Expert Opinion

Advances in using chitosan-based nanoparticles for *in vitro* and *in vivo* drug and gene delivery

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Importance of the field: This review aims to provide an overview of state-of-the-art chitosan-based nanosized carriers for the delivery of therapeutic agents. Chitosan nanocarriers are smart delivery systems owing to the possibility of their property alterations with various approaches, which would confer them with the possibility of spatiotemporal delivery features.
Areas covered in this review: The focus of this review is principally on those aspects that have not often been addressed in other reviews. These include the influence of physicochemical properties of chitosan on delivery mechanisms and chitosan modification with a variety of ligand moieties specific for cell surface receptors to increase recognition and uptake of nanocarriers into cells through receptor-mediated endocytosis. Multiple examples that demonstrate the advantages of chitosan-based nanocarriers over other delivery systems of therapeutic agents are highlighted. Particular emphasis is given to the alteration of material properties by functionalization or combination with other polymers for their specific applications. Finally, structural and experimental parameters influencing transfection efficiency of chitosan-based nanocarriers are presented for both *in vitro* and *in vivo* gene delivery.
What the reader will gain: The readers will acquire knowledge of parameters influencing the properties of the chitosan-based nanocarriers for delivery of therapeutic agents (genetic material or drugs) *in vitro* and *in vivo.* They will get a better idea of the strategies to be adapted to tune the characteristics of chitosan and chitosan derivatives for specific delivery applications.
Take home message: Chitosan is prone to chemical and physical modifications, and is very responsive to environmental stimuli such as temperature and pH. These features make chitosan a smart material with great potential for developing multifunctional nanocarrier systems to deliver large varieties of therapeutic agents administrated in multiple ways with reduced side effects.

Keywords: chitosan, drug delivery, gene delivery, nanocarrier, nanoparticle, transfection

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1. Introduction

Since its discovery by Rouget in the late 1800s [1], chitosan has been used in a vast array of applications [2]. Among them, it has emerged as an excellent candidate for designing vehicles for the delivery of a variety of compounds in different formats. However, nanosized carriers are by far the most suitable delivery system for therapeutic purposes. They are able to be internalized by cells more efficiently than microsized vehicles, and deliver their cargo more effectively into cells. Furthermore, the clearance by the reticuloendothelial system (RES) is retarded, thus providing longer *in vivo* residency time for delivery of encapsulated material [3]. The size plays an important role in the kinetics and biodistribution of nanocarriers, and should be

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Article highlights.

- Primary properties of chitosan (toxicity, biodegradability and solubility) affect the delivery capabilities of the nanocarriers made of this biopolymer.
- Multifunctional polyplexes and nanoplexes are developed using the polyelectrolyte assembly approach or grafting of various moieties to chitosan backbone.
- Chitosan chemical and physical modifications enhance its targeting and tracking abilities and allow us to understand the fate and the interaction of chitosan-based nanoparticles with tissues and cells.
- Chitosan-based nanoplexes and polyplexes provide us with the possibility of multiple modes of therapeutics delivery for both *in vitro* and *in vivo* applications.

This box summarizes key points in the article.

taken into consideration. Also, the efficacy of the chitosan particles varies depending on what the targeted tissue is. In some tissues, the higher fenestration among cells such as those found in the liver or spleen allows for greater biodistribution. In other tissues, particularly with tight junctions, such as those of the brain, the biodistribution could be compromised. This fenestration size could be altered in different contexts. For example, alterations occur in the presence of inflammation, or in some pathological cases such as cancerous tissues; this results in a change in the biodistribution of the nanoparticles. In this paper, primarily the nanoparticle carrier systems made of chitosan and its derivatives are reviewed for the delivery of therapeutic agents. The physicochemical properties of chitosan are well suited to studying delivery mechanisms and its internalization process. Alteration of material properties by functionalization or combination with other polymers is discussed to stress the relationship between the structural properties and the particle size to address a specific application. As an end note, an overview of drug and gene deliveries using chitosan-based delivery systems is presented.

2. Primary properties of chitosan: toxicity, biodegradability and solubility

Although the different polymers are frequently evaluated for gene [4] or drug delivery [5], chitosan has been revealed to combine many advantageous compared with other biopolymers, making it one of the most interesting materials for controlled release applications [6]. Its biocompatibility, biodegradation and solubility are the most critical properties that influence a chitosan-based nanocarrier's effectiveness. The physicochemical properties of chitosan also greatly control the delivery capabilities of the nanocarriers made of this biopolymer, and could drastically alter the context of its use. In addition to the advantageous properties inherent to the material, many methods are used to assemble chitosan nanocarriers to confer extra features in the resulting nanoparticles [7].

Chitosan is well known as being non-toxic, with an LD_{50} (lethal dose for 50% of test population) level in the same range as sugar or table salt [8]. *In vitro* investigations on the effect of chitosan concentrations by both Richardson *et al.* and Mao *et al.* showed relatively low cytotoxicity (< 1 mg/ml) on different cell lines [9,10]. The molecular mass also seemed to have some, but minimal, effect on cell viability. Reduced toxicity and the absence of hemolysis have been reported with shorter chitosan chain lengths [8-11]. The degree of deacetylation (DDA) of the polymer, however, had a greater effect on the toxicity. Chitosan nanoparticles with lower DDA showed lower toxicity *in vitro* [12].

Chitosan degrades through enzymatic hydrolysis by chitinases, chitosanases and other nonspecific enzymes such as lysozymes. Intermediate breakdown products of chitosan include chitooligomers and monomers, which can activate macrophages and fibroblasts, respectively [13]. Once degraded, chitosan is converted into the common sugar group *N*-acetylglucosamine through the glycoprotein pathway, or is excreted as carbon dioxide [14]. Some studies have also shown that the degradation rate of chitosan is governed by its intrinsic properties, such as the DDA [15]. When compared with non-biodegradable materials, chitosan does not show a great risk of toxicity, difficulties in removal, or long-term accumulation.

In addition to biodegradability, chitosan solubility plays a key role in the use of chitosan derivatives for the development of nanocarrier systems [2]. Qian *et al.* have noted the importance of water solubility for nanoparticle function, as more soluble chitosan may trigger the opening of tight junctions between cells, facilitating paracellular transport of hydrophilic compounds [16]. Solubility of chitosan depends on various parameters, such as molecular mass, average DDA and the distribution of acetyl groups along the chitosan backbone. Improved solubility in more alkaline solutions has been shown using lower molecular mass chitosan [10] as well as chitosan with higher DDA [17]. Kurita *et al.* have further shown that a more uniform distribution of acetyl groups increases solubility by reducing crystallinity [18].

3. Size-dependent biodistribution

Chitosan is an excellent vehicle for the oral delivery of drugs as it has the ability to adhere to the walls of the gastrointestinal pathway. When delivered orally, its mucoadhesive property is believed to increase the cellular uptake of complexes. Owing to it cationic nature, chitosan can interact electrostatically with cell membranes, which increases the uptake of nanoparticles and affects its biodistribution. This mucoadhesive ability could be altered by chitosan molecular mass, deacetylation degree, conformation and the flexibility of the polymer chain [19]. In a general review, Hagens *et al.* presented the effect of particle size on their bioactivity, toxicity and kinetics (i.e., clearance, half-life and distribution) [20]. As particle size decreases, the ratio between relative surface area and volume of the particle increases rapidly. For the same volume,

nanosized particles present a larger surface area for interaction, resulting in more electrostatic interactions (e.g., with cell membranes in the gastrointestinal tract). Hence, smaller particles are considered to be more reactive. Also, smaller particles have an easier time passing through gaps in tissues and membranes through nonspecific interactions, affecting biodistribution and circulation time in the blood (**Figure 1B**) [21].

The nanosized chitosan-based carriers possess all the features of a good carrier, namely nanometer size range, targeting capability, biocompatibility and biodegradability. As such, they offer many possibilities for delivery of cargos to cells. As illustrated in **Figure 2**, nucleic acid delivery occurs in the cytosol or in the nucleus, whereas drugs could be released to reach targets on the cell membrane, in cytosol or in the nucleus.

The biodistribution is generally dominated by the carrier size. Passive targeting of cancerous tissues through a phenomenon referred to as the 'enhanced permeation and retention' (EPR) effect is achieved with chitosan-based nanoparticles having an appropriate size. This enables them to accumulate within tumor tissues where disorganized and defective vascular networks are developed. Some studies suggest that pores can be as large as 780 nm in tumor models [22]. This is because the formation of networks increases blood flow to tumor cells and allows larger macromolecules to enter while limiting lymphatic draining from these sites. This form of passive tumor targeting introduced by the size effect can increase effectiveness of the drug and reduce systemic toxicity to other surrounding tissues. Despite the fact that the size affects the biodistribution of the nanoparticles, the EPR process is also affected by other factors such as surface thermodynamics (hydrophobicity), or by other parameters that are not yet fully understood. For example, Brannon-Peppas and Blanchette indicated that particles with more hydrophobic surfaces will preferentially be taken up by the liver, followed by the spleen and lungs (**Figure 1B**) [23].

4. Functionalized chitosan-based nanoparticle delivery system

To increase further chitosan's capabilities as ideal nanocarrier systems, it has been modified chemically and physically to improve its biocompatibility, solubility, biodistribution, circulation time and, more importantly, its DNA condensing, targeting and tracking abilities.

4.1 Chitosan functionalization for enhanced targeting abilities

Chitosan can easily be modified with a variety of ligands to increase the recognition and uptake of nanocarriers into cells through receptor-mediated endocytosis. Common categories of chitosan modification for targeting to certain cell surface receptors are summarized in **Figure 1A**. Most of these strategies use moieties specific for cell surface receptors [24]. Sugar moieties have been covalently attached to the chitosan backbone by some groups. Galactose was bonded on low-molecular-mass chitosan to target Kupffer cells of the liver and evaluated both *in vitro* and *in vivo* [25]. Galactose residues can recognize asialoglycoprotein receptors expressed on the surface of some cell membranes. To target mannose receptors on dendritic cells residing in the tumor, chitosan has been functionalized with mannose. Using these nanoparticles in the delivery of a plasmid encoding IL-12 resulted in enhanced IL-12 gene transfer efficiency, suppressed tumor growth and angiogenesis in the carcinoma BALB/c mouse model [26]. A trisaccharide branch was attached onto chitosan chain in order to target lectin on the cell surface in lung tissues [27]. This modification increased the carrier uptake and transfection efficiencies in various *in vitro* assays as well as in mouse lung tissue.

Several molecules such as folate and transferrin have also been used for their ability to target cell surface receptors known to be overexpressed, particularly in cancer cells. Chitosan-grafted folate was produced to transfect interleukin-1 receptor antagonist (IL-1Ra) in synovial mononuclear cells and $CD14^+$ cells via the targeting of the folate receptor-β [28]. Compared with unmodified chitosan or naked DNA, this system allowed for an increase in IL-1Ra expression combined with a lower cytotoxicity *in vitro,* and reinforced protection against inflammation and abnormal bone metabolism *in vivo.* Using transferrin as a ligand covalently linked to chitosan, a higher level of uptake and delivery efficiency of anticancer agents was observed *in vitro* in comparison with the control, but *in vivo* results were less convincing [29]. In an attempt to overcome the blood–brain barrier, conjugated chitosan with PEGylated OX26 antibody was used to target transferrin receptor, which is overexpressed in the brain capillary endothelium. After intravenous administration to mice, only antibody-conjugated nanoparticles were found in the brain. The absence of antibody-free nanoparticles indicated the successful targeting ability of conjugated chitosan. Cholanic acid-modified glycol chitosan, used for the delivery of anticancer drugs, was examined for encapsulation of a synthetic peptide bearing the RGD sequence to target the $\alpha_v\beta_3$ integrin on angiogenic blood vessels. Interestingly, high yield and loading efficiency were found. The authors established that this approach can be used as an indirect method for targeting tumors, by targeting surrounding blood vessels [30]. *In vivo* results showed that the intratumoral administration of nanoparticles decreased the tumor size and microvessel size in mice [31].

Few other groups have developed stimuli-sensitive systems with chitosan-based nanoparticles. An example is Chuang *et al.*, who have reported on nanoparticles made of chitosan co-polymerizied with poly(*N*-isopropylacrylamide), which was responsive to pH or temperature changes [32]. Also, biocompatible magnetic chitosan-coated nanoparticles have been used to treat malignant tumors through delivery of hyperthermia thermoseed to the diseased site in response to an external magnetic field [33].

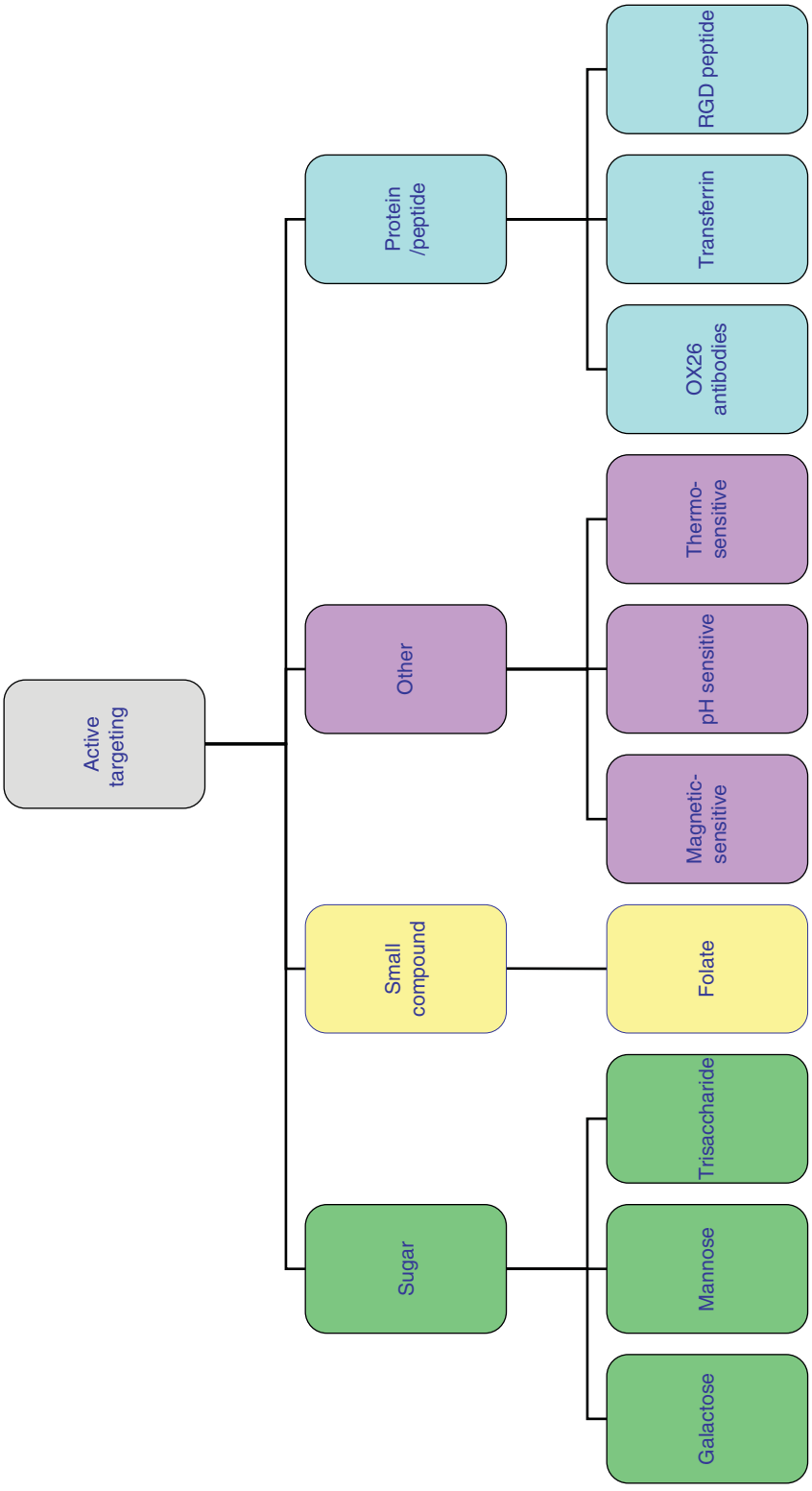

Figure 1. A. Main categories of ligands, namely sugars, peptides/proteins and the small molecules grafted to the chitosan nanocarriers for specific cell targeting. Some other small molecules, such as the magnetic nanoparticles, have also been investigated. **B.** Passive targeting as a function of particle size and hydrophobicity. For hydrophobicity, see [23]. For the height of fenestrations, see [21].

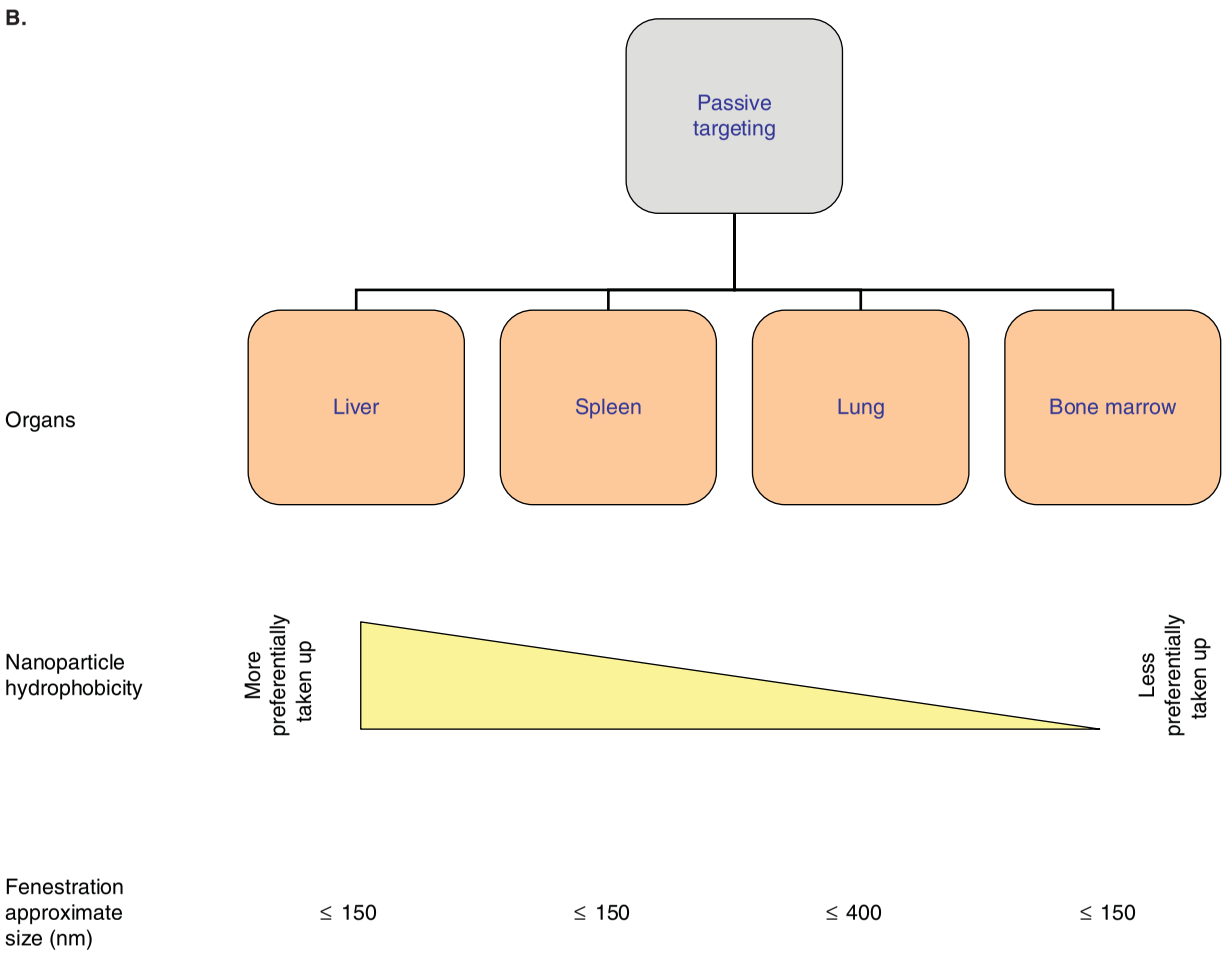

Figure 1. A (continued). Main categories of ligands, namely sugars, peptides/proteins and the small molecules grafted to the chitosan nanocarriers for specific cell targeting. Some other small molecules, such as the magnetic nanoparticles, have also been investigated. **B.** Passive targeting as a function of particle size and hydrophobicity.

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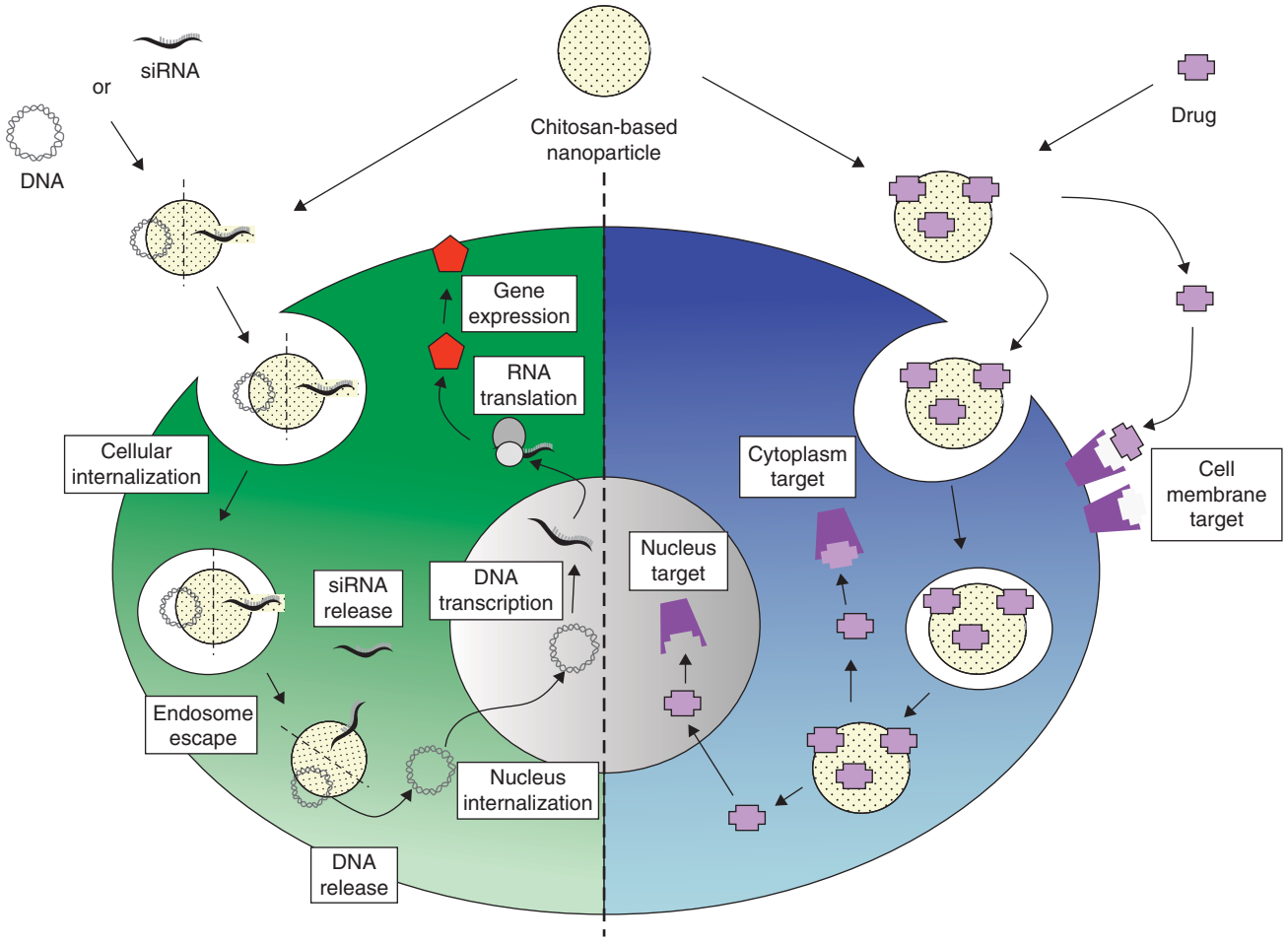

Figure 2. Schematic representation of DNA or siRNA (left) and drug (right) delivery using nanocarriers. DNA usually needs to be delivered in the nucleus as opposed to the siRNA, which is active in the cytoplasm. Targeted drug delivery depends on the nature of the active compounds and can be delivered to the cell membrane, in the cytoplasm or in the nucleus.

4.2 Chitosan functionalization for enhanced tracking abilities

To study the effectiveness of chitosan nanocarrier systems, namely their fate and interaction with tissues and cells, different labeling strategies have been developed. The most common approach is the use of fluorescein isothiocyanate (FITC) organic dye, which can be covalently bound to amine groups in chitosan backbone through a fairly simple reaction. This provides nanoparticles with the ability to be investigated by flow cytometry, and fluorescent and confocal microscopy. Using nanoparticles made of chitosan-FITC, Huang *et al.* [34] showed that nanoparticles were predominantly internalized by endocytosis, which was initiated by nonspecific interactions between nanoparticles and cell membranes. Using FITC-labeled plasmid and Texas Red dye-labeled chitosan, Ishii *et al.* [35] followed pGL3 plasmid encoding luciferase in SOJ cells. Complexes were found to enter the cells through endocytosis. Their release from endosomes was mediated by a combination of proton accumulation and the buffering capacity of chitosan–DNA complexes, causing endosomal rupture and release into the cytosol. This phenomenon depends mainly on the ability of chitosan to buffer acidic cellular compartments. This is due to that fact that the presence of amine groups governing the level of chitosan deacetylation influences the pK_a of cell microenvironment [36], not its molecular mass.

Owing to the limitations of organic dyes, such as photobleaching, a wide emission spectrum and limited resolution in imaging, quantum dots (QDs) are introduced to track the internalization process of nanoparticles. Quantum dots are nanocrystalline semiconductors with unique optical and electronic properties, such as size-tuneable light emission, superior signal brightness, resistance to photobleaching, and broad absorption spectra for simultaneous excitation of multiple fluorescence colors [37]. Deep tissue imaging *in vivo* has been demonstrated with QD-labeled chitosan [38]. Magnetite nanoparticles as tracking probes have been used both *in vitro* and *in vivo* [39]. Combination of QDs and magnetite

nanoparticles allowed for simultaneous fluorescence and magnetic resonance imaging of nanoparticles [40].

Bhattarai *et al.* studied chitosan/gold nanoparticles for carrying DNA into cells. Gold core was used here as a contrast agent to track nanoparticles to their delivery site as well as to obtain more monodispersed nanoparticles. The results indicated that gold is an efficient and biocompatible tracking agent to be introduced for both *in vitro* and *in vivo* applications of nanoparticulate delivery systems [41].

5. Chitosan polyplexes

One of the greatest advantages in using chitosan is its amenability to reaction, given the presence of three different functional groups on its backbone. In addition to facilitating the covalent bonding for targeting or tracking, these groups are often used to introduce other compounds to meld advantageous properties and to moderate or remove some undesirable chitosan characteristics.

5.1 Chitosan-based grafted polymers

Covalent bonding of chitosan to other molecules, where stable grafted polymers are formed, has been explored well in the literature. Sashiwa *et al.* produced a chitosan-*g*-dendrimer hybrid for the purposes of gene delivery. Amine groups present on the dendrimer aimed to increase transfection efficiency, although this fact could not be demonstrated experimentally. Chitosan's own amine groups might also contribute to transfection, as well as to improving biocompatibility [42]. Chitosan covalently bonded to deoxycholic acid was evaluated for transfection of pCMV-CAT in COS-1 cells [43]. Modified chitosan was able to form self-aggregates in aqueous media and mediated higher transfection than naked DNA, but still less than liposomal vectors, as shown by band intensity of the chloramphenicol acetyltransferase assay (CAT). Yu *et al.* examined the chitosan-*g*-poly- L-lysine polyplex (Ch-*g*-PLL), demonstrating higher solubility, increased DNA binding ability and higher transfection efficiency for this system (33% for Ch-*g*-PLL, 4.5% for PLL and < 1% for Ch in per cent of positive 293T cells). It also showed lower toxicity (between 40 and 70% survival with 50 µg/ml of polymer incubated 24 h on L-929 cells) as compared with PLL or poly(ethylene imine) (PEI) alone (15 – 20% cell survival) but still higher than bare chitosan (> 90% survival) [44].

The use of a chitosan-grafted co-polymer is also emerging as an area of active research in order to enhance both material and biological properties. A thermosensitive chitosan-pluronic co-polymer was prepared by grafting monocarboxyl pluronic onto chitosan for the delivery of hydrophobic drugs [45]. The pluronic polymer core allowed for the incorporation of hydrophobic compounds with a thermosensitive release feature whereas chitosan conferred mucoadhesiveness and highly biocompatible characteristics to the delivery system. Poly(caprolactone)-graft-chitosan amphiphilic co-polymer was similarly formed in order to combine the physical properties and delivery potential of the polymers for gene or drug delivery [46].

5.2 Chitosan-based polyelectrolyte complexes

Highly deacetylated chitosan contains a large number of amine groups on the polymer backbone, leading to a global positive charge that can be used to form polyelectrolyte complexes with anionic compounds, thus conferring new properties to chitosan. A great advantage of polyelectrolyte complexes is that no synthesis is needed, avoiding the use of harsh solvents and purification steps. Nanosized complexes formed with a chitosan/alginate polyelectrolyte pair have shown improved delivery properties for both drug [11] and gene deliveries [47-49] as compared with nanoparticles obtained with chitosan alone. Nanoplexes made of ultra-low-molecular-mass chitosan and hyaluronic acid (HA) showed transfection efficiencies 25 times higher than ultra-low-molecular-mass chitosan alone [50]. The nanoparticles of chitosan/dextran sulfate used to encapsulate insulin showed higher retention efficiency of insulin in simulated gastric conditions as compared with chitosan/alginate [51]. This system has also been explored for the delivery of antibiotics, namely amphotericin-B [52]. *In vivo* results showed the absence of renal toxicity that occurs with direct drug delivery.

Some groups have investigated polyelectrolyte complexes formed with non-polysaccharide compounds such as polyaspartic acid sodium salt (PAsp). Zheng *et al.* studied the loading of 5-fluorouracil (5-FU), a drug with a short half-life in plasma (10 – 20 min), into nanoplexes made of chitosan and PAsp [53]. The encapsulation of 5-FU in chitosan/PAsp complexes allowed for a slower and more targeted release of the drug. It also avoided the accumulation of carrier materials in plasma and reduced the required administration dose of the drug.

Another non-polysaccharide polyanion commonly used to form polyplex with chitosan is poly(DL-lactide-*co*-glycolide) (PLGA). PLGA is well known as an excellent drug delivery vehicle for enhanced drug uptake across various biological barriers. On its own, PLGA is not suitable for the transport of large molecules such as DNA through cell membranes, because of its strong negative charge. Combination with chitosan has been shown to offset these limitations in the delivery of antisense oligonucleotides [54]. Guan *et al.* showed the possibility of delivering DNA, but *in vitro* transfection assays still remain very modest with such a system [55].

6. Chitosan nanoparticles as drug delivery vehicle

Chitosan is an effective vehicle for drug delivery, in particular to enhance the absorption of hydrophobic macromolecular drugs, and an excipient for mucosal drug delivery and for

the transport of vaccines [56,57]. Release of encapsulated materials occurs through diffusion and a slow degradation of the polymer complex, resulting in slow release, with some protection of encapsulated materials [58]. Chitosan has also been found to improve the effectiveness of drug delivery by being able to hold the therapeutic material in closer proximity to its site of action owing to its mucoadhesive cationic nature [59]. Also, when used as nanoscale-sized particles, it allows for the diffusion and targeting to specific tissues and cells compared with larger carriers, as shown in following examples.

6.1 Chitosan nanoparticles as a delivery vehicle for insulin

A great number of publications are available on chitosan-based nanoparticles for the delivery of insulin. Different methods of administration, such as oral [60] or nasal [61] delivery, are used to improve the absorption and the bioavailability of the insulin [61]. The molecular mass [62] of the chitosan and the pH [63] have been shown to be important parameters for insulin release. Some investigations aimed at the functionalization of chitosan with poly(methyl methacrylamide) (PMMA) [64], *N*-acetyl-L-cysteine (NAC) [65], glycol (Gc) [66], thiolated-trymethyl (TTM) [67] and poly(ethylene glycol) (PEG) [68] to improve the chitosan-based nanoparticles' properties for insulin delivery.

6.2 Chitosan nanoparticles as a delivery vehicle for antitumor drugs

An increasing number of studies have been published using chitosan nanoparticles for the delivery of antitumor agents with the goals of reducing the toxicity of the drug and obtaining a controlled drug release. Examples of such studies for different classes of antineoplastic drugs are presented in the **Table 1**. The benefits of chitosan nanoparticle use in these circumstances are twofold [69]. First, chitosan itself has a cytotoxic effect on cancerous cells, both *in vivo* and *in vitro* [70]. Second, these nanoparticles could accumulate in tumoral tissue owing to the impaired lymphatic drainage, resulting in a higher drug concentration at the tumor site [71].

6.3 Chitosan nanoparticles as a delivery vehicle for ocular drugs

Agnihotri and Aminabhavi used chitosan-based nanoparticles to deliver timolol maleate, a drug used in glaucoma treatment [72]. This drug is normally administrated four to six times daily, which results in respiratory and cardiovascular side effects caused by the excessive diffusion of the drug through the nasolacrimal drainage. Chitosan nanoparticles have also been studied for the entrapment of dorzolamide hydrochloride and pilocarpine [73], another drug used in the treatment of glaucoma. These studies have shown that the use of chitosan nanoparticles provides a promising solution to decrease the frequency of administration, and consequently to reduce the drug's side effects [74].

6.4 Chitosan nanoparticles as a vehicle for the delivery of drugs through olfactory neurons

Drug delivery to the brain is major issue in drug pharmakinetics. One of the reasons is the presence of the blood–brain barrier, which protects the brain against various intrusions. Chitosan-based nanoparticles have been identified as potential carriers to the brain, delivering drugs by means of the olfactory neurons [75]. As a mucoadhesive polymer, chitosan-loaded drugs increase the residence time and the concentration gradient in the nasal mucosa. Consequently, absorption by the olfactory neuron is increased and the drug can be carried to the brain. This strategy has been used by Wang *et al.*, with the goal of delivering estradiol as a potential treatment for Alzheimer's disease. As a result, a significant improvement of estradiol concentration has been observed in the central nervous system of patients diagnosed with Alzheimer's disease [76]. Another group investigated chitosan-based nanoparticle emulsion with polycarbophil to deliver risperidone, an antipsychotic agent [77], and showed an absence of ciliotoxicity and good diffusion properties of nanoparticles *in vitro*.

6.5 Chitosan nanoparticles as a vehicle for delivery of antibiotics

Nanocarriers are used to circumvent the side effects, such as hepatic, neurologic, hematologic or nephrologic problems, associated with the administration of antibiotics. For example, amphotericin used for the treatment of fungal infection has a low solubility in the gastrointestinal tract, which can lead to serious nephrotoxicity. Tiyaboonchai and Limpeanchob have shown that the use of chitosan-based nanoparticles represents a low-cost solution to reducing the toxicity of amphotericin during treatment [52]. Chitosan nanoparticles could also be used to limit the diffusion of an antibiotic through the body, as shown with gatifloxacin [78]. Motwani *et al.* designed a chitosan alginate polyplex as an ophthalmic drug delivery system. Usually, antibiotics added to the eye are lost by drainage and high tear fluid turnover [79]. With the use of chitosan on negatively charged cornea and conjunctiva of the eyes, it was possible to increase the residency time and the concentration of the antibiotic on the extra-ocular structure. Similar works have also be performed with ciprofloxacin, and with a broad spectrum of antibiotics used to treat skin and ocular infections [80].

7. Chitosan nanoparticles for gene delivery

Chitosan is an effective carrier for gene delivery owing to its ability effectively to condense and complex DNA through the electrostatic interactions between the positively charged amino groups on glucosamine units of chitosan and the negatively charged phosphates on DNA. It is able to overcome some of the major barriers to transfection, including cellular uptake, release from endosomes into the cell and nuclear localization. Incorporation of DNA into a polyplex delivery system can provide targeting properties as well as protect

Table 1. Chemotherapeutic agents delivered using chitosan-based nanoparticles.

Anticancer drugs	Ref.	Drug category	> Highlighted information
Methotrexate	Yang *et al.,* 2008 [107]	Antimetabolite	These carriers release 50% of the methotrexane loaded in 48 h
5-Fluorouracil	Zheng *et al.,* 2007 [53]	Antimetabolite	*In vitro* and *in vivo* experiment indicated that the drug-loaded CS-PAsp nanoparticles presented a sustained release of 5FU compared with the 5FU solution
Cysplatin	Cha *et al.,* 2006 [108]	Alkylating agent	Investigated nanoparticles were able to release cysplatin drug in a sustained manner for a prolonged period of time
Doxorubicin	Janes *et al.,* 2001 [109]	Antibiotic	Encapsulated doxorubicin in chitosan-based nanoparticles could be released into the cells in its active form
Epirubicin	Wang *et al.,* 2007 [110]	Antibiotic	Entrap epirubicin into cholesterol-modified chitosan and show pH-dependent release of drug *in vitro*
Mitomycin C	Bilensoy *et al.,* 2009 [111]	Antibiotic	Poly-ε-caprolactone coated with chitosan was developed to encapsulate intravesical chemotherapeutic agent mitomycin C. These nanoparticles have been shown to selectively incorporate nanoparticle-containing molecules in the bladder cancer cell line, but not for normal bladder epithelial cells
Doxorubicin	Janes *et al.,* 2001 [109]	Antibiotic	Showed the feasibility of chitosan nanoparticles entrapping doxorubicin, a cationic drug, into chitosan via a complexation with the polyanion dextran sulfate, to mask the charge. This approach has shown the capacity to maintain cytostatic activity relative to free doxorubicin, *in vitro*
Docetaxel	Hwang *et al.,* 2008 [112]	Antimitotic	Docetaxel-loaded nanoparticles reduced drug toxicity compared with free drug, and reduced tumor volume on mice
Camptothecin	Min *et al.,* 2008 [113]	Topoisomerase inhibitor	Tumor growth inhibition was obtained with a lower dose of camptothecin loaded in chitosan-based nanoparticles compared with free drugs administration in mice

the polyplex from degradation and clearance from circulation, thus increasing its half-life within the body. Many studies are available that have investigated several parameters, namely molecular mass, DDA and plasmid length parameters, to improve the transfection efficiency of chitosan-based nanoparticles both *in vitro* and *in vivo* for gene delivery.

7.1 Chitosan for gene delivery *in vitro*

7.1.1 Effect of chitosan structural properties

The average chain molecular mass of chitosan can greatly influence transfection efficiency of chitosan/DNA nanoparticles (**Table 2**). High-molecular-mass chitosan seems to mediate the highest transfection efficiency, followed by intermediate and low-molecular-mass species [81-83]. Although the reason for such an observation is not yet clear, one reason could be that high-molecular-mass chitosan (≥ 100 kDa) improves stabilization and protection of DNA. As presented by Kiang *et al.* [82], longer polymer chains more easily entangle free DNA once the initial electrostatic interaction has occurred, and is more energetically favorable to particle formation. By contrast, some groups have reported higher transfection efficiencies with low-molecular-mass chitosan (≤ 30 kDa) [84,85], and concluded that better degradation and intracellular release of DNA could take place with low-molecular-mass chitosan. Yet some investigations have shown highest transfection efficiencies using intermediate-molecular-mass chitosan (30 – 100 kDa) [35,86,87], where a compromise between the ability to protect, carry and release the DNA effectively could be reached.

A more understandable trend is observed with the degree of deacetylation. The general consensus is that a higher DDA mediates better gene delivery [82,84,88]. A given mechanism by which chitosan interacts with negatively charged cell membranes and DNA phosphates is through its positive charge partaking in electrostatic interaction. As DDA increases, the relative number of potentially reactive amino groups also increases, resulting in a more positive global charge of the polymer.

Table 2. Parameters influencing transfection efficiency of chitosan-based nanocarriers.

Parameters	Sub-categories	Ref.	Range of studies	Optimized result	Cell line used
Molecular mass	High (≥ 100 kDa)	Kiang *et al.*, 2004 [82]	138; 209; 390 kDa	390 kDa	HEK 293; HeLa; SW756
		Huang *et al.*, 2005 [92]	10; 17; 48; 98; 213 kDa	213 kDa	A549
		Liu *et al.*, 2007 [83]	9; 12; 65; 114; 170 kDa	170 kDa‡	H1299
	Intermediate (30 – 100 kDa)	Zhao *et al.*, 2006 [114]	6; 46; 85; 200; 300; 800 kDa	85 kDa	Chondrocyte
		Ishii *et al.*, 2001 [35]	4.6; 40; 84; 110 kDa	40 kDa	SOJ
		Sato *et al.*, 2001 [115]	15; 52; 100 kDa	52 kDa	A549; HeLa
	Low (≥ 30 kDa)	Lavertu *et al.*, 2006 [93]	10; 40; 80; 150 kDa	10 kDa	HEK 293
		Koping-Hoggard *et al.*, 2003 [116]	1; 2 – 10 kDa*	35 – 50 units (*in vitro*) 15 – 21 units (*in vivo*)	
		Sato *et al.*, 2001 [115]	15; 52; 100 kDa	15 kDa	
DDA		Kiang *et al.*, 2004 [82]	DDA: 62%; 70%; 90%	DDA: 90%	HEK 293; HeLa; SW756
		Huang *et al.*, 2005 [92]	DDA: 46%; 61%; 88%	DDA: 88%	A549
		Lavertu *et al.*, 2006 [93]	DDA: 72%; 80%; 92%; 98%	DDA: 80 or 92%§	HEK 293
Size	Particle size	Lavertu *et al.*, 2006 [93]	Around 200 – 1100 nm	1000 nm	HEK 293
		Yang *et al.*, 2007 [117]	250; 580; 1300 nm	580 nm	HEK 293
		Koping-hoggard *et al.*, 2003 [116]	53 – 1016 nm	190 nm	HEK 293
	Plasmid	Xu *et al.*, 2008 [118]	4, 7 and ~ 10 kb	4, 7 kb	Chondrocytes
Zeta potential		Huang *et al.*, 2005 [92]	Between +10 and +23 mV	+23 mV	A549
		Lavertu *et al.*, 2006 [93]	Between +2 and +16 mV	Around +10 mV	HEK 293

*In this paper, the molecular mass of the chitosan is expressed by the number of monomer units forming the chitosan chain.

‡Gene silencing was measured instead of gene reporter expression.

§Depending on the other parameters used.

DDA: Degree of deacetylation.

7.1.2 Effect of plasmid length, nanocarrier size and zeta potential

The length of the encapsulated plasmids may also affect transfection efficiency. Xu *et al.* determined that plasmids between 9 to 10 kb could be condensed by chitosan nanoparticles and successfully internalized into the cells, but were not as successful at mediating efficient transfection [89]. This may have occurred as a result of stronger polyelectrolyte interactions, causing slower dissociation of the large plasmid from the chitosan particles. In addition to plasmid length, the final size of the polyplex can affect transfection, as the internalization mechanism into the cell can vary with complex size [90]. Conflicting results have concluded that particle size does not in fact have any effect on transfection efficiency. Lavertu *et al.* showed that larger complexes (~ 700 nm) were able to produce higher transfection in HEK 293 cells over smaller ones (280 nm) [84]. In this same cell line, Yang *et al.* found that particle size had no effect on the transfection efficiency of plasmid DNA/chitosan particles in the size range 250 nm – 1.3 μm [91]. These findings are worth noting as they provide a different view of the internalization mechanisms of chitosan nanocarriers. Finally, some groups have compared the zeta potential of nanoparticles made of different molecular mass or DDA with their transfection efficiency [92,93]. These results support that a higher positive zeta potential leads to more efficient transfection.

7.1.3 Effect of experimental parameters

pH is one of the external factors influencing transfection efficiency, with better transfection obtained in acidic conditions (see **Table 3**) [84,86,87]. Knowing that the pK_a of amino groups of chitosan is 6.5, this result could be expected as a slightly acidic environment increases positive charge on the polymer and allows for a better interaction with negatively charged DNA. It should be noted that, at a certain level of acidity, transfection drops [35]. The exact reason for this effect is not explained well in the literature. Some studies have reported that highly protonated chitosan is cytotoxic [84], which might explain, to a certain extent, the decrease in transfection. This might also be a result of the strong electrostatic binding of DNA with the carrier system in acidic conditions, which may prevent its release.

The change in transfection may be due to the inclusion of serum in the medium because some studies have shown an increase of transfection efficiency in the absence of serum [94]. The detrimental effect of high doses of serum has been reported in both pancreatic SOJ and human-lung carcinoma A459 cells, where transfection efficiency was found to decrease for 10 and 20% serum concentrations, respectively [35,87].

Transfection efficiency is also cell line-dependent. HEK 293 is the most used cell line to evaluate transfections *in vitro* [95]. Corsi *et al.* evaluated transfection potential of β-gal plasmid in chitosan-DNA nanoparticles in human embryonic kidney cells HEK 293, osteosarcoma MG63, and mesenchymal stem cells MSC, and showed that transfection varies with the type of cell line used. The highest efficiency was obtained with HEK 293 cells, followed by MSC and MG63 cells [96]. Variation in the composition of the cell membranes might be a factor explaining the differences observed between the cell lines, as reported by Pouton *et al.* to describe similar results observed for lipid-mediated transfections [97].

Gene delivery using chitosan-based nanoparticles has also been investigated *in vivo* using various gene delivery strategies (**Table 4**). The intranasal administration of chitosan nanoparticles holding plasmid DNA encoding for many respiratory syncytial virus (RSV) agents showed significant induction of RSV-specific IgG, nasal IgG, and nasal IgA antibodies. In addition, recruitment of cytotoxic T lymphocytes and the production of interferon-γ in the lung and splenocytes have been observed [98]. Intratracheal delivery of plasmid DNA-encoding luciferase was used to illustrate the effect of chitosan molecular mass for *in vivo* gene delivery [99]. This study also revealed that optimal parameters for *in vitro* transfection are not necessarily the same as those required for effective *in vivo* transfection. After 3 days, the *in vivo* transfection was higher in more acidic pH with high-molecular-mass chitosan than the ones with a lower molecular mass chitosan (24-mer). However, for the same period of time, *in vitro* transfection efficiency was high with the high-molecular-mass chitosan, regardless of the pH used for the transfection, illustrating that discrepancy can occur between *in vitro* an *in vivo* assays.

Further study by Leong and co-workers using DNA/chitosan nanoparticles for oral delivery showed effective modulation of murine anaphylactic responses by the introduction of *pCMVArah2,* a peanut allergen gene in mice [100]. DNA/chitosan nanoparticles have further been introduced directly into the rabbit knee joint, encoding IL-1Ra or IL-10 plasmids. The results demonstrated that transfection efficiency was closely dependent on the type of gene being delivered [101]. Modified trimethylated chitosan was examined as a vector for pEGFP on BALB/c mice. Orally administered nanoparticles induced gene expression in 100, 89, 78 and 67% of the animals evaluated, respectively, in stomach/duodenum mucosa, jejunal, ilial and large intestine mucosa [102]. *In vivo* transfection was higher than what was found with *in vitro* transfection of HCCLM6 carcinoma cells. This is possibly owing to the repeated oral administration of the complexes creating an accumulation of complexes within the mice.

8. General discussion

Investigation of chitosan nanoparticles started 20 years ago [103], with a considerable increase in the number of articles related to this topic during the last 10 years (**Figure 3**). Drug delivery is the main field that benefits from the use of chitosan nanocarriers, given their ability for cell and tissue targeting. Studies on chitosan nanocarriers for delivery of biomolecules, such as proteins, siRNA and DNA, have also been very promising for both

Table 3. Effect of experimental parameters on transfection efficiency of chitosan-based nanocarriers.

Parameters	Ref.	Range of studies	Optimized result
pH	Sato *et al.,* 2001 [115]	pH 6.9 and 7.6	pH 6.9
	Zhao *et al.,* 2006 [114]	pH 6.8; 7.0; 7.2; 7.4; 7.6	pH 6.8
	Lavertu *et al.,* 2006 [93]	pH 6.5; 6.8; 7.1; 7.4	pH 6.8
	Ishii *et al.,* 2001 [35]	pH 6.5; 7.0; 7.5; 8.0	pH 7.0
Serum	Erbacher *et al.,* 1998 [94]	0 and 10% serum	10% serum
	Ishii *et al.,* 2001 [35]	0%; 5%; 10%; 20%;30%; 40%; 50% serum	10% serum
	Sato *et al.,* 2001 [115]	0%; 10%; 20%; 30%; 40%; 50% serum	20% serum
Cell type	Corsi *et al.,* 2003 [96]	HEK293; MG63; MSC	HEK 293
	Sato *et al.,* 2001 [115]	A549; HeLa; B16	HeLa

Table 4. Examples of *in vivo* administration of chitosan-based nanocarriers.

Administration modes	Ref.	Animal models
Intraocular	Badawi *et al.,* 2008 [119]	Albino rabbits
Intranasal	Zhang *et al.,* 2008 [68]	New Zealand rabbits
Oral	Qi *et al.,* 2007 [70]	Athymic BALB/c nude mice
Intratumoral	Tokumitsu *et al.,* 2000 [120]	C57BL/6 mice
Intravenous	Zhang *et al.,* 2008 [121]	Sprague-Dawley rats/ICR mice/New Zealand albino rabbits/guinea-pigs

in vitro and *in vivo* applications. This review has demonstrated the possibilities of using chitosan-based nanocarriers for the delivery of biological therapeutics and discussed the delivery of DNA and drugs, as studies moved from *in vitro* evaluation to *in vivo* evaluation. Chitosan nanoparticles' biocompatibility, biodegradability and biodistribution present a great advantage over many other compounds for their use as a delivery system of therapeutics. Natural properties of this biopolymer can be well tailored for the delivery of therapeutic agents to different tissues and organs, such as skin, eyes, digestive track or brain. Although by no means an exhaustive review of the literature, the focus was on the intrinsic characteristics of the material and the role it plays in choosing a delivery strategy. An interesting chitosan chemical structure such as positively charged backbone and mucoadhesivity make it an ideal carrier system with high efficiency and reduced side effects. One of the greatest benefits of chitosan is, however, its ability to be modified fairly easily with other molecules, to encompass a wider range of properties. As discussed in this review, the parameters associated with the assembly of chitosan nanocarriers could affect tremendously their delivery properties, especially for DNA. More studies seem to be needed to acquire a full understanding of the role of the degree of deacetylation and the molecular mass on the delivery properties of chitosan nanoparticles.

9. Expert opinion

There is a need for multifunctional chitosan-based nanocarriers containing a combination of extra- and intracellular targeting abilities as well as the capacity to allow monitoring of their activities and cargo delivery. Recent trends also focus on the use of water-soluble chitosan and modified chitosan nanoparticles or coating for delivery of protein antigen to protect the antigen from degradation and increase its uptake through the intestinal epithelium by means of M cells [104-106]. The aim is to develop practical, economical intranasal or oral administration of vaccines to attenuate the systemic immune responses to vaccine. Although gathering all characteristics required for drug delivery or mucosal immunization in the same carrier is very challenging, chitosan-based nanocarriers seem to be prone to the development of these multifunctional delivery systems. Chitosan is a smart material, as its properties can be altered in response to a change of the medium acidity. However, the pH range in which this change occurs is not always convenient for delivery applications. To improve the system's robustness in particular circumstances, more features must be included in the system, such as grafting ligands that are able to modify the pH range in which the chitosan is soluble. Recent trends aim at including more features, such UV-cleavable bonds, gold nanoparticles and/or QDs, in chitosan nanocarriers for tuneable spatiotemporal delivery and for increased carrier targeting and tracking capabilities. Important issues related to the control of release of active agents at the target site need to be addressed for full understanding of the mechanisms governing cell behavior towards chitosan-based nanoparticle delivery systems. Partial knowledge of the internalization pathway and of ligand–target interaction is often the reason for the failure of the delivery system. Correlative imaging, in which two labeling methodologies can be used, may offer an opportunity for studying the fate of nanoparticles. For example, combination of transmission electron microscopy

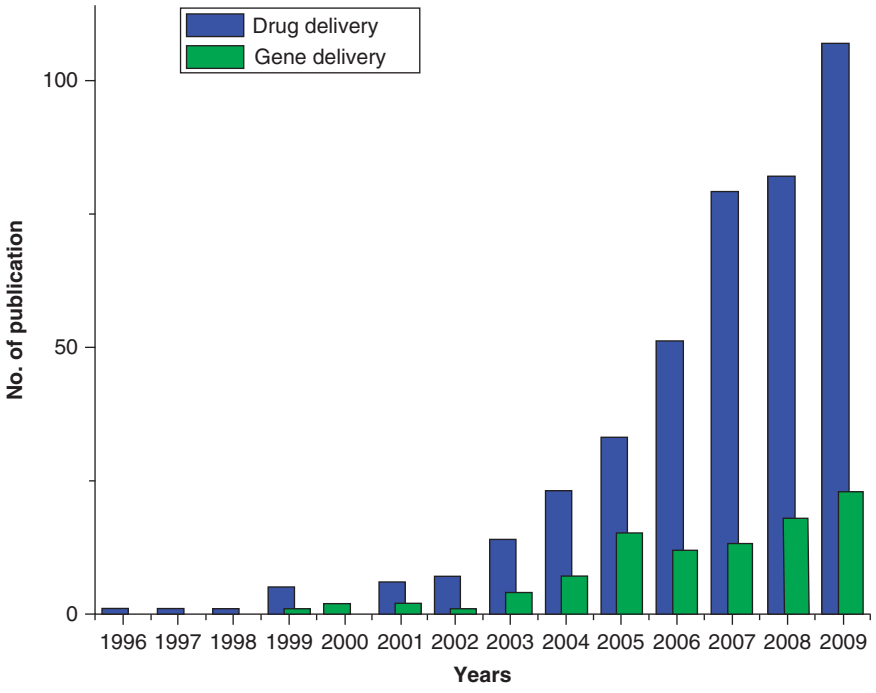

Figure 3. Number of publications on chitosan nanoparticles or nanocarriers for drug or gene delivery in the past 20 years as obtained by Scopus™ research engine. The keywords were (TITLE(chitosan*) AND TITLE(nanoparticle* OR nanocarrier*) AND TITLE-ABS-KEY(drug*)) and (TITLE(chitosan*) and TITLE(nanoparticle* OR nanocarrier*) AND TITLE-ABS-KEY(gene therap* OR transfection* OR gene deliver*)) for 'drug' and for 'gene', respectively.

with nano-secondary ion mass spectrometry or fluorescence microscopy would allow investigation of the nanoparticle internalization process. The inclusion of a fluorescent probe such as QDs, gold and/or organic dye to label plasmid and chitosan to form donor and acceptor pairs that are able to produce a fluorescence resonance energy transfer (FRET) system is another way to address some questions in the field of targeted gene and drug delivery. The latter would be very sensitive to changes in structure at the molecular level and would provide an excellent tool for studying the fate of chitosan-based nanocarriers. Research has to be pursued in order to develop sophisticated imaging and sensing tools to bring some insight to the internalization pathway along with the ligand–target interactions for both *in vitro* and *in vivo* investigations of these carriers.

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