



Translating chitosan to clinical delivery of nucleic acid-based drugs

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ABSTRACT

A number of systems based on synthetic molecules, among them cationic liposomes and poly(ethylene imine)-based polymers, have been proposed as delivery vehicles for nucleic acids. Some of these systems have even reached the market, ensuring efficient and transient transfection levels in a variety of cell types. However, toxicity issues have limited their application in vivo. In this context, chitosan, a biocompatible and biodegradable polysaccharide, has been proposed as a promising alternative for the delivery of nucleic acid-based molecules. Here we present an overview of the state of the art of chitosan-based vectors for nucleic acid delivery and the most recent data on the in vivo testing of the proposed systems. We additionally express our view on the barriers that might be hampering the translation of this knowledge into clinical practice and the challenges that need to be fulfilled for these promising vehicles to reach patients.

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Chitosan: Sources and properties

Chitosan is a linear co-polymer of D-glucosamine and N -acetyl- D-glucosamine in a β -(1–4) linkage, in which glucosamine is the predominant repeating unit (Figure 1). The molar fraction of N-acetylated units defines the degree of acetylation (DA) of a given chitosan, expressed in molar percentage of N-acetylated units. Chitosan is soluble in lightly acidic aqueous solutions. At a pH lower than its pKa, ranging from \sim 6.5 to \sim 7, chitosan is a polycation, and at pH 4.0 and below, it is completely protonated.¹ The cationic character of chitosan under mildly acidic conditions and its ability to complex and condense nucleic acid-based molecules are the basis for its use as a transfection agent.

Chitosan is mostly obtained by deacetylation of chitin. The most commonly used sources of chitin are the exoskeleton of crustaceans and squids. The chitin present in squid pens is easier to deacetylate due to the absence of inter-sheet hydrogen bonding.² To conduct the deacetylation process with chitin from crustaceans, harsher conditions need to be applied, and the resulting chitosan typically has lower molecular weight. Consequently, this has been the preferred source of chitosan for use in nucleic acid delivery, as smaller and less disperse complexes can be formed when chitosan of lower molecular weight is used.^{3,4} In either case, the fact that chitosan has an animal origin has raised some concerns, as it might be a potential source of allergens. More recently, chitosan isolated from the cell walls of mushrooms has become commercially available. As compared to chitosan obtained from marine animal resources, chitosan isolated from fungi cultured under controlled growing conditions presents higher reproducibility among batches and traceability,⁵ which makes it of interest for application in medical and pharmaceutical products. Irrespective of the original source, chitosan of ultrapure grade can now be obtained.

In addition to its ability to condense nucleic acid-based molecules and protect them from endonuclease degradation, a particular advantage of chitosan over other polycations proposed as non-viral vectors for nucleic acids relies on its low cytotoxicity and biodegradability. Besides chemical hydrolysis, enzymes can also mediate chitosan degradation *in vivo*.⁶ Among these, lysozyme has been identified as the main one being involved in this process.^{7,8} It can hydrolyze partially N-acetylated chitosans, with the active site of the enzyme binding six sugar rings, being three consecutive N -acetyl-D-glucosamine residues required for lysozyme catalytic activity.⁷ As a consequence, the susceptibility of chitosan to lysozyme depolymerization depends not only on the DA but also on the distribution of N-acetylated units along the chitosan chains.

Further contributions to the field are expected when enzymatic production of chitosan will become readily available.⁹ This will allow precise control over the resulting chain molecular weight, DA, and the distribution of the monomer units along the polymer backbone. Consequently, nanocomplex formation reproducibility and fine-tuning of biodegradation can be achieved, ultimately affecting the overall process of cell transfection.

Chitosan as a vector for nucleic acid delivery

Gene therapy is currently considered a strategy that provides cells with the genetic information required to produce specific therapeutic proteins or increase their overall expression levels, thus correcting or modulating specific pathologies. Moreover, the concept has been expanded to include specific regulation of gene expression through the use of short oligonucleotides targeting DNA or RNA, otherwise named as antisense gene therapy or oligonucleotide gene therapy.

Over the past several years, many attempts have been made to design non-viral vectors that could achieve the level of gene expression and specificity attained by viral vectors, while providing greater flexibility in terms of size of the transported nucleotide cargo, bypassing the immune system, and reducing safety concerns.²⁰ Among them, chitosan-based carriers have gained a great deal of attention for delivery of plasmid DNA (pDNA), small interference RNA (siRNA), and single-stranded oligonucleotides (ssON) (Figure 2). In the following sections, we present the state of the art of chitosan-based nanocarriers, focusing on their ability to deliver different types of nucleic acids.

Plasmid DNA

At the molecular level, pDNA can be considered to be a pro-drug that, upon cellular internalization and nuclear entry, leads to the biosynthesis of a therapeutic protein²¹ (Figure 2a). In addition to disease treatment, plasmids can be used as DNA vaccines for genetic immunization. DNA vaccines function through induction of immune response by introducing gene encoding antigens for specific pathogens.²² pDNA is a double-stranded DNA molecule (1–200,000 base pairs) that can occur in three topological forms: the compact supercoiled form, the open circular form, and the linearized form.

Supercoiling arises from inherent properties of the DNA double helix to twist (turn) and writhe (turn over itself) and allows formation of a more compact molecule with implications for complex formation and transfection capabilities, also dependent on the type of vector used.²³ The US Food and Drug Administration (FDA) guidelines require the majority of pDNA (>80%) to be in its supercoiled form for application in pDNA vaccines for infectious disease indications.¹⁴

There are a large number of reports describing the potential of chitosan and its derivatives for pDNA delivery.¹⁵ Both chitosan-pDNA complexes¹⁶ and chitosan-based nanocarriers prepared by ionic gelation^{17,18} have been described for pDNA transfection. Overall, results revealed that transfection efficiency of chitosan-based delivery vehicles is dependent on several formulation parameters, such as chitosan molecular weight, DA, and stoichiometry (N / P ratio, moles of positive charges from amino groups to moles of negative charges from phosphate groups), among others.¹⁹ Therefore, these parameters should be carefully considered and optimized when designing a pDNA delivery system. An in-depth review describing factors affecting pDNA delivery mediated by chitosan has been recently published.²⁰

Although chitosan has been proposed as an alternative to other non-viral vectors such as cationic liposomes or poly(ethylene imine) (PEI), it generally presents lower transfection efficiencies.²¹ This difference could be attributed to its limited solubility at physiological pH as well as to its inefficient cellular uptake, endolysosome escape, and polyplex dissociation (see Figure 2a). Consequently, several efforts are being made toward improving chitosan transfection efficiency by introducing different types of chemical modifications into the polymer backbone.

One common approach to enhance transfection efficiency is by improving chitosan solubility under physiological conditions. Chitosan grafted with PEI led to increased solubility, as well as improved transfection efficiency in relation to PEI alone.²² Another strategy relies on the grafting of the poly(ethylene glycol) chain (PEGylation) to chitosan, which improves polymer solubility, yielding nanostructures that are more stable in the blood stream (extending their circulation time) and mediating transfection more efficiently.^{23,24} The quaternization of chitosan by methylation of

primary amine groups was also found to improve chitosan solubility and polyplex stability in physiological conditions.²⁵

In order to overcome the endosomal barrier (see Figure 2a), conjugation of imidazole to chitosan has been explored to improve chitosan buffering capacity and endosomal escape.^{26,27} The introduction of hydrophobic moieties into the polymer backbone also led to more efficient endosomal escape²⁸ along with improvement of pDNA condensation capacity due to enhanced interaction between tethered hydrophobic domains and nucleic acids. Thiolation of chitosan also resulted in improvement of pDNA protection against degradation and formation of more stable complexes via establishment of disulfide bonds between chitosan chains. Additionally, in the reductive environment of the cytoplasm, there is an enhanced dissociation of pDNA from the polymer resulting in enhanced transgene expression.^{29, 30} Finally, an amphiphilic modification of chitosan has also been attempted. Hydrophobic modification of chitosan with linoleic acid led to improved endonuclease protection ability, promoting cell binding and enhanced pDNA dissociation. Contrarily, introduction of poly(beta-malic acid) via ester bonds led to enhanced water solubility, minimized nonspecific protein adsorption, and facilitated pDNA unloading, resulting in an overall improved transfection efficiency.³¹

As an alternative to chitosan backbone modification, the preparation of ionically cross-linked chitosan-TPP (tripolyphosphate) nanoparticles was also proposed.³² Such systems were found to improve stability, achieving comparable gene expression levels to other efficient transfection agents. A more advanced system combines chitosan, TPP, and an additional poly/oligosaccharide such as hyaluronic acid³³ or beta-cyclodextrins.³⁴

While non-viral vectors can associate with most cell types via nonspecific binding to the cell surface, specific targeting strategies based on receptor-ligand interactions are preferable to prevent widespread delivery and off-target effects. The incorporation of targeting moieties into the chitosan nanoparticles was found to modify the particle uptake, with the ligand/ receptor interaction governing the internalization pathway and the subsequent intracellular trafficking.^{29, 35, 36}

Small interfering RNA

siRNAs are short, usually 19–21 base pair double-stranded synthetic RNA molecules, which elicit the degradation of messenger RNA (mRNA), thereby achieving down-regulation of a target gene through the mechanism of RNA interference (Figure 2b).³⁷

Due to its chemical nature, siRNA is highly sensitive to degradation by nucleases and unable to cross cellular membranes. Being much smaller than pDNA, siRNA has far less charge

density, possessing an inherently different structure and topology. These differences underlie the specificity of certain synthetic vectors in achieving efficient delivery with only one type of nucleic acid molecule. Chitosan-based vectors follow this rule, hence chemical and physical parameters need to be specifically optimized for siRNA delivery.

To the best of our knowledge, the first report on the use of chitosan-based nanoparticles for siRNA delivery was published in 2006.³⁸ This study explored different methods of complexation (simple coacervation, ionic gelation, and adsorption to chitosan-TPP nanoparticles prepared by ionic gelation) using high molecular weight chitosans (110–470 kDa). The results showed that the

molecular weight did not significantly influence transfection efficiency, although smaller particles were formed with the lowest molecular weight chitosan. The method of siRNA association, in turn, was relevant for transfection efficiency, with entrapment by ionic gelation being the most effective.

Recent reports highlight the effect of chitosan molecular weight on its interaction with siRNA and the transfection efficiency of the resulting complexes. However, the results are not definite yet. Some authors indicated that low molecular weight chitosan is not able to stably interact with siRNA merely because of insufficient electrostatic interactions between the two oppositely charged molecules. This effect was observed when 10 kDa chitosan (5% DA)³⁹ was compared to that of 35 kDa (fully deacetylated).⁴⁰ In contrast, another paper showed that efficient silencing could be achieved with a chitosan of 12 kDa (8% DA).⁴¹

Overall, the conclusion at this point could be that increasing polymer molecular weight might not translate into further increases in performance.^{40,42} This could be explained by a productive interaction threshold between siRNA and the polymer chains, leading to sufficient condensation and intracellular dissociation capacity of the complexes. The previously mentioned examples also reveal that low DAs favor the interaction with siRNA and the overall vector performance. The use of chitosan with low DA is particularly crucial when preparing nanoparticles using the ionic complexation technique. In contrast, when using the ionic gelation technique, siRNA molecules, besides establishing possible ionic interactions with chitosan, are physically entrapped during the particle formation process.^{32,43}

A myriad of modifications in the polymer backbone, aimed at improving nanoparticle formation with siRNAs, have been explored. Similar to pDNA delivery, chitosan quaternization⁴⁴ and chitosan thiolation⁴⁵ have also been investigated. Hydrophobic modification of chitosan with tocopheryl (vitamin E) has also been achieved.⁴⁶ Other possible modifications include glycolchitosan, PEGylated chitosan, and guanidylated chitosan, which have basically conferred improved stability, solubility, and cellular uptake resulting in enhanced transfection capabilities.⁴⁷ Another alternative for efficient encapsulation of siRNA combines PEGylated chitosan with hyaluronic acid and the process of nanoparticle formation induced by ionic gelation using TPP.⁴⁸

Single-stranded oligonucleotides

The therapeutic potential of single-stranded ONs (ssONs) was discovered in 1978, when Zamecnik and Stephenson reported that a DNA oligonucleotide complementary to the target RNA was able to inhibit virus replication in cell culture.⁴⁹ Soon after, the idea of achieving generalized blocking of RNA function through hybridization of ssONs with the RNA started to develop and with it the field of antisense oligonucleotides (AON). As with siRNAs, these molecules can be easily degraded and have difficulty entering cells. Attempts have been made to increase their stability by chemical modification and to maintain their effectiveness upon in vivo administration; however, the high amounts of ssONs needed to achieve a good response and the lack of specific cell targeting may raise some toxicity issues.⁵⁰ Thus, the development of appropriate delivery vectors is also critical in this context (Figure 2c). As in the case of siRNA, the small size and low charge density of the molecule poses a problem for complexation. Additionally, its single-stranded character confers a slight apolar characteristic to the molecule due to exposure of aromatic nitrogenous bases.

Although the number of reports on chitosan-based vectors for ssON delivery is still low, there are some approaches that have led to promising data. Chitosan nanocomplexes showed transfection activity in a model of intranasal application, even though very high amounts (100 μ g) were needed to achieve a response.⁵¹ In another example, complexes of ssON with a galactose-modified chitosan resulted in an improved in vitro transfection efficiency as compared to the parent chitosan complexes.⁵² Moreover, in vivo galactosylation of chitosan was found to vectorize high amounts of AON to specific liver cells, which was seen as the major event leading to the increased efficiency of this vector.

The combination of chitosan with alginate for complexation of AON either by a lyophilization⁵³ or a pre-gelling method⁵⁴ has also led to interesting results. Indeed, chitosan-alginate nanoparticles prepared by lyophilization showed a capacity to transfect skin cells in vivo in an excisional wound model. Those prepared according to the pre-gelling method were found to control the AON release over a 50-hour period⁵⁵ and to transfect a human breast cancer cell line.⁵⁶

The delivery of AON from nanoparticles made by ionic gelation has also been reported in few studies.⁵⁷ A relevant example is the one showing that chitosan nanoparticles are useful carriers of AON for delivery into rat skin cells,⁵⁸ with the authors suggesting the potential of this approach for topical application on human skin. More recently, the use of thiolated chitosan in order to facilitate the dissociation of the AON from the complex was also explored.⁵⁹

Overall, the information reported so far does not yet offer a comprehensive view of the most favorable properties for ssON delivery by chitosan. Considering the enormous potential for ssON-based therapeutics, a more detailed view on the use of chitosan with ssON is warranted.

In vivo application of chitosan as a vector for nucleic acid delivery

Since initial proposals of chitosan as a nucleic acid delivery system, chitosan and its derivatives have been widely tested as nucleic acid carriers in animal models. The majority of the reported in vivo studies focus on the use of reporter gene models in order to evaluate biodistribution and transfection levels mediated by chitosan-pDNA complexes.²⁰ Following these pioneering studies, there has been an increasing number of reports evaluating the efficacy of chitosan as a delivery carrier of therapeutic genes, namely in the fields of cancer, infections, and inflammatory diseases (Table I).

Due to their mucoadhesive properties, chitosan-based nanoparticles have been applied in the development of strategies to treat diseases that affect mucosal surfaces, such as the gastrointestinal tract, lung, and eye. Kai and Ochiya reported successful oral delivery of the β -galactosidase reporter gene mediated by chitosan alone or N-acetylated to the intestine.⁶⁰ Others have reported similar findings in the gastric and intestinal mucosa. pDNA-loaded chitosan-based nanoparticles have also been explored in the field of respiratory diseases. An example is the work of Jiang et al., in which it was shown that aerosol delivery of pDNA producing small hairpin RNA, complexed to folate-chitosan-graft-PEI, specifically silenced the isoform 1 of the protein kinase B (Akt1) in lung tumor cells, resulting in lung tumorigenesis suppression.⁶¹

Hyaluronic-chitosan nanoparticles made by ionic gelation have also been proposed for the delivery of nucleic acids to the ocular mucosa, which is a thin, continuous mucous membrane layer that lines the inner surface of the eyelids and eye surface.^{62,63} In this case, the mucoadhesive properties of both

polymers were combined with the CD₄₄ targeting ability of hyaluronic acid, rendering the system very efficient for transfection of both the corneal epithelium and conjunctiva, ocular tissues that express this receptor.

Another field of application has been mucosal administration of vaccines. For example, Roy et al. reported that oral immunization with pDNA vaccine-loaded chitosan nanoparticles led to successful immunological protection in a murine peanut allergy model.⁶⁴ Another pDNA vaccine encoding for the dense granule protein 1 (GRA₁) has been proposed, intended to prevent *Toxoplasma gondii* infection. A significant immune response against the encoded protein was observed after gastric administration of chitosan nanoparticles containing GRA₁ pDNA to mice.⁶⁵ Nanovaccines have also been designed for intranasal administration. For example, a pDNA-loaded chitosan nanoparticle vaccine against the respiratory syncytial virus (RSV) infection was developed and tested in a murine model. The results showed an attenuation of pulmonary inflammation by induction of protective T helper 1 (Th₁) cell type⁶⁶ and cytotoxic T lymphocyte responses⁶⁷ in BALB/c mouse strain.

The encouraging results of chitosan in delivering pDNA molecules drove interest in it as an attractive carrier for siRNA. Compared to in vivo pDNA delivery, research on siRNA delivery mediated by chitosan-based vectors is still in its infancy, the majority of current work being basic research involving model siRNA molecules. Delivery of siRNA for therapeutic purposes has recently started to receive increasing attention, with specific emphasis in the field of infectious diseases and cancer (Table I). One example is intranasal delivery of chitosan nanoparticles loaded with siRNA targeting the viral NS₁ gene that encodes for a RSV nonstructural protein sequence with an important role in the virus replication process. The results showed a significant reduction of virus concentration (titers) in the lung and decreased pulmonary inflammation associated with RSV infection.^{68,69} On the other hand, in the field of cancer therapy, Pille et al. reported a 90% inhibition of tumor growth in a xenograft aggressive breast cancer model after intravenous administration of anti-Ras *homologous* A (Rho A) siRNA encapsulated in chitosan-coated polyisohexylcyanoacrylate nanoparticles.⁷⁰

Although being reported as one of the most promising nucleic acid carriers for clinical applications, so far chitosan-based nanoparticles have only been evaluated in pre-clinical settings. Therefore, much research is needed in order to advance toward the clinical development phase. This advancement is expected to be favored by the fact that several chitosan-based products are under clinical development, among them, an adjuvant for a Norwalk virus-like particle,⁷¹ whereas others, such as for wound dressing and as a dietary supplement, are already being marketed.⁷¹⁻⁷³

Translating chitosan into the clinic for nucleic acid delivery: progress and challenges

The use of chitosan nanocarriers for gene therapy is largely documented, and there is now a considerable body of knowledge supporting this application. Notwithstanding, there are still no reports of marketed products or current clinical trials with chitosan-based vectors for in vivo administration of nucleic acids. While trying to bring such products from bench-to bedside, a number of requisites must be taken into account. Important issues to be tackled are inherent to the use of nucleic acids as therapeutic molecules, namely the optimization of sequences to ensure robust gene expression while evading immune system surveillance (pDNA) or to minimize off-targets and reduce toxicity effects (ssONs and siRNA), as well as to determine therapeutic dosages. On the other hand, the proposed vector needs to fulfill its role as a carrier of the therapeutic agent. Some of the

strategies being pursued to improve the efficiency of chitosan for nucleic acid delivery have been discussed in the previous sections. Most of the chitosan-related limitations are common to other vectors, as well as approaches being adopted to overcome these hurdles.

It is also important to consider that while chitosan is not currently listed as an excipient in any drug formulation approved by regulatory authorities, chitosan hydrochloride has been on the list of excipients of the European Pharmacopeia since 2002⁹² and in the US Pharmacopeia and The National Formulary since 2011.⁹³ Moreover, chitosan (from shrimp and *Aspergillus niger* origin) is “generally recognized as safe” by the FDA as a direct ingredient in foods and beverages.⁹⁴ Chitosan is also being used as a dietary supplement to reduce fat adsorption and in wound dressings,⁹⁵ indicating that a good understanding of its topical and oral tolerance exists. In addition, there are a number of clinical trials reported by the FDA involving the use of chitosan for oral and nasal peptide/vaccine delivery where chitosan is being used as a vaccine adjuvant. In spite of this, there is still a need for standard guidelines describing the range of requirements, both for suppliers and product manufactures, of product quality and characterization methods.⁹⁶ The American Society for Testing and Materials (ASTM) is making a concerted effort to establish standard guidelines for tissue-engineered medical products and has recently dedicated one of these documents (F2103–11) to the characterization and testing of chitosan salts as starting materials for biomedical applications.⁹⁷

One must be aware that chitosan is not a single chemical entity but varies in compositional DA and distribution of the monomer units along the polymer backbone and molecular weight, depending on the source, extraction, and preparation method.^{96, 98, 99} For nucleic acid delivery, the polymer molecular weight and DA are very important parameters, as they will have an impact in terms of chain flexibility and the number of positive charges per chitosan molecule. Moreover, solubility, biodegradability, aggregation properties, and immune response will be affected by physicochemical properties of chitosan as well.¹⁰⁰ In this context, regulatory issues concerning polymer preparation and characterization methods will have to be addressed such that chitosan can successfully reach the clinic. In this respect, there are three main areas that must be dealt with: (1) polymer characterization and functionality, (2) product reproducibility between different batches, and (3) toxicology and long-term safety.¹⁰¹

The ASTM guideline F2103–11 suggests the use of infrared spectroscopy (IR) and nuclear magnetic resonance (NMR) to correctly identify chitosan.⁹⁷ It also establishes limits of insoluble impurities, residual protein content, inorganic matter, and presence of heavy metals in the chitosan samples. The ASTM standard F2260–03 describes the methodology for determination of chitosan DA using ¹H-NMR.¹⁰² It is based on the work of Vårum et al. and is valid for DA values from 50% down to 1%.¹⁰³ For molecular weight determination, a specific ASTM standard (F2602–08E011) has been published.¹⁰⁴ Several mobile phases are proposed for size exclusion chromatography, with many composed of not only acetic acid and sodium acetate, but also ammonium acetate. Finally, according to FDA specifications, the endotoxin content of chitosan samples should not exceed 5 EU/kg for non-intrathecal administration routes, in order for the material to be considered safe for biomedical applications.¹⁰⁵

The ASTM guidelines represent a promising attempt to surpass some of the difficulties listed previously. If properly followed, they should improve the manufacturing efficiency of chitosan-based products, with significant reduction in the cost of product development.

Another key issue to take into consideration is chitosan biodegradability. In the literature, multiple studies regarding chitosan processed in different forms (hydrogels, scaffolds, beads, or just in solution) have asserted this material as biodegradable. Most of these studies assess biodegradation upon variation of the polymer DA, since this parameter is positively related with the rate of chitosan degradation. In spite of this, little is known about the degradation of chitosan in nanoparticle systems.¹⁰⁶ The extrapolation to nanoparticle biodegradation is not straightforward, because when the polymer is complexed with nucleic acids, the exposure of acetylated groups may not favor the degradation

mediated by enzymes. Chellat and colleagues evaluated biodegradation of chitosan and chitosan complexed with xanthan (CH-X) (an anionic polysaccharide) in degradation media simulating gastric (containing pepsin) or intestinal fluids (containing pancreatin) and found that chitosan degradation was faster than CH-X microspheres.¹⁰⁷ To the best of our knowledge, *in vivo* degradation of chitosan nanoparticles containing nucleic acids has not been disclosed in the literature. It is worthwhile mentioning that this is a common limitation for most nanoparticulate systems.

Conclusions

This review highlights some of the most promising results involving the use of chitosan as a vector for nucleic acids, including examples already in pre-clinical trials that have been fueling the interest of the research community and manufacturers into use of this biopolymer. Chitosan has advantages over other non-viral carriers in terms of its nontoxicity and biodegradability. Another interesting feature widely explored is the mucoadhesive properties of chitosan, with impact on gastrointestinal, lung, and ocular applications.

While the field of gene therapy is still addressing many of the hurdles that have been hampering the widespread translation of nucleic acid-based therapies to the clinic, there are several products in clinical trials based on both viral and non-viral vectors supporting the high potential of such therapeutic strategies. Recently, two products successfully reached the market (Glybera and Gendicine) for the treatment of lipoprotein lipase deficiency and head and neck squamous cell carcinoma, respectively. Nevertheless, attaining safe and efficient delivery of the nucleic acid of interest is at the forefront of this quest, which makes chitosan and its derivatives attractive candidates.

While a number of issues that are common to all non-viral vectors remain to be addressed, there are a number of questions that are particular to chitosan. Many of these involve standardization of production and characterization methodology. While seemingly trivial, these issues have held back manufacturers and made comparison between studies difficult at times. Addressing these issues will certainly provide the basis for a deeper understanding of the behavior of this material in different biomedical settings, of which vectorization of nucleic acids is an example with great therapeutic potential. We believe that this will make pharmaceutical companies more secure to bring chitosan into clinical trials and finally to the market.

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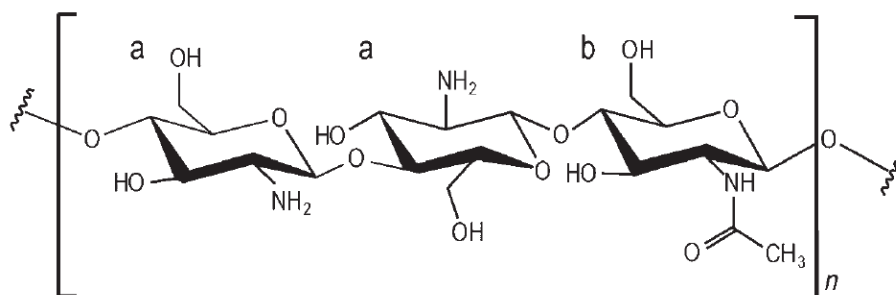


Figure 1. Chemical structure of partially deacetylated chitosan. a: D-glucosamine; b: N -acetyl D-glucosamine. Note: n , repetition unit.

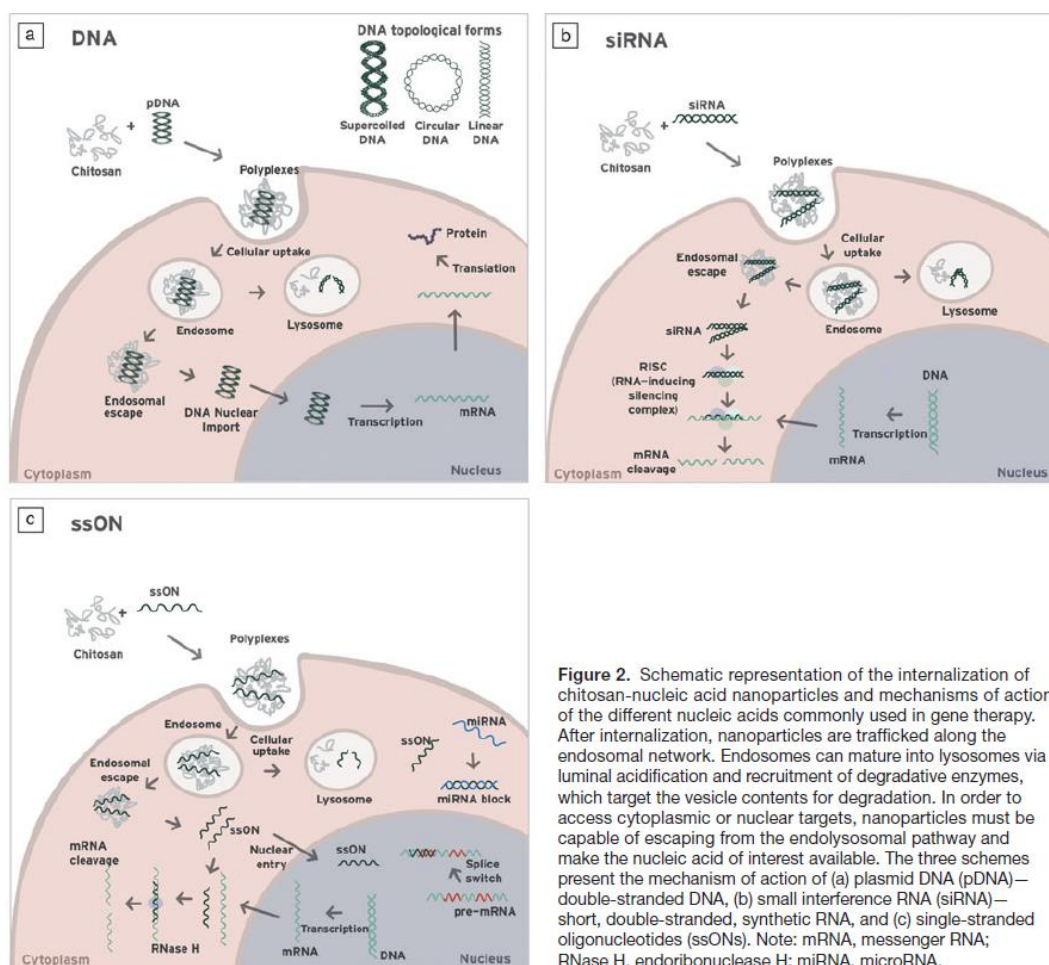


Figure 2. Schematic representation of the internalization of chitosan-nucleic acid nanoparticles and mechanisms of action of the different nucleic acids commonly used in gene therapy. After internalization, nanoparticles are trafficked along the endosomal network. Endosomes can mature into lysosomes via luminal acidification and recruitment of degradative enzymes, which target the vesicle contents for degradation. In order to access cytoplasmic or nuclear targets, nanoparticles must be capable of escaping from the endolysosomal pathway and make the nucleic acid of interest available. The three schemes present the mechanism of action of (a) plasmid DNA (pDNA) – double-stranded DNA, (b) small interference RNA (siRNA) – short, double-stranded, synthetic RNA, and (c) single-stranded oligonucleotides (ssONs). Note: mRNA, messenger RNA; RNase H, endoribonuclease H; miRNA, microRNA.

Table 1. Overview of the main *in vivo* studies using chitosan-based nanoparticles to deliver therapeutic plasmid DNA (pDNA) and small interference RNA (siRNA) molecules.

| Application | Delivery System | Gene | Animal Model | Administration Route | Main Findings | Ref. |
|-------------|----------------------------------|--|-------------------------------|------------------------------|--|------|
| Infection | Chitosan/pDNA | GRA1 | C3H/HeN mice | Intragastric | Chitosan/GRA1 pDNA vaccine induced an immune response against <i>Toxoplasma gondii</i> . The protective immune responses against <i>Toxoplasma gondii</i> are clearly associated with Th1 type responses. | 65 |
| | Chitosan/pDNA | VP1 | BALB/c (H-2d) mice | Intranasal | Chitosan/pDNA-VP1 induced CVB3-specific systemic immunity (humoral, mediated by B cells, and cellular, mediated primarily by sensitized T cells, immune responses), and protected 42.9% mice from lethal CVB3 infection. | 74 |
| | Chitosan/pDNA | 8 HLA-A2 0201-restricted T cell epitopes | HLA-A2 transgenic mouse model | Pulmonary | Pulmonary vaccination with chitosan-pDNA nanoparticles enhanced the protection against <i>Mycobacterium tuberculosis</i> infection, including the maturation of dendritic cells (antigen-presenting cells that function as sentinels) and increasing the secretion of IFN- γ from T cells. | 75 |
| | Chitosan/pDNA | 9 immunogenic RSV genes | RSV-infected BALB/c mice | Intranasal | RSV-infected mice treated intranasally with chitosan nanoparticles containing pDNA encoding for 9 RSV antigens showed a reduction in the RSV concentration levels in the lung and production of anti-RSV antibodies with neutralizing properties. | 66 |
| | Chitosan/pDNA | CTL epitope from the M2 protein of RSV | BALB/c mice | Intranasal and intradermal | The administration of chitosan-based nanoparticles (intranasal/intradermal) induced virus-specific CTL responses in mice. | 67 |
| | Chitosan/siRNA | siNS1 | Fischer 344 rats | Intranasal | The delivery of siNS1 prior to RSV exposure was effective in reducing virus titers in the lung and in preventing the inflammation and airway hyper-responsiveness associated with the infection. | 69 |
| | Chitosan/siRNA | siNS1 | BALB/c mice | Intranasal | Mice treated intranasally with siNS1 nanoparticles before or after infection with RSV showed substantially decreased virus titers in the lung and decreased inflammation and airway reactivity. | 68 |
| | Biotinylated Chitosan-biFcy-pDNA | SARS-CoV nucleocapsid (N) | BALB/c mice | Intranasal and intramuscular | The intranasal co-administration of biFcy targeted chitosan-based nanoparticles and anti-CD40 (an activation/maturation stimuli for dendritic cells) enhanced the magnitude of mucosal IgA antibodies as well as systemic IgG antibodies production against N protein. The intramuscular route showed to be better for induction of systemic IgG responses compared with the intranasal route. | 76 |
| | Chitosan/pDNA | Rho1-GTPase | C57BL/6 mice | Oral | Immunization with chitosan-based nanoparticles resulted in 47% of protection against parvovirus infection; oral immunization was not able to induce high levels of specific antibodies but induced high levels of the modulatory cytokine IL-10, resulting in a significant reduction of liver pathology. | 77 |

| Application | Gene | Animal Model | Administration Route | Main Findings | Ref. |
|----------------------------|---------------|---|---|---|------|
| Allergy | Ara12 | AKR/J mice | Oral | Immunized mice produced secretory IgA and serum IgG2a and showed a reduction in allergen-induced anaphylaxis. | 64 |
| | Der p 1 | BALB/cJ mice | Oral | In orally treated mice, a specific Der p 1 immune response was induced otherwise undetectable using parental route of administration. | 78 |
| | IFN- γ | STAT4-/- BALB/c mice | Intranasal | The intranasal administration of chitosan/IFN- γ nanoparticles reduced the functional and immunological abnormalities associated with allergen sensitization; this effect is predominantly mediated via a STAT4 signaling pathway. | 79 |
| Cancer | Der p 2 | BALB/c mice | Oral | In mice treated orally with chitosan/Der p 2 nanoparticles, a specific Th1 immune response was preferentially activated (induction of IFN- γ), and the subsequent sensitization of Th2 cell-regulated specific IgE responses was prevented. | 80 |
| | PEDF | BALB/c nude mice | Intratracheal (cortex of the anterior tuberosity of tibiae) | The delivery of PEDF mediated by chitosan nanoparticles resulted in inhibition of tumor growth at the tibial site, less bone degradation, and a reduction of number and size of pulmonary metastases. | 81 |
| | IL-21 GM-SCF | BALB/c mice | Intravenously (tail vein) | The nanoparticles exhibited hepatocyte and passive tumor specificity, increased therapeutic efficacy, promoted leukocytes to aggregate in tumor tissues, and activated the cytotoxicity of natural killer cells and cytolytic T lymphocyte. | 82 |
| | IL-12 | Ascites-bearing ICR mice | Intraperitoneal | The IL-12 expression significantly enhanced the anti-effect on ascites tumor-bearing ICR mice as compared with controls. Targeting specificity of the developed nanoparticle was demonstrated. | 83 |
| | Akt1 | A/J mice (urethane-induced lung cancer) | Aerosol | Aerosol delivery of Akt1 shRNA loaded on chitosan-poly(ethylene imine)-folate nanoparticles suppressed lung tumorigenesis in mice through the Akt signaling pathway. | 61 |
| Prostate cancer | RXFP1 | Nude mice | Intratumoral | The targeted down regulation of human RXFP1 in prostate cancer cell xenografts resulted in significantly decreased tumor growth (decreased cancer cell proliferation and increased apoptosis) and metastasis rate in nude mice. | 84 |
| | Src and Fgr | NCr-nu mice model of ovarian carcinoma (Hey/A8-MDR) | Intravenously | Dual Src and Fgr silencing decreased tumor microvessel density, cell survival (increased apoptosis), and tumor growth, consisting in an effective approach for treatment of ovarian carcinoma in animal models. | 85 |
| Melanoma and breast cancer | TG2 | NCr-nu mice models of melanoma (A375SM) and breast (MDA-MD231) cancer | Intratumoral | The intratumoral administration of chitosan-hydrogels carrying TG2-targeted siRNA significantly inhibited tumor growth (72% in melanoma and 92% in breast cancer models), without systemic side effects. | 86 |

| Application | Delivery System | Gene | Animal Model | Administration Route | Main Findings | Ref. |
|----------------|--------------------------|---------------|---|--------------------------------|---|------|
| Cancer (cont.) | Hepatocellular carcinoma | VEGF | ICR mice | Intratumoral and intravenously | The intratumoral and intravenous administration of chitosan/VEGF-shRNA nanoparticles significantly inhibited VEGF expression of hepatocarcinoma cells and liver tumor tissues. The administration of chitosan/VEGF-shRNA nanoparticles demonstrated more effective suppression of tumor growth and angiogenesis compared with naked siRNA. | 87 |
| | Cancer | VEGF | PC-3-bearing mice | Intravenously | The intravenously administration of TGCC/VEGF-siRNA resulted in VEGF gene silencing in PC-3 tumor bearing mice; tumor growth inhibition and reduced angiogenesis in tumors. No immune stimulation was observed, indicating that these nanoparticles could be safe for clinical applications. | 86 |
| Others | Hemophilia | Factor VIII | Factor VIII exon 16 knockout mice | Oral | Oral administration of chitosan/Factor VIII pDNA to hemophilia A mice resulted in thrombin generation and phenotypic correction in 77% of hemophilia A mice, although low levels of plasma Factor VIII were measured. | 89 |
| | Rheumatoid arthritis | TNF- α | C57BL/6J mice (collagen-induced arthritic mice) | Intraperitoneal | Intraperitoneal administration of chitosan/TNF- α -siRNA resulted in knockdown of TNF- α expression in systemic macrophages. The silencing of TNF- α resulted in minimal cartilage destruction and reduced inflammatory cell infiltration. | 90 |
| | Diabetes mellitus | GLP-1 | Zucker diabetic fatty rat | Intravenously | Diabetic mice treated with chitosan/GLP-1-pDNA nanoparticles showed fivefold higher plasma levels of GLP-1, leading to a threefold increase in plasma insulin concentration when compared with untreated animals and to a normalization of blood glucose level, up to 24 days after treatment. | 91 |
| | Wound healing | Smad3 | C57BL/6 mice | Wound dressing | When applied in wound mice, the smad3 ASOs incorporated in polyelectrolyte complex containing chitosan and sodium alginate led to a reduced production of TGF- β 1 via suppression of smad3 at cellular levels. Also, the healing process <i>in vivo</i> (suggested by wound closure and histological observation) was accelerated in comparison with controls. | 53 |

GRA1, dense granule protein 1; CVB3, Coxsackievirus B3; HLA-A2, human leukocyte antigen serotype-A2; IFN- γ , interferon- γ ; RSV, respiratory syncytial virus; CTL, cytotoxic T lymphocyte; SARS-CoV, severe acute respiratory syndrome coronavirus; bFGF, recombinant basic fibroblast growth factor; Der p 1, dermatophagoides pteronyssinus 1; Der p 2, dermatophagoides pteronyssinus 2; STAT4, signal transducer and activator of transcription protein 4; PEDF, pigment epithelium-derived factor; IL, interleukin; siRNA, small hairpin RNA; GM-SCF, granulocyte-macrophage colony-stimulating factor; RFXFP1, relaxin family peptide receptor 1; TGF β , tissue transglutaminase; VEGF, vascular endothelial growth factor; TNF- α , tumor necrosis factor- α ; GLP-1, glucagon-like peptide-1; TGF- β 1, transforming growth factor- β 1; ASO, antisense oligonucleotide.



Glossary

Alginate: Anionic polysaccharide present in the cell wall of brown algae.

Chitin: A high molecular weight, crystalline polysaccharide, theoretically composed entirely of N-acetylglucosamine units; it is the main component of the cell walls of fungi, the exoskeletons of arthropods such as crustaceans (e.g., crabs, lobsters, and shrimps) and insects, as well as it is present in the endoskeleton of cephalopods, including squid and octopuses.

Coacervation: The process that results in the aggregation of molecules or formation of colloidal particles under the action of electrostatic attractive forces.

Ionic gelation: The process of formation of particles driven by the reticulation (gelation) of a polymer induced by small charged molecules.

Endonuclease: Phosphodiesterase that cleaves bonds within a polynucleotide chain.

Endotoxin: Toxin present inside a bacterial cell that is released when it disintegrates; consists of lipopolysaccharide and lipoprotein complexes.

Intrathecal administration: Administration of a therapeutic agent into the subarachnoid space, which contains the cerebrospinal fluid, in the spinal cord.

pKa: Decimal logarithm of the acid dissociation constant.

Polyplex: Complex between the cationic polymer and anionic nucleic acid.

Pro-drug: Therapeutic agent administered in an inactive form that must undergo a chemical or biological conversion before becoming an active agent.

Transfection: the process of deliberately introducing nucleic acids into cells.