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Colloidal stability of chitosan/DNA polyelectrolyte complexes in presence of biological polyanions

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Natural or synthetic polycations are used in nucleic acid-based therapies as complexing agents which interact electrostatically with nucleic acids, condense them into nanoparticles, protect them and control their entry into cells. However, although the literature on the formation of nanoparticles known as complexes is well documented, fewer studies have focused on the physical chemistry behind their disassembly, especially under physicochemical conditions found in an intracellular environment. There are several theories of the disassembly of these complexes, one of them consisting in the exchange between the polycations of these particles with biological polyanions. This project is focused on the study of the complexation mechanism of chitosan and calf-thymus DNA, as well as the stability of the obtained complexes in presence biological polyanions, *i.e.*, glycosaminoglycans (GAGs). In the presence of polyelectrolyte complexes, GAGs that are present in cells are expected to compete with nucleic acids and dissociate the complex if polycation-GAG association is thermodynamically favored. It was found that chitosan/DNA complexes colloidal stability depends on its $[N^+]/[P^-]$ charge ratio (R). Furthermore, it was determined that the aggregation onset of the complexes, generated by the addition of different GAGs, depends on the structure and the charge density of the GAGs.

1. Introduction

Gene delivery systems based on synthetic nanocarriers, where nucleic acids are densely packed into polymer or lipid nanoparticles, are increasingly considered.^[1] For instance, Moderna

therapeutics uses lipid particles where the negatively charged mRNA strand is complexed with cationic lipids and packed into 100 nm nanoparticles.^[2] Other approaches are based on cationic polymers like poly(ethyleneimine) (PEI), chitosan^[3,4] or poly(L-lysine) (PLL) able to complex and condense nucleic acids into nanoparticles. Polyelectrolyte complexes (PECs) are mainly internalized by endocytosis, which is an effective means of accessing the intracellular environment. The structural models of polyelectrolyte complexes, preparation methodologies and factors affecting their formation and characteristics have been studied for many applications.^[5] However, their dissociation mechanisms in biological media, as well as the physicochemical and biological factors that trigger them, are poorly explained in the literature. Glycosaminoglycans (GAGs) such as hyaluronic acid, chondroitin sulfate and heparin, are negatively charged linear polymers having repeating disaccharides.^[6] These polyanions will compete with DNA to be able to interact with the polycation.

In this work, chitosan/DNA complexes stoichiometry was determined through ζ -potential measurements and gel electrophoresis. Then, complexes were formulated at different R ($R=[N^+]/[P^-]$, corresponding to the ratio between protonated amines from chitosan and negatively charged phosphates from DNA) and were characterized through Dynamic Light Scattering (DLS), ζ -potential and Atomic Force Microscopy (AFM) to determine their size, PDI, charge and morphology. Interactions between chitosan and GAGs were studied through ζ -potential measurements. Later, chitosan/DNA complexes colloidal stability was studied through DLS measurements in presence of heparin, hyaluronic acid and chondroitin sulfate, and was found to be dependent on the polycation structure and the charge ratio of the complex.

RESULTS AND DISCUSSION

2.1 Chitosan/DNA stoichiometry determination through ζ -potential measurements and gel electrophoresis

Gel electrophoresis and ζ -potential were used to determine chitosan/DNA complexes stoichiometry and their isoelectric point (IP). In ζ -potential measurements, the crossing point

from negative to positive ζ -potentials is related to the IP of the particle, corresponding to the stoichiometric neutralization of DNA negative charges with the polycation. **Figure 1a** shows the evolution of ζ -potential during chitosan/DNA complexes formation. For progressive additions of chitosan to a specific amount of DNA molecules in solution, a first plateau is observed around ζ -potential= -40 mV. Then, when ζ -potential equals to 0 mV the isoelectric point of the particles (IP) is reached. Finally, a second plateau is observed at ζ -potential= +22 mV, corresponding to the formation of stable and positively charged chitosan/DNA complexes. **Figure 1b** shows a gel electrophoresis assay for chitosan/peGFP complexes, in which the stoichiometry is well respected $[N^+]/[P^-]= 1$, in good agreement with ζ -potential measurements.

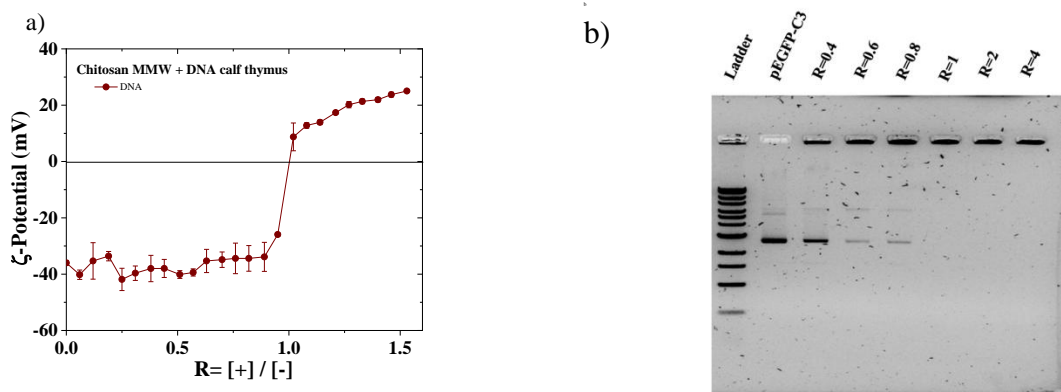


Figure 1. a) Evolution of ζ -potential as a function of charge ratio during chitosan/DNA complexes formation (MMW chitosan, $C_{DNA} = 0.03$ mg/mL, $V = 10$ mL); b) gel electrophoresis for complexes at different R (eGFP 0.005 mg/mL, MMW chitosan).

2.2 Chitosan/DNA complexes formulation and characterization

Once the real volumes of chitosan needed to reach the charge stoichiometry of chitosan/DNA complexes were determined, a set of complexes suspensions were prepared at different R between 2 and 8 for each chitosan sample. At charge ratios higher than 1, an excess of positive charges provides to the complexes the ability to interact with the cell membrane in order to get internalized. **Figure 2a** presents the evolution of sizes for chitosan/DNA complexes as a function of R , giving values between 210 and 240 nm. With the intensity distributions of the complexes (**Figure 2b**), it is possible to observe a quite homogeneous distribution for all of them, with PDI values lower than 0.38. **Figure 2c** shows an AFM micrography for

chitosan/DNA complexes, showing a globular morphology which corresponds to a dense core surrounded by shell of excess of chitosan.

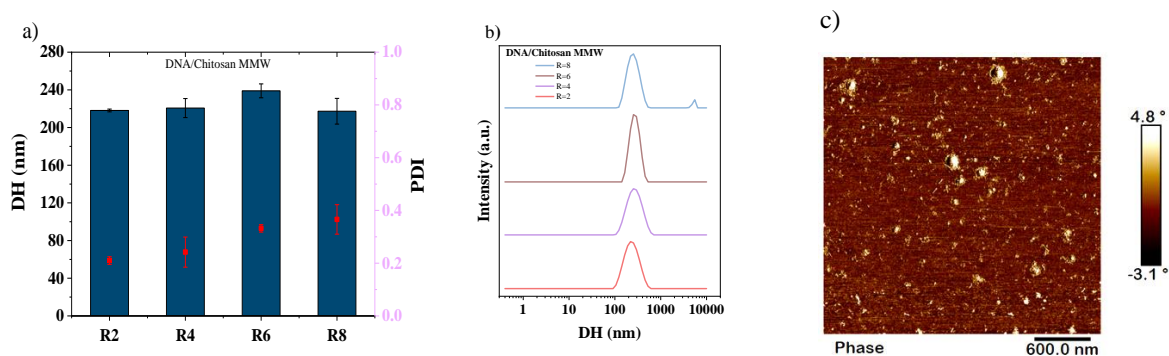


Figure 2. a) Evolution of chitosan/DNA complexes D_H as a function of R (2, 4, 6, 8) (MMW Chitosan); b) intensity distributions as a function of size (D_H) for the complexes prepared at different R; b) classical morphology of the complexes obtained through AFM measurements.

2.3 Chitosan/DNA complexes colloidal stability

Figure 3a shows, as an example, the evolution of chitosan/DNA complexes, prepared at a charge ratio of 4, with the addition of heparin and hyaluronic acid. As soon as the hydrodynamic diameters (D_H) of complexes increase in the presence of GAGs, particles get aggregated, as a signal of a disruption of the system.

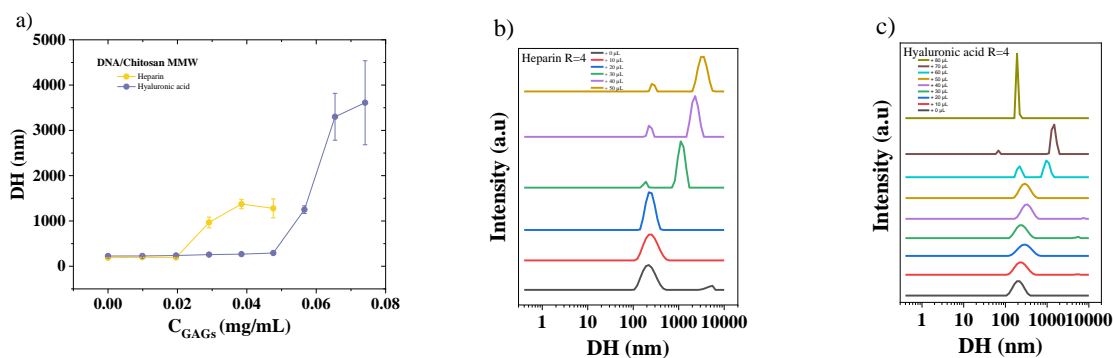


Figure 3. a) Chitosan/DNA complexes size evolution as a function of increasing concentrations of heparin and hyaluronic acid. Complexes were prepared in 1 mL of DNA (0.03 mg/mL). One charge ratio in this plot: R=4; size distribution evolution of Chitosan/DNA complexes in presence of different amounts of a) heparin and c) hyaluronic acid.

Chitosan/DNA complexes start to aggregate with less concentration of heparin than with hyaluronic acid. This could be mostly due to a higher charge density of heparin (9 905 [-]/mol), compared to hyaluronic acid (3 360 [-]/mol), as well as the binding affinity to chitosan. Heparin

is also constituted by sulfate and carboxylic groups and hyaluronic acid only by carboxylic groups. **Figures 3b** and **3c** present the size distributions of chitosan/DNA complexes in presence of different amounts of heparin and hyaluronic acid, respectively. Complexes disruption can be done in presence of competitive biological polyanions, leading to aggregation of the complexes and possible release of nucleic acids.

2.5 Conclusions

Chitosan/DNA complexes stoichiometry was determined by using 3 different chitosan samples. Chitosan/DNA complexes sizes were found to be between 100 and 300 nm, depending on chitosan MW. ζ -potential measurements between chitosan and GAGs (heparin, chondroitin sulfate and hyaluronic acid) allowed determining stoichiometry and charge density of the GAGs. Chitosan/DNA complexes colloidal stability was found to depend on the $[N^+]/[P^-]$ charge ratio of the DNA/chitosan complex. Furthermore, it was determined that the aggregation point of the complexes generated by the addition of different GAGs depends on complexes R, as well as the chemical structure and charge density of the GAGs.

4. Experimental Section/Methods

4.1 Materials and solutions preparation

A sample of calf thymus DNA with a MW of 6 559 500 g/mol, eGFP plasmid (MW= 3 100 912 g/mol) and three commercial samples of chitosan were used (low molecular weight chitosan: MW=38 484 g/mol, DA= 0.33; medium molecular weight, MW=86,750 g/mol, DA= 0.16; and high molecular weight, MW=417,600 g/mol, DA= 0.22). The materials were acquired from the Sigma Aldrich Company. DNA solutions were prepared in 0.01 M NaCl at a concentration of 1 mg/mL. Chitosan solutions were prepared at 3.33 mg/mL with the stoichiometric amount of 0.1 N HCl based on NH_2 content and were stirred during 16 hours. GAGs were prepared in water at a concentration of 1 mg/mL and were stored under refrigeration. DNA/chitosan complexes were prepared by one-shot addition with a specific amount of each chitosan (1 mg/mL) to a C_{DNA} of 0.03 mg/mL to reach the following charge ratios: 2, 4, 6 and 8.

4.2 Biological polyanions characterization

Chondroitin sulfate, heparin and hyaluronic acid were characterized by aqueous SEC and ^1H NMR in order to determine their MW, structure and purity.

4.3 DNA/chitosan complexes stoichiometry determination

Complexes stoichiometry was determined through gel electrophoresis assays with eGFP plasmid and ζ -potential measurements with calf-thymus DNA in a Malvern Zetasizer NanoZS 90 equipment at 25 °C. ζ -potential was calculated using the classical Smoluchowski expression.

4.4 Chitosan/DNA complexes characterization and stability

Particle sizes and colloidal stability were determined by DLS measurements in a Malvern Zetasizer NanoZS 90 at an angle of 90° and a temperature of 25 °C. The D_H was calculated using the Stokes-Einstein equation. Atomic Force Microscopy measurements were used to determine the morphology of chitosan/DNA complexes.

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