biocompatibility, superior biodegradability and low cell toxicity. In recent years, with more researching methods involved, a more accurate and subtle view on the process of the entry of chitosan/DNA complexes into the cell nucleus has been developed. The enabling characteristics of Chitosan-DNA nanoparticles include biocompatibility, multiple ligand affinity, and a capacity of taking up large DNA fragments, while remaining small in size (Techaarpornkul et al., 2010). Chitosan and its derivatives, as favorable non-viral vectors involved in plasmid DNA delivery, have attracted attention in the field of siRNA delivery *in vitro* and *in* vivo (Andersen et al., 2009; Howard et al., 2009). Chitosan was once believed to be less effective than most other non-viral vectors because of its low stability and buffering capacity. However, recent technological advances in the chemical modification of chitosan have instituted improvements of its transfection efficiency without disturbing its biocompatibility and biodegradability. It has been demonstrated that transfection level is closely related to the molecular weight of polymers (Godbey et al., 1999). Chitosan (10-150 kDa), with a specific degree of deacetylation, allows maximum transgenic expression *in vitro* (Lavertu et al., 2006). Another strategy for improving transfection is to take advantage of the mechanism of ligand-mediated uptake by cells to promote targeting and internalization, enhancing transfection efficiency. Ligand-mediated transfection has been shown to facilitate DNA internalization into cells via membrane receptors both *in vitro* and *in vivo.* Cell-specific ligand modification such as galactose, transferrin, folate and mannose can also effectively enhance the specificity of transfection through receptor-mediated endocytosis. Galactose ligand modification has been used to target HepG2 cells through the interaction with asialoglycoprotein receptors (ASGP-R) (Gao et al., 2003). A transferrin receptor is found on many mammalian cells, therefore it can be used as a universal ligand (Dautry-Varsat, 1986). Folate is not only over-expressed on macrophage surfaces, but is also over-expressed on many human cancer cell surfaces (Lee et al., 2006). Antigen presenting cells (APCs), the ideal targets of DNA vaccine, such as macrophages and immature dendritic cells are the target cells of mannose ligand (Kim et al., 2006). The specificity of these modifications can be demonstrated through ligand competitive inhibition experiments.

The low stability, low buffering capacity and low cell-specificity have also hindered its clinical applications. However, as a nature resource-based polysaccharide, chitosan has more functional groups that can be chemically modified than other cationic polymers, thus has many more potential chemical derivatives to overcome the deficiencies. Chitosan has been experimentally modified using hydrophilic, hydrophobic, pH-sensitive, thermosensitive and cell-specific ligand groups for enhancement of transfection efficiency (Ishii et al., 2001). The degree of deacetylation (DDA) and the molecular weight (MW) of chitosan or its derivatives, can affect the ultimate transfection efficiency. Most chitosan/DNA complexes are highly deacetylated (above 80%), because chitosan with a high degree of deacetylation exhibits an increased DNA binding efficacy (Kiang et al., 2004). Through chain entanglement, chitosan with a higher MW (longer chain length) can become more readily enmeshed with free DNA, once the initial electrostatic interaction has occurred. But it will also delay the disassociation of chitosan and DNA (Huang et al., 2005). Consequently, low MW chitosan requires a higher charge ratio to stably condense DNA for the same DDA, and a lower DDA requires a higher charge ratio to stably condense DNA at equal MW (Lavertu et al., 2006). The charge ratio for minimum complexation can be determined by agarose gel electrophoresis.

5. Chitosan structure and physicochemical behaviour

The structure of chitin and chitosan correspond to those of poly [β(1→4)-2-acetamido-2-deoxy-d-glucopyranose] and poly[β(1→4)-2-amino-2-deoxy-d-glucopyranose], respectively (Figure 1). Chitosan is mainly manufactured from crustaceans (crab, krill and crayfish) primarily because a large amount of the crustacean exoskeleton is available as a byproduct of food processing. However, depending on the organism considered chitin can adopt polymorphic structures denominated alpha (α), beta (β) and gamma (γ) chitin (Jang et al., 2004).The polymorphism of chitin is due to different arrangements of chitin chains in the lamellas that constitute the crystalline portions. Alpha (α) chitin found in arthropods corresponds to an antiparallel chain packing at which intramolecular and intermolecular hydrogen bonding is favored. β-Chitin, typically extracted from squid pens, is less widely used although it can have higher reactivity than that of α-chitin. The parallel arrangement of the lamellas is responsible for a loose-packing fashion with weak intermolecular interactions. In the gamma (γ) chitin structure arrangements, beta and alpha occur, i.e., two lamellas in a parallel arrangement is intercalated by a lamella arranged in antiparallel packing (Roberts, 1992). The source from which chitosan is prepared is considered very important since chitosan derived from β-chitin exhibits higher reactivity than that derived from α-chitin (Kuritia et al., 1994; Shimojohay 1998).

In general the isolation of chitin from crustacean shell waste consists of three basic steps: demineralization (DM-calcium carbonate and calcium phosphate separation), deproteinization (DP-protein separation), and decolorization (DC-removal of pigments). These three steps are the standard procedure for chitin production (No et al., 1989). Chitosan is obtained after hydrolysis of the acetamide groups of chitin. However in the commercialized samples both units are commonly found, since chitosans having high deacetylation degrees (DA> 99%) are obtained only through of successive hydrolysis with strong bases as KOH and NaOH, and the degree of deacetylation is strongly dependent of the alkali concentration and temperature (Figure.1). The source of chitin and the deacetylation process can change dramatically the properties of the final product and the

deacetylation in alkaline medium leads to the depolimerization (Domard & Rinaudo, 1983; Tolaimate et al., 2000). However it has been reported that chitin extracted from squid pens can be hydrolyzed under conditions that it allows obtaining chitosans of high molecular weight (Tolaimate et al., 2003).

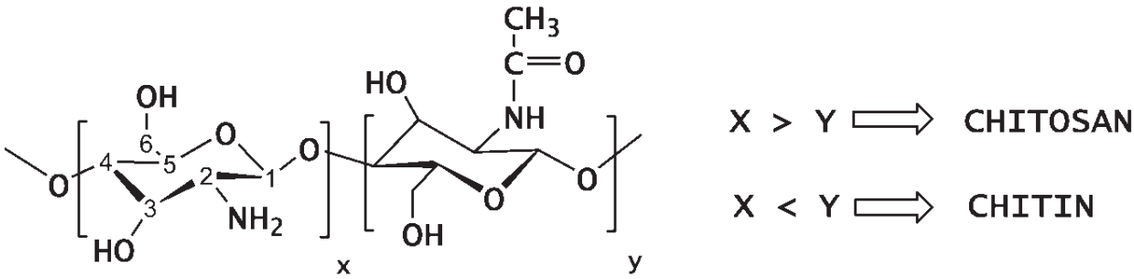


Fig. 1. Chemical structure of chitin and chitosan. In the 2-amino-2-deoxy-d-glucopyranose ring is shown the commonly used numbering for the carbon atoms.

The homopolymer is a weak base with a pKa value of the D-glucosamine residue of about 6.3 and is therefore insoluble at neutral and alkaline pH values. In acidic mediums, the amine groups will be positively charged, conferring to the polysaccharide a high charge density. As in all polyelectrolytes, the dissociation constant of chitosan is not constant, but depends on the degree of dissociation at which it is determined. The pka value can be calculated using the Katchalsky's equation (Roberts, 1992).

$$Pk_a = pH + \log [(1-\alpha)/\alpha] = pK_0 - \varepsilon\Delta\psi(\alpha)/kT$$

Where $\Delta\psi$ is the difference in electrostatic potential between the surface of the polyion and the reference, α is the degree of dissociation, k is Boltzman's constant, T is the temperature and ε is the electron charge. Extrapolation of the pK_a values to $\alpha = 1$, where the polymer is uncharged and hence the electrostatic potential becomes zero, makes possible the value of the intrinsic dissociation constant of the ionizable groups, pK_0, to be determined. The value obtained does not depend of the degree of *N*-acetylation, whereas the pKa value is dependent on this parameter, since the electrostatic potential will be varied depending of amount of the free amino groups. The pK_0 value is called the intrinsic pK_a of the chitosan. However chitosans of low molecular weight having degrees of deacetylation higher than 0.4 are also easily soluble in weakly acidic solvents such as acetic acid and formic acid (Lee et al., 1995).

The physicochemical behavior in aqueous solution is highly dependent of pH and degree of acetylation and has received more attention only recently. Bertha et al. working on chitosans from 95 to 175kDa have recently determined the radius of gyration of chitosan (R_G) (Bertha et al., 1998a; 2002b). The R_G is an alternative measure of the size of the polymer chain and it can be measured by light scattering measurements. R_G express the square mean radius of each one of the elements of the chain measured from its center of gravity. The study established the relationship between the molecular weight and radius of gyration (R_G) of chitosan in aqueous solution, and the author indicated that chitosan behaved more like a Gaussian coil instead of the worm-like chain model found in common polyelectrolytes. At the same time the presence of *N*- acetyl groups on the chitosan backbone imparts hydrophobic properties. Schatz et al. (Schatz et al., 2003) have studied homogeneous series of chitosans with different degrees of acetylation and almost the same degree of

polymerization in ammonium acetate buffer. Their results indicate that the aqueous solution behavior depends only on the degree of acetylation (DA). Three distinct domains of DA were defined and correlated to the different behaviors of chitosans: (i) a polyeletrolyte domain for DA below 20%; (ii) a transition domain between DA = 20% and 50% where chitosan loses its hydrophilicity; (iii) a hydrophobic domain for DAs over 50% where polymer associations can arise. Conformations of chitosan chains varying from 160 to 270kDa were studied by the calculations of the persistence lengths (L(p)). The average value was found to be close to 5 nm, in agreement with the wormlike chain model, but no significant variation of L(p) with the degree of acetylation was noticed. Pa et al. (Pa & Yu, 2001) have also reported that that the particle sizes of chitosan molecules in dilute acetic acid/water solutions increased with decreasing pH value. SLS data also demonstrated that the second virial coefficient (A_2) increased with decreasing pH value, suggesting that solubility of chitosan in water increased with increasing acetic acid concentration. Signini et al. (Signini et al., 2000) have also shown that acid-free aqueous solutions of chitosan hydrochloride of variable ionic strengths (0:06 M ≤ μ≤ 0.3 M) are free of aggregation as evaluated by the values of the Huggins constants ($0.31 \leq k \leq 0.63$).

As other polysaccharides the biodegradation and biocompatibility are important properties of chitosan making it an attractive polymer for a variety of biomedical and pharmaceutical applications. Besides the degradation by chitinases (Hung et al., 2002), chitosanases (Kuroiwa et al., 2003), papain (Kumar et al., 2004; Lin et al., 2002; Muzzarelli et al., 2002; Terbojevich et al., 1996) and other proteases (Kumar et al., 2004), partially acetylated chitosan may be also degraded by lyzosymes of the human serum (Varurn et al., 1997), by oxidative-reductive depolymerization (Mao et al., 2004) and by acid hydrolysis reactions (Lee et al., 1999). In the acid hydrolysis the protonation of the glycosidic oxygen is recognized as the first step of the mechanism, which leads to formation of a cyclic carbonium –oxonium ion, yielding after the addition of water the reducing sugar end group (Sinnott et al., 1990; Yip & Withers, 2004). Besides enzymatic and acid hydrolysis the alkaline treatment with ultrasonication can be used to obtain either chitosan of decreasing molecular weight (Tang et al., 2003) or oligomers having a few glucosamine units (Tsaih et al., 2003).

6. General strategies for chitosan modification

In the chitosan structure two groups are particularly susceptible to react through nucleophilics attacks, i.e., the free amine and/or acetamide groups, and the hydroxyl groups linked to the glucopyranose ring. The hydroxyl groups can be modified by substitution of the hydrogen atoms but their reactivities are smaller than that of the amino group. Various procedures targeting the hydroxyl groups employ a sequence of protection/deprotection reactions aiming to obtain derivatives with a well defined structure (Kuitra, 2001). On the other hand under appropriated conditions a variety of other reactions can be easily conducted to selectively modify the free amine groups. The literature presents a wide range of procedures to target the amine group aiming to improve the properties of chitosan for a particular purpose. The modifications include those aiming the separation technologies of chiral molecules (Franco et al., 2001), recovery of metals (Guibal, 2004; Varma et al., 2004), antimicrobial activity (Rabea et al., 2003), anti tumoral carriers (Kato et al., 2004), biomedical applications (Berge et al., 2004a; 2004b) and vectors for gene therapy (Janes et al., 2001; Sinha et al., 2004; Liu et al., 2002; Borchard et al., 2001). Kumar et al (Kumar et al., 2004) and Kurita (Kurta, 2001) reviewed the procedures for the modification of chitosan.

Many strategies have been deployed to improve transfection efficiency, taking into account the biological steps involved in gene delivery. Modifications of chitosan structure to impart properties to NPs, such as to increase endosomal escape (Jiang et al., 2010; Yu et al., 2010), attaching of ligands to mediate cell internalization or to promote the nuclear entry of DNA, are among the most common ways. Figure 2 shows representative structures from these chitosan derivatives tested as carriers for gene therapy. A variety of nucleophilic reactions targeting the groups linked to the glucopyranose ring have been employed to improve the properties of chitosan.

Poly (ethylene glycol) (PEG) has been widely used for attaching to chitosan due to its hydrophilicity and biocompatibility. In general, the terminal hydroxyl group of methoxy poly(ethylene glycol) is modified to generate PEG derivatives able to promote nucleophilic displacements targeting the amino groups of chitosan (Harris et al., 1984; Aiba et al, 1993; Saito et al., 1997; Ouchi et al., 1998). Chitosan nanospheres modified by introducing PEG_{5000} chains to amine groups were more stable during lyophilization (Leong et al., 1998). These chitosan-DNA nanospheres were effective in tranfecting 293 cells but not HeLa cells, and tranfection efficiency was not affected by PEG derivatization.

Polymers can also be attached to the chitosan main chain using different routes. Poly(vinyl pyrrolidone) (PVP) was also grafted on galactosylated chitosan (GCPVP) and displayed improved physicochemical properties over unmodified chitosan (Park et al., 2003). PVP with a single terminus carboxylic group was coupled to galactosylated chitosan via formation of an amide bond between the amino *complex* group of GC and the terminal carboxyl group of the PVP. The terminal carboxyl group of PVP was activated by the *N*-hydroxysuccinimide (NHS)/EDC. The binding strength of GCPVP 10k/DNA was superior to that of GCPVP 50k/DNA, which was attributed to its higher flexibility because of its smaller size. However, DNase I protection of GCPVP 10k/DNA complex was inferior to that of GCPVP 50k/DNA. The DNA-binding property was shown to be dependent on the MW of chitosan and the composition of PVP (Park et al., 2003). The reaction of chitosan with methoxy poly(ethylene glycol) iodide (mPEG, Mn 2 kDa) in an alkalinized suspension was recently used by Yu *et al.* to attach PEG (Yu et al., 2010). This derivative was subsequently modified by attaching poly(ethylenimine) to the amino groups (Figure 2). Other approaches successfully employed to attach PEI to chitosan were an imine reaction between periodate-oxidized chitosan and amine groups of low MW PEI (Jiang et al., 2007) and the cationic polymerization of aziridine in the presence of water-soluble oligo-chitosan (Wong et al., 2006).

A series of new degradable cationic polymers composed of biocompatible chitosan backbones and poly((2-dimethyl amino) ethylmethacrylate) (P(DMAEMA)) side chains were recently synthesized via atom transfer radical polymerization (ATR) (Ping et al., 2010). This synthesis was carried out by introducing alkyl halide initiators onto chitosan, followed by the reaction with DMAEMA. Bromoisobutyryl-terminated chitosan (CS-Br initiators) was prepared via the reaction of primary amines of chitosan with carboxyl group of 2-bromo-2-methylpropionic acid (BMPA), which was previously converted into reactive esters (succinimidyl intermediates) in the presence of EDAC and NHS. The reactive esters underwent nucleophilic substitution reactions with the amine groups of chitosan to form a stable amide linkage and produce the resultant CS-Br initiators for DMAE polymerization (Ping et al., 2010).

The activation of carboxylic groups is one of the most commonly used procedures to attach different ligands and peptides to chitosan chain. Arginine-modified trimethylated chitosans

labeled with folic acid have been prepared by activation of the acid group of arginine using EDC/NHS (Morris et al., 2010). The same procedure was utilized by Gao et al and it has proven to increase the transfection efficiency (Gao et al., 2008) and chitosan properties (Liu et al., 2004). A similar procedure was utilized to attach a short peptide (SP) (Sun et al., 2010) to chitosan. The peptide was further combined with GFP/luciferase reporter gene pDNA to form SP-CS/DNA complex. The NPs were able to transfect multiple cell lines, and the results revealed that, compared with CS, SP-CS could intensively augment transfection efficiency nearly to the level of Lipofectamine 2000 (Sun et al., 2010). Reactions targeting the hydroxyl groups are uncommon, however Sato et al. have prepared 6-Amino-6-deoxychitosan from 6-deoxy-6-halo-N-phthaloylchitosan via 6-azidation. The product had high stereoregularity because of the effective and regioselective reactions (Saito et al., 2004; Satoh et al., 2007).

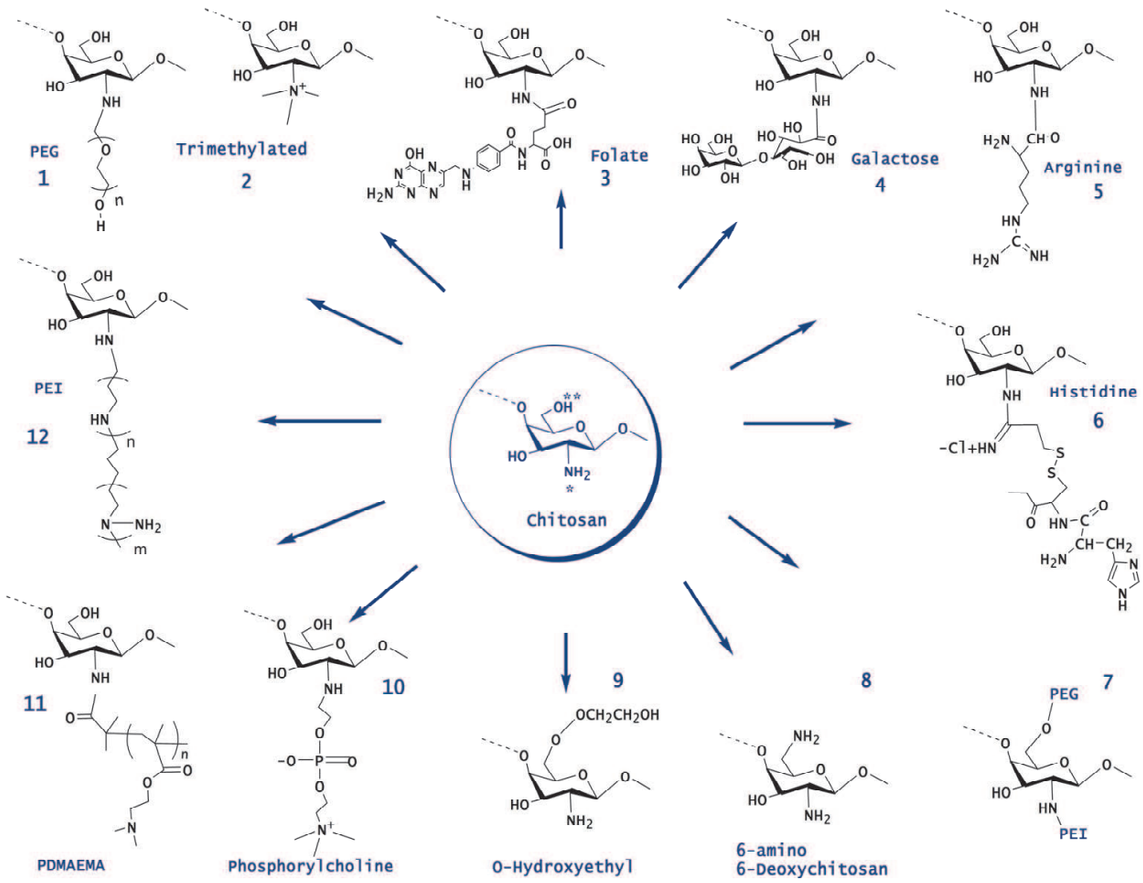


Fig. 2. Chemical structures of chitosan and its derivatives; **1**. PEG (Harris et al., 1984); **2**. trimethylated (Zeng et al., 2007); **3**. folic acid (Mansouri et al., 2006; Fernandes et al., 2008); **4**.galactosylated (Park et al., 2001); **5**. Arginine (Morris et al., 2010); **6**. histidine; **7**. PEI and PEG grafts (Yu et al., 2010); **8.** 6-amino 6-deoxychitosan (Saito et al., 2004; Satoh et al., 2007); **9**. O-hydroethyl (Kwon et al., 2003); **10**. Phosphorylcholine (Case et al., 2009; Tiera et al., 2006); **11**. grafted PDMAEMA (Ping et al., 2010); **12**. PEI (Jiang et al., 2007; Wong et al., 2006).

7. Chitosan-DNA delivery system

Chitosan-DNA gene delivery methods must achieve sufficient efficiency in the transportation of therapeutic genes across various extracellular and intracellular barriers. These barriers include interactions with blood components, vascular endothelial cells and uptake by the reticuloendothelial system. Furthermore, the degradation of therapeutic DNA by serum nucleases is a potential obstacle for functional delivery to target cells. DNA should escape from endosomes and traffic to enter the nucleus. Many factors, including the degree of deacetylation (DDA) and the molecular weight (MW) of the chitosan, the pH of the serum, the charge ratio (in some conditions, it equals the ratio of N/P, 'N': the content of Nitrogen atom in cationic polymer; 'P': the content of Phosohorus atom in DNA or RNA) of chitosan to DNA or RNA and the cell type can all affect the transfection efficiency of chitosan during each step of the process. The pKa value of chitosan is around 6.3-6.4, below which the protonated amines in the chitosan structure facilitate their binding to negatively charged DNA. Sato et al. showed the highest transfection efficiency can be obtained at pH 6.8 to 7.0. When pH of the transfection medium increases to 7.4, the transfection efficiency dramatically decreases due to the dissociation of the free plasmid from the complex (Sato et al., 2001).

Even if chitosan/DNA complexes display high transfection efficiency *in vitro*, their transfection efficiency *in vivo* may be low. Chitosan and its derivatives have become of great interest in the field of controlled release due to their favorable biocompatibility and biodegradability. Thiolated chitosan, which can be oxidized to form inter- and intra-molecular disulfide bonds, allowing the crosslinking of chitosan, shows a significant enhancement of transfection over that of lipofectin (Lee et al., 2007). Chitosan microspheres for micro-encapsulation of adenoviral vectors has been achieved by ionotropic coacervation of chitosan, using bile salts as counter-anions (Lameiro et al., 2006). A 3-D scaffold composed of chitosan-gelatin complexes with entrapped DNA has been proposed as a promoter of cartilage regeneration (Xia et al., 2004).

Although hydrophobic modification is not cell-specific, it can also enhance the attachment of complexes on cell surfaces and the subsequent cell uptake. Amphiphobic deoxycholic acid-modified chitosan oligosaccharide (DACO) nanoparticles showed superior gene condensation and high gene transfection efficiency, even in the presence of serum (Chae et al., 2005). After endocytosis, the endosome containing the complexes has to fuse with a lysosome to form an endolysosome. At this point, the complexes will meet a harsh acidic and multienzymatic environment. Nanocomplexes that are successfully protected against dissociation and degradation will finally escape from the endolysosome and enter the cytoplasm. PEI, a classic synthetic polymer with many amino groups to absorb protons (called a proton sponge mechanism), was found to have a better endolysosome buffering ability and caused a quicker release from the endolysosome in its intact form than did chitosan (Kim et al., 2005). The chemical modifications, such as urocanic acid (UA) (Kim et al., 2003), PEI-graft-chitosan (Wong et al., 2006), chitosan-graft-PEI (Jiang et al., 2007), poly(propyl acrylic acid) (PPAA) (Jones et al., 2003), trimethyl chitosan (Germershaus et al., 2008), have similar effects to PEI. Such modifications can be called pH-sensitive modifications that will not only enhance the escape of chitosan/DNA complexes from endolysosome but also enhance the stability of complexes in different pH situations.

The dissociation of chitosan/DNA complexes and subsequent release of DNA is also a very important step for its rate-limiting effect (Schaffer et al., 2000). Hydrophobic modification,

such as deoxycholic acid modification (Lee et al., 1998), or 5β-cholanic acid modification (Yoo et al., 2005), can attenuate the electrostatic attractions between cationic polymers and anionic DNA. It is actually a contradiction between the stability and dissociation ability of complexes. A temperature-sensitive modification of poly(N-isopropylacrylamide) (PNIPAAm) can control the dissociation of PNVLCS (N-isopropylacrylamide/vinyl laurate copolymer with chitosan) complexes with DNA by a temporary reduction in the culture temperature to 20℃ (Sun et al., 2005)

The cytoplasm, a mesh-like network of microfilaments and microtubules, will limit the diffusion of complexes or DNA about 500-1000-fold. Adenovirus particles naturally bind to dynein and are actively transported towards the nuclear pore complexes once they are inside the cytoplasm. Prior to entry into the nucleus, the viruses dissociate into smaller structures and use their attached transport factors such as importins or karyopherins which have nuclear localization signals (NLS) to recognize the nuclear pore complex (NPC) (Whittaker & Helenius, 1998). Justin Hanes et al. used a new method called multiple particles tracking (MPT) to quantify the intracellular transport of non-viral DNA nanocarriers. They found that PEI/DNA complexes can accumulate in the perinuclear area through a subdiffusive transport, which is a combination of diffusive transport and active transport. This discovery is a dispute to the common belief that non-viral vectors go through the cell cytoplasm in a slow random way. Further investigation showed that actively transported complexes of PEI/DNA are in endosomes undergoing motor protein-driven movement guided by microtubules or physically associated with the motor proteins themselves (Suh et al., 2003). As to chitosan and its derivatives, however, few studies have examined how they pass through the highly structured cytoplasm and eventually enter into the nucleus.

8. Chitosan-siRNA delivery system

siRNA silencing technology is exploited in a wide range of biological studies, but has also become one of the most challenging therapeutic strategies. However, because of its poor delivery and susceptibility to nuclease degradation, siRNA-based approaches need a protective delivery system. A variety of polymer formulations have been proposed in the literature as potential carriers (De Fougerolles 2008; Gary et al., 2007; Zhang et al., 2007). Polymer molecular weight, change density, N/P ratio (ratio of protonatable polymer amine groups to nucleic acid phosphate groups) and ionic strength of the medium can affect electrostatic binding between siRNA and cationic polymers. Research over the years has revealed that chitosan is one of the desirable polymeric carriers of siRNA because of its natural biocompatibility, biodegradability, nontoxicity, and high nuclease resistance. The effects of different chitosan (114-kDa or more)-siRNA complexes on transfection activity have been observed previously (Katas et al., 2008; Katas & Alpar, 2006; Liu et al., 2007; Rojanarata et al., 2008). Higher MW and DDA are desirable characteristics for the formation of chitosan nanoparticles, as higher MW chitosan molecules are long and flexible while higher DDA enhances its electrostatic interaction with siRNAs, thus synergically reducing the size of complexes and increasing their stability (Liu et al., 2007). A high charge ratio also enhances the stability of complexes because the loosely bound excess chitosan on the outer surface of nanoparticles can promote binding and uptake across anionic cell surfaces and also provide subsequent protection against siRNA degradation within endosome compartments (Liu et al., 2007). The method of complexation also affects the gene-silencing

activity of chitosan/siRNA complexes. Haliza Katas et al. studied the difference between simple complexation, ionic gelation (siRNA entrapment) and adsorption of siRNA onto the surface of preformed chitosan nanoparticles. Ionic gelation gave the strongest stability and the most efficient gene-silencing activity among the three methods tested. For the involvement of tripolyphosphate (TPP) ions during the complexation of ionic gelation, pH became one of the factors that mostly affected the gene-silencing activity. The decrease of pH resulted in a reduction in the charge number of TPP, which subsequently led to the need for more TPP ions for cross-linking of the chitosan by electrostatic forces (Katas & Alpar, 2006). Rojanarata et al. reported that chitosan-thiamine pyrophosphate (TPP)-mediated siRNA enhanced green fluorescent protein (EGFP) gene silencing efficiency depended on the molecular weight and weight ratio of chitosan and siRNA. The chitosan-TPP-siRNA complex with the lowest molecular weight of chitosan (20 kDa) at a weight ratio of 80 showed the strongest inhibition of gene expression (Rojanarata et al., 2008). A novel study of chitosan/siRNA nanoparticles with fluorescent quantum dots was taken to silence HER2/neu and achieved desirable silencing effects (Tahara et al., 2008; Tan et al., 2007). In the field of controlled release, chitosan coating PLGA nanospheres with a high loading efficiency of siRNAs were found to reduce the initial burst of nucleic acid release and to prolong release at later stages, without changing the release pattern (Tahara et al., 2008). Kenneth A. Howard found that the chitosan-based system had the ability for endosome escape through the proton sponge mechanism, because the endosomolytic agent chloroquine did not increase the effect of RNA interference (Howard et al., 2006). In terms of *in vivo* administration of chitosan/siRNAs complexes, only a few studies are available. Nasal administration to silence EGFP expression of the endothelial cells distributed in the bronchioles of transgenic EGFP mouse model has been successfully achieved without showing any adverse effects (Howard et al., 2006). Cross-linking of hyaluronan and chitosan has proven to have a higher efficiency of transfection in ocular tissue over unmodified chitosan (de la Fuente et al., 2008).

9. Potential application of chitosan-DNA/siRNA nanoparticles

Gene therapy offers new possibilities for the clinical management of different disease conditions that are difficult to treat by traditional surgical or medical means. In the last decade, extensive improvements have been made to optimize gene therapy and have been tested on several disease conditions. The success of chitosan-DNA nanoparticles for delivery plasmid DNA to mucosal surfaces such as the oral and nasal mucosa has already shown (Bivas-Benita et al., 2003; Chen et al., 2004; Khatri et al., 2008). Oral delivery is most attractive due to easy administration. The oral delivery of peptide, protein, vaccine and nucleic acid-based biotechnology products is the greatest challenge facing the drug delivery industry. Mice were fed with plasmid pCMVβ (containing LacZ gene), whether it was wrapped by chitosan or no. The study demonstrated that oral chitosan-DNA nanoparticles can efficiently deliver genes to enterocytes, and may be used as a useful tool for gene transfer (Chen et al., 2004). Hepatitis B virus infection is a major global health concern and is the most common cause of chronic liver disease, new generation of HBV vaccines are urgently needed in order to overcome problems encountered with the immunization of immunocompromised people and more importantly with the potential of using active immunotherapy in treating chronic patients. DNA vaccines have the potential to eliminate many of the limitations of current vaccine technologies. Chitosan nanoparticles loaded with

plasmid DNA encoding surface protein of Hepatitis B virus. Nasal administration of such 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 (Khatri et al., 2008). Particulate mucosal delivery systems that encapsulate protein or plasmid DNA encoding antigens have been widely explored for their ability to induce an immune response. Oral delivery of vaccines using chitosan as a carrier material appears to be beneficial for inducing an immune response against *Toxoplasma gondii*. Chitosan microparticles as carriers for GRA-1 protein vaccine were prepared. It was shown that priming with secreted dense granule protein 1 (GRA1) protein vaccine loaded chitosan particles and boosting with GRA1 pDNA vaccine resulted in high anti-GRA1 antibodies, characterized by a mixed IgG2a/IgG1 ratio (Bivas-Benita et al., 2003). The application of chitosan-based delivery system as ocular gene carriers, there is evidence of their ability to transfect the ocular cells in vitro. This capacity of chitosan nanoparticles to transfect the cells, was found to be highly dependent on the molecular weight of chitosan. Only chitosan of low molecular weight (10-12 kDa) was able to transfect plasmid DNA in both cell lines derived from the human cornea and the conjunctives (De la Fuente et al., 2008). In Utero delivery of chitosan-DNA results in postnatal gene expression, and shows promise for non-viral gene transfer in animal models of fetal gene therapy (Yang et al., 2010). The intravenous and intratracheal solutions and the intratracheal powder of pCMV-Muβ encoding murine interferon-β were administered the day after the inoculation of mice with CT26 cells. Lung weight and the number of pulmonary nodules at day 21 were significantly suppressed by the three formulations at a dose of 10 μg (N/P = 5). Reducing the dose to 1 μg resulted in a loss of effect by the intravenous solution (Okamoto et al., 2010). These findings showed that therapeutic gene powders are promising for gene therapy to treat lung cancer or metastasis.

siRNA gene therapy research has focused on several types of viral vectors: adeno-associated viruses (AAV), adenoviruses, retroviruses, lentiviruses, and herpes simplex viruses. siRNA therapeutics have been assessed in numerous diseases, including genetic and viral diseases, cancer, as well as non-lethal disorders, such as arthritis and osteoporosis. Among these viral vectors, lentiviruses have progressed to clinical trials on metastatic melanoma and HIV infection (Baker, 2010a; 2010b). siRNA-based gene therapy has already been tested in clinical trials dealing with the treatment of age-related macular degeneration, viral infection, skin disorders and cancer. Cancer treatment is by the most important proposed application of gene therapy and many clinical trials using gene therapy are under investigation. Non-viral vectors including chitosan derivatives have been used in animal model, but clinical trials are lagging due to low transfection efficiency. Anderson et al. (Andersen et al., 2008) demonstrated that silencing of pro-inflammatory TNFα in the RAW 246.7 murine macrophage cell line was achieved by using lyophilized chitosan/siRNA. Compared to research in vitro with chitosan-based systems, in vivo research is still in the developmental stage. Only a few studies are avaible which *in vivo* demonstration of chitosan/siRNA nanocomplexes in silencing gene expression in animals. Howard et al. (Howard et al., 2009) demonstrated that chitosan nanopaticles contains an anti-TNFα siRNA knock downed efficiently of TNFα expression in primary peritoneal macrophages in vitro. Downregulation of TNFα-induced inflammatory responses arrested systemic and local inflammation in collagen-induced arthritic mice after intraperitoneal injection of chitosan/anti-TNFα siRNA nanoparticles, thereby presenting a novel strategy for arthritis treatment.

10. Conclusion

Clinical trials on gene therapy are limited to naked DNA or plasmid DNAs/siRNA delivered by viral vectors. Among non-viral vectors for DNA and siRNA delivery, chitosan and its derivatives are promising alternatives to viral vectors for targeting DNA and siRNA to specific cells. Chitosan has once been considered as an attractive gene transfer candidate for its superior biocompatibility, superior biodegradability and low cell toxicity, but the low stability, low buffering capacity and low cell-specificity have also hindered its clinical applications. To date, however, no clinical trials of chitosan-DNA or siRNA therapy have been performed. Chitosan-based gene therapy remains in the experimental stage due to low transfection efficacy. Many key challenges were involved in DNA and siRNA delivery to targeted cells using chitosan-based carriers. As a nature resource-based polysaccharide, chitosan has more functional groups that can be chemically modified than do the other cationic polymers, thus has many more potential chemical derivatives to make up these deficiencies. Parameters are critical to achieve favourable transfection efficiency and include degree of deacetylation, molecular weight, pH and N/P ratio. For example, a low molecular weight, high degree of deacetylation, small particle size and a moderate, positive, surface zeta potential along with a high N/P ration are advantageous to achieve high siRNA transfection efficiency. Recent technological advances in the chemical modification of chitosan have instituted improvements of its transfection efficiency without disturbing its biocompatibility and biodegradability.

Our work on gene coding for IL-lRa in dogs (Pelletier et al., 1997) and rabbits (Fernandes et al., 1999) was our previous study with the protein itself. We have improved a non-viral intraarticular transfection technique using lipofection and have tested it in osteoarhtritis animal models. These were the very first published articles in the literature that demonstrated the efficacy of gene therapy in osteoarthritis models *in vivo*. Our recent work on polymeric nanoparticles has led us to develop a much safer and effective system for in vitro transfection of embryonic kidney cells, as well as adult mesenchymal stem cells (Corsi et al., 2003). This new system has been successfully tested in muscle and skin tissues *in vivo* in mice and holds great promise for future application on the field of gene therapy and tissue engineering. We developed a second-generation nanovector by successfully coupling folic acid to the polymer (Mansouri et al., 2006). One strategy for improving transfection is to take advantage of the mechanism of folate-mediated uptake by cells to promote targeting and internalization, hence improving transfection efficiency. Folate-mediated transfection has been shown to facilitate DNA internalization into cells via membrane receptors both *in vitro* and *in vivo* (Sudimack & Lee, 2000). Expression of folate receptor (FR)-β in synovial mononuclear cells and CD14+ cells from patients with RA was described by 1999 (Nakashima-Matsushita et al., 1999). Articular macrophages isolated from rats with adjuvant-induced arthritis overexpress FRs and exhibit significantly higher binding capacity for folate conjugates than macrophages obtained from healthy rats (Turk et al., 2002). The wide distribution of FRs at the surface of activated macrophages in rheumatoid arthritis allows the use of folate as potential ligand for folate-targeted chitosan gene therapy. Our laboratory demonstrated that folate-chitosan DNA nanoparticle containing IL-1Ra has been shown to play a role to prevent abnormal osteoblast metabolism and bone damage in this adjuvant-induced arthritis model (Fernandes et al., 2008). It also allows a significant decrease of the inflammation in the rats' paw compared to untreated rats, proving indirectly the efficacy of the IL-1Ra protein treatment. Various inflammation markers (IL-1β and PGE_2)

showed a significant decrease in muscle and serum after the injection of the IL-1Ra protein demonstrating by direct evidence the efficacy of the administration technique to deliver efficient nanoparticles. Therefore, we have already shown it is possible to do gene therapy with IL-1Ra to decrease arthritis and have a positive effect on inflammation.

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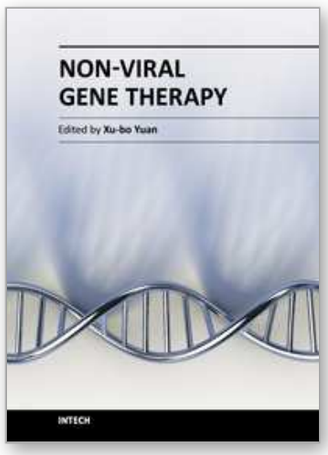
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This book focuses on recent advancement of gene delivery systems research. With the multidisciplinary contribution in gene delivery, the book covers several aspects in the gene therapy development: various gene delivery systems, methods to enhance delivery, materials with modification and multifunction for the tumor or tissue targeting. This book will help molecular biologists gain a basic knowledge of gene delivery vehicles, while drug delivery scientist will better understand DNA, molecular biology, and DNA manipulation.

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