



HAL
open science

Chitosan-DNA polyelectrolyte complex: Influence of chitosan characteristics and mechanism of complex formation

Lourdes Mónica Bravo-Anaya, Karla Gricelda Fernández-Solís, Julien Rosselgong, Jesrael Luz Elena Nano-Rodríguez, Francisco Carvajal, Marguerite Rinaudo

► To cite this version:

Lourdes Mónica Bravo-Anaya, Karla Gricelda Fernández-Solís, Julien Rosselgong, Jesrael Luz Elena Nano-Rodríguez, Francisco Carvajal, et al.. Chitosan-DNA polyelectrolyte complex: Influence of chitosan characteristics and mechanism of complex formation. *International Journal of Biological Macromolecules*, 2019, 126, pp.1037-1049. 10.1016/j.ijbiomac.2019.01.008 . hal-03486009

HAL Id: hal-03486009

<https://hal.science/hal-03486009>

Submitted on 20 Dec 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

Chitosan-DNA polyelectrolyte complex: influence of chitosan characteristics and mechanism of complex formation

Lourdes Mónica Bravo-Anaya^{a,b*}, Karla Gricelda Fernández-Solís^{a,c}, Julien Rosselgong^b,
Jesrael Luz Elena Nano-Rodríguez^{a,c}, Francisco Carvajal^d, Marguerite Rinaudo^{e**}

^a Universidad de Guadalajara, Departamento de Ingeniería Química. Blvd. M. García Barragán #1451, C.P. 44430, Guadalajara, Jalisco (México), monik_ayanami@hotmail.com.

^b University of Bordeaux/Bordeaux INP, ENSCBP and CNRS, Laboratoire de Chimie des Polymères Organiques (UMR5629), 16 avenue Pey-Berland, Pessac 33607 (France), j.rosselgong@gmail.com

^c Centro Universitario UTEG, Departamento de Investigación, Héroes Ferrocarrileros #1325, Guadalajara, Jalisco, C.P. 44460 (México), karlag.fersol@hotmail.com, jesraelrodriguez27@gmail.com

^d CUTonalá, Departamento de Ingenierías, Universidad de Guadalajara, Nuevo Periférico # 555 Ejido San José Tatepozco C.P.45425, Jalisco (México), iq_fcr@yahoo.com.mx

^e Biomaterials applications, 6 rue Lesdiguières. 38000 Grenoble (France), marguerite.rinaudo38@gmail.com

Corresponding authors: * monik_ayanami@hotmail.com, Tel: +33-656-886-206. ** marguerite.rinaudo38@gmail.com, Tel.: +33-611-434-806.

Abstract

Polyelectrolyte complexes formed between DNA and chitosan present different and interesting physicochemical properties combined with high biocompatibility; they are very useful for biomedical applications. DNA in its double helical structure is a semi-rigid polyelectrolyte chain. Chitosan, an abundant polysaccharide in nature, is considered as one of the most attractive vectors due to its biocompatibility and biodegradability. Here we study chitosan/DNA polyelectrolyte complex formation mechanism and the key factors of their stability. Compaction process of DNA with chitosan was monitored in terms of the ζ -potential and hydrodynamic radius variation as a function of charge ratios between chitosan and DNA. The influence of chitosan degree of acetylation (DA) and its molecular weight on the stoichiometry of chitosan/DNA complexes characteristics was also studied.

It is shown that the isoelectric point of chitosan/DNA complexes, as well as their stability, is directly related to the degree of protonation of chitosan (**depending on** pH), to the DA and to the external salt concentration. It is demonstrated that DNA compaction process corresponds to an *all or nothing* like-process. Finally, since an important factor in cell travelling is the buffering effect of the vector used, we demonstrated the essential role of free chitosan on the proton-sponge effect.

Keywords: Polyelectrolyte complexes; DNA-chitosan complex; electrostatic interaction; DNA compaction.

1. Introduction

Mixing oppositely charged polyelectrolytes results in the formation of a polyelectrolyte complex (PEC) based on electrostatic interactions in dependence of pH and external salt concentration [1-3]. Electrostatic complexes involving natural biopolymers are being widely developed recently for ~~new biomaterials production and~~ new biomedical applications [4,5]. Strong electrostatic interactions between oppositely charged systems are also involved at **the** interface to stabilize or destabilize liposomes for drug release [5,6], for formation of lipoplexes especially with DNA [7-9], or to stabilize a colloidal dispersion [10,11]. Layer by layer formation between oppositely charged polyelectrolytes is an important development of polyelectrolyte complexes. It was applied to form capsules by deposition on liposomes and their stabilization [12,13], to stabilize the biological activity of peptide hormones [14] or to coat blood vessel using chitosan and hyaluronan [15].

~~More specifically,~~ In the domain of bioactive systems, the attention was focused on cationic polymers able to **complex and** compact DNA [16-26]. ~~and to be used as non-viral vectors for gene delivery [16-26]. The particles formed are used as non-viral vectors for gene delivery.~~ The polycationic entities usually examined are polylysine, polyethylenimine (PEI) and chitosan. Low immunogenicity, biocompatibility and minimal cytotoxicity of chitosan is actually recognized and proposed to develop a better alternative to viral or lipid vectors. Chitosan may also be associated with cationic emulsion/DNA, allowing the reduction of the size of the gene delivery vehicle and enhances in vitro transfection [27].

The first report about chitosan as a possible carrier for gene therapy was made by *Mumper et al.*, in 1995 [28]. As recognized, chitosan is a pseudo-natural polysaccharide, biocompatible, biodegradable, mucoadhesive, and non-toxic. In addition of their immunogenicity, chitosan molecules condense efficiently with DNA forming polyplexes and preventing its degradation by DNAses or in serum [17, 23,29, 30]. These electrostatic complexes are formed reversibly without changing DNA double helix conformation, as tested with low molar mass chitosan (MW=40 000; degree of acetylation, DA= 0.01 1%) [31,32]. ~~Chitosan-DNA complex is based on electrostatic interactions between the protonated chitosan -NH₂ and the charged phosphate groups of DNA [33].~~ The parameters that control complexes formation influencing the particles size and stability include chitosan molecular weight (MW; N being the number of total -NH₂), DNA or plasmid concentration (characterized by the phosphate content P) and the charge ratio (N⁺/P⁻) [19,21,33-40]. ~~In these works, It~~ was demonstrated that the size of the formed particles decreased when the molar mass of chitosan decreased but that the stability of the complex increased when the MW increased; also, the size of the complex increased when plasmid concentration increased. In addition, higher molecular weight chitosan samples associated more strongly with plasmids. Those complexes were more stable to salt and serum challenge [21]. ~~In fact, chitosan characteristics are mostly important in complex formation since they control the condensation and their stability. Its~~ Chitosan linear structure is based on poly (β-(1-4)-N-acetyl-D-glucosamine) partially deacetylated, allowing easy specific chemical modification on the -NH₂ position [20,41-44]. Many characteristics of chitosan have been previously discussed in the literature [45] and summarized as follows: i) chitosan molar masses MW (and samples with different MW may be prepared by partial depolymerization with sodium nitrite) [21], ii) chitosan degrees of acetylation (DA usually between 0.05 and 0.30 to be soluble in acidic conditions) and iii) their acetyl groups distribution along the chains (usually not examined in complex formation) **depending on the conditions of chitin deacetylation.**

The influence of MW and DA of chitosan on complex formation with DNA have been covered by few papers [19, 20, 35-39] ~~using often electrophoretic mobility (and zeta potential), Dynamic Light Scattering (DLS), Atomic Force Microscopy (AFM), potentiometry or microcalorimetry. In these works, the main techniques used are often electrophoretic mobility (and zeta potential), Dynamic Light Scattering (DLS), Atomic Force Microscopy (AFM), potentiometry or microcalorimetry.~~

Since chitosan is a weak base with a $pK_0=6.5$, pH is an important parameter to control the degree of protonation of the amino groups (as $-NH_3^+$) controlling complex formation with the highly negatively charged DNA (P^-) [19, 33, 46]. ~~the positive fraction of charge ($-NH_3^+$) being the factor controlling complex formation with the highly negatively charged DNA (P^-) [19, 33, 46]. The higher is the degree of chitosan protonation, the stronger is the stability of complex formed with DNA and the condensation of the complex [19].~~ Strand *et al.* showed that a minimum of 6 to 9 monomeric units is necessary to complex DNA [19], however, the stability of the complex tends to be poor, dissociating at $pH > 6.5$ or in excess of salt. Clearly, the stability of the complex depends on pH, N^+/P^- charge ratio and salt concentration *in vitro*. ~~The most important parameter after chitosan characteristics is~~ Usually, the ratio N/P (or chitosan units/phosphate units) is used in literature. For progressive additions of chitosan at a pH lower than 6.5 to a dilute DNA solution, N^+/P^- increases while complex is formed, being negatively charged up to an isoelectric point followed by the charge inversion. ~~The N^+/P^- charge ratio at null charge is usually found around the charge stoichiometry when only the protonated fraction is considered [33].~~ For gene delivery, it is important to use a positive complex ($N^+/P^- > 1$) in the nanometric range of particle diameters able interacting with the negatively charged cell membrane and then, entering the cell through endocytosis and/or pinocytosis to allow transfection [18-20, 35, 39]. It was claimed that N/P molar ratio=3 gives the highest transfection activity in serum using a MW=70 000 chitosan. In addition, compared with other polycations often proposed for gene therapy, it was found that chitosan had a lower toxicity than polylysine and that after 96 hours, this polycation was 10 times more efficient than PEI [42].

Another important and delicate point is testing the transfection efficiency of chitosan/DNA systems *in vivo*. It depends on several factors such as chitosan characteristics (DA and MW), local pH and salt composition, charge ratio of chitosan to DNA (N^+/P^-) playing on complex stability. It also depends on the cell type, nanoparticle size, enzyme and protein interactions, and interactions with membranes. It was reported that the transfection efficiency at pH 6.5 was higher than at pH 7.4 [47]. At pH=8, the complex was fairly insoluble and did not penetrate the membrane [42]. Nevertheless, the transfection efficiency based on DNA/chitosan complex was not yet fully understood. ~~It was necessary to control the different steps of the mechanistic pathway for gene transfection. It firstly includes the collapse of extended DNA chains into compact nanometric particles. This process, known as DNA condensation, has received considerable attention for production~~

~~of gene delivery vehicles [22] and for this step, chitosan is a good and adequate candidate [18]. Then,~~ The positive particles of DNA compacted by polycations are able to interact with the anionic proteoglycans at the cell surface and can be transported by endocytosis. The cationic agents have a buffering capacity in the endosomal pH range (pH 4.5 to 7.5) inhibiting the degradation of DNA by lysosomal enzymes [20, 48]. The mechanism was fully described for PEI/DNA complexes [49-51]. On this point, it was shown that the buffering capacity or proton sponge effect was larger for chitosan than for PEI for the same number of cationic sites [48]. The presence of excess of free chitosan increased the osmotic pressure and destabilized the endosome, releasing DNA and translocating it into the nucleus, where it can decondense after or by separation from the cationic delivery vehicle and can regulate gene expression [17,20,48, 52-54]. ~~In fact, the last limiting step is the unpacking of DNA from the complex following localization in the nucleus. It was shown with labelled polylysine having different molecular weights that the complex formed with lower molecular weight dissociates more rapidly both *in vitro* and *in vivo* [40].~~ Interestingly, polyplexes formed with PEI and chitosan are able to protect pDNA from serum degradation at the same level and that they have a comparable transgene expression in Human Embryonic Kidney (HEK) 293 cells [30]. The question of the stability of the complex *in vivo* is important but not yet solved: higher stability hampers the transfection efficiency [30,34]. It was found that there is a fine balance between extracellular DNA protection (better with high MW and lower DA) and ability of efficient intracellular unpacking (better with low MW) in order to get a large level of transfection [20, 24, 32, 37, 39]. Especially, a relation was demonstrated between transfection efficiency and polyplex dissociation rate and it was shown that complex with high MW and low DA do not dissociate even after 24h in HEK 293 cells [55].

In a previous work, chitosan/DNA complex stoichiometry, net charge, dimensions, conformation and thermal stability were determined and discussed [33]. ~~It was found that the isoelectric point of chitosan/DNA complexes is directly related to the protonation degree of chitosan and it was also demonstrated that electrostatic interactions between DNA and chitosan are the main phenomena taking place in the solution up to the stoichiometric charge ratio $N^+/P^-=1$.~~ It was found that the isoelectric point of chitosan/DNA complexes is directly related to the stoichiometric charge ratio $N^+/P^-=1$. ~~Here~~ In the present work, we study chitosan-DNA polyelectrolyte complex mechanism and the key factors of their stability. DNA concentration was selected in the dilute regime, *i.e.*

around 10 times lower than the average value of the overlap concentration C^* (0.23 mg/mL), previously determined from viscosity measurements [56]. Different techniques (ζ -potential, fluorescence, AFM) are used to determine the role of chitosan characteristics added on the complexes formed in a wide range of N/P ratios. Furthermore, the mechanism of complex formation is proposed through the study of several physicochemical and biophysical data obtained from complementary techniques, *i.e.* UV-Vis, DLS and gel electrophoresis.

~~Different techniques are used to determine the role of characteristics of chitosan added on the formed complex in a wide range of N/P ratios.~~

~~The influence of chitosan protonation in DNA/complexes stability, as well as chitosan DA and chitosan molecular weight (MW) influence on the stoichiometry of the formed polyplexes is studied in this work. Furthermore, chitosan/DNA complex formation mechanism is also proposed through the analyses of several physical chemical and biophysical techniques, *i.e.* UV Vis and DLS measurements, and also by gel electrophoresis assays.~~

2. Materials and methods

2.1. Materials

Calf-thymus DNA sample with an average molecular weight of 6,559,500 g/mol [56], NaOH in pellets with impurities ≤ 0.001 % and anhydrous NaCl were supplied by Sigma-Aldrich Company. DNA encoding for Enhanced Green Fluorescent Protein (pEGFP) was transformed into *E. coli* bacterial strain and extracted from the culture pellets using the Midiprep Macherey Nagel protocol and stored at -20 °C. The purified plasmid was dissolved in deionized sterile water. Their purity and concentration were determined by UV-Vis measurements by measuring absorbance at 260/280 nm. AcOH, AcONa, and HCl 0.1 N (Titrisol) was supplied by Merck Millipore. Several chitosan samples were used to study the influence of their degree of acetylation (DA) and molecular weight (MW) on complex formation: two commercial samples from Sigma-Aldrich Company, High Molecular Weight (HMW chitosan (Ref: 41,941-9) and Medium Molecular Weight (MMW) chitosan (Ref: 44,887-7); and seven chitosan samples (ChitoClear®) from Northern coldwater shrimp, *Pandalus borealis* provided by Primex, Iceland. A

characterization of chitosan samples from Sigma-Aldrich Company and Primex samples are presented in this paper. Furthermore, two chitosan samples (1C: MW=84 000, DA=0.41 and 1E: MW=75 000, DA=0.61) were obtained by random reacylation by Roberts and were characterized previously [57].

2.2. DNA and chitosan solutions preparation

DNA solutions were prepared at a concentration of 1 mg/mL in water and in a series of NaCl solutions with different concentrations (5×10^{-3} , 10^{-2} , 10^{-1} , 1.0 and 2.0 M NaCl). The purified EGFP plasmid was diluted to 1 mg/mL in deionized sterile water. The vials were closed and sealed with Parafilm® to prevent water evaporation and changes in the polymer concentration. For complex formation, all nucleic acids solutions (Calf-thymus DNA and pEGFP) were prepared by dilution of the 1 mg/mL DNA solution to around 0.03 mg/mL, to avoid experimental difficulties such as turbidity during chitosan addition. Calf-thymus DNA solutions were stored in a refrigerator at a temperature of 4 °C and pEGFP solutions were stored at -20 °C to prevent degradation.

Initial chitosan solutions were prepared at a certain concentration (between 4.17 and 6.25 mg/mL, depending on chitosan DA) by dissolving a known amount of polysaccharide with the stoichiometric amount of HCl 0.1N on the basis of NH_2 content to get the fully protonated chitosan. The experimental concentration of chitosan used to prepare complexes was obtained by dilution of the previous solution with water to 1 mg/mL. The final pH after dissolution was found around 3.6. The solutions were placed under constant stirring for one night at room temperature, until complete dissolution. Finally, all the solutions were stocked at a temperature of 4 °C before use and filtered with a 0.2 μm porous membrane. For modification of the degree of protonation, chitosan solutions were adjusted to the following pH values: 4.5, 5.0, 5.5, 6.0 and 6.5 with a solution 0.1N NaOH before addition into DNA solution.

Chitosan Labelling. In order to quantify chitosan free chains after chitosan/DNA complex formation at different charge ratios by fluorescence spectroscopy, one chitosan sample was labeled with a fluorescent probe. For this, an amount of 1 g of chitosan (Primex 6, Mw=160 000) was dispersed in 40 mL of distilled water and dissolved by addition of 60 mL of 0.1 M HCl. Then 25 mL of dried methanol (MeOH) was added to the solution,

followed by the addition of 50 mL of a solution of MeOH containing 52 mg of fluorescein isothiocyanate (FITC). The mixture was stirred during 3 h at ambient temperature and then diluted with water up to a final volume of 600 mL. The solution was neutralized with a 0.1 M NaOH solution to pH 7, to precipitate the labelled chitosan. The precipitate was then recovered by filtration, washed with an ethanol/water (70/30 v/v ~~volume-ratio~~) mixture until the filtrate was clear, and finally dried under vacuum at ambient temperature. The degree of substitution was determined by comparing its fluorescence in dilute sample in acidic solution with a dilute solution of FITC at a controlled concentration as reference [58]. A low degree of labelling was chosen to avoid any kind of modification of chitosan interactions with DNA, as well as chitosan/DNA physicochemical properties, as shown in Fig. S1 (available in the supplementary information). An amount of around 4×10^{-3} fluorescent groups per chitosan sugar repeat units was determined through fluorescence measurements.

2.3. Chitosan characterization: DA and MW

DA determination. The DA, determined by the fraction of N-acetylated glucosamine units, was determined by conductometric titration [2] and ^1H NMR [45]. The fraction of free $-\text{NH}_2$, equal to 1-DA, in chitosan samples was obtained by conductivity measurements, dissolving a sample of neutral chitosan with a little excess of HCl based on the stoichiometry of the solution, followed by the neutralization of the protonated $-\text{NH}_2$ groups by the addition of NaOH. For this experiment, successive volumes of 25 μL NaOH (0.1M) were added into a 20 mL chitosan solution and the conductivity of the solution was taken at each addition. Conductivity measurements were performed in a Conductivity Meter Thermo Scientific Orion 3-Star from Thermo Fisher Scientific Company at a temperature of 25 °C. ^1H NMR measurements were performed at 298 °K on a Bruker AVANCE I instrument equipped with a 5 mm probe operating at 400.2 MHz. An amount of 3 mg of polymer was dissolved in 0.5 mL of D_2O containing the stoichiometric amount of DCl to dissolve chitosan, and 32 scans were recorded at a temperature of 80 °C.

MW determination The intrinsic viscosity of Sigma Aldrich chitosan samples was determined through capillary viscometry measurements, from which it was possible to calculate their viscosity-average molecular weight M_v by using the Mark-Houwink expression. Viscosity measurements were carried on in an automatic viscometer AMVn

from ~~the company~~ Anton Paar at a temperature of 25 °C. Chitosan samples were prepared at a concentration of 3 mg/mL in AcOH 0.3 M/AcOH 0.2 M [57]. A series of dilutions was prepared for each chitosan sample in a concentration range between 0.02 and 1.5 mg/mL. Viscosity measurements were carried out at three different angles (30°, 50° and 60°).

The weight-average molecular weights of each sample were also determined through Size Exclusion Chromatography (SEC) measurements. The solvent used was a buffer with 0.3M acetic acid and 0.25M sodium acetate. Two columns in series from Tosoh Bioscience were used, *i.e.* TSK gel G 3000 and G 6000 PWXL associated with a pre-column TSK gel PWXL. A light scattering detector Treos from Wyatt was associated with a differential refractometer. The selected flow rate was 0.5 mL/min at 30°C, the dn/dc was equal to 0.190 and chitosan concentration injected was 0.25 mg/mL.

2.4. Chitosan/DNA complex formation

Chitosan/DNA complex net charge determination. Zeta potential measurements were performed with a Malvern Zetasizer NanoZS at a temperature of 25 °C. The complex formation was followed through zeta potential measurements by adding a given volume of chitosan 1 mg/mL into a DNA solution (0.03 mg/mL) at controlled pH, under continuous stirring. After stabilization of the obtained chitosan-DNA complex (*i.e.* 5 min), 1 mL of the solution was injected into the Zetasizer Nano cell for the measurement. After each measurement, all the solution was collected from the cell and re-introduced into the bulk solution before the addition of the next volume of chitosan solution. The Malvern Zetasizer NanoZS instrument measured the electrophoretic mobility of the particles and calculates the zeta potential using the Smoluchowski expression.

Size measurements through Dynamic Light Scattering (DLS). DLS measurements were performed in a Malvern Zetasizer NanoZS 90 at 25 °C and carried out at an angle of 90°. The hydrodynamic radius (R_H) was calculated using the Stokes–Einstein equation proposed for spherical particles. The evolution of R_H during complex formation was monitored during progressive additions of chitosan.

Atomic Force Microscopy (AFM) measurements. AFM measurements were carried out in a Nanosurf instrument, model Easyscan2 (Nanosurf AG, Switzerland), using a tapping mode with tips TAP 190Al-G from Budget Sensors and at a vibrational frequency of 600 mV.

Chitosan/DNA complexes centrifugation assays. Chitosan/DNA complexes were prepared at different charge ratios using a DNA solution 0.03 mg/mL in NaCl 0.01 M selected in order to preserve the double helical conformation ~~and to be able enabling~~ to accurately quantify DNA concentration by UV-spectroscopy in the supernatant after centrifugation. They were incubated during 30 min and then DLS measurements were performed in order to determine the size and the scattered intensity of the complexes. After recording this procedure, complexes were centrifuged during 45 min at 15 000 G and at 25 °C. The supernatant was then analyzed with DLS and UV-Vis spectroscopy. DNA concentration was measured by using the absorbance A_{260} in a Nanodrop 1000 Spectrophotometer from Thermo Scientific.

Free DNA quantification through gel electrophoresis. Chitosan/ DNA binding assays evaluated through gel electrophoresis were performed as follows: 100 ng of the plasmid DNA (eGFP) were mixed with increasing amounts of chitosan (0.1 mg/mL) in 20 μ L of a mixture of buffer (5% glycerol, 10mM Tris–HCl with a pH of 8.0, 1mM EDTA, 1mM dithiothreitol and 20 mM KCl) and water (at 1/9 v/v ratio). After stabilization of chitosan/DNA complexes under stirring during 30 min at room temperature, 3 μ L of loading buffer (0.05% bromophenol blue, 0.1 mM EDTA, 50% glycerol) was added to the mixture, then an aliquot of 10 μ L was applied to a 0.6% agarose gel electrophoresis in 1x TAE buffer (40 mM Tris, 20 mM Acetic acid, 1 mM EDTA) containing SYBR[®] Safe (Life Technologies, Carlsbad, CA, USA). The fraction of free DNA was determined from the intensity of the migrating fraction of DNA by using the Image Lab Software[®] in a Bio-Rad Gel Doc EZ Imager.

Free chitosan quantification through fluorescence measurements. Chitosan/DNA complexes were prepared at different charge ratios using a DNA solution 0.03 mg/mL in water. The complexes were stabilized during 30 min before centrifugation ~~during for~~ 45 min at 15 000 G and at 25 °C. The supernatant fluorescence was then determined with a Jasco Spectrofluorometer FP-8500 using calibration in the considered conditions (water, NaCl at different concentration, HCl).

Stability of the complexes in presence of SDS. Taking into consideration the experimental conditions given in literature [30], sodium dodecyl sulphate (SDS) was used to test the ability of complexes to dissociate as an index of their stability. Complexes were prepared as mentioned previously in the section of *Free DNA quantification through gel*

electrophoresis. After stabilization of chitosan/DNA complexes under stirring during 30 min at room temperature, before adding 3 μ L of loading buffer, 3 μ L of SDS 0.5 % wt were added to induce a partial decompaction of chitosan/DNA complexes. Then, same protocol as described before was followed to apply the aliquot for agarose gel electrophoresis. As well as mentioned before, fraction of free DNA is determined from the intensity of the migrating fraction of DNA by using the Image Lab Software® in a Bio-Rad Gel Doc EZ Imager.

Stability of the complexes in presence of external salt. Gel electrophoresis assays were performed in presence of NaCl at different concentrations (from 0 to 0.2 M). DNA/complexes were prepared as mentioned previously in the section of *Free DNA quantification through gel electrophoresis* but in presence of the selected NaCl concentration (from 0 to 0.2 M). After stabilization of chitosan/DNA complexes under stirring during 30 min at room temperature, 3 μ L of loading buffer was added to the mixture. Then, same protocol as described previously was followed by applying the aliquot to the agarose gel electrophoresis. Free DNA fractions were also determined from the intensity of the migrating fraction of DNA by using the Image Lab Software® in a Bio-Rad Gel Doc EZ Imager.

Complex stability in acidic medium and buffering capacity. Complex stability in acidic medium was followed through zeta potential measurements and potentiometric titration by progressive addition of given volumes of HCl 0.01 N into a chitosan/DNA complex suspension at a given charge ratio, $N^+/P^-=1$ and a ratio **N/P=3 times the N/P ratio corresponding to $N^+/P^-=1$** , and at an initial pH around pH=7.5. After stabilization of initial pH, 1 mL of the solution was injected into the Zetasizer Nano cell for zeta potential measurement. After each measurement, all the solution was collected from the cell and reintroduced into the bulk solution before the addition of the next volume of HCl. From these measurements, the influence of excess of chitosan is demonstrated. Same procedure was used for water and for chitosan solutions having the same concentration than the one used to reach the ratios $N^+/P^-=1$ and a ratio **N/P=3 times the N/P ratio corresponding to $N^+/P^-=1$** , all of them at the same initial pH. These experiments allowed demonstrating the role of bound and free chitosan on pH stabilization in presence of the complex. At end, the composition of the complex was determined in acidic conditions.

3. Results and discussion

3.1. Electrostatic complexes formation

3.1.1. Chitosan samples characterization

DA values of chitosan samples from Sigma-Aldrich and Primex were determined through conductivity [2] and ^1H NMR [48] measurements and are presented in Table 1. With both techniques it is possible to determine a reliable value, but still, the most precise technique remains the ^1H NMR if the polymer is perfectly soluble.

The viscosity-average MW was determined from the intrinsic viscosity ($[\eta]$) of the tested samples. Then, the Mark-Houwink relation ($[\eta]= K \times M^a$) was applied, using the constants determined by Brugnerotto *et al.* [57] to calculate the MW of Sigma Aldrich chitosan samples. The weight-average MWs for all Primex samples were determined by steric exclusion chromatography (SEC).

Chitosan samples were then characterized through ζ -potential measurements and potentiometric titrations. Fig. 1 shows, as an example, the ζ -potential and potentiometric titration with 0.1 N NaOH for the MMW chitosan sample from Sigma-Aldrich. The solution of fully protonated chitosan, dissolved in presence of the stoichiometric amount of HCl 0.1 N on the basis of NH_2 content, presents an initial ζ -potential around + 75 mV at a pH of 3.6, showing that under these conditions, chitosan is strongly positively charged. While pH increases for progressive addition of NaOH solution, the fraction of $[\text{NH}_3^+]$ in the solution of chitosan decreases to null charge when the ζ -potential decreases down to zero value (Fig. 2a).

From these curves, it is easy to determine the $-\text{NH}_2$ content (corresponding also to DA determination) at null ζ -potential and potentiometric transition at $\text{pH} \sim 7.5$ when a given amount of chitosan is dissolved in a known excess of HCl. From Fig. 1, the degree of protonation of chitosan can also be determined at a given pH. Then, it will allow expressing the charge ratio N^+/P^- characterizing the complex formation in presence of highly negatively charged DNA, enabling the discussion on the mechanism of complex formation.

3.1.2. Evidence of electrostatic complex formation

When positively charged chitosan is added under stirring into a dilute DNA solution, using DNA solutions in water, a complex is formed as evidenced from ζ -potential measurements shown in Fig. 2a and by AFM measurements presented in Fig. 2b.

Fig. 2a shows the ζ -potential as a function of the total $-\text{NH}_2$ amount added and expressed as (N/P). DNA molecules in solution give an initial ζ -potential of around -40 mV (1) which reaches the ζ -potential=0 mV (3) corresponding to the isoelectric point (IP) of the complexed particles during progressive addition of chitosan. The last zone of the curve (4) corresponds to an excess of chitosan in the chitosan/ DNA complex suspension and the potential turns to positive values (ζ -potential= +26 mV). AFM measurements (Fig. 2b) demonstrate the particles formation due to electrostatic interactions between DNA and chitosan, as discussed previously.

In Fig. 2a, it is noticed that a plateau is observed around the ζ -potential= -30 mV (2) corresponding to the formation of chitosan/ DNA complexes (at a charge ratio between 0.5 and 0.9 considering the initial partial protonation of chitosan at pH=6.5) having an average hydrodynamic radius R_H of 150 ± 20 nm. These complexes correspond to partial neutralization of the ionic phosphate sites (the complex remaining negatively charged). At a ζ -potential equal to the isoelectric point of the particles, all the negative charges of the DNA are neutralized by the positive charges of the chitosan and the following relation is obtained: $N/P=5.3$. In fact, as shown previously [33], the isoelectric point corresponds to the stoichiometric charge ratio $N^+/P^- = 1$, which is imposed by the degree of chitosan protonation. In Fig. 2a, the molar ratio N/P included the total concentration of chitosan amino groups $[\text{NH}_2]_T = ([\text{NH}_2]_{\text{free}} + [\text{NH}_3^+]_{\text{protonated}})$ and the ionic concentration of phosphates/L (where $[P^-]$ is supposed to be completely ionized under normal conditions). In the example given in Fig. 2a, the degree of chitosan protonation was 0.20 at pH=6.5, allowing to calculate the charge ratio $N^+/P^- = 1$ from the molar ratio $N/P = 5.3$ (see Table 2). These isoelectric conditions (at zero charge for the complex with complete DNA compaction) correspond to the stoichiometric charge ratio 1/1 taking into account the degree of chitosan protonation. This conclusion agrees with data given in literature [19, 20, 33] even if it is not confirmed in some pieces of work [35].

3.1.3. Influence of the chitosan degree of protonation and pH on complex formation

The protonation behavior of polycations such as chitosan is one interesting feature controlling endosomal escape of polyplexes, since it determines the density of cationic charges that affects the interaction with cell membranes as well as stability of the complex [20]. In the present work, the influence of the chitosan degree of protonation on chitosan/DNA complexes formation was firstly studied using a series of chitosan solutions at different initial pHs (3.6, 5.0, 5.5 and 6.0) and a DNA solution in water at a constant pH=6. The experimental results are given in Fig. 3a.

Fig. 3a shows the ζ -potential variation as a function of the molar ratio between chitosan and DNA dissolved in water for different initial pH chitosan values. The IP of the particles corresponds to the stoichiometric neutralization of negative DNA charges and positive charges of chitosan, which varies with the degree of protonation imposed by the initial pH of the chitosan solution. For chitosan/DNA complexes formed between the fully protonated chitosan (pH=3.6) and DNA negatively charged (at pH=6.0), the charge ratio of N^+/P^- is equal to $N/P=1$. At pH= 5.0, 5.5 and 6.0, chitosan is partially positively charged, since respectively 82%, 63% and 50% of the amino groups are protonated (see Table 2). Those values are obtained from the titration curve as given in Fig. 1. For chitosan/ DNA complexes obtained from the electrostatic interactions between partially protonated chitosan (pH = 5.0, 5.5, 6.0 and 6.5) and DNA (pH = 6.0), the IP corresponds to the equivalence $N^+/P^-=1$ calculated by the percentage of protonated amino groups, as shown in Table 2. These data demonstrate clearly that the PEC is based on electrostatic interactions as discussed previously [33].

To complete the study of complex formation and test the degree of compaction of DNA with the stoichiometry of the complex, the hydrodynamic radius of the particles formed in different conditions was determined by DLS measurements. The results are given in Fig. 3b and 3c.

Fig. 3b shows the variation of the particle size of DNA/chitosan complexes as a function of the stoichiometric ratio $[N^+]/[P^-]$. Within the range of charge ratio N^+/P^- from 0.5 to 0.7, it is observed that the particle size is almost constant, with an average value of 125 nm. Then, when approaching to the charge stoichiometry, the particle size slightly increases to 135 nm. It was previously found that maximum size of the complex occurs at the isoelectric point [35]. Fig. 3c shows the particle size of the chitosan/ DNA complexes formed at a constant charge ratio $N^+/P^- = 0.7$, using chitosan solutions at different initial pH

values. It is observed that the hydrodynamic radius obtained is around 125 nm for the four initial pH values used, *i.e.* 3.6, 4.5, 5.0, 5.5 and 6.0. Therefore, the pH variation of the initial chitosan solution does not affect the particle size of chitosan/DNA complexes, *i.e.* the degree of compaction of DNA in the complexes formed at a given charge ratio between the positively charged chitosan at different initial degrees of protonation and the strongly negative charged DNA independently of the amount of chitosan bound to DNA (even when $N/P > 1$).

Fig. 4a shows the results obtained for ζ -potential as a function of added chitosan solutions prepared at initial pH=3.6 with different molecular weights (chitosan samples 1 to 4 from Primex). It is possible to observe that the charge ratio for complex formation is nearly independent from the molecular weight, which varies from 550 000 to 180 000 g/mol. For the same chitosan samples, we report DLS measurements results, from which chitosan/DNA complexes sizes were determined at two chitosan/DNA ratios. **Fig. 4b** shows the results obtained for the R_H of chitosan samples at different Mw and with different amount of added chitosan. In these experiments, chitosan/DNA complexes were prepared separately by adding the specific amount of chitosan to the DNA solution in order to reach the desired charge ratio avoiding passing slowly through the isoelectric point of the particles for $N^+/P^-=1$, at which the system is instable and tends to produce larger particles [35]. At $N^+/P^-=0.8$, it is confirmed that the dimension is slightly larger for higher MW. This conclusion agrees with the results obtained by Alatorre-Meda *et al.* on three different MW in relation with the strong binding affinity between chitosan and DNA [38]. In excess of added chitosan ($N/P=5$), for positively charged particles, the dimensions are lower than for $N^+/P^-=0.8$ in relation with the higher ζ -potentials. In addition, the zeta potential is slightly higher for higher MWs (even if the DA varies from 0.30 to 0.05). This indicates a larger degree of compaction of DNA [20] and consequently a larger stability of chitosan/DNA complexes with high MW chitosan [36,38].

3.1.4. Influence of chitosan DA on the stoichiometry of chitosan/DNA complexes

At last, the influence of the degree of acetylation is studied on different samples selected in a large range of DA but moderate MW variation. Chitosan solution is added in DNA solution in water at pH=6 starting with chitosan solutions at 1 mg/mL and in slight excess of HCl corresponding to complete protonation with any possible DA.

From the volume of added chitosan (Fig. 5), the total amount of -NH_3^+ added into DNA solution is determined and demonstrated that the isoelectric point is obtained at a charge ratio $\text{N}^+/\text{P}^- = 1$. These results obtained in a large range of DA (0.05 up to 0.61) agree with the majority of the studies concerned by the influence of chitosan characteristics on the complex formed [37]. Using isothermal titration microcalorimetry, Ma *et al.* determined the binding constant and showed that it increased when pH and salt concentration decreased but also showed that this constant increased when DA decreased (especially when $\text{DA} < 0.2$) and MW increased [37]. This affinity, different from the stoichiometry is directly related to the stability of the complexes related with protection from Deoxyribonuclease (DNase) and with the transfection efficiency [20, 37].

To conclude, from these experimental results it is clearly confirmed that the net charge of chitosan is the main factor involved in complex formation whatever the DA and the molecular weight with the fully dissociated DNA in dilute solution. In addition, the MW has no influence on the complex formation, since for all samples, the isoelectric point corresponds to a charge ratio $\text{N}^+/\text{P}^- = 1$, however, it has a slight influence on the dimensions of the complexed nanoparticles.

3.2. Mechanism of chitosan/ DNA complex formation

It should be interesting to establish the mechanism of chitosan /DNA interaction in relation with the composition or ratio N/P since this point has been rarely covered. In the present work, it is clearly demonstrated that the ionic interaction between the negative phosphate sites and positive -NH_3^+ from chitosan is essential for complex formation. Then, two hypotheses may be suggested: i) the complexation occurs randomly in solution, *i.e.* chitosan associated on each DNA chains in the charge ratio N^+/P^- ; ii) a DNA chain is saturated by cooperative interactions with positively charged chitosan and the complementary DNA fraction remains free of chitosan as long as $\text{N}^+/\text{P}^- \leq 1$. A remaining question concerns the case when $\text{N}^+/\text{P}^- \geq 1$: when the complex becomes positively charged, is there an excess of chitosan fixed on the complex to control this charge inversion?

Such question was previously discussed for the interaction of a surfactant with a polyelectrolyte in dependence of the distance between ionic sites on the polyelectrolyte [59, 60]. In this case, as soon as the distance was lower than around 0.6 nm, cooperative

interactions with oppositely charged surfactant occur and one chain is saturated and located at the air/aqueous solution interface when free polyelectrolytes remain in solution. This process is related to the large effect of polyelectrolyte-surfactant association on the decrease of the air/water interfacial activity compared to only surfactant role [61].

To access to the exact composition of the chitosan/DNA complexes, different techniques were used to separate the complexed fraction and free fraction of DNA or chitosan in the supernatant in the different ranges of charge ratio.

3.2.1. Complex composition of chitosan/ DNA mixtures

It is important to determine the composition of chitosan/DNA complexes formed in different conditions. The complexes were formed and after stabilization, the system was characterized by DLS obtaining a relative intensity of the suspension and the hydrodynamic radius of the formed nanoparticles. Then, after centrifugation, the composition of the supernatant was determined by UV spectroscopy to determine the amount of free DNA. The obtained data are given in Table 3.

From light scattering results (Table 3), it is shown that particles start to be formed at $N^+/P^- = 0.3$ and that after centrifugation, the intensity in the supernatant remains very low due to the separation of nearly all the particles formed. From UV-Vis measurements, DNA concentration is determined and compared with the initial value 0.03 mg/mL. The fraction of free DNA determined in the supernatant from these experiments decreases when the charge ratio increases; additionally, it is demonstrated that this decrease is in good agreement with the amount of free DNA calculated after complex formation in the hypothesis of cooperative interaction. This indicates that DNA is saturated with charged chitosan forming particles.

A complementary experiment concerns electrophoresis of DNA on complexed systems: chitosan/DNA binding assays evaluated through gel electrophoresis allowed determining the percentage of free DNA chains in chitosan/DNA complexes at different molar ratios (0, 0.3, 0.4, 0.6, 0.8, 1.0 and 3.0) through the quantification of the amount of DNA chains migrating in the gel (obtained by using the Image Lab Software®). This technique allows covering a large charge ratio range. **Fig. 6a** presents the gel electrophoresis assay showing the effect of increasing amounts of chitosan on the electrophoretic mobility of plasmid

DNA (eGFP). The determined values of migrating DNA are in fair agreement with the theoretical percentage of free DNA chains at a specific charge ratio up to 1, when chitosan is initially fully protonated (*i.e.* at pH=3.6) assuming strong cooperative electrostatic interactions. These results are presented in Table 4.

From these data it is confirmed that all DNA is complexed at stoichiometric charge ratio equal to 1, as previously shown after 10 minutes of complex formation [56]. These results are in good agreement with the results obtained for the free fraction of DNA determined by UV spectroscopy (Table 3).

To confirm the composition of the complex, the migration of free DNA on gel was tested at charge ratio $N^+/P^- = 1$ for three chitosan samples having different DA (*i.e.* 0.05, 0.25 and 0.41). The results shown in Fig. 6b confirm that all DNA is complexed in the presence of the stoichiometric amount of fully protonated chitosan for every DA value.

Chitosan complexes formed with chitosan at 1 mg/mL, at initial pH of 3.6 and different DA at charge ratio =1 are uncharged, meaning that all DNA is involved in the complex formed. To definitively conclude on the complex composition, it was examined if some chitosan is free in the supernatant after centrifugation of suspension. It ~~is~~ was tested as a function of the stoichiometric molar ratio N/P. For that purpose, labelled chitosan was used to easily identify free chitosan after centrifugation of the chitosan/DNA complexes. Free labelled chitosan solution at 1×10^{-3} mg/mL ~~is~~ was used as reference; its fluorescence intensity is 28 which should allow predicting a fluorescence value equals to 30.2 for the chitosan volume added at $N^+/P^- = 1$ if all the chitosan was free. The obtained results are given in Table 5 for a series of molar ratios from 0.5 to 10. At $N/P < 1$, the fluorescence is close to zero, indicating the association of all the protonated chitosan added. Over $N/P = 1$, the molar ratio N/P corresponds to an excess of chitosan added on the basis of DNA phosphate (P⁻) content.

From the data provided in Table 5, it is shown that nearly all chitosan is complexed by DNA up to the stoichiometric charge ratio equal to 1, corresponding to a ζ -potential= 0 mV (experimentally it is found as a value of around +3 mV in Table 5, which in fact fluctuates around 0). For higher chitosan content, an additional chitosan fraction is bound to the particles corresponding to the overcharging chitosan/DNA complexes, becoming positively charged. In the same time, the zeta potential level off to around +40 mV over $N/P = 3$. At these conditions, the pH is lower than 7, corresponding to a slightly positive

charged chitosan, which is able to associate to previously complexed chitosan, bound by H-bonds and/or hydrophobic interactions. The amount of **monomeric units of** chitosan complexed with **phosphate ionic sites of** DNA levels to 1.6 to 2.3 indicating that a large amount of chitosan is free as soon as $N/P > 3$. This conclusion agrees with previously obtained results by Ma *et al.* [53]. In large excess of initially fully protonated chitosan ($pH=3.6$), the pH at equilibrium decreases causing a slight decrease of the amount of chitosan bound due to the increase of the degree of protonation of chitosan bound in excess of stoichiometry, in the same time as the positive zeta potential of the complex increases. Then, over $N/P=1$, positively charged particles are able to interact with the negatively charged cell membrane and then to enter into cells ~~to~~ **allowing** transfection. Another important conclusion is that over $N^+/P^- = 1$, free chitosan is in equilibrium with the complexed particles, which is related to its role on the proton sponge effect. This amount of free chitosan at the equilibrium is easily obtained from these fluorescence experiments. The presence of excess of free chitosan was proposed to increase the osmotic pressure and to destabilize the endosome.

These results clearly confirm, for the first time, that chitosan/DNA interaction is mainly based on cooperative electrostatic interaction up to $N^+/P^-=1$, where no free DNA nor free chitosan are present. In addition, the composition of the complexes is established over $N^+/P^-=1$, allowing to estimate the fraction of chitosan associated with the complex (1.6 to 2.3) and then, the free fraction of chitosan able to be protonated in acidic medium as a basis of the proton sponge effect [48].

3.2.2. Stability of chitosan/ DNA complex

3.2.2.1. Influence of DA on stability

Three chitosan samples with different DA (0.05, 0.25 and 0.41) were used to prepare complexes with DNA at a stoichiometric ratio $N^+/P^-=1$ (see **Fig. 5 and 6b**). Then, complex stability was tested in the presence of SDS (**Fig. 6c**).

From these data, it is shown that the chitosan/DNA complex stability is preserved in a relatively large excess of added chitosan and for the concentration of SDS used for the test (0.65 mg/mL). No influence of the DA is observed in these conditions and all DNA remains complexed.

3.2.2.2. Influence of external salt on complex formation and stability

As discussed in the literature [3], the stability of polyelectrolyte complexes depends on the external salt concentration and pH when one of the polyelectrolyte engaged is a weak base or acid. It was then interesting to analyze the role of external salt (NaCl from 0 to 2M) on chitosan/DNA complex formation and on its stability. Firstly, zeta potential was used but with a limitation imposed by conductivity of the solution (Fig. 7a). At higher ionic concentrations, the relative intensity of light scattered was determined (Fig. 7b).

When salt concentration increases, more chitosan is needed to obtain the isoelectric point as shown in Table 6. This is due to a decrease of the degree of protonation of the initial solution as tested by potentiometric titration in relation with the electrostatic screening effect.

In addition, the positive ζ -potential over the isoelectric point decreases progressively due to the lower degree of protonation of chitosan as shown by the higher amount of chitosan needed to get IP. A large excess of NaCl over 1M causes flocculation of the particles due to screening of the electrostatic repulsions between particles. To confirm the influence of external salt on chitosan/DNA complex formation, gel electrophoresis experiment at different molar ratios (0 to 3.0) was performed in presence of NaCl 0.1 M. Free DNA for each N/P was then quantified, as shown in Fig. 7c.

From these results, it is confirmed that the isoelectric point needs the addition of a larger amount of chitosan, as indicated by 16% of DNA free at a molar ratio equal to 1 (Fig. 7d), representing the amount of chitosan fully protonated to get the isoelectric point in absence of salt. To conclude, in presence of external salt, due to a decrease of the degree of protonation of chitosan prepared at a given pH, more chitosan is needed to get the isoelectric point. Then, for positive nanoparticles, N/P larger than 1 has to be adopted depending on the amount of salt. This behavior seems to be related to the decrease of stability mentioned in the literature [20].

Chitosan/DNA complexes stability was tested in presence of SDS at different salt content and is presented in Fig. 8. From these data, it is confirmed that the complex formed in water or 0.01M NaCl has nearly the same composition at N/P=2 and the same stability as around 40% of DNA is dissociated in presence of SDS (Fig. 8a and 8b). Then, DNA is partly dissociated when external salt concentration increases (at NaCl concentration \geq

0.1M), confirming our results given in Fig. 7. In the same time, the stability of chitosan/DNA complexes is also shown to decrease in presence of a given SDS concentration.

When the external salt concentration increases, the fraction of chitosan bound in the complex was determined after centrifugation using the FITC-labelled chitosan. The results are given in Table 7. From these data, which complete Table 6, it comes that more chitosan is fixed in the complex in the presence of external salt, and at the same time, some DNA is dissociated. This is attributed to the decrease of the net charge of chitosan when the pK tends to pK_0 due to the electrostatic screening effect.

3.2.2.3. Influence of HCl excess on composition and stability of the complex

It is important to study the behavior of complexes in acidic medium as they encounter in the cells where the pH range of endosomal release varies from 4.5 to 7. This is expressed by the buffering capacity of the complex. In that respect, chitosan was proved to have higher buffering capacity than PEI in the endosomal pH range for same charge number [48]. For this mechanism, it is important to fix a N/P ratio larger than 3 to have free chitosan in presence of the complex (N/P=3 times the N/P ratio corresponding to $N^+/P^- = 1$). The proton sponge effect or buffering capacity was analyzed with comparison with the case of $N^+/P^- = 1$. In Fig. 9a, the pH and ζ -potential are measured during progressive addition of HCl in presence of chitosan/DNA complex and excess of chitosan. The ζ -potential increases progressively to higher positive values corresponding to a better stability of the nanoparticles suspension. At the same time, the pH of the medium slightly decreases between 7 and 5, corresponding to the pH range inside the cells. This step corresponds also to chitosan protonation.

In comparison, the complex formed at $N^+/P^- = 1$ was examined in the same conditions. The variations of the pH of the suspension are compared in Fig. 9b. In absence of chitosan excess, the pH decreases more rapidly; nevertheless, it remains more stable than when the same acid is added in water. These data confirm the role of the excess of free chitosan on the buffering effect. In addition, the composition of the complex in acidic conditions (pH=3.50) was estimated from fluorescence measurements. At $N^+/P^- = 1$, it was found that the fraction of chitosan bound is slightly depressed from 1 to 0.84 in relation with the increase of protonation at the final pH=3.50. At the ratio N/P=3 times the N/P ratio corresponding

to $N^+/P^-=1$, the fraction of chitosan in the complex slightly increases from 2.26 to 2.68 indicating a good stability in excess of acid (pH= 3.72).

To conclude, it is shown that the composition of the complex depends on the electrostatic interaction imposed by the protonation degree of chitosan and on the screening effect of external salt concentration. In the same time, the stability depends on the same parameters; this is important because it is also related to the transfection efficiency of the complex, especially on the unpackaging step of DNA from its vector after nuclear location [36, 37, 39].

4. Conclusions

This paper describes the study ~~of the role of chitosan considering the~~ of chitosan/DNA complex formation mechanism ~~often proposed as a gene transfer vector~~. Firstly, a series of chitosan samples were characterized in molar mass (MW) and degree of acetylation (DA). Then, the roles of MW and DA on complex formation were established demonstrating that the main factor is the electrostatic interaction between negatively charged DNA and positively charged chitosan ~~based on its protonation~~ as $[-NH_3^+]$. Two regimes were clearly determined: i) for charge ratio <1 , complexes were negatively charged up to the isoelectric point (IP) at $N^+/P^-=1$; ~~obtained for DNA charge neutralization; for partial chitosan protonation depending on the initial pH of the medium, the molar ratio N/P was larger than the charge ratio. Nanoparticles with radius in the range of 130 nm were formed at charge ratio ≥ 0.3 ;~~ ii) further addition of chitosan, ~~over the isoelectric point~~, expressed as $N/P > N^+/P^-=1$, the complex turned to positively charge nanoparticles able to interact with cell membranes. The influence of DA and MW was low when the net charge of chitosan added was correctly taken into account.

~~Secondly, the cooperative mechanism of complex formation is demonstrated and their stability is tested in salt excess or presence of SDS using specific determination of chitosan and DNA contents in the complex formed. Secondly, the cooperative mechanism of complexes formation as well as their stability was established using mainly chitosan with very low DA for better homogeneity. From specific determination of chitosan and DNA content in the complex formed and in the supernatant obtained after nanoparticles separation by centrifugation;~~ It is concluded that DNA was saturated with charged chitosan

in equilibrium with free DNA for $N^+/P^- < 1$. At the isoelectric point, neither free chitosan nor DNA was present independently of DA and MW. Clearly, external salt plays a role on the stabilization of the chitosan/DNA complex due to electrostatic screening decreasing the net charge of chitosan. ~~Over the IP, more chitosan was fixed on the complex and the particles became positively charged. In this regime, bound chitosan amount increased when N/P increases. The stability of the complexes formed in excess of chitosan was tested in presence of SDS and /or external salt excess. In presence of 0.1 M NaCl, the IP was obtained at $N^+/P^- = 1 > N/P = 1$ due to the electrostatic screening on chitosan protonation and no DNA was decomplexed up to $N/P = 3$. Then, clearly, external salt played a role on the stabilization of the chitosan/DNA complex due to change of the net charge of chitosan, which needs to be considered.~~ At $N/P = 2$, a small fraction of DNA was decomplexed in the presence of SDS and in absence of external salt, but it increased in 0.2 M NaCl. At the same time, the fraction of labelled chitosan bound slightly increased. At $N/P = 3$, the complexes were stable in the presence of SDS with any value of DA. ~~To conclude~~ Then, positively charged complexes formed at $N/P > 3$ had a good stability in the presence of external salt and the SDS tested.

Thirdly, the influence of acidic medium on complex stability was investigated. The importance of free chitosan was clearly ~~shown on the low pH variation in the range of endosomal pH (between 7 and 4.5) in agreement with a proton-sponge effect. was clearly demonstrated by comparison between N^+/P^- equal to 1 and $N/P = 3$ times N^+/P^- during progressive additions of HCl.~~ In the same time, a large increase of the positive charge of the particles due to progressive protonation of the complexed chitosan stabilizing the particle suspension. To conclude, the compositions and main properties of chitosan complexed with DNA were developed *in vitro* using complementary techniques, allowing in the future to take advantage of biocompatibility and structural stability of chitosan for development in the field of gene transfer and especially to test for the transfection efficiency.

~~The pH variation was low in the range of endosomal pH (between 7 and 4.5) indicating that the proton sponge effect appears in excess of free chitosan. At acidic pH, the composition of the complex remained nearly unchanged (with a slight increase of the chitosan bounded). However, a large increase of the positive charge of the particles due to progressive protonation of the chitosan involved in the complex is observed. These results~~

~~demonstrated the good buffering property of free chitosan involved in stabilization and protection of the complex inside the cells.~~

~~To conclude, the compositions and main properties of chitosan complexed with DNA were developed *in vitro* using complementary techniques, allowing in the future to take advantage of biocompatibility and structural stability of chitosan for development in the field of gene transfer and especially to test for the transfection efficiency.~~

Acknowledgements

The authors acknowledge Mrs H.L. Lauzon from Primex Cy (Iceland) and G. Roberts (England) for the gift of chitosan samples and E. Bayma from CERMAV (CNRS) laboratory (Grenoble, France) for the SEC experiments. L.M. Bravo-Anaya acknowledges the fellowship grant given by CONACYT (CVU 350759), the collaboration established through this grant with the LCPO laboratory (University of Bordeaux, France), as well as technical support obtained there. F. Carvajal and L.M. Bravo-Anaya acknowledge the technical support given by Dr. J.F. Armando Soltero Martínez from Laboratory of Rheology (University of Guadalajara, México).

List of abbreviations

DNA: deoxyribonucleic acid

PEI: polyethylenimine

PEC: polyelectrolyte complex

IP: isoelectric point

N: number of chitosan NH_2

N^+ : number of protonated NH_3^+

P^- : number of phosphate from DNA or plasmid

N^+/P^- : charge ratio

N/P: molar ratio

MW: molecular weight

Mw: weight average molecular weight

Mv: viscosity average molecular weight

References

[1] V. Kabanov, Fundamentals of polyelectrolyte complexes in solution and the bulk, in G. Decher, J. B. Schlenoff (Eds.) Multilayer thin films: sequential assembly of nanocomposite materials, Wiley-VCH Verlag GmbH & Co., Weinheim (Germany) 2003, pp. 47-86, <https://doi.org/10.1002/3527600574.ch2>

[2] L. Rusu-Balaita, J. Desbrières and M. Rinaudo. Formation of a biocompatible polyelectrolyte complex: chitosan-hyaluronan complex stability, Polym. Bull. 50 (2003) 91-98. <https://doi.org/10.1007/s00289-003-0144-1>.

[3] V. A. Kabanov and A. B. Zezin. Soluble interpolymeric complexes as a new class of synthetic polyelectrolytes, Pure Appl. Chem. 56 (3) (1984) 343-354. <https://doi.org/10.1351/pac198456030343>

[4] Y. Jeong, D. Lee, K. Choe, H. Ahn, P. Kim, J. H. Park and Y. C. Kim. Polypeptide-based polyelectrolyte complexes overcoming the biological barriers of oral insulin delivery, J. Ind. Eng. Chem. 48 (2017) 79-87. <https://doi.org/10.1016/j.jiec.2016.12.022>

[5] D. Volodkin, H. Mohwald, J. C. Voegel and V. Ball. Coating of negatively charged liposomes by polylysine: drug release study, J. Control. Release 117(1) (2007) 111-120. <https://doi.org/10.1016/j.jconrel.2006.10.021>

[6] F. Vial, S. Rabhi and C. Tribet. Association of octyl-modified poly (acrylic acid) onto unilamellar vesicles of lipids and kinetics of vesicle disruption, Langmuir 21(3) (2005) 853-862. <https://doi.org/10.1021/la048039v>

[7] T. Serikawa, N. Suzuki, H. Kikuchi, K. Tanaka and T. Kitagawa. A new cationic liposome for efficient gene delivery with serum into cultured human cells: A quantitative analysis using two independent fluorescent probes, Biochim. Biophys. Acta 1467 (2000) 419-430. [https://doi.org/10.1016/S0005-2736\(00\)00239-X](https://doi.org/10.1016/S0005-2736(00)00239-X)

- [8] D.J. Stephan, Z.Y. Yang, H. San, R.D. Simari, C.J. Wheeler, P.L. Felgner, D. Gordon, G.J. Nabel, E.G. Nabel, A new cationic liposome DNA complex enhances the efficiency of arterial gene transfer in vivo, *Hum. Gene Ther.* 7 (1996) 1803–1812. <https://doi.org/10.1089/hum.1996.7.15-1803>
- [9] S. Bochicchio, A. Dalmoro, A. Barba, G. Grassi and G. Lamberti. Liposomes as siRNA Delivery Vectors, *Curr. Drug Metab.* 15 (2015) 882–892. <https://doi.org/10.2174/1389200216666150206124913>
- [10] A. Domard, M. Rinaudo and C. Terrassin. Adsorption of chitosan and a quaternized derivative on kaolin, *J. Appl. Polym. Sci.* 38 (1989) 1799-1806. <https://doi.org/10.1002/app.1989.070381002>
- [11] E. Pefferkorn, The role of polyelectrolytes in the stabilisation and destabilisation of colloids. *Adv. Colloid Interfac.* 56 (1995) 33-104. <https://doi.org/10.1002/app.1989.070381002>
- [12] Y. Fukui and K. Fujimoto. The preparation of sugar polymer-coated nanocapsules by the layer-by-layer deposition on the liposome, *Langmuir* 25(17) (2009) 10020-10025. <https://doi.org/10.1021/la9008834>
- [13] G. Angelini, S. Boncompagni, P. De Maria, A. Fontana, C. Gasbarri and G. Siani. Kinetic evaluation of the effect of layer by layer deposition of polyelectrolytes on the stability of POPC liposomes, *Colloid Surface A.* 322(1-3) (2008) 234-238. <https://doi.org/10.1016/j.colsurfa.2008.03.015>
- [14] J. Chluba, J.C. Voegel, G. Decher, P. Erbacher, P. Schaaf and J. Ogier, Peptide hormone covalently bound to polyelectrolytes and embedded into multilayer architectures conserving full biological activity, *Biomacromolecules* 2(3) (2001) 800-805. <https://doi.org/10.1021/bm015529i>
- [15] B. Thierry, F. M. Winnik, Y. Merhi and M. Tabrizian, Nanocoatings onto arteries via layer-by-layer deposition: toward the in vivo repair of damaged blood vessels, *J. Am. Chem. Soc.* 125(25) 2003) 7494-7495. <https://doi.org/10.1021/ja034321x>

- [16] A. V. Kabanov and V. A. Kabanov, DNA complexes with polycations for the delivery of genetic material into cells, *Bioconjugate Chem.* 6(1) (1995) 7-20. <https://doi.org/10.1021/bc00031a002>
- [17] S.W. Richardson, H.J. Kolbe and R. Duncan, Potential of low molecular mass chitosan as a DNA delivery system: biocompatibility, body distribution and ability to complex and protect DNA, *Int. J. Pharmaceut.* 178(2) (1999) 231-243. [https://doi.org/10.1016/S0378-5173\(98\)00378-0](https://doi.org/10.1016/S0378-5173(98)00378-0)
- [18] H. Q. Mao, K. Roy, V. L. Troung-Le, K. A. Janes, K. Y. Lin, Y. Wang, J. Thomas August and K.W. Leong, Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency, *J. Control. Release* 70(3) (2001) 399-421. [https://doi.org/10.1016/S0168-3659\(00\)00361-8](https://doi.org/10.1016/S0168-3659(00)00361-8)
- [19] S. P. Strand, S. Danielsen, B.E. Christensen and K.M. Vårum, Influence of chitosan structure on the formation and stability of DNA–chitosan polyelectrolyte complexes, *Biomacromolecules* 6(6) (2005) 3357-3366. <https://doi.org/10.1021/bm0503726>
- [20] M.D. Buschmann, A. Merzouki, M. Lavertu, M. Thibault, M. Jean and V. Darras, Chitosans for delivery of nucleic acids, *Adv. Drug Deliver. Rev.* 65(9) (2013) 1234-1270. <https://doi.org/10.1016/j.addr.2013.07.005>
- [21] F.C. MacLaughlin, R.J. Mumper, J. Wang, J. M. Tagliaferri, I. Gill, M. Hinchcliffe and A.P. Rolland, Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery, *J. Control. Release* 56(1-3) (1998) 259-272. [https://doi.org/10.1016/S0168-3659\(98\)00097-2](https://doi.org/10.1016/S0168-3659(98)00097-2)
- [22] V. Vijayanathan, T. Thomas and T.J. Thomas, DNA nanoparticles and development of DNA delivery vehicles for gene therapy, *Biochemistry* 41(48) (2002) 14085-14094. <https://doi.org/10.1021/bi0203987>
- [23] K.W. Leong, H.Q. Mao, V.L. Truong-Le, K. Roy, S.M. Walsh and J.T. August, DNA-polycation nanospheres as non-viral gene delivery vehicles, *J. Control. Release* 53(1-3) (1998) 183-193. [https://doi.org/10.1016/S0168-3659\(97\)00252-6](https://doi.org/10.1016/S0168-3659(97)00252-6)
- [24] D. Luo and W.M. Saltzman, Synthetic DNA delivery systems, *Nat. Biotechnol.* 18(1) (2000) 33. <https://doi.org/10.1038/71889>

- [25] M.J. Tiera, F.M. Winnik and J.C. Fernandes, Synthetic and natural polycations for gene therapy: state of the art and new perspectives, *Curr. Gene Ther.* 6(1) (2006) 59-71. <https://doi.org/10.2174/156652306775515510>
- [26] B. Santos-Carballal, E. Fernández Fernández and F. M. Goycoolea, Chitosan in Non-Viral Gene Delivery: Role of Structure, Characterization Methods, and Insights in Cancer and Rare Diseases Therapies, *Polymers* 10(4) (2018) 444. <https://doi.org/10.3390/polym10040444>
- [27] M.K Lee, S.K. Chun, W.J. Choi, J K. Kim, S.H. Choi, A.K. Kim, K. Oungbho, J.S, Park, W.S. Ahn and C.K. Kim, The use of chitosan as a condensing agent to enhance emulsion-mediated gene transfer, *Biomaterials* 26(14) (2005) 2147-2156. <https://doi.org/10.1016/j.biomaterials.2004.07.008>
- [28] R.J. Mumper, J. Wang, J.M. Claspell and A.P. Rolland, Novel polymeric condensing carriers for gene delivery, *Proceedings Controlled Release Society* (22) (1995) 178-179.
- [29] I.R.C. Hill, M.C. Garnett, F. Bignotti and S.S. Davis, Determination of protection from serum nuclease activity by DNA–polyelectrolyte complexes using an electrophoretic method, *Anal. Biochem.* 291(1) (2001) 62-68. <https://doi.org/10.1006/abio.2001.5004>
- [30] M. Köping-Höggård, I. Tubulekas, H. Guan, K. Edwards, M. Nilsson, K.M. Vårum and P. Artursson, Chitosan as a nonviral gene delivery system. Structure–property relationships and characteristics compared with polyethylenimine in vitro and after lung administration in vivo, *Gene Ther.* 8(14) (2001) 1108. <https://doi.org/10.1038/sj.gt.3301492>
- [31] K.V.H. Prashanth, S.M. Dharmesh, K.S.J. Rao and R.N. Tharanathan, Free radical-induced chitosan depolymerized products protect calf thymus DNA from oxidative damage, *Carbohydr. Res.* 342(2) (2007) 190-195. <https://doi.org/10.1016/j.carres.2006.11.010>
- [32] W. Liu, S. Sun, Z. Cao, X. Zhang, K. Yao, W.W. Lu and K.D.K. Luk, An investigation on the physicochemical properties of chitosan/DNA polyelectrolyte complexes, *Biomaterials* 26(15) (2005) 2705-2711. <https://doi.org/10.1016/j.biomaterials.2004.07.038>
- [33] L.M. Bravo-Anaya, J.F.A. Soltero, M. Rinaudo, DNA/chitosan electrostatic complex, *Int. J. Biol. Macromol.* 88 (2016) 345–353. <https://doi.org/10.1016/j.ijbiomac.2016.03.035>

- [34] M. Alatorre-Meda, P. Taboada, F. Hartl, T. Wagner, M. Freis and J. R. Rodríguez, The influence of chitosan valence on the complexation and transfection of DNA: the weaker the DNA–chitosan binding the higher the transfection efficiency, *Colloid Surface B*. 82(1) (2001) 54-62. <https://doi.org/10.1016/j.colsurfb.2010.08.013>
- [35] F. Amaduzzi, F. Bomboi, A. Bonincontro, F. Bordi, S. Casciardi, L. Chronopoulou, M. Diociaiuti, F. Mura, C. Palocci and S. Sennato, Chitosan–DNA complexes: charge inversion and DNA condensation, *Colloid Surface B* 114 (2014) 1-10. <https://doi.org/10.1016/j.colsurfb.2013.09.029>
- [36] F. Bordi, L. Chronopoulou, C. Palocci, F. Bomboi, A. Di Martino, N. Cifani, B. Pompili, F. Ascenzioni and S. Sennato, Chitosan–DNA complexes: effect of molecular parameters on the efficiency of delivery, *Colloid Surface A* 460 (2014) 184-190. <https://doi.org/10.1016/j.colsurfa.2013.12.022>
- [37] P.L. Ma, M. Lavertu, F.M. Winnik and M.D. Buschmann, New Insights into chitosan–DNA interactions using isothermal titration microcalorimetry, *Biomacromolecules* 10(6) (2009) 1490-1499. <https://doi.org/10.1021/bm900097s>
- [38] M. Alatorre-Meda, P. Taboada, J. Sabín, B. Krajewska, L.M. Varela and J.R. Rodríguez, DNA–chitosan complexation: a dynamic light scattering study. *Colloid Surface A* 339(1-3) (2009) 145-152. <https://doi.org/10.1016/j.colsurfa.2009.02.014>
- [39] M. Lavertu, S. Methot, N. Tran-Khanh and M.D. Buschmann, High efficiency gene transfer using chitosan/DNA nanoparticles with specific combinations of molecular weight and degree of deacetylation, *Biomaterials* 27(27) (2006) 4815-4824. <https://doi.org/10.1016/j.biomaterials.2006.04.029>
- [40] K.A. Howard, U.L. Rahbek, X. Liu, C.K. Damgaard, S.Z. Glud, M.Ø. Andersen, M. B. Hovgaard, A. Schmitz, J.R. Nyengaard, F. Besenbacher and J. Kjems, RNA interference in vitro and in vivo using a novel chitosan/siRNA nanoparticle system, *Mol. Ther.* 14(4) (2006) 476-48. <https://doi.org/10.1016/j.ymthe.2006.04.010>
- [41] G. Borchard, Chitosans for gene delivery, *Adv. Drug Deliver. Rev.* 52(2) (2001) 145-150. <https://www.ncbi.nlm.nih.gov/pubmed/11718938>
- [42] P. Erbacher, S. Zou, T. Bettinger, A.M. Steffan and J.S. Remy, Chitosan-based vector/DNA complexes for gene delivery: biophysical characteristics and transfection

ability, *Pharmaceut. Res.* 15(9) (1998) 1332-1339.
<https://doi.org/10.1023/A:1011981000671>

[43] B. Layek and J. Singh. Caproic acid grafted chitosan cationic nanocomplexes for enhanced gene delivery: effect of degree of substitution, *Int. J. Pharmaceut.* 447(1-2) (2013) 182-191. <https://doi.org/10.1016/j.ijpharm.2013.02.052>

[44] B. Layek and J. Singh, N-hexanoyl, N-octanoyl and N-decanoyl chitosans: Binding affinity, cell uptake, and transfection, *Carbohydr. Polym.* 89(2) (2012) 403-410. <https://doi.org/10.1016/j.carbpol.2012.03.021>

[45] M. Rinaudo, Chitin and chitosan: Properties and applications, *Prog. Polym. Sci.* 31(7) (2006) 603-632. <https://doi.org/10.1016/j.progpolymsci.2006.06.001>

[46] T. Sato, T. Ishii and Y. Okahata, In vitro gene delivery mediated by chitosan, Effect of pH, serum, and molecular mass of chitosan on the transfection efficiency, *Biomaterials* 22(15) (2001) 2075-2080. [https://doi.org/10.1016/S0142-9612\(00\)00385-9](https://doi.org/10.1016/S0142-9612(00)00385-9)

[47] S. Nimesh, M.M. Thibault, M. Lavertu and M.D. Buschmann, Enhanced gene delivery mediated by low molecular weight chitosan/DNA complexes: effect of pH and serum, *Mol. Biotechnol.* 46(2) (2010) 182-196. <https://doi.org/10.1007/s12033-010-9286-1>

[48] I. Richard, M. Thibault, G. De Crescenzo, M.D. Buschmann and M. Lavertu, Ionization behavior of chitosan and chitosan–DNA polyplexes indicate that chitosan has a similar capability to induce a proton-sponge effect as PEI, *Biomacromolecules* 14(6) (2013) 1732-1740. <https://doi.org/10.1021/bm4000713>

[49] A. Akinc, M. Thomas, A.M. Klivanov and R. Langer, Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis, *J. Gen. Med.* 7(5) (2005) 657-663. <https://doi.org/10.1002/jgm.696>

[50] R.V. Benjaminsen, M.A. Matthebjerg, J.R. Henriksen, S.M. Moghimi and T.L. Andresen, The possible “proton sponge” effect of polyethylenimine (PEI) does not include change in lysosomal pH, *Mol. Ther.* 21(1) (2013) 149-157. <https://doi.org/10.1038/mt.2012.185>

- [51] P. Neuberg and A. Kichler, In *Advances in Genetics*, Elsevier, Academic Press. Recent developments in nucleic acid delivery with polyethylenimines, 2014, vol.88, pp. 263-288. <https://doi.org/10.1016/B978-0-12-800148-6.00009-2>
- [52] M. Thibault, M. Astolfi, N. Tran-Khanh, M. Lavertu, V. Darras, A. Merzouki, and M. D. Buschmann. Excess polycation mediates efficient chitosan-based gene transfer by promoting lysosomal release of the polyplexes, *Biomaterials* 32(20) (2011) 4639-4646. <https://doi.org/10.1016/j.biomaterials.2011.03.010>
- [53] P.L. Ma, M.D. Buschmann and F.M. Winnik, Complete physicochemical characterization of DNA/chitosan complexes by multiple detection using asymmetrical flow field-flow fractionation, *Anal. Chem.* 82(23) (2010) 9636-9643. <https://doi.org/10.1021/ac100711j>
- [54] P.L. Ma, M.D. Buschmann and F.M. Winnik, One-step analysis of DNA/chitosan complexes by field-flow fractionation reveals particle size and free chitosan content, *Biomacromolecules* 11(3) (2010) 549-554. <https://doi.org/10.1021/bm901345q>
- [55] M. Thibault, S. Nimesh, M. Lavertu and M.D. Buschmann, Intracellular trafficking and decondensation kinetics of chitosan-pDNA polyplexes, *Mol. Ther.* 18(10) (2010) 1787-1795. <https://doi.org/10.1038/mt.2010.143>
- [56] L.M. Bravo-Anaya, F. Pignon, F.A. Soltero and M. Rinaudo, Conformation and Rheological Properties of Calf-Thymus DNA in Solution, *Polymers* 8 (2016) 51. <https://doi.org/10.3390/polym8020051>
- [57] J. Brugnerotto, J. Desbrieres, G. Roberts and M. Rinaudo, Characterization of chitosan by steric exclusion chromatography, *Polymer* 42 (2001) 9921-9927. [https://doi.org/10.1016/S0032-3861\(01\)00557-2](https://doi.org/10.1016/S0032-3861(01)00557-2)
- [58] F. Quemeneur, M. Rinaudo and B. Pépin-Donat, Influence of Molecular Weight and pH on Adsorption of Chitosan at the Surface of Large and Giant Vesicles, *Biomacromolecules* 9 (2008) 396-402. <https://doi.org/10.1021/bm700943j>
- [59] H. Okuzaki and Y. Osada, Effects of Hydrophobic Interaction on the Cooperative Binding of a Surfactant to a Polymer Network, *Macromolecules* 27 (1994) 502-506. <https://doi.org/10.1021/ma00080a025>

[60] L. Chen, S. Yu, Y. Kagami, J. Gong and Y. Osada. Surfactant binding of polycations carrying charges on the chain backbone: cooperativity, stoichiometry and crystallinity, *Macromolecules* 31(3) (1998) 787-794. <https://doi.org/10.1021/ma971228i>

[61] V. Babak, I. Lukina, G. Vikhoreva, J. Desbrieres and M. Rinaudo, Interfacial properties of dynamic association between chitin derivatives and surfactants, *Colloid Surface A* 147 (1999) 139-148. [https://doi.org/10.1016/S0927-7757\(98\)00752-3](https://doi.org/10.1016/S0927-7757(98)00752-3)

Captions

~~Fig. 1. ζ potential as a function of added volume of chitosan for $C_{DNA}=0.03$ mg/mL dissolved in water (pH=6.0) and $C_{Chit}=1.0$ mg/mL (pH=3.6). Chitosan Primex 6, labeled (\bullet) with FITC and unlabeled (\blacksquare).~~

~~Fig. 2. Fig. 1. a) ζ -potential and b) potentiometric titration of 1.0 mg/mL chitosan at initial pH=3.6 with 0.1 N NaOH. MMW sample from Sigma Aldrich having a Mv of 8.6×10^4 g/mol, DA= 0.22.~~

~~Fig. 3. Fig. 2. a) ζ -Potential as a function of N/P during the formation of chitosan/DNA complexes at a temperature of 25 °C. $C_{DNA}= 0.03$ mg/mL at pH=6.0 in water and added $C_{Chit}= 1$ mg/mL at pH=6.5. b) AFM images chitosan/DNA complexes formed at a charge ratio of 0.8. Chitosan Primex 6 with Mw= 160 000, DA= 0.05~~

~~Fig. 4. Fig. 3. a) ζ -potential as a function N/P for $C_{DNA}=0.03$ mg/mL dissolved in water (pH=6.0) and $C_{Chit}=1.0$ mg/mL (pH=3.6, 5.5, 5.0 and 6.0). MMW sample from Sigma Aldrich, having a Mv= 8.6×10^4 g/mol, DA= 0.22. b) Hydrodynamic radius as a function of the N^+/P^- ratio. $C_{DNA}=0.03$ mg/mL (pH=6.0), $C_{Chit}=1.0$ mg/mL (initial pH=3.6). c) Hydrodynamic radius as a function of initial pH of chitosan solutions with $[N^+]/[P^-]=0.7$. $C_{DNA}=0.03$ mg/mL (pH=6.0), $C_{Chit}=1.0$ mg/mL (pH=3.6, 4.5, 5.0, 5.5 and 6.0). MMW sample from Sigma Aldrich, having a Mv= 8.6×10^4 g/mol, DA= 0.22.~~

~~Fig. 5. a) Hydrodynamic radius as a function of the N^+/P^- ratio. $C_{DNA}=0.03$ mg/mL (pH=6.0), $C_{Chit}=1.0$ mg/mL (initial pH=3.6). b) Hydrodynamic radius as a function of initial pH of chitosan solutions with $[N^+]/[P^-]=0.7$. $C_{DNA}=0.03$ mg/mL (pH=6.0), $C_{Chit}=1.0$ mg/mL (pH=3.6, 4.5, 5.0, 5.5 and 6.0). MMW sample from Sigma Aldrich, having a Mv= 8.6×10^4 g/mol, DA= 0.22.~~

~~Fig. 6. Fig. 4. a) ζ -potential as a function of chitosan added for $C_{DNA}=0.03$ mg/mL (pH=6.0) dissolved in water and $C_{Chit}=1.0$ mg/mL (initial pH=3.6). Chitosan samples 1 to 4 from Primex. b) Hydrodynamic radius for each chitosan sample (1 to 6 from Primex) with the charge ratio $N^+/P^- = 0.8$ and molar ratio $N/P=5.0$.~~

~~Fig. 7. Fig. 5. ζ -potential as a function of volume of fully protonated chitosan with different DA added in 10 mL DNA solution for $C_{DNA}=0.03$ mg/mL (pH=6.0) dissolved in~~

water. Chitosan samples were prepared in presence of slight excess of HCl and at a concentration of 1 mg/mL.

Fig. 8. Fig. 6. a) DNA-binding properties of chitosan. Electrophoresis gel assays showing the effect of increasing concentrations of chitosan (sample Primex 6 at initial pH=3.6) on the electrophoretic mobility of plasmid DNA (eGFP). The molar ratio N/P equals to N^+/P^- when $N/P < 1$. M is the molecular weight markers (SmartLadder MW-1700-10 Eurogentec). **b)** DNA-binding properties of chitosan in dependence of the degree of acetylation (DA): electrophoretic mobility of free DNA for 0.03 mg/mL plasmid DNA (eGFP) added of chitosan at charge ratio $N^+/P^- = 1$. DNA/chitosan complex formed with chitosans Primex 4, Primex 6 and Chitosan 1C at 1 mg/mL at an initial pH=3.6. **c)** Electrophoresis gel assays showing the stability test for DNA/chitosan complex formed with chitosan samples having different DA (0.41, 0.25 and 0.05) and having a $N/P = 3 \times (N/P)$ at $N^+/P^- = 1$. $C_{DNA} = 0.03$ mg/mL (pH=7.4) and $C_{Chit} = 1.0$ mg/mL (initial pH=3.6). M is the molecular weight markers (SmartLadder MW-1700-10 Eurogentec). SDS concentration in the total volume of DNA/chitosan complexes is 0.65 mg/mL.

~~**Fig. 9.** DNA binding properties of chitosan in dependence of the degree of acetylation (DA): electrophoretic mobility of free DNA for 0.03 mg/mL plasmid DNA (eGFP) added of chitosan at charge ratio $N^+/P^- = 1$. DNA/chitosan complex formed with chitosans Primex 4, Primex 6 and Chitosan 1C at 1 mg/mL at an initial pH=3.6.~~

~~**Fig. 10.** Electrophoresis gel assays showing the stability test for DNA/chitosan complex formed with chitosan samples having different DA (0.41, 0.25 and 0.05) at N/P equals 3 times the charge ratio $N^+/P^- = 1$. $C_{DNA} = 0.03$ mg/mL (pH=7.4) and $C_{Chit} = 1.0$ mg/mL (initial pH=3.6). M is the molecular weight markers (SmartLadder MW 1700 10 Eurogentec). SDS concentration in the total volume of DNA/chitosan complexes is 0.65 mg/mL.~~

Fig. 11. Fig. 7. a) ζ -potential as a function of the total volume of chitosan solutions added. $C_{DNA} = 0.03$ mg/mL (pH=6.0) for different ionic concentrations in H₂O, NaCl 0.005 M, 0.01 M and 0.1 M. $C_{Chit} = 1.0$ mg/mL (initially at pH=3.6) **b)** Scattered intensity as a function of the ratio N/P for NaCl concentrations of 1.0 and 2.0 M. HMW chitosan sample from Sigma Aldrich, $M_v = 4.2 \times 10^5$ g/mol, DA=0.16. **c)** NaCl effect on DNA-binding properties of chitosan. Gel retardation assay showing the effect of increasing concentrations of chitosan on the electrophoretic mobility of plasmid DNA (eGFP) in NaCl 0.1 M. Ratios indicate the increasing chitosan-to-plasmid amounts in terms of the molar ratio N/P. M represents the

molecular weights of the marker (SmartLadder MW-1700-10 Eurogentec). d) Percentage of DNA migrating in electrophoresis gel assays for chitosan/DNA complexes in NaCl 0.1 M at different molar ratios. $C_{\text{DNA}}=0.03$ mg/mL (pH=7.4) and $C_{\text{Chit}}=1.0$ mg/mL (initial pH=3.6). Chitosan Primex 6 at 1 mg/mL and initial pH=3.6.

~~**Fig. 12.** a) NaCl effect on DNA binding properties of chitosan. Gel retardation assay showing the effect of increasing concentrations of chitosan on the electrophoretic mobility of plasmid DNA (eGFP) in NaCl 0.1 M. Ratios indicate the increasing chitosan to plasmid amounts in terms of the molar ratio N/P. M represents the molecular weights of the marker (SmartLadder MW 1700 10 Eurogentec). b) Percentage of DNA migrating in electrophoresis gel assays for chitosan/DNA complexes in NaCl 0.1 M at different molar ratios. $C_{\text{DNA}}=0.03$ mg/mL (pH=7.4) and $C_{\text{Chit}}=1.0$ mg/mL (initial pH=3.6). Chitosan Primex 6 at 1 mg/mL and initial pH=3.6.~~

~~**Fig. 13. Fig. 8.** a) NaCl effect on DNA-binding properties of chitosan at N/P=2. Gel electrophoresis assay showing the effect of increasing concentrations of NaCl on the amount of 0.03 mg/mL plasmid DNA (eGFP) released from chitosan/DNA complex with the addition of SDS. b) Percentage of DNA that migrated in electrophoresis gel assays for chitosan/DNA complexes at different NaCl concentrations (0, 0.01 and 0.2 M) without and with SDS (0.65 mg/mL). Chitosan Primex 6 at 1 mg/mL at an initial pH=3.6.~~

~~**Fig. 14. Fig. 9.** a) Potentiometric titration and ζ -potential of chitosan/DNA complex (V=5 mL) with a fixed molar ratio **N/P= 3 times the N/P ratio corresponding to $N^+/P^-=1$** , during progressive addition of 0.01 N HCl. $C_{\text{DNA}}= 0.03$ mg/mL at pH=7.5 and added chitosan $C_{\text{Chit}}= 1$ mg/mL. b) Potentiometric titration of chitosan/DNA complexes (V=5 mL) with $N^+/P^-=1$ and **N/P= 3 times the N/P ratio corresponding to $N^+/P^-=1$** , as well as for water during progressive addition of 0.01 N HCl. $C_{\text{DNA}}= 0.03$ mg/mL, around pH=7.5 and added chitosan $C_{\text{Chit}}= 1$ mg/mL Labeled chitosan, Primex 6, DA=0.05 prepared at pH=3.6.~~

Tables

Table 1. DA values determined by conductivity and ^1H NMR measurements. Intrinsic viscosity and MW values determined through viscosity measurements using AcOH 0.3 M/AcOH 0.2 M solvent and SEC with elution by AcOH 0.3 M/AcOH 0.25 M.

Table 2. $[\text{NH}_3^+]$ fraction and N/P molar ratio for chitosan/DNA complexes obtained in absence of external salt at the isoelectric point as a function of initial pH of chitosan solution

Table 3. Scattered intensity, size and DNA concentration before and after centrifugation assays of chitosan/DNA complexes at different charge ratios (0, 0.3, 0.5 and 0.7). Chitosan/DNA complexes were formed using chitosans 1, 5, 6 and 7 from Primex. $C_{\text{DNA}}=0.03$ mg/mL (pH=6.0) dissolved in NaCl 0.01 M and $C_{\text{Chit}}=1.0$ mg/mL (initial pH=3.6).

Table 4. Calculated percentage of free DNA at different charge ratios of chitosan/DNA complexes and the percentage of DNA that migrated in electrophoresis gel assays (in buffer solution at pH=7.4). $C_{\text{DNA}}=0.03$ mg/mL and $C_{\text{Chit}}=1.0$ mg/mL (initial pH=3.6, Primex 6, DA=0.05).

Table 5. Fraction of complexed chitosan units as a function of the molar ratio N/P and equilibrium pH and ζ -potential. $C_{\text{DNA}}=0.03$ mg/mL and $C_{\text{Chit}}=1.0$ mg/mL (initial pH=3.6, Primex 6, DA=0.05). Fluorescence is determined on the supernatant.

Table 6. Influence of the salt concentration on the complex formation: increase of the volume of chitosan needed for isoelectric point and ζ -potential in excess of chitosan added.

Table 7. Fraction of chitosan included in the complex at different external salt concentration and different N/P ratio. $C_{\text{DNA}}=0.03$ mg/mL dissolved in H_2O , NaCl 0.01 M, 0.1 M and 0.2 M, and FITC-labelled chitosan sample, $C_{\text{Chit}}=1.0$ mg/mL (initial pH=3.6, Primex 6, DA=0.05). Fluorescence is determined on the supernatant.

Fig. 2.-Fig. 1

International Journal of Biological Macromolecules

Lourdes Mónica Bravo-Anaya, Karla Gricelda Fernández-Solis, Julien Rosselgong, Jesrael

Luz Elena Nano-Rodríguez, Francisco Carvajal, Marguerite Rinaudo

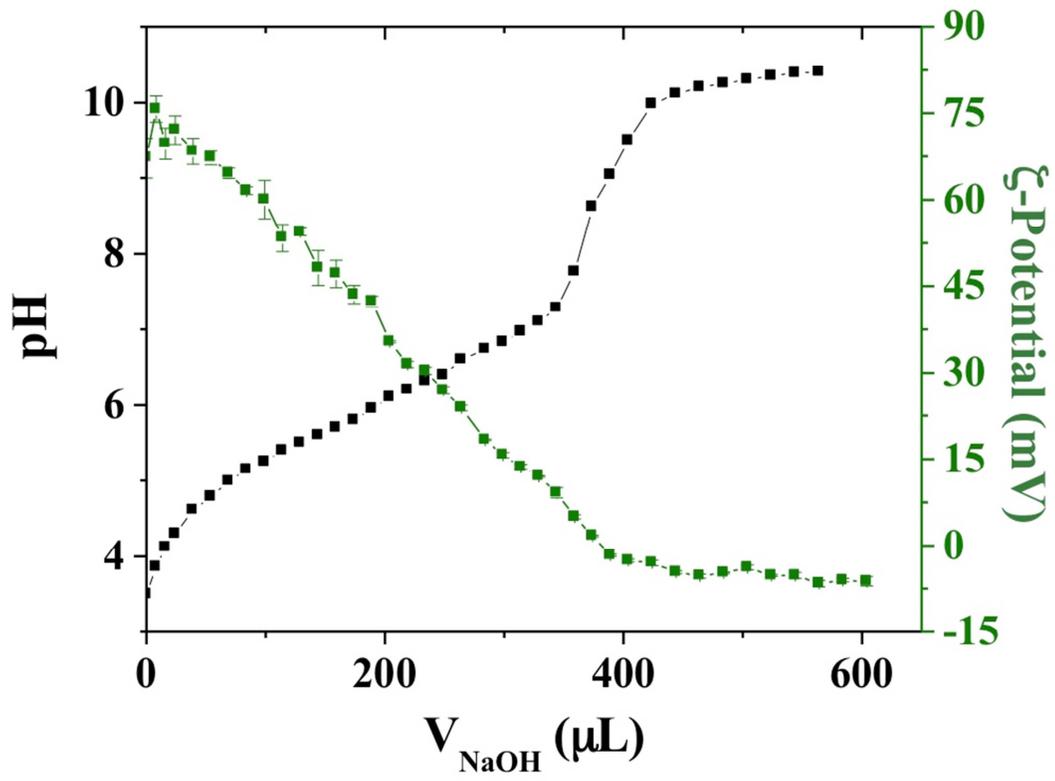


Fig. 3. Fig. 2.

International Journal of Biological Macromolecules

Lourdes Mónica Bravo-Anaya, Karla Gricelda Fernández-Solis, Julien Rosselgong, Jesrael Luz Elena Nano-Rodríguez, Francisco Carvajal, Marguerite Rinaudo

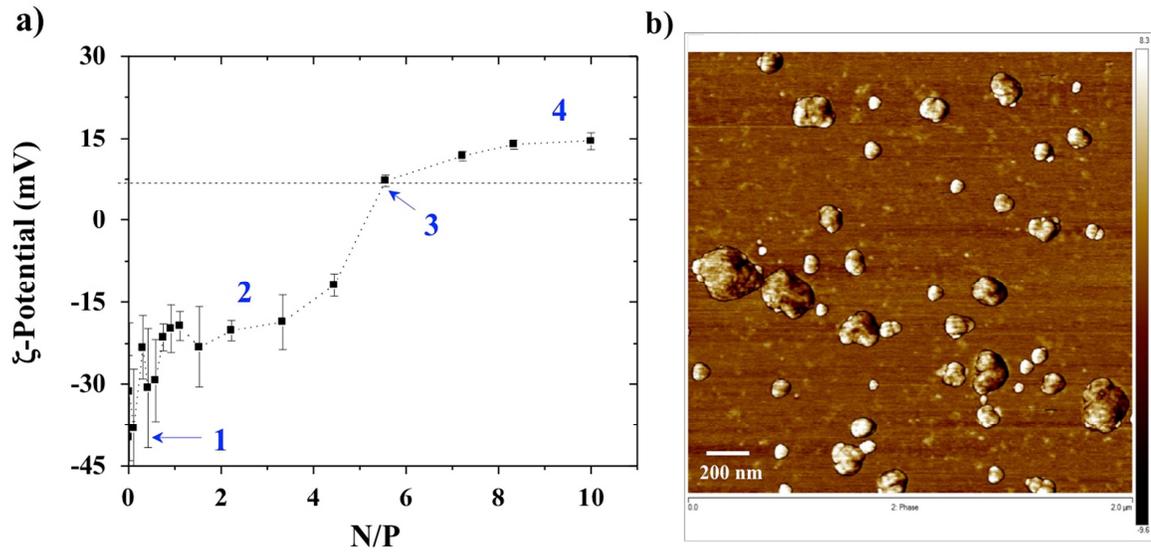


Fig-4. Fig. 3

International Journal of Biological Macromolecules

Lourdes Mónica Bravo-Anaya, Karla Gricelda Fernández-Solis, Julien Rosselgong, Jesrael

Luz Elena Nano-Rodríguez, Francisco Carvajal, Marguerite Rinaudo

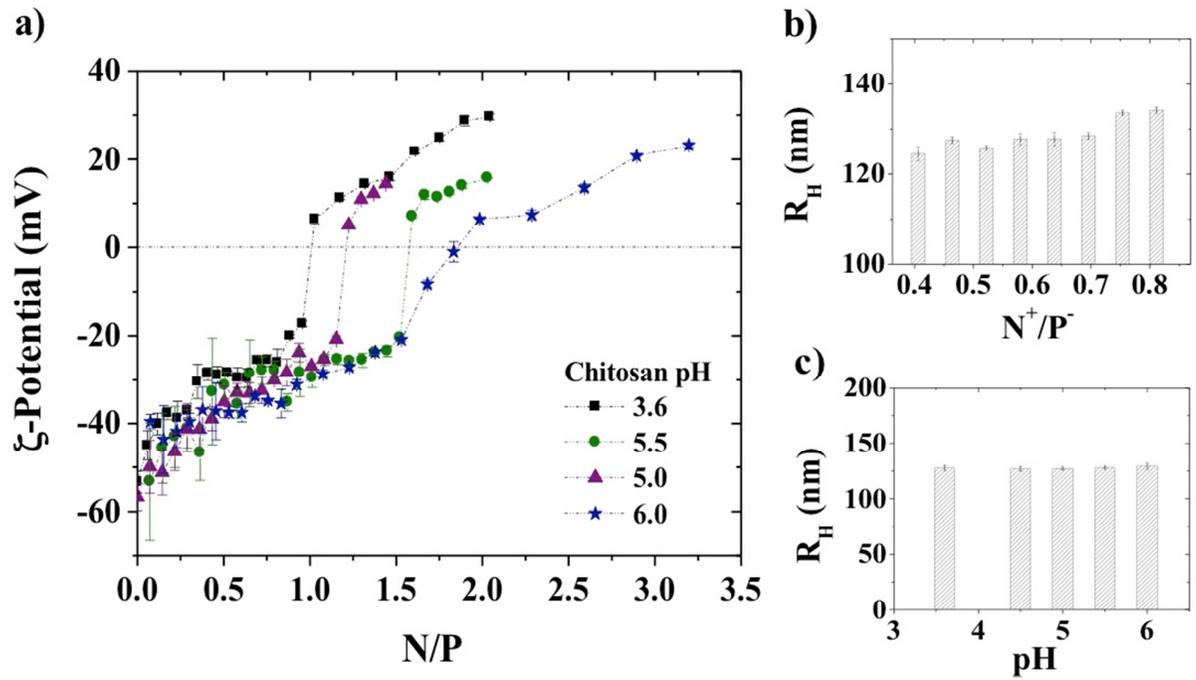


Fig. 5. Fig. 4

International Journal of Biological Macromolecules

Lourdes Mónica Bravo-Anaya, Karla Gricelda Fernández-Solis, Julien Rosselgong, Jesrael

Luz Elena Nano-Rodríguez, Francisco Carvajal, Marguerite Rinaudo

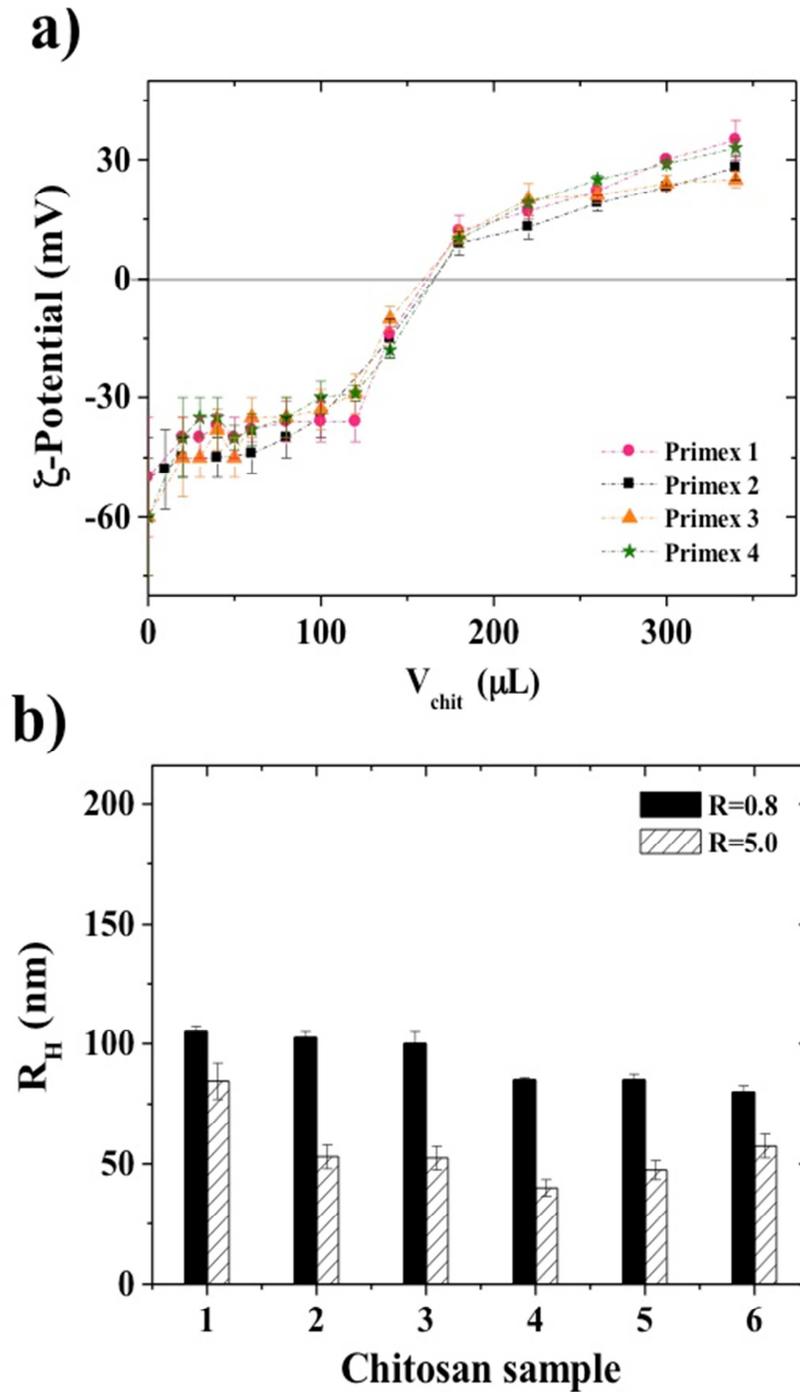


Fig. 5.

International Journal of Biological Macromolecules

Lourdes Mónica Bravo-Anaya, Karla Gricelda Fernández-Solis, Julien Rosselgong, Jesrael

Luz Elena Nano-Rodríguez, Francisco Carvajal, Marguerite Rinaudo

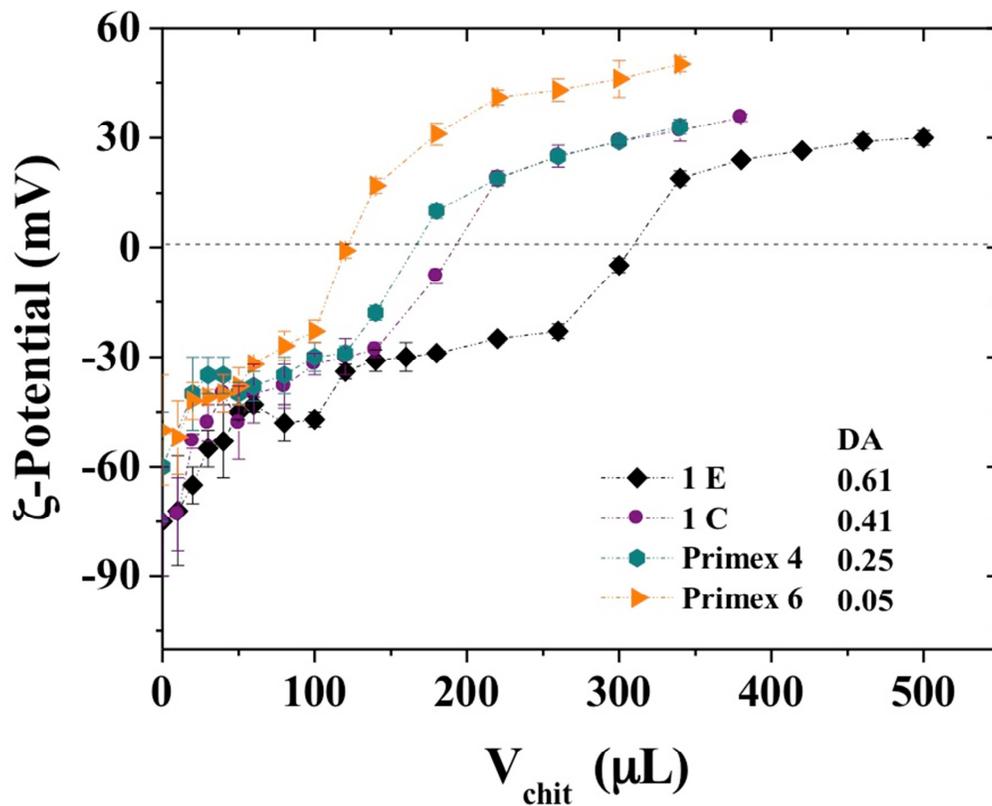


Fig-8. Fig. 6.

International Journal of Biological Macromolecules

Lourdes Mónica Bravo-Anaya, Karla Gricelda Fernández-Solis, Julien Rosselgong, Jesrael

Luz Elena Nano-Rodríguez, Francisco Carvajal, Marguerite Rinaudo

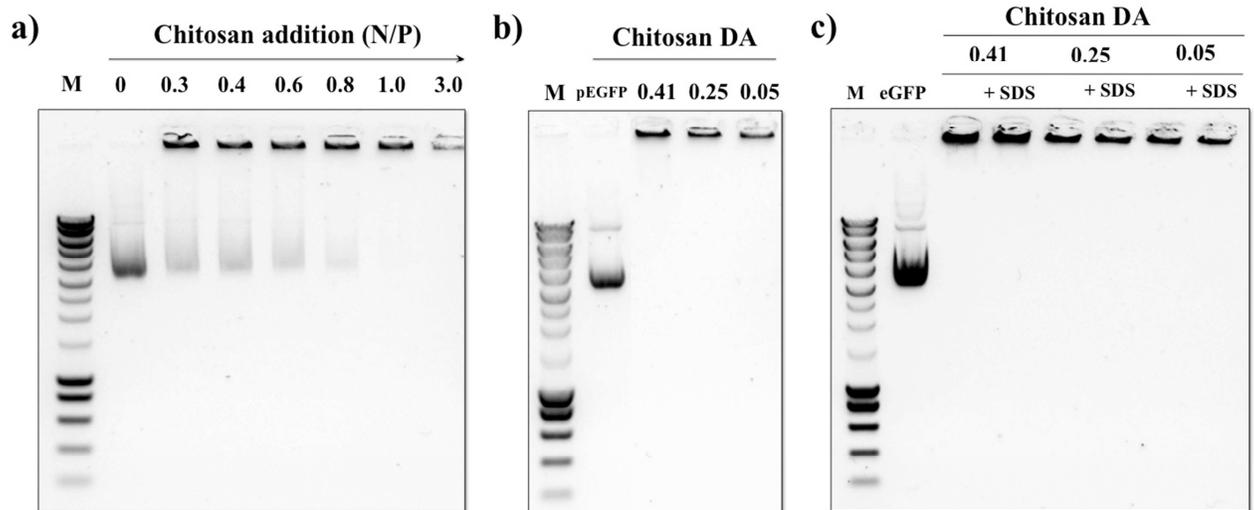


Fig.-9. Fig. 7.

International Journal of Biological Macromolecules

Lourdes Mónica Bravo-Anaya, Karla Gricelda Fernández-Solis, Julien Rosselgong, Jesrael Luz Elena Nano-Rodríguez, Francisco Carvajal, Marguerite Rinaudo

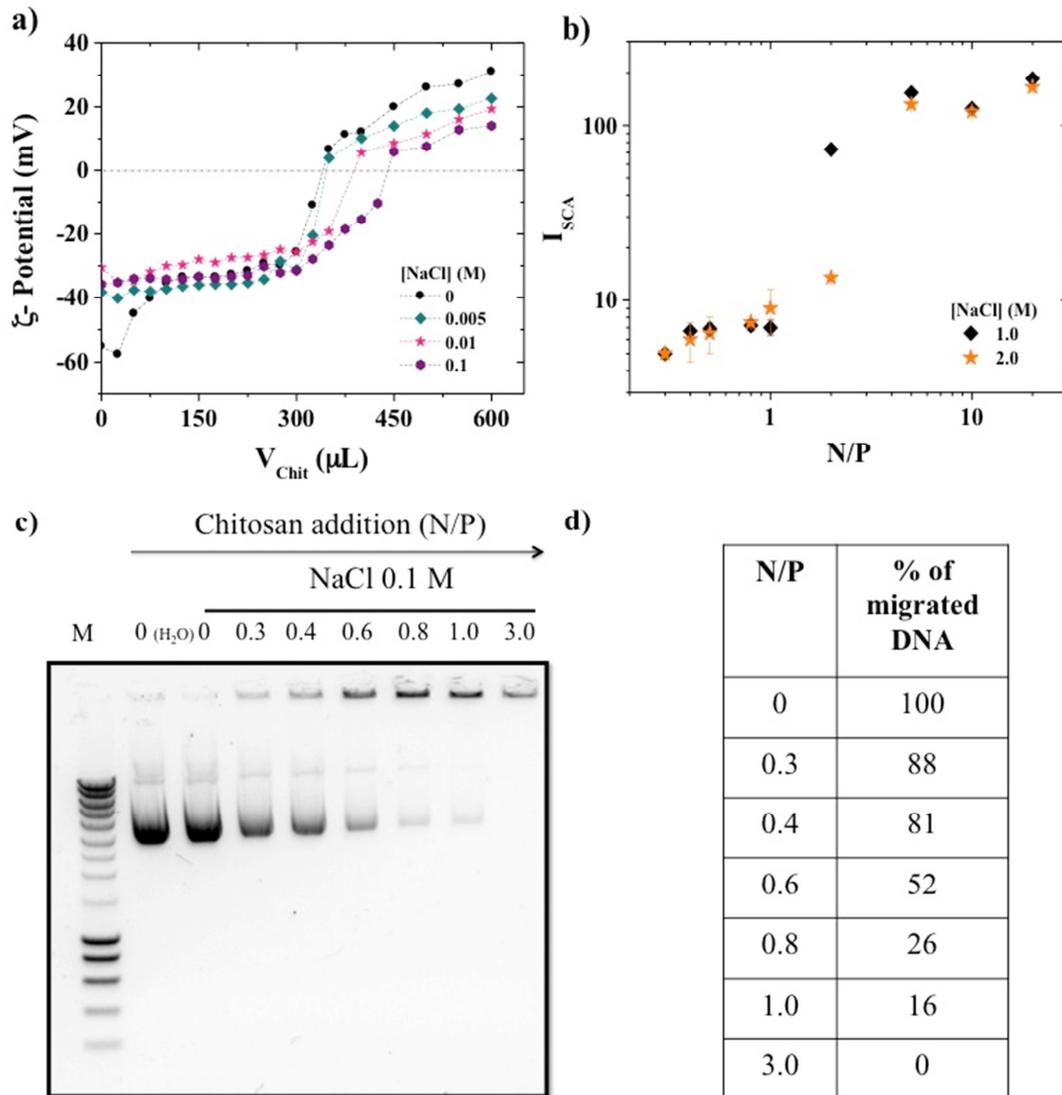


Fig-10. Fig. 8.

International Journal of Biological Macromolecules

Lourdes Mónica Bravo-Anaya, Karla Gricelda Fernández-Solis, Julien Rosselgong, Jesrael

Luz Elena Nano-Rodríguez, Francisco Carvajal, Marguerite Rinaudo

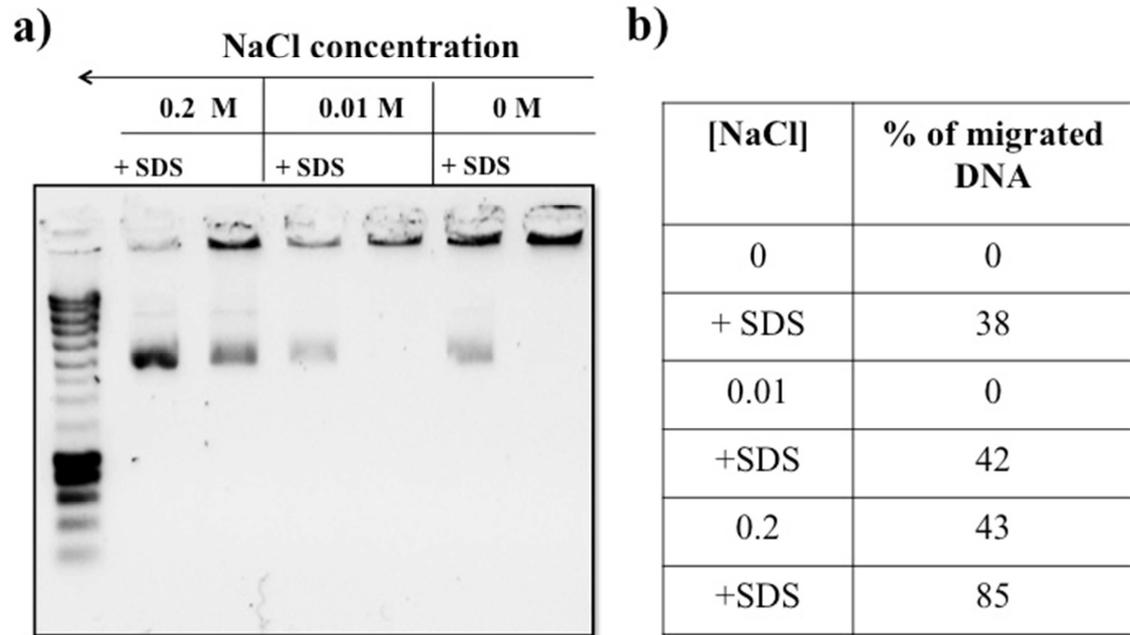


Fig. 11. Fig. 9.

International Journal of Biological Macromolecules

Lourdes Mónica Bravo-Anaya, Karla Gricelda Fernández-Solis, Julien Rosselgong, Jesrael

Luz Elena Nano-Rodríguez, Francisco Carvajal, Marguerite Rinaudo

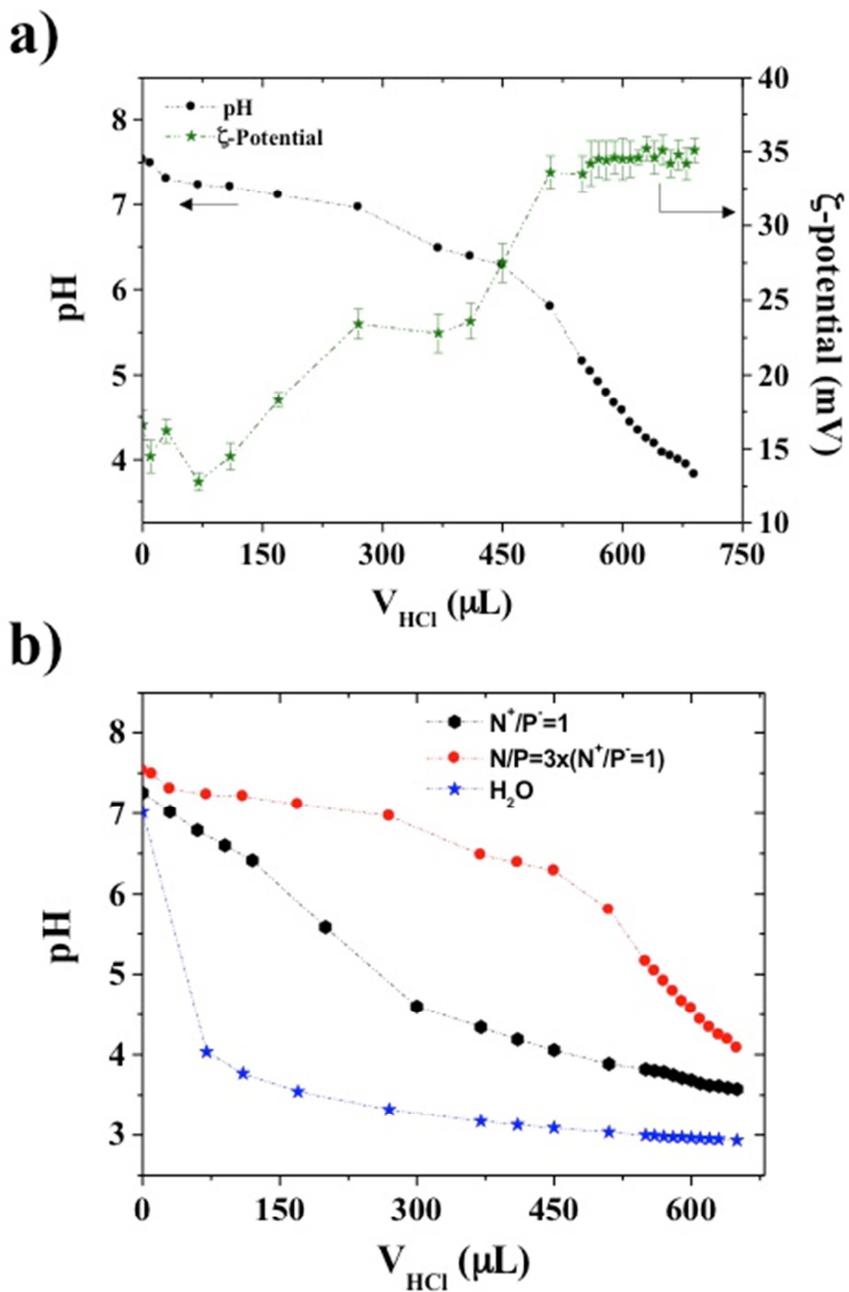


Table 1.

International Journal of Biological Macromolecules

Lourdes Mónica Bravo-Anaya, Karla Gricelda Fernández-Solis, Julien Rosselgong, Jesrael

Luz Elena Nano-Rodríguez, Francisco Carvajal, Marguerite Rinaudo

Chitosan sample	Supplier	DA from conductivity	DA from ¹H NMR	Intrinsic viscosity (mL/g)	Viscosity-average Molecular weight (g/mol)
High molecular weight (HMW)	Sigma Aldrich	0.16	0.16	2500	417 600
Medium molecular weight (MMW)	Sigma Aldrich	0.20	0.22	700	86 750
Chitosan sample	Supplier	DA from conductivity	DA from ¹H NMR	<i>Mw from SEC (g/mol)</i>	
ChitoClear 1	Primex	0.31	0.30	540 000	
ChitoClear 2	Primex	0.27	0.28	350 000	
ChitoClear 3	Primex	0.27	0.29	240 000	
ChitoClear 4	Primex	0.24	0.25	180 000	
ChitoClear 5	Primex	0.03	0.03	150 000	
ChitoClear 6	Primex	0.04	0.05	160 000	
ChitoClear 7	Primex	0.13	0.13	220 000	
Chitosan 1C	G. Roberts	NA	0.41	84 000	
Chitosan 1E	G. Roberts	NA	0.61	75 000	

Table 2.

International Journal of Biological Macromolecules

Lourdes Mónica Bravo-Anaya, Karla Gricelda Fernández-Solis, Julien Rosselgong, Jesrael

Luz Elena Nano-Rodríguez, Francisco Carvajal, Marguerite Rinaudo

pH	Fraction of [NH₃⁺] (%)	N/P
	Solution in water	
3.6	100	1.0
5.0	82	1.2
5.5	63	1.6
6.0	50	2.0
6.5	20	5.3

Table 3.

International Journal of Biological Macromolecules

Lourdes Mónica Bravo-Anaya, Karla Gricelda Fernández-Solis, Julien Rosselgong, Jesrael

Luz Elena Nano-Rodríguez, Francisco Carvajal, Marguerite Rinaudo

Chitosan sample # 1						
Before centrifugation			Supernatant analysis after centrifugation			
	DLS		UV-Vis analysis	DLS	% DNA chains	
N⁺/P⁻	I_{SCA}	Size (nm)	Concentration (mg/mL)	I_{SCA}	Experimental	Calculated
0	10	-	0.03	3	100	100
0.3	26	166	0.022	8	73.3	70
0.5	134	200	0.016	10	53.3	50
0.7	144	177	0.01	10	33.3	30
Chitosan sample # 5						
Before centrifugation			Supernatant analysis after centrifugation			
	DLS		UV-Vis Analysis	DLS	% DNA chains	
N⁺/P⁻	I_{SCA}	Size (nm)	Concentration (mg/mL)	I_{SCA}	Experimental	Calculated
0	18	-	0.03	2	100	100
0.3	90	190	0.02	8	66.7	70
0.5	180	180	0.015	8	50	50
0.7	360	240	0.005	2	16.7	30
Chitosan sample # 6						
Before centrifugation			Supernatant analysis after centrifugation			
	DLS		UV-Vis Analysis	DLS	% DNA chains	
N⁺/P⁻	I_{SCA}	Size (nm)	Concentration (mg/mL)	I_{SCA}	Experimental	Calculated
0	10	-	0.03	3	100	100
0.3	50	150	0.02	8	66.7	70
0.5	133	160	0.014	8	46.7	50
0.7	133	160	0.007	3	23.3	30
Chitosan sample # 7						
Before centrifugation			Supernatant analysis after centrifugation			
	DLS		UV-Vis Analysis	DLS	% DNA chains	
N⁺/P⁻	I_{SCA}	Size (nm)	Concentration (mg/mL)	I_{SCA}	Experimental	Calculated
0	18	-	0.03	2	100	100
0.3	92	190	0.019	13	63.3	70
0.5	176	190	0.015	9	50	50
0.7	452	230	0.006	2	20	30

Table 4.

International Journal of Biological Macromolecules

Lourdes Mónica Bravo-Anaya, Karla Gricelda Fernández-Solis, Julien Rosselgong, Jesrael

Luz Elena Nano-Rodríguez, Francisco Carvajal, Marguerite Rinaudo

N/P	Calculated % of free DNA from R ratio	% of free DNA
0	100	100
0.3	70	55
0.4	60	50
0.6	40	30
0.8	20	15
1.0	0	0
3.0	0	0

Table 5.

International Journal of Biological Macromolecules

Lourdes Mónica Bravo-Anaya, Karla Gricelda Fernández-Solis, Julien Rosselgong, Jesrael

Luz Elena Nano-Rodríguez, Francisco Carvajal, Marguerite Rinaudo

N/P	Equilibrium pH (± 0.1) on supernatant	Equilibrium ζ -potential (± 0.2) on suspension	Fluorescence intensity*	Fraction of bound chitosan on P- basis
0.5	7.00	-35	~ 0	~ 0.5
0.8	6.90	-28	~ 0	~ 0.8
1	6.83	+ 3	~ 0	~ 1
2	6.82	+22	25	1.10
3	6.68	+32	21.5	2.23
5	6.60	+36	100.1	1.45
6	6.50	+36	105.5	2.28
7	6.40	+38	176.2	0.84
8	5.94	+38	190	1.21
10	5.45	+40	232.9	1.79

*Fluorescence is very low and difficult to determine precisely. It means that all the chitosan is fixed at $N/P \leq 1$.

Table 6.

Lourdes Mónica Bravo-Anaya, Karla Gricelda Fernández-Solis, Julien Rosselgong, Jesrael Luz Elena Nano-Rodríguez, Francisco Carvajal, Marguerite Rinaudo

Solvent	Added volume of chitosan (μL) to a 20 mL solution of DNA at IP	ζ -potential at $V_{\text{chit}}=500 \mu\text{L}$ (mV) added to a 20 mL solution of DNA
H ₂ O	340	+26
NaCl 0.005 M	345	+18
NaCl 0.01 M	388	+12
NaCl 0.1 M	440	+ 8

Table 7.

International Journal of Biological Macromolecules

Lourdes Mónica Bravo-Anaya, Karla Gricelda Fernández-Solis, Julien Rosselgong, Jesrael

Luz Elena Nano-Rodríguez, Francisco Carvajal, Marguerite Rinaudo

Molar ratio N/P	H ₂ O	0.01M NaCl	0.1M NaCl	0.2M NaCl
2	1.19	1.45	1.54	1.77
3	2.26	2.38	2.44	2.68