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Review article

Chitosan-DNA nanoparticles as non-viral vectors in gene therapy: strategies to improve transfection efficacy

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Abstract

Currently, the major drawback of gene therapy is the gene transfection rate. The two main types of vectors that are used in gene therapy are based on viral or non-viral gene delivery systems. The viral gene delivery system shows a high transfection yield but it has many disadvantages, such as oncogenic effects and immunogenicity. However, cationic polymers, like chitosan, have potential for DNA complexation and may be useful as non-viral vectors for gene therapy applications. Chitosan is a natural non-toxic polysaccharide, it is biodegradable and biocompatible, and protects DNA against DNase degradation and leads to its condensation. The objective of this paper was to summarize the state of the art in gene therapy and particularly the use of chitosan to improve the transfection efficiency in vivo and in vitro.

Keywords: Non-viral vectors; Chitosan; Nanoparticles; Transfection efficiency; Gene therapy

1. Introduction

The basic concept underlying gene therapy is that human disease may be treated by the transfer of genetic material into specific cells of a patient in order to correct or supplement defective genes responsible for disease development. Gene therapy is currently being applied in many different health problems such as cancer, AIDS, and cardiovascular diseases [1]. Several trials employing gene therapy protocols have already been successfully completed in patients with cystic fibrosis [2,3], and adenosine deaminase deficiency [4]. However, in some cases, the effectiveness of the protocols was not well achieved, mainly owing to the inefficiency of the gene transfer vectors that were used.

There are several systems that can be used to transfer foreign genetic material into the human body. In order to do so, the DNA to be transferred must escape the processes that affect the disposition of macromolecules. These processes include the interaction with blood components, vascular endothelial cells and uptake by the reticuloendothelial system. Furthermore, the degradation of therapeutic DNA by serum nucleases is also a potential obstacle for functional delivery to the target cell [5].

Therefore, the ideal gene delivery system must be capable of protecting the DNA until it reaches its target. To do so, the system must be small enough to allow internalization into cells and passage to the nucleus, it must have flexible tropisms for applicability in a range of disease targets, and it must be capable of escaping endosome–lysysome processing and of following endocytosis (Fig. 1). Currently, transport of exogeneous DNA to cells can be achieved using vectors, which can be separated into two categories: viral and non-viral vectors.

2. Gene therapy vectors

2.1. Direct injection of naked DNA

The simplest non-viral gene delivery system simply uses naked DNA. Direct injection of free DNA into certain

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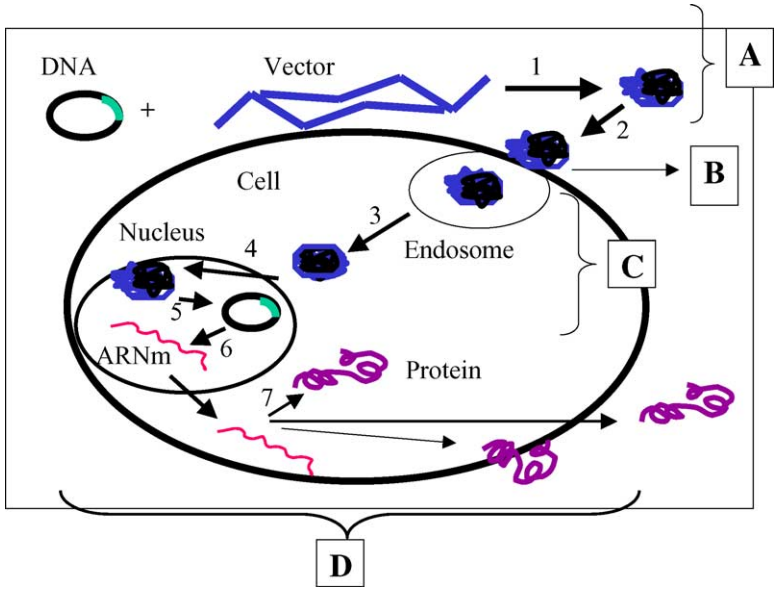

Fig. 1. Simplified gene therapy mechanism. (A) Extracellular trafficking (1) insertion of the gene in the vector; (B) internalization contact and crossing of the cell membrane; (C) intracellular trafficking (2) uptake of the vector complex into intracellular endosome; (3) complex release from the endosome into cytoplasm; (4) uptake of the complex in the nucleus; (D) gene expression; (5) DNA dissociation from the vector; (6) ARNm transcription from the gene; (7) protein translation from ARNm. The protein can be secreted out of the cell, be released into the cytoplasm, or fixed onto the membrane.

tissues, particularly muscle, has been shown to produce high levels of gene expression, and the simplicity of this approach has led to its adoption in a number of experimental protocols [6]. Direct cutaneous injection in patients suffering from lamellar ichthyosis, a genetic skin condition caused by a loss of transglutaminase 1 (TGase1) expression, showed that skin regeneration was possible following repeated injections of DNA encoding TGase1 [7]. Further analysis, however, revealed that the pattern of expression was non-uniform and failed to correct the underlying histological and functional abnormalities of the disease.

Although direct injection of DNA has been shown to lead to gene expression, the overall level of expression is much lower than with either viral or liposomal vectors. Naked DNA is also unsuitable for systemic administration due to the presence of serum nucleases. As a result, direct injection of DNA seems to be limited to tissues that are easily accessible to direct injection such as skin and muscle.

2.2. Viral vectors

The most common viral vectors used today are retrovirus, herpes simplex virus, lentivirus, adenovirus and adeno-associated virus, each having their own characteristics [8]. The advantage of using viral vectors is their natural ability to enter the cells and express their own proteins. This type of vector allows a high transfection rate and a rapid transcription of the foreign material inserted in the viral genome. However, the use of viruses in gene therapy could be limited by various factors. First and foremost, security issues have been raised following the death of a patient during a clinical trial that investigated the potential of gene therapy using viral vectors [9,10]. Second, gene therapy using viral vectors is limited by the fact that only small sequences of DNA can be inserted in the virus genome, while large-scale production may be difficult to achieve. Third, viruses present a variety of potential problems to the patients such as toxicity, immune responses, and inflammatory responses. Lastly, insertional mutagenesis and oncogenic effects can occur when used in vivo [11]. The limitations of viral vectors, particularly regarding safety concerns, have led to the evaluation and development of alternative vectors based on non-viral systems.

2.3. Non-viral vectors

Cationic phospholipids and cationic polymers are the two major types of non-viral gene delivery vectors currently investigated. Because of their permanent cationic charge, both types interact electrostatically with negatively charged DNA and form complexes (lipo- or polyplexes). Although liposomes formed from cationic phospholipids offer several advantages over viral gene transfer, e.g. low immunogenicity and ease of preparation [12], the success of the liposomal approach is limited. Toxicity of the cationic lipids and the relatively low transfection efficiency compared to viral gene delivery vectors [8] are the main disadvantages. DNA/polymer complexes involving cationic polymers, on the other hand, are more stable than cationic lipids [13]. However, compared to viral vectors, the efficiency of gene delivery by cationic polymers is still relatively low [14]. Cationic polymers have been used to condense and deliver DNA both in vitro and in vivo. Several cationic polymers have been investigated that lead to increased transfection

efficiencies [9,10]. They show structural variability and versatility including the possibility of covalent binding of targeting moieties for gene expression mediated through specific receptors [9,15].

2.3.1. Cationic lipids

Cationic lipids have been used for the delivery of encapsulated drugs, as well as for vectors for gene therapy [16,17]. They are able to interact spontaneously with negatively charged DNA to form clusters of aggregated vesicles along the nucleic acid [18]. At a critical liposome density, the DNA is condensed and becomes encapsulated within a lipid bilayer. However, there is some evidence that cationic liposomes do not actually encapsulate the DNA, but instead bind along the surface of the DNA, maintaining its original size and shape. Cationic liposomes can also interact with negatively charged cell membranes where fusion between cationic vesicles and cell surfaces might result in delivery of the DNA directly across the plasma membrane [16]. Nevertheless, it is also well documented that liposome/DNA complexes are directly cytotoxic in vitro [19].

Cationic liposomes can be formed from a variety of cationic lipids, though they usually incorporate a neutral lipid such as dioleoylphosphatidyl-ethanolamine (DOPE) into the formulation in order to facilitate membrane fusion [20]. A variety of cationic lipids have been developed to interact with DNA, but perhaps the best known are *N*-1(-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammoniumethyl sulfate (DOTAP) and *N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride (DOTMA).

There have been several studies investigating the systemic use of liposome/DNA complexes in vivo [2,21]. Complexes formed between the cationic lipid and DNA are rapidly cleared from the bloodstream and have been found to be widely distributed in the body [20,22]. The transfection efficiency of liposome/DNA complexes in vivo has been shown to be relatively low, especially when compared to viral vectors [23]. One explanation for the relatively poor transfection efficiency of liposome/DNA complexes is that they are susceptible to disruption by serum proteins [20,22]. Various proteins are known to bind to liposomes in vitro and in vivo, which may lead to membrane destabilization. Targeting of the liposomes to specific cell types has also been investigated as a means of improving the transfection efficiency.

2.3.2. Cationic polymers

Generally, cationic carriers are widely accepted because of their ability to efficiently condensate DNA and interact with cell [24]. DNA/polymer complexes involving cationic polymers are more stable than those involving cationic lipids. The ability of a vector to transport gene into targeted cells is the premise of improving gene transfer efficiency. The mechanism of gene transfer across the cell membrane is not well understood, and to date, the cationic polymers explored as non-viral vectors are relatively poor in carrying DNA molecules across membrane. Cationic polymers are able to condense more DNA than lipids. They form complexes with DNA and protect it against nuclease degradation [24]. To reach cells, the complexes must readily diffuse through the capillary network, escape macrophages surveillance and interact with the cell membrane [25]. They must be internalized, possibly through endocytosis, then exit the endosome in the cytoplasm and reach the nucleus where it can be transcribed [26].

Cationic polymers include gelatin [27–29] polyethyleneimine [30], poly(L-lysine) [31,32], polybrene [33], tetraminofullerene [34], poly(L-histidine)-graft-poly(L-lysine) [35]and chitosan [36]. Although PEC systems have some advantages over virus vector, e.g. low immunogenicity and easy manufacture [12], several problems such as toxicity, lack of biodegradability, low field of gene transfection, biocompatibility and in particular, low transfection efficiency need to be solved prior to practical use features in shuttling gene into cell [37,38].

3. Chitosan

Chitosan is a non-toxic biodegradable polycationic polymer with low immunogenicity [39]. It is a good candidate for gene delivery system because positively charged chitosan can be complexed with negatively charged DNA [40,41]. Chitosan can effectively bind DNA and protect it from nuclease degradation [42,43]. It has advantages of not necessitating sonication and organic solvents for its preparation, therefore minimizing possible damage to DNA during complexation. DNA-loaded chitosan microparticles were found to be stable during storage [29]. The application of DNA–chitosan nanospheres has advanced in vitro DNA transfection research and data have been accumulating that shows their usefulness for gene delivery [36,44].

Chitosan (a (1 → 4) 2-amino-2-deoxy-β-D-glucan(is obtained by the alkaline deacetylation of chitin. Chitin is a polysaccharide found in the exoskeleton of crustaceans and insects [45]. The chitosan molecule is a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine. This polymer is a weak base with a pK_a value of the D-glucosamine residue of about 6.2–7.0 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 [46]. Chitosan excels in enhancing the transport of drugs across the cell membrane. Its cationic polyelectrolyte nature provides a strong electrostatic interaction with mucus, negatively charged mucosal surfaces and other macromolecules such as DNA [46,47]. Chitosan has been used as a delivery vehicle for nasal, ocular and peroral drug delivery in order to prolong contact time and improve drug absorption, and it has also been used for gene delivery [45]. Intrinsic

characteristics of chitosan makes it an attractive polymer for a variety of biomedical and pharmaceutical applications.

4. Strategies to improve gene transfection efficacy

4.1. Chitosan/DNA complexes

We have previously demonstrated that chitosan–DNA complexes appear spherical with a mean size inferior to 100 nm and a homogenous distribution of DNA is established within the particle. Our results are in agreement with previous studies [43]. The size of the complexes is of crucial importance to cellular uptake. Illum et al. have synthesized chitosan–DNA nanoparticles ranging from 20 to 500 nm thus our complexes are smaller than other polymeric systems [43]. The smaller size complexes have the advantage of entering the cells through endocytosis and/or pinocytosis, therefore increasing the transfection rate. The transfection efficacy seen in vitro is cell type-dependent, and cell viability studies following incubation with nanoparticles confirmed the lack of toxicity of chitosan compared to cationic lipids such as lipofectamine [36,48,49].

One major pitfall of in vitro studies is their lack of reproductibility in vivo. We addressed this question recently by injecting either a 0.15 M NaCl (control) or 1 μg of naked or complexed VR1012 plasmid coding for β-gal gene (Vical Inc., Anaheim, CA, USA) with chitosan or lipofectamine in the anterior tibialis muscle of 6-week-old male Balb/c mice (Fernandes et al., unpublished data). One major finding of this pilot study was that chitosan–DNA nanoparticles administered in the anterior tibialis mice muscle revealed a high signal corresponding to β-gal gene expression within 48 h (Fig. 2). In contrast, the administration of naked or DNA/Lipofectamine in the anterior tibialis muscle did not reveal any β-gal gene expression. From these preliminary data, we proposed that chitosan–DNA nanoparticles have the potential ability to transfect muscle cells in vivo and lead to protein synthesis. Work is presently under way in our laboratory to complete a larger series of in vivo experiments using this technology.

4.2. Modified chitosans for gene therapy

4.2.1. Lactosylated chitosan

Erbacher et al. investigated chitosan and lactosylated chitosan vectors for their transfection efficiencies in vitro [44]. In this study, the transfection efficiency of chitosan in HeLa cells in the presence of 10% fetal calf serum was found comparable to that of another cationic polymer, polyethyleneimine (PEI). It was shown that these vectors were poorly efficient in transfecting galactose-specific membrane lectin (HepG2 cells). This was probably caused by aggregation of the complexes due to decrease of zeta potential after lactosylation, accompanied by lower affinity of the lactosylated polymer to DNA. Presence of chloroquine, a weak base preventing lysosome acidification [50] did not improve transfection efficiency.

4.2.2. Galactosylated chitosan-graft-polyethylenglycol (GCP)

Park et al. have used galactosylated chitosan-graft-polyethylenglycol (GCP) as a DNA vector [51]. The particle size of GCP/DNA complexes is small, with a minimal value of about 27 nm. DNA complexed with GCP is stable and protected against enzyme degradation with DNase. However, the transfection efficiency using GCP/DNA complexes is very low, mainly because of interaction with plasma leading to dissociation of GCP/DNA complexes. This study was challenged by the data of Gao et al. in different cell lines, which supported a positive use of low molecular weight chitosan (LMWCs) as a modified vector for gene therapy [52]. The galactosylated-LMWCs (Gal-LMWCs) were prepared and complexed with plasmids containing the pSV-gal reporter gene. The complex chitosan/DNA was prepared using the coacervation process. The transfection efficiency was evaluated by a β-gal assay and compared with lipofectamine and calcium

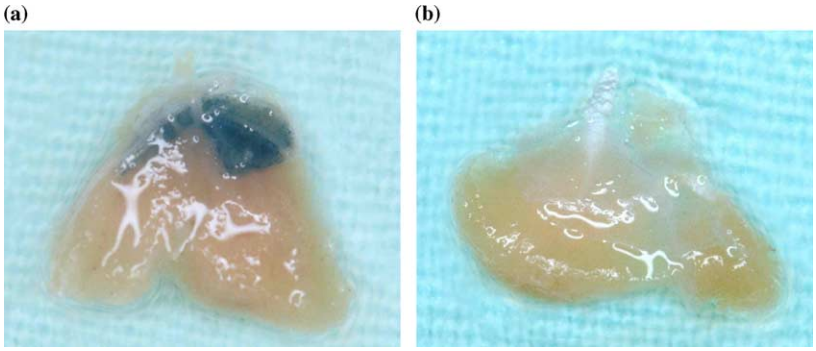

Fig. 2. β-gal gene expression under x-gal substrate incubation 48 h post-injection in the anterior tibialis muscle of balb-c mice of (a) 1 μg chitosan–DNA nanoparticles; (b) 1 μg naked DNA in the right anterior tibialis muscle (control group). The plasmid VR1012 coding for the β-gal gene was a generous gift of Vical Inc, Anaheim, CA, USA). Note the strong staining in the group transfected with the plasmid/chitosan complex.

phosphate (CaP). The high molecular weight chitosan (HMWC) and LMWC were found to be less cytotoxic as compared with lipofectamine and CaP in both HepG2 and HeLa. The results showed that the transfection efficiency increases when the amino group/phosphate group (N/P) ratio goes up from 0.94:1 to 5.6:1, with a maximum rate of transfection at an N/P ratio of 5.6:1. When the galactose density of the complex increases with the N/P ratio, the complexes can interact with the receptors on the cell surface more easily, resulting in an increase of transfection efficiency. The ability of Gal–LMWCs/DNA complexes to transfect human hepatocytes more effectively (HepG2, L-02 and SMMC-7721) than HeLa indicate that Gal–LMWC vectors can transfect gene selectively into hepatocyte via a receptor-mediated endocytosis pathway. Compared with other vectors, the transfection efficiency of Gal–LMWC/DNA complex in HepG2 cells is higher than that of naked DNA, CaP/DNA, HMWC/DNA, LMWC/DNA complexes and lower than that of lipofectin/DNA [52].

4.2.3. Quaternization of oligomeric chitosan

Another approach to increase transfection rate using chitosan as a vector, consists of preparing trimethylated chitosan oligomers (TMO) through quaternization of oligomeric chitosan as demonstrated by Thanou et al. [49]. This process is based on a reductive methylation procedure using methyl iodide in an alkaline environment [49]. These polycations (PC) were characterized and tested for their efficiency on transfecting COS-1 cells (kidney) and Caco-2 cells (epithelial). When chitosan and quaternized chitosan oligomers were mixed with DNA, they spontaneously formed complexes. All synthesized derivatives showed excellent solubility in water at different pH values. At both 2:100 and 2:10 DNA/oligomer ratios, the TMOs formed smaller complexes compared to unmodified chitosan oligomers [49]. The complexes were tested for specific targeting to Hep-G2 cells and for expression of the β-gal reporter gene. The complexes efficiently transfected the Hep-G2 cells, possibly by internalization via the galactose receptor present on the cellular surface of HepG-2 cells, as proposed by Murata [53].

4.2.4. Chitosan/DNA/ligand complexes

Another approach to increase transfection rate consists to investigate the usefulness of ligands to target polymer/DNA complexes to specific cell surface receptors. The transferrin receptor was one of the first to be exploited for receptor-mediated gene delivery. The number of transferrin molecules attached to each nanoparticle surface varies according to the molecular weight of the polymer [48]. This system is based on transferring receptor-mediated endocytosis to carry exogenous DNA into cells and yields a higher transfection rate. Compared to non-modified chitosan, the method results in a fourfold enhanced transfection efficiency, depending on the cell line. In a similar pattern, conjugation of KNOB (C-terminal globular domain of the fiber protein) to chitosan–DNA nanoparticles resulted in a 130-fold increase in the transfection efficiency in HeLa cells and several fold in H3K293. The presence of 10% fetal bovine serum did not interfere with the transfection yield, showing the lack of interaction when systemic administration is used [48]. Transfection efficiency depended on the cell type and the level of surface transferrin receptor expression, and was enhanced when an endosomalytic agent such as chloroquine was used. Generally, endosomalytic agents are used to promote endosomal release and subsequent cytoplasmic delivery of complexes internalized through a receptor-mediated endocytosis mechanism [54]. The treatment with these agents resulted in a fourfold increase in receptor number and a fivefold increase in reporter gene expression.

4.2.5. Dodecylated chitosan vector

Yau and Liu have proposed a DNA/N-dodecylated chitosan complex and salt-induced gene delivery (CS-12) from dodecyl bromide and chitosan (average molecular weight, 700 kDa), assembled with DNA (salmon testes, average molecular weight, 2 kbp) to form a polyelectrolyte complex (DNA–CS-12 PEC) [55,56]. Incorporating dodecylated chitosan can enhance the thermal stability of DNA. Pure DNA in the absence of dodecylated chitosan is hydrolyzed by DNase and can been broken into fragments. On the other hand, DNA dissociated from the complex is well protected and remains intact due to the protection from DNase offered by alkylated chitosan. Although an interesting research approach, no analysis of transfection efficacy in vitro or in vivo has been published by the authors at the moment [15].

4.2.6. Deoxycholic acid modified-chitosan vector

Lee and Kim et al. [11,57] proposed to hydrophobically modify chitosan ($M_v = 7.0 \times 10^4$, degree of deacetylation 80%) with deoxycholic acid. Deoxycholic acid is a main component of bile acid, which is biologically the most detergent-like molecule in the body. Since bile acid can assemble in water, the deoxycholic acid-modified chitosan also self-associates to form micelles of a mean diameter of 160 nm. The transfection of COS-1 cells (monkey kidney) with chitosan self-aggregate–DNA complexes was examined using the plasmid encoding chloramphenicol acetyltransferase (CAT). The transfection efficiency of the complexes is enhanced compared to that achieved by naked DNA but lower than that achieved by Lipofectamine. This system still lacks more efficacy in transfection compared to standard chitosan/DNA formulations.

5. Potential clinical applications

5.1. Mucosal genetic immunization

To test potential mucosal genetic immunization, Kumar et al. utilized a strategy involving an intranasal gene

transfer, referred to as IGT, complexed with chitosan–DNA nanospheres containing a cocktail of DNA encoding nine immunogenic syncitial virus (RSV) antigens. This system was tested against acute RSV infection in a BALB/c mouse model. The effectiveness and mechanism of this IGT strategy were investigated, and results demonstrated that IGT is safe and effective against RSV as well as it significantly attenuates pulmonary inflammation induced by RSV infection [58].

This system is a novel gene transfer approach against RSV infection, which at a single dose of about 1 mg/kg body weight, is capable of decreasing viral titers by two orders of magnitude (100-fold) on primary infection. The immunologic mechanisms for the effectiveness of this prophylaxis include the induction of high levels of both serum IgG and mucosal IgA antibodies, the generation of an effective control response, and elevated lung-specific production of interferon-γ with antiviral action. Although effective as a single-dose, it is possible that dose escalation and prime-booster strategies of IGT vaccine might further enhance its effectiveness. In addition, IGT significantly decreases pulmonary inflammation and does not alter airway hyperresponsiveness, thus making it safe for in vivo use [58].

5.2. Pulmonary gene delivery

In order to investigate the potential of chitosan in the form of an inhaled powder as a gene delivery system, Okamoto et al. prepared powders using pCMV-Luc as a reporter gene and a low molecular weight chitosan (M_w 3000–30,000) as a cationic vector with supercritical CO_2. The obtained chitosan–DNA powders were administered to mice lungs. The transfection efficiency of these powders was compared with that of DNA solutions and DNA powders without the cationic vector. The gene powder with the cationic polymer was found to be an excellent gene delivery system to the lungs, as evidenced by a high transfection rate and a high expression of the luciferase protein. The benefits of the chitosan–DNA powders examined were summarized as follows: (a) the addition of chitosan suppressed the degradation of cytomegalovirus-luciferase plasmid (pCMV–Luc) during the supercritical CO_2 process; (b) the addition of chitosan increased the yield of powders; and (c) the chitosan–DNA powders increased the luciferase activity in the mouse lung compared with pCMV–Luc powders without chitosan or pCMV–Luc solutions with or without chitosan [59].

6. Conclusions

As described in this review, several non-viral vectors, including cationic polymers, have been developed for DNA delivery. The transfection efficiency may depend on several factors such as chemical structure of polycations, size and composition of complexes, interaction between cells and complexes and the cell type. One such cationic polymer, namely chitosan, is considered to be one of the best candidates for DNA delivery. A number of in vitro and in vivo studies, including ours, showed that chitosan is a suitable material for efficient non-viral gene therapy. Several groups, including ours, are conducting studies using different strategies that can be linked to chitosan–DNA nanoparticles (including targeting cell membrane receptors) for a higher transfection yield.

Thus, the high solubility and low toxicity of chitosan as well as its bioactive properties at neutral pH allow its use in a wide variety of domains, namely, pharmaceutical and food industries, and gene therapy.

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