Polymers **2014**, *6*, 1727-1755; doi:10.3390/polym6061727

OPEN ACCESS

polymers

ISSN 2073-4360

www.mdpi.com/journal/polymers

Review

Low Molecular Weight Chitosan (LMWC)-based Polyplexes for pDNA Delivery: From Bench to Bedside

Mireia Agirre [1,2], Jon Zarate [1,2], Edilberto Ojeda [1,2], Gustavo Puras [1,2], Jacques Desbrieres [3] and Jose Luis Pedraz [1,2,*]

1 NanoBioCel Group, University of the Basque Country, Vitoria-Gasteiz, 01006, Spain; E-Mails: mireia.agirrediez@gmail.com (M.A.); jon.zarate@ehu.es (J.Z.); edi082@hotmail.com (E.O.); gustavo.puras@ehu.es (G.P.)

2 Networking Research Center of Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Zaragoza, 50018, Spain

3 Institute of Analytical Sciences and Physico-Chemistry for Environment and Materials (IPREM) (UMR CNRS 5254), University of Pau and Adour Countries, Hélioparc Pau Pyrénées, Pau, 64000, France; E-Mail: jacques.desbrieres@univ-pau.fr

* Author to whom correspondence should be addressed; E-Mail: joseluis.pedraz@ehu.es; Tel.: +34-945-013-091; Fax: +34-945-013-040.

Received: 14 February 2014; in revised form: 21 May 2014 / Accepted: 26 May 2014 / Published: 16 June 2014

Abstract: Non-viral gene delivery vectors are emerging as a safer alternative to viral vectors. Among natural polymers, chitosan (Ch) is the most studied one, and low molecular weight Ch, specifically, presents a wide range of advantages for non-viral pDNA delivery. It is crucial to determine the best process for the formation of Low Molecular Weight Chitosan (LMWC)-pDNA complexes and to characterize their physicochemical properties to better understand their behavior once the polyplexes are administered. The transfection efficiency of Ch based polyplexes is relatively low. Therefore, it is essential to understand all the transfection process, including the cellular uptake, endosomal escape and nuclear import, together with the parameters involved in the process to improve the design and development of the non-viral vectors. The aim of this review is to describe the formation and characterization of LMWC based polyplexes, the *in vitro* transfection process and finally, the *in vivo* applications of LMWC based polyplexes for gene therapy purposes.

Keywords: low molecular weight chitosan (LMWC); pDNA; polyplex; non-viral vector; gene delivery; transfection efficiency

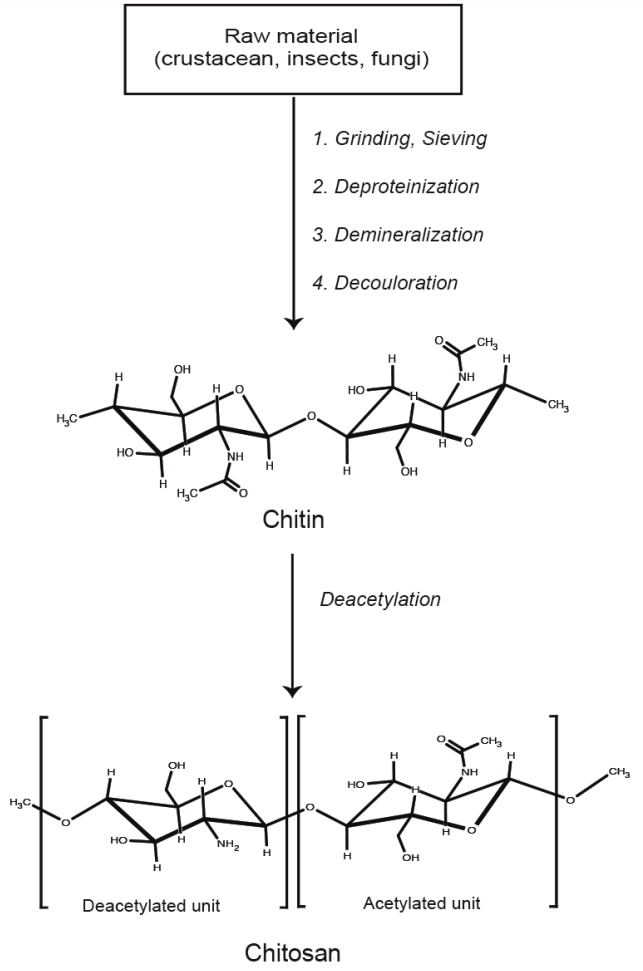
1. Introduction

The success of gene therapy relies on ensuring that the therapeutic gene enters into the target cell and transcribes without being biodegraded. Thus, a delivery vehicle, referred to as vector, is required to carry the therapeutic gene into the target cell, protecting it from nucleases degradation and facilitating its entry across the cellular membrane. Nowadays, gene therapy is still dominated by viral vectors, which present higher transfection efficiencies compared to non-viral gene delivery systems. However, safety issues such as immunogenicity and mutagenicity hinder the clinical applications of the viral gene delivery systems [1,2]. At this point, non-viral vectors are emerging as a safer alternative to viral vectors for the delivery of nucleic acids, since they are non-pathogenic and non-immunogenic [3,4]. Non-viral vectors can be classified into two main groups: (a) lipoplexes, based on cationic lipids; (b) polyplexes, which are elaborated with cationic polymers. Although cationic lipids are the most studied vectors at the moment, natural cationic polymers have shown promising results and they are being studied as a less toxic alternative to lipid based non-viral systems [5]. Among natural polymers, chitosan (Ch) is one of the most studied polysaccharides [6–10] as it is biocompatible, biodegradable and non-toxic [11]. Chitin is a nitrogenous polysaccharide derived from the exoskeleton of crustacean, insects and fungi. It is the second most ubiquitous natural polymer after cellulose on earth and tones of chitin are produced as food industry waste each year [12], making it an economical and renewable material. However the use of chitin for biomedical uses is hampered as it is considered chemically inert and insoluble in water and organic solvents. Deacetylation of chitin leads to its main derivative, Ch a natural linear cationic polysaccharide that consists of β-D-glucosamine and β-*N*-acetyl-D-glucosamine subunits connected by a (1–4) glycosidic bond. The amine groups of the Ch have a pKa value of 6.5 [13–15], making it a pH responsive polymer, which is protonated at pH values below physiological pH. Thanks to the positively charged amine groups, it is possible to form polyplexes by electrostatic interactions with the negatively charged phosphate groups of DNA. The size of the Ch chain length and thus, the molecular weight (M_w) can be modified by a depolymerization process, which consists of the cleavage of glycosidic linkage between saccharide units [16]. Figure 1 shows a schematic representation of Ch manufacturing process.

Numerous factors affect the stability and transfection efficiency of Ch based vectors, being the most relevant ones the molecular weight (M_w) and the degree of deacetylation (DDA) of the polymer [17]. The M_w of the polymer is an important factor because it influences the particle size of the polyplexes, the binding affinity between the polymer and the nucleic acid, the cellular uptake, the dissociation of the DNA and thus, the transfection efficiency. For a successful non-viral gene therapy an optimized balance between the DNA protection and intracellular DNA unpacking is required [18]. Polyplexes elaborated with high molecular weight chitosans (HMWC) (>150 KDa) [10] show better DNA binding, which is beneficial for the protection of DNA. However, the release of the DNA cargo once inside the cell is restricted due to the high stability of the polyplexes. Polyplexes based on low molecular weight chitosans (LMWC) (<150 KDa) are less stable and thus, the protection of the DNA

seems to be less effective than the protection obtained with HMWC based polyplexes [19]. However, it is reported in the literature the ability of LMWC to form plasmid/LMWC complex as effectively as HMWC [20,21]. In addition, Kopping-Hoggard and colleagues reported the capacity of chitosan oligomers with a degree of polymerization (DP) 15 to 24 to retain pDNA, protect it against DNase degradation and efficiently release and deliver the intact transgene [22].

Figure 1. Schematic representation of low molecular weight chitosan (LMWC) manufacturing process.



DDA is the second physicochemical parameter of Ch that influences the transfection efficiency of the vectors. The DDA refers to the percentage of deacetylated primary amine groups in the Ch molecule backbone, and thus it determines the average amount of amines available to interact with nucleic acids, and thus, the positive charge of the polymer. High DDA (>90%) results in an increased positive charge, enabling the formation of stable polyplexes with a great binding capacity between the polymer and the DNA. Decreased DDA has been shown to reduce the strength of the electrostatic interactions and cause an easier release and degradation of the DNA [19]. Kiang *et al.* reported decreased overall *in vitro* gene expression levels with low DDA Ch due to the instability of the particles [23]. Consequently, taking both the M_w and the DDA into account, binding affinities between Ch and DNA should be modulated by using Ch with the appropriate M_w and DDA values, in order to obtain a balance between DNA protection and unpacking. In fact, there seems to be an optimal DDA value for each particular M_w chitosan, which indicates the coupling effect between the two parameters,

being the binding constant between Ch and DNA significantly influenced by M_w and DDA [24]. LMWC, particularly, presents more interesting characteristics for its clinical use, such as higher solubility at physiological pH [20]. In addition, LMWC have antimicrobial, inmunostimulant, antioxidant, and cancer growth inhibitory effects [25]. Therefore, this review will describe the formation, characterization, *in vitro* and *in vivo* transfection process and the future prospects of LMWC based polyplexes as promising non-viral vectors for gene therapy purposes.

2. Formation and Characterization of LMWC-pDNA Polyplexes

2.1. LMWC-pDNA Polyplexes Formation Process

The basic method for the formation of LMWC-pDNA polyplexes is the direct mixing of the positively charged Ch and negatively charged DNA, via a process mainly driven by electrostatic interactions. It has been reported that the mixing technique of Ch and pDNA and the incubation conditions influence the final gene expression, therefore, several groups have worked in the optimization of this process. According to Lavertu and colleagues the best mixing technique was found to be adding Ch over pDNA, pippeting up and down and tapping the tube gently [26]. Other authors, however, have reported that for the correct formation of LMWC-pDNA polyplexes a more vigorous vortex agitation is preferred [27–30]. The incubation time required for the correct formation of the polyplexes is another factor to consider. LMWC-pDNA polyplexes tend to aggregate after several hours of incubation due to the physical instability of aqueous suspensions over time [31,32]. Therefore, an incubation time between 15 and 30 min has been chosen by several authors as the optimal for the correct formation of the polyplexes avoiding the formation of aggregates [27,30,33,34]. Nevertheless, according to Lavertu *et al.* there were no observed differences in the gene expression with incubation times in the range of 30 and 120 min [26].

Much attention is paid to the mixing stoichiometry in the elaboration process of Ch based polyplexes. The N/P ratio is used to define the ratio between the protonable amines in the Ch backbone (N) and the negatively charged phosphate groups in nucleic acid. In order to ensure high transfection efficiencies, polyplexes elaborated at different N/P ratios are tested by the researchers. Generally, Ch-pDNA complexes are prepared using an excess of Ch *vs.* pDNA (N/P ratios above one). The influence of the N/P ratio on the physicochemical characteristics and transfection efficiency of the LMWC-pDNA polyplexes is described below (Section 3.5).

Based on the literature, the pH of the solution is a very important mixing parameter that affects Ch ionization and therefore physico-chemical properties (size, mass, density, morphology) [35] of the formed polyplexes. Apart from the pH, other parameters such as the ionic strength, and the mixing regime seem to be also important in the formation of the polyplex. The mixing concentration of nucleic acid has also a clear influence on the resulting polyplexes properties: size of polyplexes increase with the increment of the nucleic acid concentration. However, the two main structural parameters that affect the final properties of the formed polyplexes are the M_W and the DDA of the Ch, as it has been explained in the introduction section, being highly deacetylation LMWC the most suitable for gene therapy purposes [22,36]. Therefore, a more detailed study of their mechanisms of formation is needed to circumvent this situation, which will resolve several contradictory results reported in the literature regarding the influence of formulation parameters on the resulting polyplexes

properties [10]. There are also a significant number of works that present an incomplete description of the polyplex preparation method, which limits comparison with other studies.

In addition, Ch polyplexes, and non-viral gene delivery systems in general, are usually prepared by the manual mixing of small volumes of Ch and pDNA solution. Moreover, the mixing procedure is performed in diluted conditions in order to ensure a stable and homogeneous colloidal suspension. Thus, the clinical applicability of non-viral vectors is hampered, since the administrable volumes limit the deliverable dose. An approach to overcome this limitation is explained below (Section 5). Taking all this considerations into account, polyplexes elaboration must be carried out considering all the factors that can influence the final characteristics of the resulting polyplexes, in order to development the desired vector for gene delivery.

2.2. Physico-Chemical Characterization of LMWC-pDNA Polyplexes

LMWC-pDNA carriers are characterized by size, zeta potential, morphology, binding-affinity, buffering capacity and colloidal stability. These physicochemical characteristics are used to, somehow, predict the transfection efficiency of the polyplexes. The methods used for physicochemical characterization and the role of these physicochemical characteristics in the transfection process of LMWC-pDNA polyplexes is described below. In addition, the most commonly employed characterization techniques are summarized in Table 1.

Table 1. Characterization methods of low molecular weight chitosan (LMWC)/pDNA polyplexes.

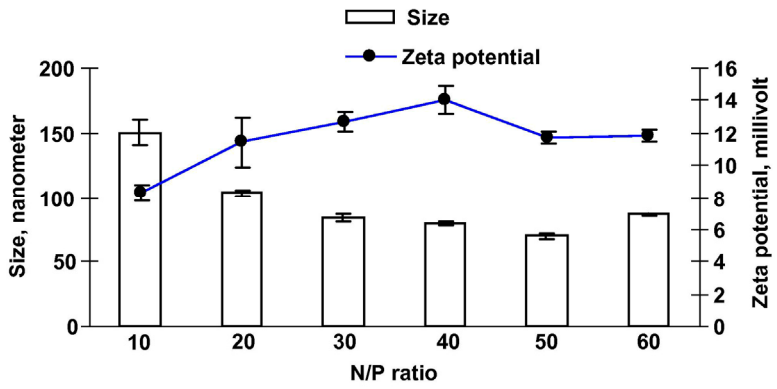
Characterization technique	**Parameter**
Acid-base titration	Buffering capacity
Agarose gel electrophoresis	Binding affinity Release capacity Protection against endonuclease Stability
Asymmetrical flow field-flow fractionation (AF4) coupled with light scattering	Size Stoichiometry
Atomic force microscopy	Size Morphology
Electronic microscopy	Size Morphology
Dynamic light scattering	Size Colloidal stability
EtBr displacement assay	Stability
Laser Doppler velocimetry	Zeta potential
Isothermal titration calorimetry	Binding affinity Stability
Nanoparticle tracking analysis	Size M_w Concentration
Orange II dye depletion assay (AF4 results confirmation)	Stoichiometry
Polyanion competition assay	Stability
Potentiometric titration	Buffering capacity
Static light scattering	M_w

2.2.1. Size

Typically, the size of Ch based polyplexes is measured by dynamic light scattering (DLS). It is described in the literature that Ch-pDNA polyplex size can range from a few ten nm [18] to a few hundred nm [30] depending on several factors such as the mixing concentration, the N/P ratio, the pH of the medium and properties of the chosen Ch and pDNA. Electron microscopy has also been employed to measure particle size, but smaller results in particle size are obtained compared to DLS. Some authors have attributed this increment in particle size by DLS characterization due to the fact that, particles are fully hydrated when characterized by DLS [37] or that they are not spherical [38].

The concentration of the mixing solutions of Ch and pDNA has a clear influence on the particle size. It is described in the literature an increase of polyplexes size with increasing the concentration of the nucleic acid solution [17,39]. According to the influence of the M_w on the polyplexes size, contradictory results are described in the literature. Koping-Hoggard *et al.* observed a size increase from 68 to 174 nm when chitosan Mw increased from 2 to 7 kDa for polyplexes prepared at N/P ratio of 60 [22]. Our research group has recently described that particle size of HMWC is around 4-hold higher than the size of polyplexes based on LMWC (5.3 kDa; 7kDa), prepared at N/P of 20 [30]. However, other authors have reported a decrease in size of polyplexes with increased Ch M_w [19]. These differences in the results could be explained in part due to the fact that different methods have been employed for the preparation of the polyplexes. In relation to the N/P ratio, it is no clear how it affects the particle size of Ch based polyplexes. Some authors have reported that, as the N/P ratio increases, so do the ability of the Ch to complex and compact the pDNA, and thus a decreased size is observed. Nevertheless, our research group has not found a significant influence of the N/P ratio on the size of polyplexes elaborated with oligochitosans, probably because both aforementioned effects equilibrate the final particle size (Figure 2) [29,30,33]

Figure 2. Effect of N/P ratio on the size of oligochitosan/pDNA polyplexes suspended in HEPES medium 10 mM, pH 7.1. Reproduced from [33] with permission from Elsevier.



The particle size of the polyplexes will determine the ability of the vectors to reach various organs. For example, particles smaller than 100 nm are required to cross liver fenestrae and target hepatocytes, while particles with 200 nm will be trapped in the lung capillaries [40]. For this reason, polyplexes with the appropriate sizes must be elaborated in order to deliver the genetic material to the desired target organ.

2.2.2. Zeta Potential

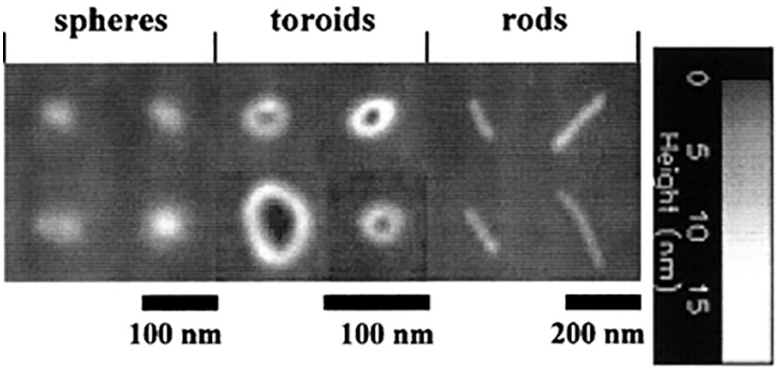
The zeta potential of the nanometric polyplexes is measured by laser doppler velocimetry (LDV) and it indicates the value of the polyplexes surface charge. This parameter determines the colloidal stability of the polyplexes aquous suspension and it plays a crucial role in the viability, cellular uptake and transfection efficiency of LMWC-pDNA polyplexes, as it is widely reported in the literature [30,41].

Several factors influence the zeta potential of LMWC based polyplexes and therefore, they should be modulated in order to obtain the desired surface charge. Since the pKa of Ch is about 6.5, the pH strongly influences the zeta potential of Ch based polyplexes. As reported in the bibliography, an increment of the surface charge is observed when the pH of the medium is decreased from 7.4 to 6.5. Recently, our research group has described an increase of around 6 mV in the zeta potential of ultrapure LMWC [30,41]. Considering the zeta potential has an important influence on the cellular uptake of the vectors, as it is explained below, much attention must be paid to the elaboration of the polyplexes in order to get the appropriate surface charge.

2.2.3. Morphology

Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM) have been extensively used to determine the morphology of LMWC based polyplexes. According to the bibliography, Ch-pDNA polyplexes can adopt different structures, depending mainly on the fractional content of acetylated units and the degree of polymerization [42]. Some authors have reported the spherical shape of the complexes [33,43] while toroids, rodlike and globular particles have also been developed [38,39]. Danielsen *et al.* reported that the interactions are important for determining the shape of the polyplexes. Moreover, they revealed that Ch with high and low DP yielded the same structure, but higher amounts of the low DP Ch were needed for this purpose [42]. Figure 3 shows the different morphological shapes that LMWC based polyplexes can adopt.

Figure 3. Spherical, toroidal and rod shapes of polyplexes base on fractionated chitosan oligomers. The polyplexes were formed at a concentration 13.3 μg/mL and a charge ratio of 60:1 and examined in the atomic force microscope. The horizontal bars indicate 100 nm (spheres and toroids) and 200 nm (rods). The vertical bar gives the height of the polyplexes. Reproduced from [22] with permission from Nature.

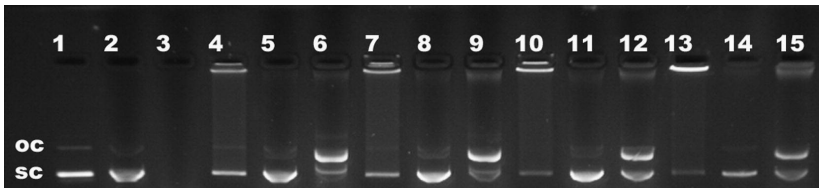


2.2.4. Binding Affinity

The binding affinity between LMWC and pDNA determines the ability of the polymer to complex, release and protect the nucleic acid from degradation, and consequently the transfection efficiency of the corresponding polyplex.

Several techniques have been employed in order to establish the binding affinity of LMWC for DNA. One of the most commonly used methods to qualitatively analyze this parameter is the gel retardation assay. This type of electrophoresis-based assay has been used to monitor the pDNA dissociation from polyplexes formed at different N/P ratios (Figure 4). As reported in the bibliography, LMWC-pDNA polyplexes with low DDA elaborated at low N/P ratio are less stable, and thus they dissociate the pDNA more easily [18,22]. However, the ability of LMWC to complex, release and protect the DNA has been established by several authors [29,34]. Ethidium bromide (EtBr) displacement is another method that indirectly measures the binding affinity between LMWC and DNA. EtBr intercalates between the base pairs of the DNA double helix yielding a highly fluorescent DNA. Upon the addition of the polycation, EtBr is expelled from the DNA, which results in a decrease of fluorescence. The displacement degree of EtBr provides information about the relative strength of the interaction between the polycation and the pDNA [27].

Figure 4. Binding efficiency between oligochitosans and DNA at differnet N/P ratios, and protection capacity from DNase I enzymatic digestion visualized by agarose gel electrophoresis. OC: open circular form, SC: supercoloid form. Lanes 1–3 correspond to free DNA; lanes 4–6, N/P 5; lanes 7–9, N/P 10; lanes 10–12, N/P 20; lanes 13–15, N/P 30. Polyplexes were treated with sodium dodecyl sulfate (SDS) (lanes 2, 5, 8, 11 and 14) and DNase I (lanes 3, 6, 9 and 15). Reproduced from [29] with permission from Elsevier.

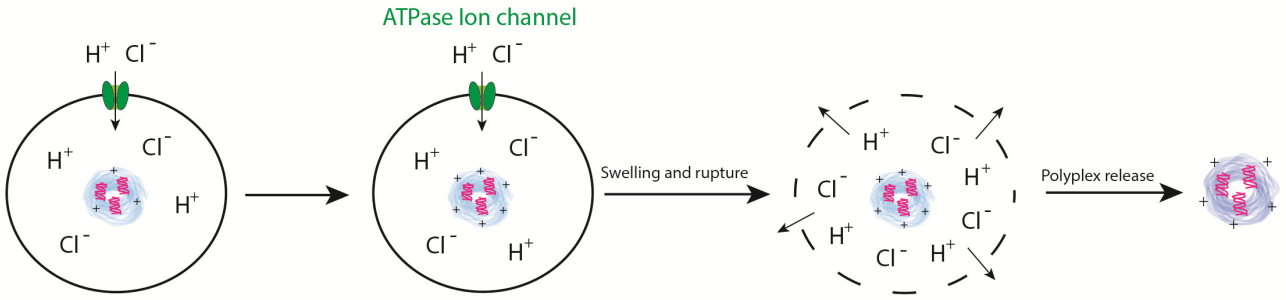


Another technique employed to quantify the binding affinity of Ch to pDNA is the isothermal titration calorimetry (ITC) combined with the single set of identical sites model (SSIS). This technique allows the quantification of the binding constants of Ch to DNA, which is influenced by the pH, the ionic strength and structural properties of Ch [24].

2.2.5. Buffering Capacity

It has been reported that the mechanism by which polyplexes escape from the endosome, once inside the cell, is the commonly referred *proton sponge effect*, which is determined by the buffering capacity of the Ch. This mechanism is based on the acidification of the endosome or lysosome by the pumping of protons accompanied by the influx of chloride ions that compensates the proton influx into the vesicle. This ion influx causes an increase in the osmotic pressure inside the vesicle and thus a swelling process ruptures the endosome/lysosome, allowing the escape of the polyplexes before being degradated (Figure 5).

Figure 5. Schematic representation of the proton sponge effect that takes place after the cellular uptake of the polyplexex through an endosomal pathway.



The buffering capacity of Ch has been determined by acid-base titration assay [30,44]. Some authors have concluded that it is unlikely that Ch escape the endosome by proton sponge effect due to its low buffering capacity compared to other cationic polymers with high buffering capacity, such as polyethilenimine (PEI). However, Bushcmann *et al.* have recently reported that comparing Ch and PEI in a molar charge basis, instead of in a mass concentrations basis, the natural polymer has a larger buffering capacity than PEI in the endosomal/lysosomal relevant pH ranges [45]. Interestingly, our research group has established a M_w dependent buffering capacity, being significantly higher for oligochitosans compared to HMWC, which could explain in part the flattering properties of LMWC for the transfection purposes when compared to their HMWC counterparts [30].

2.2.6. Colloidal Stability in Physiological Conditions

One of the main drawbacks related to LMWC-pDNA polyplexes is their poor colloidal stability in physiological conditions [46]. Thus, the presence of polyanions such as blood proteins, heparin or glycosaminoglycans (GAG) in the body may cause the dissociation of the polyplex and the early release of the pDNA before it reaches the desired target tissue [47,48]. For this reason, researchers have focused their attention in the development of more stable vectors, as it is the case of modified Ch (mCh) based polyplexes (mCh-pDNA).

For the characterization of the colloidal stability, the hydrodynamic size is measured along the time, in different mediums that mimic physiological conditions. It has been reported that the size of LMWC-pDNA polyplexes increased from 100 nm to more than 1 micron after 1 h of incubation in phosphate buffered saline (PBS) [46]. In addition, bovine serum albumin (BSA)-induced aggregation of polyplexes has been assessed by measuring changes in the turbidity of the solution after their incubation with BSA. A significant increase in turbidity was observed after the incubation of Ch-DNA polyplexes with 2 mg/mL BSA. However, lactosylated-Ch based complexes were stable even after the incubation for 24 h [49].

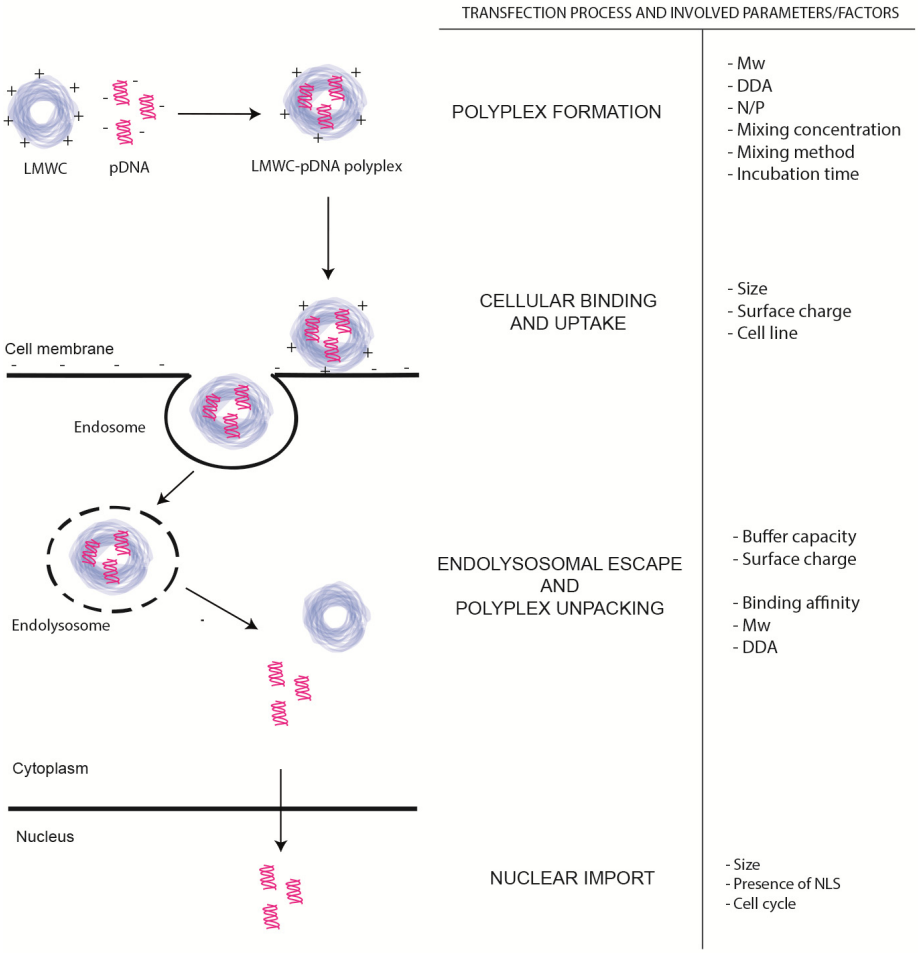
The limited colloidal stability of LMWC based polyplexes may influence their behavior *in vivo*, especially after systemic administration. In order to improve the stability and increase the circulation lifetime of the polyplexes, PEGylation [50], quaternization [51] and glycolyzation [52] of the polymer have been proposed.

3. *In Vitro* Evaluation of LMWC-pDNA Polyplexes Transfection Process

Transfection efficiency refers to the ability of a pDNA carrier, LMWC based polyplexes in this case, to induce transgene expression. Most researchers have focused their attention in the determination of the final gene expression, employing traceable reporter genes such as genes that express Green fluorescente protein (GFP), luciferase and galactosidase. One of the most commonly employed techniques to evaluate the transfection efficiency of the non-viral vectors is flow cytometry. This technique allows the quantitative assessment of the percentage of cells expressing a fluorescent transgene, such as GFP. Moreover, the quantification of luciferase activity by luminometry provides the level of gene expression. Qualitative techniques based on microscopy are also valuable to determine the expression of the codified reporter protein.

The transfection process is a complex multi-step process influenced by several parameters (Figure 6). Previous to the protein synthesis, there are several barriers that the non-viral vectors must overcome: binding to the cell membrane and uptake, endolysosomal escape and polyplex unpacking and finally, the import of pDNA into the nucleus. Apart from the determination of the final gene expression, considerable attention must be paid to these intracellular barriers to understand the possible limitations of LMWC based polyplexes in order to improve the design of these promising polymers for gene therapy applications.

Figure 6. Schematic representation of the transfection process and the parameters/factors involved in each step.

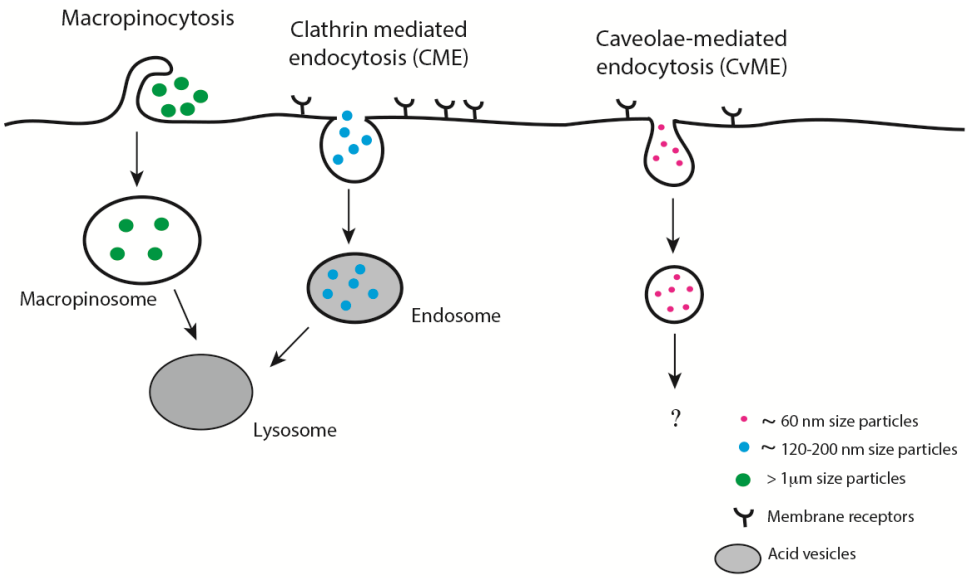


3.1. Cellular Binding and Uptake

Once in the proximity of the target cells, the polyplexes are bound to the cell surface. Unless the polyplexes are modified with a specific targeting ligand, the binding of polyplexes to the cell membrane occurs by unspecific electrostatic interactions between the positive charge of the polyplexes and the negative charge of the cell surface [53]. The microenvironment around the target cells has to be considered, since it has been described that acidic environments favor the cellular binding and uptake of LMWC-pDNA polyplexes due to an increase in the zeta potential value of the polyplexes at low pH values as a consequence of the protonated amines [30,41,54].

Polyplexes enter the cell mainly via endocytosis. Five types of endocytic pathways have been reported: phagocytosis, macropinocytosis, clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME) and clathrin- and caveolin- independent endocytosis (Figure 7) [55]. The usage of phagocytosis and macropinocytosis seems very limited, as it only exists in specialized cells such as macrophages monocytes, dendritic cells or antigen presenting cells. Thus, CME and CvME are the best-characterized types of endocytosis in non-viral gene therapy field, although CvME pathway is less well understood [56]. It is described that LMWC based polyplexes could enter the cell via these two endocytic pathways, however, there is a lack of consensus in the scientific area [53,57]. The diversity that exists in the published results may be attributed to the fact that many factors determine the uptake pathway of Ch-pDNA polyplexes. Depending on the particle size, endocytosis will occur by one endocytic route or another. It is described that small particles (around 60 nm) are internalized through CvME, whereas intermediate (120 nm) and larger (around 200 nm) particles are taken up by CME or macropinocytosis (>1 μm) [58]. The surface charge also affects the cellular uptake level of the polyplexes. Positively charged polyplexes will interact efficiently will the cell membrane and favor the internalization [54]. Finally, the endocytic pathway is cell type dependent. Depending on the variations on the membrane phenotype and the types of receptor, each cell line will take up the complexes via different endocytic routes. According to Douglas *et al.* Ch polyplexes (around 150 nm) are internalized via CME and CvME in 293T cells, but only via CvME in Chinese hamster ovary (CHO) cells [59].

Figure 7. Schematic representation of different endocytic pathways involved in gene delivery.



Although internalization of the polyplexes is the first cellular barrier, is has been shown not to be the unique key step in the transfection process. Several authors have reported no correlation between polyplex uptake and transfection efficiency [18,30,53] indicating that internalized polyplexes must overcome other important barriers previous to gene expression.

3.2. Endolysosomal Escape and Polyplex Dissociation

The endocytic pathway whereby the polyplexes are internalized will determine the intracellular route of the corresponding non-viral vector and therefore the transfection efficiency. Regardless of the mechanism of internalization, polyplexes must escape from the endosome before being degraded in the lysosome. Polyplexes that enter the cell via CME are confined within endosomes that will suffer a maturation process involving the compartment acidification resulting in late endosomes and finally, lysosomes [56]. The release of the pDNA cargo from these vesicles seems to be the bottleneck in the transfection process [57], since the acidic environment inside lysosome may lead to the degradation of the pDNA if it does not escape from the endosome on time. However, routing through endolysosomal pathway might be essential for endosomal escape of Ch based vectors. It has been described that the endolysosomal escape of LMWC based polyplexes occurs by the "proton sponge" mechanism (described in Section 2.2.5), which is only possible when the vectors are internalized through acidic vesicles, as it is the case of CME. Another way for endosomal escape of Ch based vectors is membrane destabilization. Acidic environment of the endolysosome increases the charge of the polyplex causing anionic phospholipids in the endosomal membrane to flip from the cytoplasmatic face to the intra-endosomal face. This provokes a membrane disorganization allowing the escape of the polyplexes [60]. Non-endosomal pathways, such as CvME, deprive the opportunity for pH-responsive polymers, like Ch, to promote membrane destabilization [61] or endosomolysis by proton sponge effect. Thus, the polyplexes are entrapped inside the vesicle, preventing the release of the pDNA and leading to its degradation [62].

DNA unpacking is an important rate-limiting step in the transfection process of Ch based polyplexes. Thibault *et al.* revealed that the kinetics of polyplex dissociation is the most critical formulation-dependent intracellular process, indicating the relationship between Ch physicochemical characteristics (M_w and DDA) and transfection efficiency [53]. A high affinity between Ch and pDNA may be a limiting step in the successful dissociation of the plasmid from the carrier. At this point, LMWC offer an advantage over HMWC, since the polyplexes formed with the lower M_w polymers tend to form less stable polyplex leading to an easier unpacking of the DNA.

3.3. Tools for the Study of Uptake Pathways

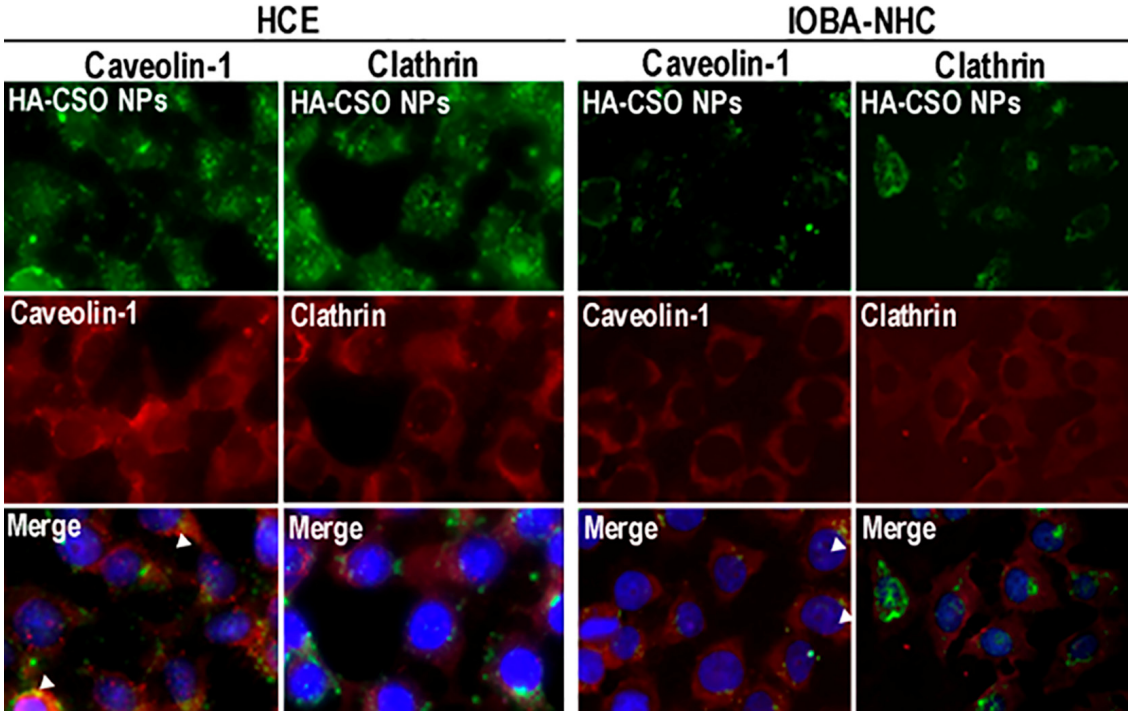
The study of the uptake mechanisms a mandatory issue for the design of an adequate non-viral vector, as it will determine their intracellular fate and thus they final gene expression level. Nowadays, several tools are available for the study of the uptake pathways of a determinate pDNA carrier.

The use of endocytosis inhibitors is an effective way to determine if a specific endocytic pathway plays an important role in the uptake of the polyplex to be examined. Firstly, endocytosis must be distinguished from non-endocytic pathways. The most direct way to determine if the complexes are being endocyted is to use the energy depletion method (lowering the incubation temperature of the cells

to 4 °C), since most endocytic pathways are energy dependent [57,63]. To distinguish the phagocytic and macropinocytic pathways from CME and CvME, the most commonly used inhibitors are amiloride (inhibitors of sodium-proton spongue) and wortmannin (inhibitor of phosphoinositide). CME could be inhibited by using hypertonic medium, cytosol acidification or chlorpromazine drug. According to CvME, genestein, nystatin and filipin are the most commonly used specific inhibitors [57,63,64]. Inhibitors for the study of intracellular fates of complexes are also available. Monensin, bafilomycin A prevent the maturation and fusion of endosomes into lysosomes by inhibiting the acidification of the endosome. Moreover, cloroquine can be used to swell and disrupt endocytic vesicle by osmotic effects [65].

Molecular probes, markers and dyes can also be used to corroborate the information obtained with the cellular uptake pathways inhibitors. Transferrin is commonly used as a probe of CME and so is cholera toxin beta subunit for CvME. These can be combined with fluorophores in order to visualize them under a fluorescent microscope. The colocalization of these probes with the fluorescently labelled polyplexes can provide information about the internalization route of the corresponding vector (Figure 8). Organelle specific dyes such as Lysotracker and Lyso Sensor (lysosomal dyes) and Cell light (dyes for early endosomes) are other ideal tools for the detection and colocalization of the intracellular trafficking of the non-viral vectors.

Figure 8. Caveolin-1 and clathrin immunofluorescence in HCE and IOBA-NHC cells after incubation with hyaluronic acid-chitosan oligomer-based nanoparticles (HA-CSO NP). Merged images showed co-localization of HA-CSO NP with caveolin (staining at arrowheads). Reproduced from [64]. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium.



3.4. Nuclear Import

The last barrier before gene expression is the internalization of the pDNA across the nuclear membrane. The nuclear membrane is composed of a double lipid bilayer with tightly regulated aqueous channels forming the nuclear pore complex (NPC). The NPC has 10 nm of diameter in its relaxed state, which allows the passive diffusion of small molecules. However, active transport of larger molecules is possible thanks to the recognition of nuclear localization signals (NLS). Particle size of LMWC based polyplexes is generally >100 nm, therefore, it is unlikely that they diffuse through the nuclear membrane. Therefore, the incorporation of NLS to the polyplex formulation is a strategy that can be followed in order to enhance the internalization of the pDNA to the nucleus.

However, nuclear delivery can still take place during the cell division process. It is well described that the transfection efficiency correlates with cell division and cell growth [66]. Thus, the transfection efficiency in proliferating cells is supposed to be higher than in cells that do not undergo mitosis regularly. At this point, the nuclear import is a poorly characterized step in the transfection process, which should be further investigated in order to better understand the exact mechanisms whereby LMWC-pDNA polyplexes are internalized.

3.5. Factors Involved in the Transfection Efficiency of LMWC based Polyplexes

Transfection efficiency of highly deacetylated LMWC based vectors is influenced by several formulation parameters such as, serum content, pH of the transfection medium, N/P ratio, pDNA concentration, and also by the employed cell type. These parameters will define the physico-chemical characteristics of the polyplexes (described in Section 3.3) and thus the transfection efficiency.

There is no consensus in the scientific community regarding the influence of serum in the transfection efficiency of LMWC based polyplex. According to some authors, the presence of serum enhances the transfection efficiency of Ch based polyplexes [41,67,68]. The reason for the increment of transfection in the presence of serum is not clear. Some authors argue that serum content might improve the cell function including cell division and endocytosis. Nimesh *et al.* found an increment in the cellular uptake when cells where expose to polyplexes with serum content, probably because the Ch forms small hydrodynamic diameter complexes with the negatively charged serum proteins which can be efficiently uptaken [41]. Although several studies have demonstrated the advantage of chitosan based vectors for transfecting cells in serum containing medium, there are still some authors that preferred to use serum free Opti-MEM (full name) transfection medium, making clear the controversy that exists about the influence of the serum content on the transfection process [28,29].

Another important parameter to consider is the pH of the transfection medium. Ch is a pH responsive polymer, which means that its charge density depends on the pH value of the solutions. It is well described in the scientific literature that medium acidification enhanced the cellular uptake and the transfection efficiency of LMWC based polyplexes [30,41]. Our research group has recently reported that medium acidification from 7.4 to 6.5 increases the zeta potential and decreases the size of ultrapure oligochitosan bases polyplexes. Positively charged complexes can efficiently bind to negatively charged cell membranes facilitating the cellular uptake and improving the transfection efficiency. These results suggest the possible application of this kind of vectors in cells with an acidic environment, such as tumor cells.

The N/P ratio used to elaborate the polyplex also influences the transfection efficiency. The polyplexes must be stable enough to retain the pDNA, but they must be able to release the cargo once inside the cell. The required N/P ratio to obtain this balance between DNA packing and release will depend on the Mw and DDA of the used Ch. Lavertu *et al.* reported that LMWC would require a higher N/P ratio to completely condense DNA [69].

4. Chemical Modifications of Ch to Overcome Transfection Barriers

In order to ensure an efficient transfection process, the elaborated non-viral vectors must overcome all the biological barriers described above: cellular binding and uptake, endolysosomal escape and nuclear import. Apart from the biological difficulties, the enhancement of the complexes colloidal stability is a key issue for high transfection efficiencies.

With the aim of addressing the obstacles previously mentioned, LMWCs could be modified by several ways. Among the modification reactions, chemical grafting of molecules or polymers on the C2 amine and the C6 hydroxyl are the most usual. Although the C6 hydroxyl is reactive and easy to be functionalized, modifications on the C2 amine are normally employed in a large number of research works.

The principal drawback in the use of chitosan's amine functional groups for modification is that this chemical reaction may generate steric hindrance and decrease the number of ionisable amines that bind to pDNAs. Thus, the nonbonding pair of electrons on the primary amine of the chitosan is nonetheless a good candidate for nucleophilic attack and it allows several reactions. Thus, quaternization, amidation and reductive *N*-alkylation are the main chemical reactions used to overcome the barriers in the transfection process.

Quaternization of chitosan has been performed to improve solubility at physiological pH. For this purpose two methods of quaternization have been performed: N-trialkylation usually with CH_3I [51,70]. Another technique based on glycidyltrimethylammonium chloride reaction is also used for Ch quaternization [71].

Amidation is another widely employed technique to graft molecules and polymers to the amine group of chitosan. It can be achieved using carboxylic compounds with carbodiimides such as EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) or DCC (dicyclohexyl carbodiimide) coupled with NHS (*N*-hydroxysulfosuccinimide) to accelerate the reaction [72]. Amidation of chitosan could overcome several drawbacks related to the transfection process, depending on the chosen molecule. In this respect, the grafting of molecules such as urocanic acid, imidazole-4-acetic acid and histidine [73–75] could improve the endosomal escape of Ch based vectors. Moreover, grafting Ch with arginine has been proven to increase cell uptake and PEG-succinimidyl ester or Folate–PEG–COOH molecules could enhance pharmacokinetics [76]

Finally, the reductive *N*-alkylation method serves to graft an aldehyde derivative on the amine of Ch. Grafted species include fluorescent probes as 9-anthraldehyde [77], phosphorylcholine [78,79], oligosaccharides [18,46,52,80] and dextran like polymers [81]. Normally, reductive *N*-alkylation is directed to reduce cytotoxicity and increase transfection efficiency. In the case of dextran molecule, it is used with the aim of improving complexes stability. Succinoyl groups have also been grafted to Ch for gene delivery applications [82].

5. Freeze-Drying of Polyplexes

The poor long-term stability of aqueous suspensions [31] of polyplexes is one of the major limitations for their wide scale clinical application. Aggregation of polyplexes over time provokes a loss of transfection, and thus a fresh preparation of the polyplexes prior to their administration is required. However, day-to-day preparation of the vectors leads to batch-to-batch variations in the product quality, safety and transfection rates [83]. Thus, the development of stable and transfection competent polyplexes is an important step from a promising technological formulation to its clinical application.

Freeze-drying or lyophilization is the most common method for preparing dry formulations, which ensures a long-term conservation of the preformed complexes [32,84,85]. Freeze-drying process can be divided in three steps: freezing, primary drying (ice sublimation) and secondary drying (desorption of unfrozen water). These are stress factors that could cause damage either in the polymer or in the DNA. The final lyophilized product should preserve the original chemical and physical characteristics and be stable over time. In order to avoid the possible damages caused by the freezing or drying processes, cryo/lyo protective agents are generally added to the complexes. The most popular cryoprotectants reported in the literature are sugars such as, trehalose, sucrose, glucose and mannitol, which are also called stabilizers [86].

Compared to *in vitro* studies, clinical trials and *in vitro* experiments require higher doses of DNA, formulated in a volume suitable for each administration. It is well know that the mixing conditions of the polyplex components play an important role in the final characteristics of the non-viral vector, and is very complicated to elaborate formulations at high concentrations with the conventional preparation methods of polyplexes. The use of polyplexes *in vivo*, and of course in clinical trials can be hampered if the dose needed to obtain a therapeutic effect is very high, due to the low concentration of the polyplexes suspension obtained from a diluted polymer and plasmid solutions. Thus, polyplex suspensions need to be concentrated. At this point, lyophilization could be considered as an advantageous method to concentrate vectors suspensions and thus obtain higher pDNA concentrations, as the final dried formulation can be reconstituted in reduced volumes of the desired diluent [87,88]. Up to now there are not many works related to the lyophilization of LMWC-pDNA polyplexes, thus, according to the positive results obtained with other complexes, the possibility of freeze-drying LMWC based vectors should be considered.

6. *In Vivo* Applications of LMWC based Vectors for pDNA Delivery

Once the polyplexes are elaborated, characterized and their transfection efficiency is proven *in vitro*, the next step is to determine the efficiency and toxicity of the vectors *in vivo.* Due to its well-characterized biocompatibility and biodegradability, LMWC has been widely used for *in vivo* gene therapy purposes (Table 2). In addition, Ch is a highly mucoadhesive polymer, which makes it an excellent candidate for ocular and lung delivery of nucleic acids [89,90].

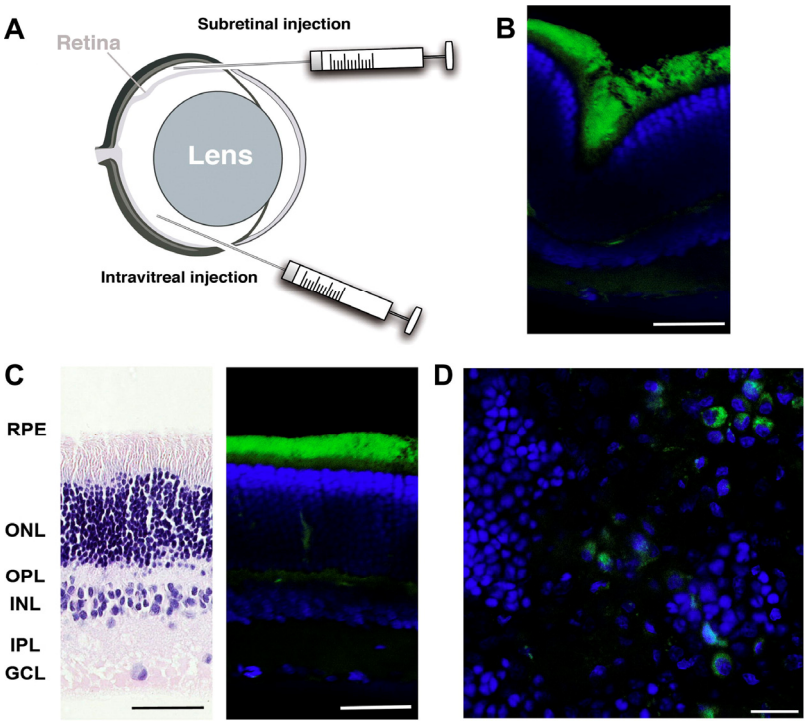
6.1. Ocular Delivery

The ocular system is a privilege organ for the localized delivery of non-viral vectors to specific ocular tissues, and it is affected by many well understood genetic-based diseases. Several research

groups have investigated the potential use of LMWC based vectors for the delivery of pDNA to different ocular tissues.

Novafect O15 and O25 oligochitosans have been used to transfect corneal and retinal cells *in vivo*. Klausner *et al.* injected Ch-DNA nanoparticles, elaborated with various plasmids with different characteristics, to the corneal stroma. They found that these LMWC based polyplexes were able to transport pCpG-GFP plasmid to keratocytes in the cornea, and that the cells could efficiently express high levels of GFP [28,34]. Our research group has also used Novafect O15 and O25 for the delivery of pDNA to the retina. The study showed for the first time that polyplexes based on these LMWC at N/P ratio of 10 are able to transfect different cells of the retina rats depending on the administration route. After subretinal injection of the polyplexes gene expression was observed mainly in the cells of the retinal-pigmented epithelium (RPE). Polyplexes were also administered by intravitreal injections, obtaining GFP expression in the ganglion cell layer and in the inner layers of the rats' retina (Figure 9) [29,33]. Bioadhesive hyaluron-Ch nanoparticles loaded with a reporter plasmid encoding the enhanced green fluorescent protein (pEGFP) were topically administrated to the cul-de sac of rabbits. Positive transfection results were observed in the corneal epithelium for up to 7 days [91].

Figure 9. *In vivo* gene expression of GFP after administration of oligochitosan/DNA polyplexes at N/P ratio 10 to rats. (**A**) Schematic drawing of the subretinal and intravitreal injection. (**B**) Cross-section of a treated retina close to the place of the subretinal injection. Enhanced green fluorescent protein (EGFP) expression with Hoechst 33342 staining for cell nuclei. Scae bar = 50 µm. (**C**) Hematoxylin-eosin rat retina cross-section, showing the different layers of the retina. RPE (Retinal Pigment Epithelium layer), ONL (Outer nuclear layer), OPL (Outer plexiform layer), INL (Inner nuclear layer), GCL (Ganglion cell layer) and fluorescent microscopy image of a 5-µm treated retina cross-section. Scale bar = 50 µm. (**D**) Whole-mount views of several sections of the retina focused at the RPE layer. Scale bar = 100 µm. Reproduce from [33] with permission from Elsevier.



6.2. Lung Delivery

Non-viral gene delivery to the lung holds therapeutic potential for the treatment of diseases such as cystic fibrosis and lung cancer. The mucoahdesive properties of Ch offer advantages for the delivery of pDNA to this target tissue. One of the most non-invasive approaches to target the lung is via inhalation. Mohammadi *et al.* nebulized Ch-DNA nanoparticles attached to Fibronectin Attachment Protein of Mycobacterium bovis (FAP-B) for the delivery of pGL3-control plasmid encoding lucifrase to mice lung. They reported high levels of luciferase gene expression in the dissected lungs after 48 h [92].

Intratracheal administration, a more invasive route, has also been used to target the lung. According to Kopping-Hoggard *et al.* LMWC based polyplexes showed a 10–20 fold higher *in vivo* transfection efficiency compared to HMWC based vectors, after intratracheal administration. This occurs as a result of the weaker association of pDNA to Ch and the retained capacity of the polymer to protect the plasmid against DNase degradation, enabling an efficient release and delivery of the intact transgene [22]. Trisaccharide-sustituted Ch oligomers have been also administered via the trachea, observing luciferase gene expression in the mouse lung 24 hours post administration [52].

6.3. Other Delivery Routes

The nasal delivery has attracted researcher's attention since it has been established that nasally administered DNA vaccines can induce effective humoral and cellular responses. In addition, it is a non-painful administration route, which could increase patient compliance. Yang *et al.* investigated the effect of Ch M_w on *in vivo* delivery efficiency of the plasmid encoding the Human cholesteryl ester transfer protein (CETP). They found that nasal vaccination of LMWC/pDNA polyplexes elicited specific anti-CET IgG antibodies whose presence lasted for more than 21 weeks [93]. More recently, mannosylated Ch nanoparticles have been administered by the nasal mucosa route to elicit serum anti-GFP IgG anitibodies and inhibit the growth of gastrin-releasing peptide (GRP) dependent tumor cells. The results confirmed that these Ch based carriers are feasible in DNA vaccine delivery and they offer a possibility for efficient tumor immunotherapy [94].

Other administration routes such as the subcutaneous (SC) and intramuscular (IM) have also been used for the delivery of pDNA. Jean *et al.* found that SC administration of Ch/pVax1-4sFGF-2 or Ch/pVax1-PDGF-bb complexes lead to the expression and distribution of high levels of FGF-2 and PDGF-BB recombinant proteins in serum, and the IM administration induced a rapid production of specific antibodies, which is promising for the development of prophylactic or therapeutic vaccines [95]. The same research group described the ability of Ch based complexes to deliver the pVax1-GLP-1 plasmid encoding the native recombinant GLP-1 protein in a diabetic rat model [96].

In relation to cancer gene therapy, the most direct approach to achieve the expression of genes in tumor tissues is the injection of polyplex solution into the tumor foci. Ch/pCMV-Luc polyplexes have been intratumorally administered to subcutaneously generated tumors, resulting in acceptable transfection efficiencies [97]. Finally, transfection efficiency of LMWC based polyplexes has also been investigated after their systemic administration. This route allows the possibility of targeting concrete organs and and tissues that may not be reached via a local administration. Zhang *et al.* elaborated hydrophobically modified LMWC vectors for the delivery of pCMV-Luc reporter gene. After 3 days of the systemic administration in the tail vein, a very strong expression of the gene was

observed in the kidney of the treated mice (Figure 10) [98]. LMWC functionalized with bPEI and conjugated with tuftsin for macrophage targeting has also been proven to be an adequate carrier for the systemic administration of pDNA. An increased gene expression was observed in mice spleen, heart and brain 7 days post administration of the polyplexes, compared to HMWC based counterparts [99].

Figure 10. Gene expression in mice kidneys after systemic injection LMWC/pDNA complexes. Complexes of pUC 19 DNA with (**A**) LMWC; (**B**) 3% hydrophobically modified LMWC; (**C**) 18% hydrophobically modified LMWC and (**D**) branched PEI at N/P 5 were injected into the tail vein of the mice. The kidneys were harvested 3 days after administration. (**E**) Naked plasmid DNA injected as a control. Magnification, ×200. Reproduced from [98] with permission from Wiley.

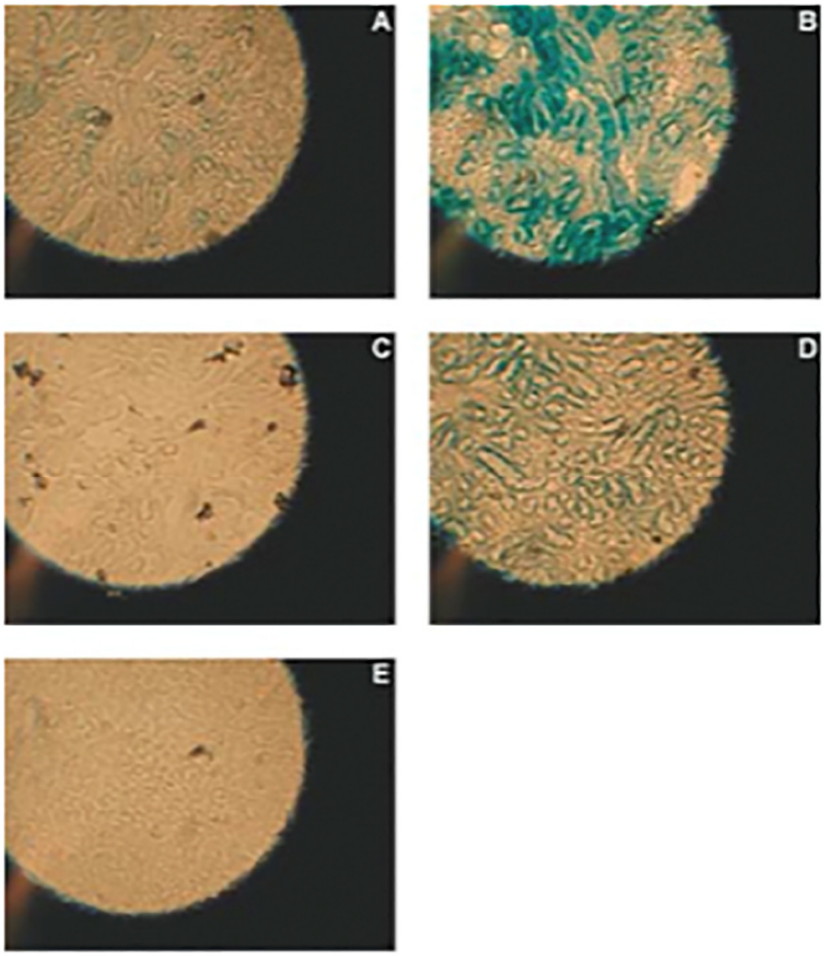


Table 2. Selected studies using LMWC based polyplexes for pDNA delivery.

Route of Administration	Animal Model	Chitosan	pDNA	Comments	Objective	Reference
Corneal injection	Sprague-Dawley rats	Novavect O15 (5.7 kDa, DDA 99%); Novafect O25 (7.3 kDa, DDA 99%)	gWiz-Luc (luciferase encoding reporter gene) 1.5 μg; gWiz-GFP (GFP encoding reporter gene) 1.5 μg	–	Treatment of acquired and inherited corneal disorders	[27]
Corneal injection	Sprague-Dawley rats	Novafect O15 (5.7 kDa, DDA 99%)	gWiz,-Luc, pCpG-Luc, pEPI-CMV, pEPI-UbC (encoding for luciferase) 1.5 μg; gWiz-GFP, pCpG-GFP (encoding for GFP) 1.5 μg	–	Treatment of acquired and inherited corneal disorders	[33]
Subretinal, intravitreous injection	Sprague-Dawley rats	Novafect O15 (5.7 kDa, DDA 99%)	pCMS-EGFP reporter gene (100 ng)	–	Treatment of retinal disorders	[28]
Subretinal, intravitreous injection	Sprague-Dawley rats	Novafect O25 (7.3 kDa, DDA 99%)	pCMS-EGFP reporter gene (100 ng)	–	Treatment of retinal disorders	[32]
Topic administration	Rabbits	Ultrapure Ch hydrochloride salt (113 kDa)	pEGFP reporter gene (25 μg, 50 μg, 100 μg)	Ch was mixed with HA salt, and NPs were prepared by ionotropic gelification	Treatment of ocular diseases	[89]
Aerosol	Balb/c mice	Ch Chitoclear (126 kDa, DDA 98%)	pGL3-control plasmid encoding luciferase	Electrostatially formed polyplexes were conjugated with FAP-B	Lung targeting	[90]
Intratracheal administration	Balb/c mice	UPC, Protasan UPG 210	gWiz-Luc, pCMV-Luc (luciferase encoding reporter genes) 5 μg, 10 μg, 25 μg	Fully deacetylated Ch was depolymerized to obtain oligomers with number average DPn 25 and 18	Lung targeting	[21]
Intratracheal administration	Balb/c mice	Fully de-*N*-acetylated Ch (3.6–7 kDa)	gWiz-Luc, pCMV-Luc (luciferase encoding reporter genes)	Ch oligomers were substituted with trisaccharides, obtaining oligormes with 7%, 23%, 40% of substituted amines	Lung targeting	[50]

Table 2. *Cont.*

Route of Administration	Animal Model	Chitosan	pDNA	Comments	Objective	Reference
Intranasal administration	Sprague-Dawley rats	Ch M_w: 5, 173 kDa	pEGFP-C3 encoding GFP; pDNA encoding CETP-C	–	Immunotherapeutic DNA vaccine for atherosclerosis treatment	[93]
Intranasal administration	C57BL/6 mice	Ch M_w: 115 kDa, DDA 95%	pGRP (0.5 mg)	Ch was conjugated with D-mannose	Production of anti-GRP IgG and inhibition of tumor growth	[94]
Intramuscular and subcutaneous administration	Balb/c mice	Depolymerized Ch 92–10 and 80–80 (M_w-DDA)	pVax1-4sFGF-2 and pVax1-PDGF-BB (encoding for FGF-2 and PDGF recombinant proteins)	–	Enhancing the repair of cartilage lesions or enhancing bone defect fill	[95]
Intramuscular and subcutaneous administration	Zucker Diabetic Fatty rats	Depolymerized Ch 92–10 and 80–80 (M_w-DDA)	pVax1-GLP1 encoding for the recombinant GLP-1 (165 μg)	–	Type 2 diabetes treatment	[96]
Intratumoral administration	C.B-17/Icr-scid-bg mice	Ch M_w 15.5 kDa, DDA 75%–85%	pAcEGFP1-C1 and Luc reporter plasmids encoding GFP and luciferase (100 μg)	–	Cancer treatment	[97]
Intravenous administration	Mice	–	pUC 19 encoding β-galactosidase reporter gene	Hydrophobically modified LMWC	–	[98]
Intravenous administration	Balb/c mice	Depolimerized Ch (7 kDa and 10 kDa)	pGL3 luciferase reporting gene (25 μg)	Ch was conjugated with bPEI and further with tuftsin	–	[99]

Abbreviations: Ch, chitosan; HA, hyaluronate salt; NP, nanoparticles; M_w, molecular weight; DDA, deacetylation degree; DP, depolymerisation degree; FAP-B, Fibronectic Attachment Protein of Mycobacterium bovis; GFP, green fluorescent protein; CETP-C, cholesteryl ester transfer protein C-terminal fragment; GRP, gastrin releasing peptide; GLP-1, glucagon like peptide; FGF-2, fibroblast growth factor-2; PDGF, platelet-derived growth factor; bPEI, branched polyethylenimine.

7. Future Prospects

Although LMWC have been investigated and developed for the delivery of DNAs for fifteen years, the efficient expression of the desired genes into the target cells is not as close as we wish. The resolution of many of the challenges associated with the production of the non-viral vectors, and their accurate analyses, has been the key for the use of LMWC based carriers in biomedical applications and particularly, for gene delivery purposes [100,101].

The physicochemical and biological basis for the successful *in vitro* function of these systems have also been partly elucidated, resulting in the improvement of the susceptibility to degradation of DNA by DNAses, the low cellular membrane permeability and the low solubility and stability at physiological pH [22,29]. Nevertheless, polyplexes elaborated with LMWC face multiple obstacles that still remain unsolved for its *in vivo* success. In order to increase the translation from the raw material to the clinic, efforts are focusing on the chemical and biological modifications of Ch. Specific ligands attached to the LMWC-based nanocomplexes should enhance their *in vivo* targeting toward the desired tissue after systemic administration. Nevertheless, aggregation of the polyplexes in the presence of biological polyanions, due to their high superficial charge, represents the bottleneck in the development of effective polymeric non-viral vectors for systemic administration. Against this major problem, LMWC grafted with neutral polymers have been extensively studied [51].

Due to the drawbacks associated with intravenously administered non-viral vectors, in our opinion, future clinical trials will be focused in the direct administration of the carriers into the target tissue. However, we should point out that with a systemic administration a wider dissemination of the plasmid would be achieved, which is beneficial for the treatment of several pathologies like multi-compartment localized tumors or tumors with metastasis.

In conclusion, pending challenges to obtain successful treatments for human genetic diseases include the production of adequate quantities of highly characterized nanoplexes with features that satisfy regulatory agencies, and the matching of current capabilities in protein expression, targeting and safety profile to specific clinical indications.

Acknowledgments

The authors wish to acknowledge the financial supports of the University of the Basque Country (UPV/EHU, Unidad de Formación e Investigación UFI 11/32), Basque Government (Department of Education, University and Research, predoctoral BFI-2011-2226 grant), and Mexican Government (Grant of the National Council of Science and Technology (CONACYT))

Author Contributions

All authors contributed extensively to the work presented in this review. Mireia Agirre wrote the main manuscript. Edilberto Ojeda provided general advice in relation to the cellular uptake and intracellular trafficking of the polyplexes. Jacques Desbrieres provided data and gave general advice related to chitosan modifications for the review. Gustavo Puras gave general advice related to the *in vivo* administrations of the polyplexes and corrected the final manuscript. Jon Zarate designed,

coordinated, gave general advice and corrected the manuscript. Jose Luis Pedraz supervised and managed the whole work as the director of the research group.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Dewey, R.A.; Morrissey, G.; Cowsill, C.M.; Stone, D.; Bolognani, F.; Dodd, N.J.; Southgate, T.D.; Klatzmann, D.; Lassmann, H.; Castro, M.G.; *et al.* Chronic brain inflammation and persistent herpes simplex virus 1 thymidine kinase expression in survivors of syngeneic glioma treated by adenovirus-mediated gene therapy: Implications for clinical trials. *Nat. Med.* **1999**, *5*, 1256–1263.
2. Fox, J.L. Gene-therapy death prompts broad civil lawsuit. *Nat. Biotechnol.* **2000**, *18*, doi:10.1038/81104.
3. Li, S.D.; Huang, L. Gene therapy progress and prospects: Non-viral gene therapy by systemic delivery. *Gene Ther.* **2006**, *13*, 1313–1319.
4. Ibraheem, D.; Elaissari, A.; Fessi, H. Gene therapy and dna delivery systems. *Int. J. Pharm.* **2014**, *459*, 70–83.
5. Lv, H.; Zhang, S.; Wang, B.; Cui, S.; Yan, J. Toxicity of cationic lipids and cationic polymers in gene delivery. *J. Control. Release* **2006**, *114*, 100–109.
6. Tong, H.; Qin, S.; Fernandes, J.; Li, L.; Dai, K.; Zhang, X. Progress and prospects of chitosan and its derivatives as non-viral gene vectors in gene therapy. *Curr. Gene Ther.* **2009**, 496–502.
7. Mao, S.; Sun, W.; Kissel, T. Chitosan-based formulations for delivery of DNA and siRNA. *Adv. Drug Deliv. Rev.* **2010**, *62*, 12–27.
8. Garcia-Fuentes, M.; Alonso, M.J. Chitosan-based drug nanocarriers: Where do we stand? *J. Control. Release* **2012**, *161*, 496–504.
9. Raftery, R.; O'Brien, F.; Cryan, S. Chitosan for gene delivery and orthopedic tissue engineering applications. *Molecules* **2013**, *18*, 5611–5647.
10. Buschmann, M.D.; Merzouki, A.; Lavertu, M.; Thibault, M.; Jean, M.; Darras, V. Chitosans for delivery of nucleic acids. *Adv. Drug Deliv. Rev*. **2013**, *65*, 1234–1270.
11. Kean, T.; Thanou, M. Biodegradation, biodistribution and toxicity of chitosan. *Adv. Drug Deliv. Rev.* **2010**, *62*, 3–11.
12. Jo, G.H.; Park, R.D.; Jung, W.J. Enzymatic production of chitin from crustacean shell waste. In *Chitin, Chitosan, Oligosaccharides and Their Derivatives*; Kim, S.K., Ed.; CRC Press: Boca Raton, FL, USA, 2011; pp. 37–45.
13. Filion, D.; Lavertu, M.; Buschmann, M.D. Ionization and solubility of chitosan solutions related to thermosensitive chitosan/glycerol-phosphate systems. *Biomacromolecules* **2007**, *8*, 3224–3234.
14. Sorlier, P.; Denuziere, A.; Viton, C.; Domard, A. Relation between the degree of acetylation and the electrostatic properties of chitin and chitosan. *Biomacromolecules* **2001**, *2*, 765–772.
15. Strand, S.P.; Tommeraas, K.; Varum, K.M.; Ostgaard, K. Electrophoretic light scattering studies of chitosans with different degrees of *N*-acetylation. *Biomacromolecules* **2001**, *2*, 1310–1314.

16. Aam, B.B.; Heggset, E.B.; Norberg, A.L.; Sorlie, M.; Varum, K.M.; Eijsink, V.G. Production of chitooligosaccharides and their potential applications in medicine. *Mar. Drugs* **2010**, *8*, 1482–1517.
17. Romoren, K.; Pedersen, S.; Smistad, G.; Evensen, O.; Thu, B.J. The influence of formulation variables on *in vitro* transfection efficiency and physicochemical properties of chitosan-based polyplexes. *Int. J. Pharm.* **2003**, *261*, 115–127.
18. Strand, S.P.; Lelu, S.; Reitan, N.K.; de Lange Davies, C.; Artursson, P.; Vårum, K.M. Molecular design of chitosan gene delivery systems with an optimized balance between polyplex stability and polyplex unpacking. *Biomaterials* **2010**, *31*, 975–987.
19. Huang, M.; Fong, C.; Khor, E.; Lim, L. Transfection efficiency of chitosan vectors: Effect of polymer molecular weight and degree of deacetylation. *J. Controll. Release* **2005**, *106*, 391–406.
20. Lee, M.; Nah, J.W.; Kwon, Y.; Koh, J.J.; Ko, K.S.; Kim, S.W. Water-soluble and low molecular weight chitosan-based plasmid DNA delivery. *Pharm. Res.* **2001**, *18*, 427–431.
21. Duceppe, N.; Tabrizian, M. Factors influencing the transfection efficiency of ultra low molecular weight chitosan/hyaluronic acid nanoparticles. *Biomaterials* **2009**, *30*, 2625–2631.
22. Koping-Hoggard, M.; Varum, K.M.; Issa, M.; Danielsen, S.; Christensen, B.E.; Stokke, B.T.; Artursson, P. Improved chitosan-mediated gene delivery based on easily dissociated chitosan polyplexes of highly defined chitosan oligomers. *Gene Ther.* **2004**, *11*, 1441–1452.
23. Kiang, T.; Wen, J.; Lim, H.W.; Leong, K.W. The effect of the degree of chitosan deacetylation on the efficiency of gene transfection. *Biomaterials* **2004**, *25*, 5293–5301.
24. Ma, P.L.; Lavertu, M.; Winnik, F.M.; Buschmann, M.D. New insights into chitosan-DNA interactions using isothermal titration microcalorimetry. *Biomacromolecules* **2009**, *10*, 1490–1499.
25. Kim, S.; Rajapakse, N. Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review. *Carbohydr. Polym.* **2005**, *62*, 357–368.
26. Lavertu, M.; Methot, S.; Buschmann, M. Composition Method for Efficient Delivery of Nucleic Acids to Cells using Chitosan. Patents WO2007059605-A1; EP1948810-A1; US2009075383-A1; CA26283131-A1, May 31, 2007.
27. Strand, S.P.; Danielsen, S.; Christensen, B.E.; Varum, K.M. Influence of chitosan structure on the formation and stability of DNA-chitosan polyelectrolyte complexes. *Biomacromolecules* **2005**, *6*, 3357–3366.
28. Klausner, E.A.; Zhang, Z.; Chapman, R.L.; Multack, R.F.; Volin, M.V. Ultrapure chitosan oligomers as carriers for corneal gene transfer. *Biomaterials* **2010**, *31*, 1814–1820.
29. Puras, G.; Zarate, J.; Aceves, M.; Murua, A.; Díaz, A.R.; Avilés-Triguero, M.; Fernández, E.; Pedraz, J.L. Low Molecular Weight Oligochitosans for Non-Viral Retinal Gene Therapy. *Eur. J. Pharm. Biopharm.* **2012**, *2012*, doi:10.1016/j.ejpb.2012.09.010.
30. Agirre, M.; Zarate, J.; Puras, G.; Ojeda, E.; Pedraz, J.L. Improving transfection efficiency of ultrapure oligochitosan/DNA polyplexes by medium acidification. *Drug Deliv.* **2014**, in press.
31. Anchordoquy, T.J.; Koe, G.S. Physical stability of nonviral plasmid-based therapeutics. *J. Pharm. Sci.* **2000**, *89*, 289–296.
32. Pfeifer, C.; Hasenpusch, G.; Uezguen, S.; Aneja, M.K.; Reinhardt, D.; Kirch, J.; Schneider, M.; Claus, S.; Friess, W.; Rudolph, C. Dry powder aerosols of polyethylenimine (PEI)-based gene vectors mediate efficient gene delivery to the lung. *J. Controll. Release* **2011**, *154*, 69–76.

33. Puras, G.; Zarate, J.; Díaz-Tahoces, A.; Avilés-Trigueros, M.; Fernández, E.; Pedraz, J.L. Oligochitosan polyplexes as carriers for retinal gene delivery. *Eur. J. Pharm. Sci.* **2013**, *48*, 323–331.
34. Klausner, E.A.; Zhang, Z.; Wong, S.P.; Chapman, R.L.; Volin, M.V.; Harbottle, R.P. Corneal gene delivery: Chitosan oligomer as a carrier of CpG rich, CpG free or S/MAR plasmid DNA. *J. Gene Med.* **2012**, *14*, 100–108.
35. Rungsardthong, U.; Ehtezazi, T.; Bailey, L.; Armes, S.P.; Garnett, M.C.; Stolnik, S. Effect of polymer ionization on the interaction with DNA in nonviral gene delivery systems. *Biomacromolecules* **2003**, *4*, 683–690.
36. Köping-Höggård, M.; Mel'nikova, Y.S.; Vårum, K.M.; Lindman, B.; Artursson, P. Relationship between the physical shape and the efficiency of oligomeric chitosan as a gene delivery system *in Vitro* and *in Vivo*. *J. Gene Med.* **2003**, *5*, 130–141.
37. Lou, Y.; Peng, Y.; Chen, B.; Wang, L.; Leong, K.W. Poly(ethylene imine)-G-chitosan using EX-810 as a spacer for nonviral gene delivery vectors. *J. Biomed. Mater. Res. A* **2009**, *88A*, 1058–1068.
38. Erbacher, P.; Zou, S.; Bettinger, T.; Steffan, A.M.; Remy, J.S. Chitosan-based vector/DNA complexes for gene delivery: Biophysical characteristics and transfection efficiency. *Pharm. Res.* **1998**, *15*, 1332–1339.
39. MacLaughlin, F.C.; Mumper, R.J.; Wang, J.; Tagliaferri, J.M.; Gill, I.; Hinchcliffe, M.; Rolland, A.P. Chitosan and depolymerized chitosan oligomers as condensing carriers for *in vivo* plasmid delivery. *J. Controll. Release* **1998**, *56*, 259–272.
40. Schroeder, A.; Heller, D.; Winslow, M.; Dahlman, J.; Pratt, G.; Langer, R.; Jacks, T.; Anderson, D. Treating metastatic cancer with nanotechnology. *Nat. Rev. Cancer* **2011**, *12*, 39–50.
41. Nimesh, S.; Thibault, M.; Lavertu, M.; Thibault, M. Enhanced gene delivery mediated by low molecular weight chitosan/DNA complexes: Effect of pH and serum. **2010**, *46*, 182–196.
42. Danielsen, S.; Varum, K.M.; Stokke, B.T. Structural analysis of chitosan mediated DNA condensation by AFM: Influence of chitosan molecular parameters. *Biomacromolecules* **2004**, *5*, 928–936.
43. Corsi, K.; Chellat, F.; Yahia, L.; Fernandes, J.C. Mesenchymal stem cells, MG63 and HEK293 transfection using chitosan-DNA nanoparticles. *Biomaterials* **2003**, *24*, 1255–1264.
44. Lu, B.; Wang, C.; Wu, D.; Li, C.; Zhang, X.; Zhuo, R. Chitosan based oligoamine polymers: Synthesis, characterization, and gene delivery. *J. Control. Release* **2009**, *137*, 54–62.
45. Richard, I.; Thibault, M.; De Crescenzo, G.; Buschmann, M.; Lavertu, M. Ionization behavior of chitosan and chitosan-DNA polyplexes indicate that chitosan has a similar capability to induce a proton-sponge effect as PEI. *Biomacromolecules* **2013**, *14*, 1732–1740.
46. Strand, S.P.; Issa, M.M.; Christensen, B.E.; Varum, K.M.; Artursson, P. Tailoring of chitosans for gene delivery: novel self-branched glycosylated chitosan oligomers with improved functional properties. *Biomacromolecules* **2008**, *9*, 3268–3276.
47. Ruponen, M.; Honkakoski, P.; Tammi, M.; Urtti, A. Cell-surface glycosaminoglycans inhibit cation-mediated gene transfer. *J. Gene Med.* **2004**, *6*, 405–414.
48. Danielsen, S.; Strand, S.; de Lange Davies, C.; Stokke, B.T. Glycosaminoglycan destabilization of DNA–chitosan polyplexes for gene delivery depends on chitosan chain length and GAG properties. *Biochim. Biophys. Acta* **2005**, *1721*, 44–54.

49. Hashimoto, M.; Morimoto, M.; Saimoto, H.; Shigemasa, Y.; Sato, T. Lactosylated chitosan for DNA delivery into hepatocytes: The effect of lactosylation on the physicochemical properties and intracellular trafficking of pDNA/chitosan complexes. *Bioconjug. Chem.* **2006**, *17*, 309–316.
50. Jiang, X.; Dai, H.; Leong, K.W.; Goh, S.; Mao, H.; Yang, Y. Chitosan-G-PEG/DNA complexes deliver gene to the rat liver via intrabiliary and intraportal infusions. *J. Gene Med.* **2006**, *8*, 477–487.
51. Thanou, M.; Florea, B.I.; Geldof, M.; Junginger, H.E.; Borchard, G. Quaternized chitosan oligomers as novel gene delivery vectors in epithelial cell lines. *Biomaterials* **2002**, *23*, 153–159.
52. Issa, M.M.; Köping-Höggård, M.; Tømmeraas, K.; Vårum, K.M.; Christensen, B.E.; Strand, S.P.; Artursson, P. Targeted gene delivery with trisaccharide-substituted chitosan oligomers in vitro and after lung administration *in vivo*. *J. Controll. Release* **2006**, *115*, 103–112.
53. Thibault, M.; Nimesh, S.; Lavertu, M.; Buschmann, M. Intracellular trafficking and decondensation kinetics of chitosan-pDNA polyplexes. *Mol. Ther.* **2010**, *18*, 1787–1795.
54. Yue, Z.; Wei, W.; Lv, P.; Yue, H.; Wang, L.; Su, Z.; Ma, G. Surface charge affects cellular uptake and intracellular trafficking of Chitosan-Based Nanoparticles. *Biomacromolecules* **2011**, *12*, 2440–2446.
55. Khalil, I.A.; Kogure, K.; Akita, H.; Harashima, H. Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. *Pharmacol. Rev.* **2006**, *58*, 32–45.
56. Xiang, S.; Tong, H.; Shi, Q.; Fernandes, J.C.; Jin, T.; Dai, K.; Zhang, X. Uptake mechanisms of non-viral gene delivery. *J. Controll. Release* **2012**, *158*, 371–378.
57. Garaiova, Z.; Strand, S.P.; Reitan, N.K.; Lélu, S.; Størset, S.Ø; Berg, K.; Malmo, J.; Folasire, O.; Bjørkøy, A.; de Lange Davies, C. Cellular uptake of DNA–chitosan nanoparticles: The role of clathrin- and caveolae-mediated pathways. *Int. J. Biol. Macromol.* **2012**, *51*, 1043–1051.
58. Conner, S.D.; Schmid, S.L. Regulated portals of entry into the cell. *Nature* **2003**, *422*, 37–44.
59. Douglas, K.L.; Piccirillo, C.A.; Tabrizian, M. Cell line-dependent internalization pathways and intracellular trafficking determine transfection efficiency of nanoparticle vectors. *Eur. J. Pharm. Biopharm.* **2008**, *68*, 676–687.
60. Fang, N.; Chan, V.; Mao, H.; Leong, K. Interactions of phospholipid bilayer with chitosan: Effect of molecular weight and pH. *Biomacromolecules* **2001**, *2*, 1161–1168.
61. Hsu, C.; Uludağ, H. Nucleic-acid based gene therapeutics: Delivery challenges and modular design of nonviral gene carriers and expression cassettes to overcome intracellular barriers for sustained targeted expression. *J Drug Target* **2012**, *20*, 301–328.
62. Douglas, K.L. Toward development of artificial viruses for gene therapy: A comparative evaluation of viral and non-viral transfection. *Biotechnol. Prog.* **2008**, *24*, 871–883.
63. Chiu, Y.; Ho, Y.; Chen, Y.; Peng, S.; Ke, C.; Chen, K.; Mi, F.; Sung, H. The characteristics, cellular uptake and intracellular trafficking of nanoparticles made of hydrophobically-modified chitosan. *J. Controll. Release* **2010**, *146*, 152–159.
64. Contreras-Ruiz, L.; de la Fuente, M.; Párraga, J.; López-García, A.; Fernández, I.; Seijo, B.; Sánchez, A.; Calonge, M.; Diebold, Y. Intracellular trafficking of hyaluronic acid-chitosan oligomer-based nanoparticles in cultured human ocular surface cells. *Mol. Vis.* **2011**, *17*, 279–290.
65. Thibault, M.; Astolfi, M.; Tran-Khanh, N.; Lavertu, M.; Darras, V.; Merzouki, A.; Buschmann, M.D. Excess polycation mediates efficient chitosan-based gene transfer by promoting lysosomal release of the polyplexes. *Biomaterials* **2011**, *32*, 4639–4646.

66. Männistö, M.; Rönkkö, S.; Mättö, M.; Honkakoski, P.; Hyttinen, M.; Pelkonen, J.; Urtti, A. The role of cell cycle on polyplex-mediated gene transfer into a retinal pigment epithelial cell line. *J. Gene Med.* **2005**, *7*, 466–476.
67. Ishii, T.; Okahata, Y.; Sato, T. Mechanism of cell transfection with plasmid/chitosan complexes. Biochim. *Biophys. Acta* **2001**, *1514*, 51–64.
68. Sato, T.; Ishii, T.; Okahata, Y. *In vitro* gene delivery mediated by chitosan. Effect of pH, serum, and molecular mass of chitosan on the transfection efficiency. *Biomaterials* **2001**, *22*, 2075–2080.
69. Lavertu, M.; Méthot, S.; Tran-Khanh, N.; Buschmann, M.D. High efficiency gene transfer using chitosan/DNA nanoparticles with specific combinations of molecular weight and degree of deacetylation. *Biomaterials* **2006**, *27*, 4815–4824.
70. Kean, T.; Roth, S.; Thanou, M. Trimethylated chitosans as non-viral gene delivery vectors: cytotoxicity and transfection efficiency. *J. Controll. Release* **2005**, *103*, 643–653.
71. Seong, H.; Whang, H.S.; Ko, S. Synthesis of a quaternary ammonium derivative of chito-oligosaccharide as antimicrobial agent for cellulosic fibers. *J. Appl. Polym. Sci.* **2000**, *76*, 2009–2015.
72. Hermanson, G. *Bioconjugate Techniques*; Academic Press: Waltham, MA, USA, 2008.
73. Kim, T.H.; Ihm, J.E.; Choi, Y.J.; Nah, J.W.; Cho, C.S. Efficient gene delivery by urocanic acid-modified chitosan. *J. Controll. Release* **2003**, *93*, 389–402.
74. Chang, K.L.; Higuchi, Y.; Kawakami, S.; Yamashita, F.; Hashida, M. Efficient gene transfection by histidine-modified chitosan through enhancement of endosomal escape. *Bioconjug. Chem*. **2010**, *21*, 1087–1095.
75. Pires, L.R.; Oliveira, H.; Barrias, C.C.; Sampaio, P.; Pereira, A.J.; Maiato, H.; Simoes, S.; Pego, A.P. Imidazole-grafted chitosan-mediated gene delivery: *In vitro* study on transfection, intracellular trafficking and degradation. *Nanomedicine* **2011**, *6*, 1499–1512.
76. Morris, V.B.; Sharma, C.P. Folate mediated in vitro targeting of depolymerised trimethylated chitosan having arginine functionality. *J. Colloid Interface Sci.* **2010**, *348*, 360–368.
77. Tømmeraas, K.; Strand, S.P.; Tian, W.; Kenne, L.; Vårum, K.M. Preparation and characterisation of fluorescent chitosans using 9-anthraldehyde as fluorophore. *Carbohydr. Res.* **2001**, *336*, 291–296.
78. Tiera, M.J.; Qiu, X.P.; Bechaouch, S.; Shi, Q.; Fernandes, J.C.; Winnik, F.M. Synthesis and characterization of phosphorylcholine-substituted chitosans soluble in physiological pH conditions. *Biomacromolecules* **2006**, *7*, 3151–3156.
79. Case, A.H.; Dalla Picola, I.P.; Zaniquelli, M.E.; Fernandes, J.C.; Taboga, S.R.; Winnik, F.M.; Tiera, M.J. Physicochemical characterization of nanoparticles formed between DNA and phosphorylcholine substituted chitosans. *J. Colloid Interface Sci.* **2009**, *336*, 125–133.
80. Tommeraas, K.; Koping-Hoggard, M.; Varum, K.M.; Christensen, B.E.; Artursson, P.; Smidsrod, O. Preparation and characterisation of chitosans with oligosaccharide branches. *Carbohydr. Res.* **2002**, *337*, 2455–2462.
81. Park, I.K.; Park, Y.H.; Shin, B.A.; Choi, E.S.; Kim, Y.R.; Akaike, T.; Cho, C.S. Galactosylated chitosan-graft-dextran as hepatocyte-targeting DNA carrier. *J. Controll. Release* **2000**, *69*, 97–108.

82. Ercelen, S.; Zhang, X.; Duportail, G.; Grandfils, C.; Desbrières, J.; Karaeva, S.; Tikhonov, V.; Mély, Y.; Babak, V. Physicochemical properties of low molecular weight alkylated chitosans: A new class of potential nonviral vectors for gene delivery. *Colloids Surf. B Biointerfaces* **2006**, *51*, 140–148.
83. Clement, J.; Kiefer, K.; Kimpfler, A.; Garidel, P.; Peschka-Süss, R. Large-scale production of lipoplexes with long shelf-life. Eur. *J. Pharm. Biopharm.* **2005**, *59*, 35–43.
84. Kasper, J.C.; Schaffert, D.; Ogris, M.; Wagner, E.; Friess, W. Development of a Lyophilized Plasmid/LPEI Polyplex Formulation with Long-Term stability—A Step Closer from Promising Technology to Application. J. Controlled Release **2011**, *151*, 246–255.
85. Kasper, J.C.; Troiber, C.; Küchler, S.; Wagner, E.; Friess, W. Formulation development of lyophilized, long-term stable siRNA/oligoaminoamide polyplexes. *Eur. J. Pharm. Biopharm.* **2013**, *85*, 294–305.
86. Abdelwahed, W.; Degobert, G.; Stainmesse, S.; Fessi, H. Freeze-drying of nanoparticles: formulation, process and storage considerations. *Adv. Drug Deliv. Rev.* **2006**, *58*, 1688–1713.
87. Anchordoquy, T.J.; Armstrong, T.K.; Molina, M.d.C. Low molecular weight dextrans stabilize nonviral vectors during lyophilization at low osmolalities: Concentrating suspensions by rehydration to reduced volumes. *J. Pharm. Sci.* **2005**, *94*, 1226–1236.
88. Vauthier, C.; Cabane, B.; Labarre, D. How to concentrate nanoparticles and avoid aggregation? *Eur. J. Pharm. Biopharm.* **2008**, *69*, 466–475.
89. Csaba, N.; Garcia-Fuentes, M.; Alonso, M.J. The performance of nanocarriers for transmucosal drug delivery. *Expert Opin. Drug Deliv.* **2006**, *3*, 463–478.
90. De la Fuente, M.; Ravina, M.; Paolicelli, P.; Sanchez, A.; Seijo, B.; Alonso, M.J. Chitosan-based nanostructures: A delivery platform for ocular therapeutics. *Adv. Drug Deliv. Rev.* **2010**, *62*, 100–117.
91. De la Fuente, M.; Seijo, B.; Alonso, M. Bioadhesive hyaluronan-chitosan nanoparticles can transport genes across the ocular mucosa and transfect ocular tissue. *Gene Ther.* **2008**, *15*, 668–676.
92. Mohammadi, Z.; Dorkoosh, F.A.; Hosseinkhani, S.; Gilani, K.; Amini, T.; Najafabadi, A.R.; Tehrani, M.R. *In vivo* transfection study of chitosan-DNA-FAP-B nanoparticles as a new non viral vector for gene delivery to the lung. *Int. J. Pharm.* **2011**, *421*, 183–188.
93. Yang, X.; Yuan, X.; Cai, D.; Wang, S.; Zong, L. Low molecular weight chitosan in DNA vaccine delivery via mucosa. *Int. J. Pharm.* **2009**, *375*, 123–132.
94. Yao, W.; Peng, Y.; Du, M.; Luo, J.; Zong, L. Preventative vaccine-loaded mannosylated chitosan nanoparticles intended for nasal mucosal delivery enhance immune responses and potent tumor immunity. *Mol. Pharm.* **2013**, *10*, 2904–2914.
95. Jean, M.; Smaoui, F.; Lavertu, M.; Methot, S.; Bouhdoud, L.; Buschmann, M.D.; Merzouki, A. Chitosan-plasmid nanoparticle formulations for IM and SC delivery of recombinant FGF-2 and PDGF-BB or generation of antibodies. *Gene Ther.* **2009**, *16*, 1097–1110.
96. Jean, M.; Alameh, M.; De Jesus, D.; Thibault, M.; Lavertu, M.; Darras, V.; Nelea, M.; Buschmann, M.D.; Merzouki, A. Chitosan-based therapeutic nanoparticles for combination gene therapy and gene silencing of in vitro cell lines relevant to type 2 diabetes. *Eur. J. Pharm.Sci.* **2012**, *45*, 138–149.

97. Yang, S.; Chang, S.; Tsai, K.; Chen, W.; Lin, F.; Shieh, M. Effect of chitosan-alginate nanoparticles and ultrasound on the efficiency of gene transfection of human cancer cells. *J. Gene Med.* **2010**, *12*, 168–179.
98. Zhang, X.; Ercelen, S.; Duportail, G.; Schaub, E.; Tikhonov, V.; Slita, A.; Zarubaev, V.; Babak, V.; Mély, Y. Hydrophobically modified low molecular weight chitosans as efficient and nontoxic gene delivery vectors. *J. Gene Med.* **2008**, *10*, 527–539.
99. Tripathi, S.K.; Goyal, R.; Kashyap, M.P.; Pant, A.B.; Haq, W.; Kumar, P.; Gupta, K.C. Depolymerized chitosans functionalized with bPEI as carriers of nucleic acids and tuftsin-tethered conjugate for macrophage targeting. *Biomaterials* **2012**, *33*, 4204–4219.
100. United States Pharmacopeial Convention. Chitosan. In *United States Pharmacopeial and The National Formulary (USP–NF)*; United States Pharmacopeial Convention: Rockville, MD, USA, 2011; Volume 29, pp. S5361–S5365.
101. American Society for Testing and Materials. *Standard Guide for Characterization and Testing of Chitosan Salts as Starting Materials Intended for Use in Biomedical and Tissue-Engineered Medical Product Applications*; ASTM Standard F2103; American Society for Testing and Materials (ASTM): West Conshohocken, PA, USA, 2011.