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Counterion of chitosan influences thermodynamics of association of siRNA with a chitosan-based siRNA carrier.

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Running title: Chitosan salt influences thermodynamics of association with siRNA

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Abstract

Purpose: The work aimed to compare quality of a siRNA carrier prepared with chitosan of two different sources having similar degree of deacetylation and molecular weights. Differences were analyzed from thermodynamic characteristics of interactions with siRNA. **Methods:** The siRNA carrier (chitosan-coated poly(isobutylcyanoacrylate) nanoparticles) was prepared with home-prepared, CS_{Lab}, and commercial, CS_{Com}, chitosans. Chitosan counterion was identified and chitosans CS_{Com}mod1 and CS_{Com}mod2 were obtained from CS_{Com} exchanging counterion with that found on CS_{Lab}. Carrier quality was checked considering the size, zeta potential and siRNA association capacity by gel electrophoresis. Thermodynamic parameters of interactions between siRNA and chitosans in solution or immobilized at the carrier surface were determined by (ITC). **Results:** CS_{Lab} and CS_{Com}mod2 having a high content of acetate counterion associated better siRNA than CS_{Com} and CS_{Com}mod1 which counterion included mainly chloride. ITC measurements indicated that siRNA interactions with chitosan and the siRNA carrier were driven by entropic phenomena including dehydration, but thermodynamic parameters of interactions clearly differed according to the nature of the counterion of chitosan. The influence of chitosan counterions was interpreted considering their different lyotropic character. **Conclusion:** Association of siRNA with our siRNA carrier was influenced by the nature of counterions associated with chitosan. Driven by entropic phenomena including dehydration, interactions were favored by acetate counterion. Although more work would be needed to decipher the influence of the counterion of chitosan during association with siRNA, it was pointed out as a new critical attribute of chitosan to consider while formulating siRNA carrier with this polysaccharide.

Key words: siRNA, chitosan salt, siRNA carrier, counterion, lyotropic, thermodynamics

List of Abbreviation ad symbols

CS: generic abbreviation for chitosans used in this work

CS_{Lab}: chitosan prepared in our lab.

CS_{Com}: Commercial chitosan

CS_{Com}-mod1: Commercial chitosan modified with method 1

CS_{Com}-mod2: Commercial chitosan modified with method 2

DeltaH: normalized enthalpy

ΔH_{fit} : interaction enthalpy deduced from the fit of the curve and using equation 5

ΔH_{graph} : interaction enthalpy evaluated from graphs

ΔG : difference in free energy

ΔS : entropy of the reaction

D_H : hydrodynamic diameter

ITC: isothermal titration calorimetry

K_a : association constant

K_d : dissociation constant

NP CS_{xxx} : nanoparticles prepared with the specified chitosan

NP CS_{xxx}-P: nanoparticles prepared with the specified chitosan and poloxamer 188

NP P: nanoparticles prepared with poloxamer 188

PACA: poly(alkylcyanoacrylate)

siRNA: small interfering RNA.

$-T\Delta S$: entropic therms of the reaction

1 INTRODUCTION

The discovery of siRNA at the dawn of 2000 [1,2] has paved the way for the development of breakthrough treatments for cancers [3-9], viral infections [5,10,11], metabolic disorders [12] and neuromuscular [13] and neurodegenerative diseases [14]. In these treatments, siRNAs are designed to trigger the degradation of messenger RNAs (mRNAs) that encode for proteins which expression needs to be down regulated. mRNAs are located within the cell cytoplasm where active siRNAs need to be delivered to achieve their therapeutic potential. The poor stability of siRNAs in biological media and their incapacity to diffuse across cell membranes are tremendous hurdles for the development of siRNA-based treatments [10,15-17]. However, proof of success of siRNA-based therapeutic methods have been provided in numerous works having considered siRNA formulated nanomedicines [6,7,10,16-18]. Many of these nanomedicines were designed with chitosan, a polysaccharide derived from chitin which forms complexes with nucleic acids [5,9,17-33]. SiRNA associates with the nanomedicines thanks to the formation of a complex with chitosan involving electrostatic interactions between the negative charges of the phosphates of siRNA and the positive charges of the amino group of glucosamine residues found in chitosan [26,34,35]. The association is influenced by the molecular weight and degree of deacetylation of chitosan and by conditions in which it is performed including the pH of the medium and the amine to phosphate ratio [26,29,36-38]. The molecular weight of chitosan and its degree of deacetylation were reported to also influence the delivery performance of active siRNA *in vitro* and *in vivo* [29,37,39-42].

In previous works, we have reported the delivery of siRNA in intravenous treatments of two xenografted tumor models caused by junction oncogenes in mice using a chitosan-coated polymer nanoparticle designed from the poly(alkylcyanoacrylate) nanoparticle technologies [43,44]. The promising therapeutic perspectives shown by this nanomedicine encouraged to start its development preparing its clinical translation. The scale up of the carrier preparation is one of the first critical step which can compromise the development [45-52]. Hurdles may include methodological issues but also difficulties to find sustainable source of chemicals of the required quality. Production of clinical batches of poly(alkylcyanoacrylate) (PACA) nanoparticles was achieved during the development of Livatag®, a nanomedicine considered in a phase III clinical trial for the treatment of hepatocellular carcinoma in human [53-55]. Thus, scaling-up the preparation process of the PACA nanoparticle-based siRNA carrier should not be a major difficulty for the development. In contrast, shifting the home-prepared chitosan to a commercial product was identified as a possible risk. Indeed, performances of siRNA carriers greatly depend on chitosan characteristics. While obtained from chitin, a natural compound, the quality of chitosan is subjected to variability which in turn can affect activities in biomedical applications [26,41,56,57]. The most cited characteristics of chitosan influencing *in vitro* and *in vivo* delivery and activity of nucleic acids including siRNA are the molecular weight and degree of deacetylation [26,36-41,58-60]. The influence of the type of salt of chitosan was studied in very few works giving mitigated results. It was considered to have a marginal effect [10,29,40,61,62].

Having found a commercial chitosan which quality criteria in terms of degree of deacetylation and molecular weight that fitted those of our home-prepared chitosan, the present work was aimed to transpose the preparation of the pristine siRNA carrier made with the home-prepared chitosan with that using the chitosan of commercial source. Chitosans were characterized determining their molecular weight, degree of deacetylation, type of salt, ionization after dissolution in water. The quality of corresponding batches of nanoparticles were compared investigating their size, zeta potential, capacity to associate a siRNA and thermodynamic parameters of the interactions between the siRNA and the siRNA carrier by isothermal titration calorimetry (ITC). Additional chitosans were prepared from the commercial source changing the type of counterion towards the one present in the home-prepared chitosan. This was done to explain the

difference in siRNA association capacity of nanoparticles prepared with the commercial chitosan compared with that of the pristine nanoparticles.

2 Materials and Methods

2.1 Materials

Isobutylcyanoacrylate (IBCA) was purchased from Orapi (Paris, France). Chitosan (20kDa, DDA 80-85%, yellow powder) was supplied by Amicogen (Seoul, South Korea) and chitosan (medium viscosity, slightly yellow powder) by Aldrich (France). Poloxamer 188 (Pluronic® F68) a non-ionic surfactant composed of poly(oxyethylene)₈₀-poly(oxypropylene)₂₇-poly(oxyethylene)₈₀ [63], was provided as a gift by BASF. The average molecular weight of poloxamer 188 is 8.400 g/mol [64]. Cerium (IV) ammonium nitrate and nitric acid were purchased from Fluka.

The siRNA was obtained by duplex formation from the complementary single strands. (Sens/antisens strand: 5'-GCUACGGGCAGCAGAACCCd(TT)-3'; 3' -(GT)CGAUGCCCGUCGUCUUGGG-5'). The solution of 20 μM of sense and antisense RNAs in annealing buffer (Eurogentec) was heated at 95 °C for 5 min and then incubated at 37 °C for 1 h. The siRNA was stored at -20 °C until use.

2.2 Methods

2.2.1 Preparation of the different chitosan

The different chitosans used in the study were prepared according to the following procedures leading to chitosans CS_{Lab}, CS_{Com}, CS_{Com}mod1, CS_{Com}mod2.

Chitosan 1 (CS_{Lab}) or home-prepared chitosan: chitosan medium viscosity at 2% in solution in acetic acid (0.1N) was hydrolyzed in conditions described by Huang *et al.* [36] adapted by Bertholon *et al.* [65] with a concentration in sodium nitrite of 11 g/L. The hydrolysis was performed at room temperature for 1 hour. The hydrolyzed chitosan was recovered by precipitation raising the pH at 9 with sodium hydroxide. The precipitate was recovered by centrifugation and lyophilized for 24 h (Alpha 2-4 LD plus, Bioblock Scientific) after freezing with liquid nitrogen.

Chitosan 2 (CS_{Com}) or commercial chitosan: was used as received from Amicogen (Seoul, South Korea)

Chitosan 3 (CS_{Com}mod1) was prepared from CS_{Com} applying a method of purification based on a single dialysis (method 1). CS_{Com} (2 g) was dissolved for 30 min in acetic acid solution (0.1 N, 50 mL) and purified by dialysis (Spectra/Por membrane with molecular weight cut off (MWCO) of 3 500g/mol), twice during 1 h and once overnight against 1 L of distilled water. Lyophilization was performed for 24 h (Alpha 1-2 LD plus, Bioblock Scientific) after freezing with liquid nitrogen to recover CS_{Com}mod1.

Chitosan 4 (CS_{Com}-mod2) was prepared from CS_{Com} applying a method of purification by precipitation followed by a dialysis (method 2). CS_{Com} (2 g) was dissolved in acetic acid solution (6 %, 50 mL) and stirred at room temperature for 1 h. The pH was increased by adding a sodium hydroxide solution (4 N) until a precipitate of

chitosan appeared (pH 7-8). The precipitate was recovered by centrifugation (10 min, 10 °C, 4 000*g, rotor A-4-44, Eppendorf 5804R) and subsequently washed with water (40 mL). After final centrifugation in same condition as before, it was frozen at -80°C and lyophilized for 24 h (Alpha 1-2 LD plus, Bioblock Scientific). The obtained lyophilized powder was then dissolved again in acetic acid (0.1 N, 40 mL), and purified by dialysis (Spectra/Por membrane, MWCO 3 500g/mol), twice during 1 h and once overnight against 1 L of distilled water. Lyophilization was performed (Alpha 1-2 LD plus, Bioblock Scientific) after freezing at -80°C for 24 h to recover CS_{Com-mod2}.

2.2.2 Characterization of chitosan

2.2.2.1 Determination of the nature of the counter ion

Elemental analysis was used to identify inorganic counterions. The different lyophilized chitosans were analyzed by elemental analysis for their content in chloride and phosphorus (Service central d'analyse, CNRS, Solaize, France).

Acetate counter ion was identified from the presence of corresponding signal appearing around 1.8 ppm on ¹H NMR spectra recorded using a spectrometer 300 MHz (Brucker, Wissenbourg, France). Chitosan solutions were prepared at a concentration of 20 mg/mL in D₂O. The percentage of acetate counterion was evaluated using **equation 1** by the integration on peaks at 1.8 ppm corresponding to 3 hydrogens and the signal at around 3 ppm corresponding to the signals of H₂ protons integrated for 2 hydrogens (Figure 1A).

$$\text{Acetate counterion}(\%) = 100 * \left(\frac{\frac{1}{3}HAc}{\frac{1}{2}H2} \right) \quad \text{Equation 1}$$

2.2.2.2 Determination of the molecular weight by viscosimetry

The molecular weight of chitosan was determined by viscometry according to the procedure described by **Bertholon et al. [65]**. Chitosan was dissolved in a 0.1M acetic acid and 0.2M NaCl solution at concentrations ranging from 1 to 2.5 g/L. The intrinsic viscosity (cm³.g⁻¹) was deduced from the measurement of reduced viscosity of solutions at 5 concentrations in chitosan at 25.0°C using a viscometer AVS400 equipped with a Ubbelohde tube (53710/1 Schott Geräte). The molecular weight was determined using the Mark Houwink Sakurada equation using coefficients of the literature ($K = 1.81 \times 10^{-3} \text{ cm}^3.\text{g}^{-1}$, $a = 0.93$) [66,67].

2.2.2.3 Determination of the degree of deacetylation of chitosan by ¹H NMR spectroscopy:

¹H NMR spectra were recorded using a spectrometer 300 MHz (Brucker, Wissenbourg, France) on solutions of chitosan prepared at a concentration of 20 mg/mL in D₂O. The deacetylation degree (DDA) was evaluated from the ¹H NMR spectra by the method proposed by Hirai et al. [68] using equation 2.

$$DDA(\%) = \left(1 - \frac{\frac{1}{3}HAc}{\frac{1}{6}H2-H6} \right) * 100 \quad \text{Equation 2}$$

where HAc, H₂-H₆ referred to the proton position in the chemical structure of chitosan (Figure 1A).

Figure 1: Chemical structure of chitosan (A) and ^1H NMR spectra (B) of the different chitosans in deuterium oxide solutions at a concentration of 20 mg/mL. Spectra were recorded using a spectrometer 300 MHz. Signal appearing at 1.8 ppm was attributed to the acetate counterion.

2.2.2.4 Determination of the ionization of the glucosamine residue by pH metric

The percentage of ionized glucosamine residues found on chitosan was evaluated by pH metric by measuring the pH of chitosan solution and performing a titration with sodium hydroxide until precipitation occurred. Chitosan was dissolved in 10 mL of Milli-Q® water at a concentration of 2.5 mg/mL. The pH of the solution was measured at 23°C (pH 210, Hanna Instruments). The solution was then titrated with a sodium hydroxide solution at 0.1M until a precipitation occurred. The amount of sodium hydroxide required to produce the precipitation was calculated. This provided the number of hydroxyl ions, N (moles), required to neutralize the positive charged of the chitosan assuming that the precipitation of the macromolecule occurred when it became neutral in charge. Knowing the amount of chitosan dissolved in the solution and its degree of deacetylation, the fraction of ionized glucosamine residue (F_{i-glc}) could be calculated applying equation 3.

$$F_{i-glc} = \frac{N}{M_{g\ln c}} \quad \text{Equation 3}$$

With M_{gInc} : number of glucosamine residues contained in chitosan in the solution calculated from the degree of deacetylation, the amount of chitosan in the solution and the molar masses of glucosamine residues (MM: 161 g/mol) and of N-acetyl glucosamine residues (MM: 203 g/mol).

2.2.3 Preparation of the siRNA carrier by redox radical emulsion polymerization of isobutylcyanoacrylate.

The siRNA carrier was prepared by redox radical emulsion polymerization according to the protocol used by De Martimprey et al. [43] and Ramon et al. [44]. Chitosan (0.068 g) and poloxamer 188 (3.5 % w/v) were dissolved in a solution of nitric acid 0.2 N (4 mL) at 45°C under vigorous magnetic stirring and nitrogen atmosphere. After 10 min, a solution of cerium (IV) ammonium nitrate 80 mM (1 mL) in nitric acid 0.2 M and IBCA (0.25 mL) were successively added. Nitrogen bubbling was maintained for an additional 10 min and the polymerization was continued at constant temperature for 50 min. At the end of the preparation, the obtained dispersion was cooled down to + 4 °C to avoid aggregation.

After cooling, the dispersion was purified by dialysis using a Spectra/Por membrane with molecular weight cut off of 100 000 g/mol, three times during 1 h and once overnight against 0.5 L of Milli-Q® water. This purification was efficient to remove all remaining free chitosan while it removed around 85 % of the non-associated fraction of poloxamer 188 [68].

For some experiments, nanoparticles were synthesized with only chitosan or poloxamer 188. The same procedure was followed but corresponding media of polymerization were prepared without only poloxamer 188 or chitosan respectively.

A systematic nomenclature was used to name the different nanoparticles: NP meant nanoparticles. The letter code attached indicated whether the particles were prepared with chitosan (CS meant any type of chitosan while CS_{Lab}, CS_{Com}, CS_{Com}mod1 or CS_{Com}mod2 designated a specific chitosan) and/or poloxamer 188 (P). According to this nomenclature, the formulation corresponded to the siRNA carrier was designated by the nanoparticles named NP-CS_{Lab}P.

2.2.4 Characterization of the nanoparticles

2.2.4.1 Size

The mean hydrodynamic diameter of the nanoparticles, D_H , was evaluated by dynamic light scattering (Nano ZS-90, Malvern, Orsay, France) by diluting 5 to 60 µL of each nanoparticle dispersion in acetic acid 0.1 N (1 mL). All measurements were performed at least three times on different preparations.

2.2.4.2 Concentration

The mass concentration in nanoparticles in the dispersions, C (mg/cm³), was evaluated by gravimetry after lyophilization of three aliquots (200 µL) of the nanoparticle dispersions (Alpha 1-2 LD plus, Fisher Scientific, Illkirch, France).

The concentration of nanoparticles was also expressed in surface per volume unit, S (cm²/cm³). It was calculated from the mass concentration deduced from gravimetry measurements and the mean hydrodynamic diameter, D_H , of the nanoparticles determined by dynamic light scattering. For this calculation,

the volumetric mass of the nanoparticles, ρ (g/cm³), was taken at 1.14 consistently with the experimental value determined for poly(isobutylcyanoacrylate) nanoparticles in a previous work [70]. The concentration of the nanoparticles in surface per volume unit calculated using equation 4

$$S = \frac{6C}{\rho D_H} \quad \text{Equation 4}$$

2.2.4.3 *Determination of the content in chitosan of the nanoparticles by elemental analysis*

The percentage in weight of chitosan in the nanoparticles was determined by elemental analysis performed on lyophilized nanoparticles according to the methods reported previously [69]. Elemental analysis was performed by the Microanalysis facility of the Faculty of Pharmacy from the University of Paris Sud (Chatenay Malabry, France).

2.2.4.4 *Determination of the association of siRNA with chitosan in solution and immobilized at the surface of the nanoparticles by gel electrophoresis.*

siRNA was incubated at a concentration of 40 µg/mL with a solution of chitosan at 2 mg/mL for 15 min at room temperature. Presence of remaining free siRNA in an aliquot of the incubation medium (20 µL + 9 µL Glycerol) was revealed by running an electrophoresis for 1 h at 190 V in a 2% agarose gel plate containing ethidium bromide (Sigma-Aldrich). Gels were exposed to UV light in a UV light box operating at 254 nm to reveal the presence of siRNA thanks to the fluorescence with ethidium bromide. The same procedure was followed to investigate association of siRNA with nanoparticles using an incubation medium prepared with 0.17 mg/mL siRNA and 4.9 mg/mL nanoparticles. Consistently with the whole study, all solutions and dispersions of nanoparticles included in the incubation media were prepared with Milli-Q® water.

2.2.5 **Study of the thermodynamic characteristics of the interactions of siRNA with chitosan in solution and immobilized at the surface of nanoparticles by Isothermal titration calorimetry (ITC):**

Thermodynamic characteristics of the interactions occurring during association between siRNA and chitosan either in solution or immobilized at the surface of PIBCA nanoparticles were investigated using a VP-ITC microcalorimeter (MicroCal, Malvern Panalytical) with a sample cell volume of 1.441 mL at 25 °C, under constant stirring at 394 rpm. Before any measurement, the different parts of the microcalorimeter were washed with RNaseZAP (Aldrich) and rinsed several times with Milli-Q® water to remove RNase. The solution of chitosan (2 mg/mL) in water or the dispersion of nanoparticles (dispersed in water and used as produced) was introduced into the titration syringe. The thermostated cell containing either Milli-Q® water or the solution of siRNA (100 nM) in water was titrated by the water-based chitosan solution or dispersion. Each titration began with an initial injection of 5 µL followed by 27 other injections of 10 µL each with an interval of 400 s between the five first injections and 250 s for the following ones. Background of titration consisted on injecting the chitosan aqueous solutions or dispersion in MilliQ® water placed in the sample cell. The corresponding heat flow recorded as a function of time accounts for dilution effect.

All solutions and dispersions of nanoparticles were freshly prepared before use in ITC measurements.

Data were analyzed as follow. Raw ITC data were recorded with VP viewer 2000 ITC (version 1.4.12) software and processed with Microcal LLC, ITC software based on Origin (version 7.0) software. The dilution effect was subtracted from the titration enthalpogram. For the evaluation of the molar ratio N/P, only the deacetylated part of chitosans including glucosamine residues was considered to be available to interact with the siRNA. The concentration of chitosan was then given as a concentration of glucosamine residues expressing the

concentration in primary amine function based on the fact that one glucosamine residue contains one primary amine function. The concentration of siRNA was expressed as a concentration of phosphate to calculate the N/P ratio corresponding to the molar ratio between primary amine function of chitosan and phosphate groups of siRNA. The normalized enthalpy, ΔH_{fit} , could be calculated in kJ.mol^{-1} of primary amines and as the function of the N/P ratio. In experiments performed with solutions of poloxamer 188 and nanoparticles prepared with poloxamer 188 only, nanoparticles NP-P, concentrations in poloxamer 188 were expressed in molarity of poloxamer 188.

Based on the concentration of the two interacting species, the software used nonlinear least-squares algorithm to fit the corrected titration curve to provide with the binding constant (K_a) and the interaction enthalpy (ΔH_{fit}). The difference in free energy (ΔG) and the entropy (ΔS) were then deduced from these values according to the Van't Hoff equation (Equation 5).

$$\Delta G = -RT \ln K_a = \Delta H_{fit} - T\Delta S \quad \text{Equation 5}$$

The dissociation constant, K_d , was calculated from K_a deduced from equation 4 with equation 6.

$$K_d = 1/K_a \quad \text{Equation 6}$$

A graphical evaluation of the interaction enthalpy, ΔH_{graph} , was achieved from all integrated curves of titration in taking the difference between the higher and the lower enthalpy.

For experiments performed with nanoparticles, the interaction enthalpy was also expressed in μJ per surface unit (cm^2) of nanoparticle available for the interaction with siRNA.

3 Results

3.1 Characterization of chitosan

Results of the characterization of chitosans were reported in table 1. The molecular weight and degree of deacetylation of CS_{Lab} and CS_{Com} were similar. Based on these parameters, the quality of CS_{Com} was confirmed with the expectation. The pH of CS_{Lab} and CS_{Com} solutions obtained by simple dissolution of the polysaccharides in water were different as well as the degree of ionization of the glucosamine residues calculated from the neutralization of the charged carried by the polysaccharides. The two chitosans also differed from the nature of their salt.

Table 1: Characteristics of the chitosans

Chitosan	Mv ¹ (g/mol)	Counterions ²				¹ H-NMR spectra Signal NH ₃ ⁺ (ppm)	DDA ³ (%)	pH of solution	Titration (mMol NaOH/g chitosan)	Ionized glucosamine residue (% mol/mol)
		A ⁻	Cl ⁻ (% wt/wt)	PO ₄ ³⁻	molar ratio AcO ⁻ /NHAc					
<u>CS_{Lab}</u>	20,000	<u>AcO⁻</u>	0	< DL ⁴	5	2.80	90	5.60	1.47	27.0
<u>CS_{Com}</u>	15,000	Cl ⁻	12.9	< DL ⁴	0	2.96	92	5.29	3.67	65.6
<u>CS_{Com-mod1}</u>	31,000	<u>AcO⁻/Cl⁻</u>	10.6	< DL ⁴	1	2.98	90	4.81	3.41	62.6
<u>CS_{Com-mod2}</u>	33,000	<u>AcO⁻/Cl⁻</u>	0.5	< DL ⁴	6	2.82	87	5.35	1.84	35.3

¹ Determined by viscometry, ²AcO⁻ was revealed by ¹H-NMR spectra (signal at 1.8 ppm). Mineral counter ion including Cl⁻ and PO₄³⁻ were revealed by elemental analysis, ³ DDA: degree of deacetylation determined from the ¹H-NMR spectra. ⁴ < DL: bellow the detection limit

CS_{Lab} was an acetate chitosan salt as revealed by the presence of the signal at 1.8 ppm attributed to proton of an acetate counterion on the ¹H NMR spectra (Figure 1B spectrum A). No chloride and phosphate were found in this chitosan from elemental analysis. The chitosan CS_{Com} was a hydrochloride chitosan salt. No phosphorus was detected by elemental analysis and the ¹H NMR spectra did not show any signal corresponding to acetate (Figure 1B spectrum B). Because of the difference in the nature of the counterion in CS_{Lab} and CS_{Com}, it was decided to prepare new chitosans from CS_{Com} exchanging the chloride counterion by the acetate counterion found in CS_{Lab}. Two procedures were applied producing CS_{Com}mod1 and CS_{Com}-mod2. Determinations of the counterion found in CS_{Com}mod1 and CS_{Com}-mod2 indicated that they both contained chloride and acetate counterions (Figure 1B spectra C and D) but in different proportions. The procedure used to prepare CS_{Com}mod1 did not replace much chloride and CS_{Com}mod1 still contained a large amount of chloride counterion (10.6%). In contrast, the content in chloride found in CS_{Com}-mod2 was low (0.5%) while its acetate content (molar ratio AcO⁻/NHAc = 6) appeared closed to the acetate content found in CS_{Lab} (molar ratio AcO⁻/NHAc = 5). The pH of solutions prepared with CS_{Com}mod1 and CS_{Com}-mod2 (4.81 and 5.31 respectively) differed from those of solutions prepared with CS_{Lab} and CS_{Com} (5.60 and 5.29 respectively) even though all solutions were prepared identically (Table 1). Regarding the ionization of the glucosamine residues, chitosan acetate salts, CS_{Lab} and CS_{Com}-mod2, showed the lowest ionization (27.0 and 35.3 %mol/mol respectively) while chitosan hydrochloride salts, CS_{Com} and CS_{Com}mod1, displayed the highest ionization (65.6 and 62.6 %mol/mol respectively). A linear relationship was highlighted with the square root of the chloride content (Figure 2). It seemed to us interesting to report this even if we have no explanation yet to provide.

Figure 2: Percentage of ionized glucosamine residues in the different chitosan according to their content in chloride counter ion. Equation of the linear relationship is given by $y = 10,829 x + 27,684$ with a correlation coefficient of $R^2 = 0.9995$.

3.2 Quality control of nanoparticles obtained with home prepared chitosan, CS_{Lab} , and the commercially available chitosan, (CS_{Com}).

In the prospect of the upscale of the production of a siRNA carrier made of chitosan-coated poly(alkylcyanoacrylate) nanoparticles, the home-prepared chitosan, CS_{Lab} , was changed by a commercially available chitosan, CS_{Com} . As indicated in table 1, both chitosans showed similar quality based on their molecular weight and degree of deacetylation that are the most cited parameters influencing siRNA delivery performances of chitosan-based nanomedicines [36,37,39-41]. At first, it was verified that the quality of nanoparticles obtained at the lab scale with CS_{Com} was comparable with quality of a batch of nanoparticles obtained with CS_{Lab} . The two batches of nanoparticles displayed very similar size, zeta potential and contents in glucosamine and poloxamer 188 (Table 2).

Table 2: Characteristics of nanoparticles and nanoparticle dispersions.

Nanoparticles	D_H (nm)	Zeta potential (mV)	[Nanoparticles] (mg/mL)	[chitosan] (mmol glucosamine/g nanoparticles)	[poloxamer 188] (mmol/g nanoparticles)
$NP-CS_{Lab}$	208 ± 25	$+ 40 \pm 1$	12	1.25	-
$NP-CS_{Com}$	381 ± 65	$+ 37 \pm 3$	12	1.29	-
$NP-P$	60 ± 7	$- 18 \pm 2$	16	-	0.032
$NP-CS_{Lab}P$	42 ± 8	$+ 11 \pm 2$	49	0.27	0.018
$NP-CS_{Com}P$	53 ± 12	$+ 11 \pm 1$	36	0.28	0.018
$NP-CS_{Com}mod1P$	50 ± 12	nd	40	0.27	0.018
$NP-CS_{Com}mod2P$	55 ± 15	$+ 13 \pm 3$	41	0.26	0.018

The figure 3 reports results of the evaluation of the association of the siRNA assessed by gel electrophoresis. The intensity of the fluorescent bands appearing on the gels informed about the amount of remaining free siRNA in samples. A reference sample given the intensity of the band in case no association occurred was provided from the analysis of a solution of siRNA at the same concentration than that used in samples incubated with nanoparticles or chitosan (see figure 3, rows siRNA). Intensities of bands shown by samples containing nanoparticles or chitosan in solutions were much lower than that of the free siRNA reference indicated that at least part of the siRNA associated with the tested material. The results indicated a good association of the siRNA with the carrier or the chitosan. The low intensity of the band corresponded to free siRNA remaining in the siRNA carrier containing sample agreed with previous quantitative determinations showing that our siRNA carrier could associated around 95 % of siRNA [43,44]. A lower intensity of the bands could be highlighted considering the siRNA carrier prepared with CS_{Lab} compared with that prepared with CS_{Com} . No fluorescent band appeared on the gel for the sample prepared with a solution of CS_{Lab} indicating that this chitosan associated the total amount of siRNA while provided under the form of a solution. Results of the analysis of the remaining free fraction of siRNA in samples by gel electrophoresis revealed that the quality of the nanoparticles prepared with CS_{Lab} and CS_{Com} differed regarding their capacity to associate siRNA. In solution, chitosan CS_{Com} associated clearly less siRNA than CS_{Lab} while association experiments were performed in identical conditions.

Figure 3: Association of the siRNA with chitosan in solution (A) and immobilized at the surface of the siRNA carrier (B) as evaluated by gel electrophoresis. CS_{Lab}, CS_{Com}, CS_{Com}mod1, CS_{Com}mod2 referred to the type of chitosan used to prepare the solutions or the siRNA carrier. Presence of siRNA in the gel was revealed by fluorescence using ethidium bromide. At the migration position of the siRNA, the darker the migration bands appeared, the highest was the amount of free siRNA in samples containing either chitosan in solution or siRNA carriers. The lighter was this band, the higher the amount of siRNA was retained in the complex with chitosan in the solutions or chitosan immobilized at the surface of the siRNA carrier

3.3 Influence of the type of chitosan salt on siRNA association

The figure 3 showed that the association of siRNA with samples prepared with CS_{com}-mod2 appeared similar to that observed with samples prepared with CS_{Lab} while the association of siRNA with samples prepared with CS_{Com} and CS_{Com}mod1 were comparable. Samples prepared with chitosan acetate salt (solutions of CS_{Lab} and CS_{Com}-mod2 and nanoparticles NP- CS_{Lab} P and NP- CS_{Com}-mod2P) associated slightly better siRNA than those prepared with chitosan hydrochloride salts (solutions of CS_{Com} and CS_{Com}mod1 and nanoparticles NP- CS_{Com}P and NP- CS_{Com}mod1P).

3.4 Thermodynamic characteristics of the interactions between siRNA and chitosan

Thermodynamic characteristics of the interactions between siRNA and chitosan were investigated by ITC. In the present study, chitosan occurred as free solubilized polymer chains in solutions, CS_{Lab} to CS_{com}-mod2, or as a polymer chain immobilized on the surface of nanoparticles. The nanoparticle surface of siRNA carriers, i.e. nanoparticles NP- CS_{Lab}P, NP- CS_{Com}P, NP- CS_{Com}mod1P and NP- CS_{Com}-mod2P, included one type of chitosan and poloxamer 188 [69]. Some experiments were performed with simpler nanoparticle formulations with only chitosan at the surface, nanoparticles NP- CS_{Lab} and NP- CS_{Com}, or poloxamer 188, nanoparticles NP- P. A solution of poloxamer 188 was considered as well in these experiments.

The figure 4 presents representative graphs of the heat rate monitored as a function of time during titration experiments of different samples prepared with chitosan CS_{Lab} and poloxamer 188. Same trends were observed considering corresponding samples prepared with CS_{Com}. Sharp positive peaks recorded during titration performed with solutions of chitosan (see for instance Figure 4A) and dispersions of nanoparticles having only chitosan on the surface (nanoparticles NP- CS_{Lab} and NP- CS_{Com}) (see for instance Figure 4C) indicated endothermic interactions which can result from the occurrence of dehydration phenomena. Negative peaks monitoring exothermic events were recorded during titrations involving samples containing only poloxamer 188. The low variation of amplitude from the beginning and to the end of the titration suggested that interactions having generated these signals were negligible (Figure 4B,D). Raw signals obtained from titrations performed with the siRNA carrier (Figure 4E) monitored the occurrence of an endothermic event (positive peaks) followed by an exothermic event (negative peaks). The amplitude of the exothermic signal was 5 μ Watt which was twice higher than the amplitude monitored studying interactions of siRNA with nanoparticles NP-P. This suggested that siRNA interactions with nanoparticles prepared with chitosan and poloxamer 188 involved a distinct phenomenon. The exothermic signals acknowledged the occurrence of specific interactions, i.e. formation of hydrogen or electrostatic bonds, Van der Waals interactions.

Figure 4: Example of raw ITC signal recovered from the titration of siRNA with CS_{Lab} in solution (A); poloxamer 188 in solution (B); NP- CS_{Lab} (C); NP-P nanoparticles (D) and NP- CS_{Lab}P nanoparticles (E).

Graphs of the figure 5 present the normalized experimental interaction heat change, ΔH , measured by ITC for the different experiments. The variation of ΔH monitored for interactions occurring between siRNA and poloxamer 188 solutions or NP-P nanoparticles was near zero confirming that interactions detectable by ITC were negligible but the existence of possible athermal interactions cannot be excluded according to Ma et al. [71] (Figure 5Aa, Ba). Experiments performed with chitosan solutions and nanoparticles coated with both chitosan and poloxamer (NP- CS_{Lab}P, NP- CS_{mod}P, NP- CS_{Com}-mod1P, NP- CS_{Com}-mod2P) provided curves that followed a biphasic model (Figure 5Ab, Ac, Bb, Bc). The endothermic part of the curves (second part of the curves) could be fitted with the one binding site model providing the interaction enthalpy, ΔH_{fit} (J.mol⁻¹), and the dissociation constant, K_d (M). The results were included in table 3 and figure 6.

Figure 5: Normalized heat change per injection of the interaction occurring between siRNA and chitosan in solution (column A) or chitosan immobilized at the surface of nanoparticles with or without poloxamer 188 (columns B in KJ/mol and C in μJ/cm²). (Aa) solution of poloxamer 188, (Ab) solutions of CS_{Lab} (■) and CS_{Com}-mod2 (◆), (Ac) solutions of CS_{Com} (●) and CS_{Com}mod1 (▲); (Ba and Ca) nanoparticles NP-P, (Bb and Cb) nanoparticles NP-CS_{Lab} (□), NP-CS_{Lab}P (■), NP- CS_{Com}-mod2P (◆); (Bc and Cc) nanoparticles NP-CS_{Com} (○), NP- CS_{Com}P (●), NP- CS_{Com}mod1P (▲); (Aa, Ba, Ca) poloxamer 188/P corresponded to the molar ratio between poloxamer 188 and phosphate groups of the siRNA; (Ab, Bb, Cb) N/P corresponded to the molar fraction between the amine functions of glucosamine residues of chitosan and phosphate groups of the siRNA.

Table 3: Thermodynamic characteristics of interactions between siRNA and chitosan occurring as a solution (A) or immobilized on the surface of nanoparticles (B)

A Solutions	Counterions ¹	K_a^* (M ⁻¹)	K_d^{**} (M)	ΔH_{graph} (kJ.mol ⁻¹)
<i>CS_{Lab}</i>	AcO ⁻	$1.4 \pm 0.3 \cdot 10^6$	$0.8 \pm 0.2 \cdot 10^{-6}$	170
<i>CS_{Com}</i>	Cl ⁻	$1.1 \pm 0.1 \cdot 10^6$	$0.9 \pm 0.1 \cdot 10^{-6}$	830
<i>CS_{Com}mod1</i>	Cl ⁻ /AcO ⁻	$3.6 \pm 0.7 \cdot 10^6$	$0.28 \pm 0.05 \cdot 10^{-6}$	1100
<i>CS_{Com}mod2</i>	AcO ⁻ /Cl ⁻	$1.6 \pm 0.2 \cdot 10^6$	$0.59 \pm 0.04 \cdot 10^{-6}$	230
<i>poloxamer 188</i>	-	-	-	0

B Nanoparticles	K_a^* (M ⁻¹)	K_d^{**} (M)	ΔH_{graph} (kJ.mol ⁻¹)	$\Delta H'_{\text{graph}}$ (μJ.cm ⁻²)
<i>NP-CS_{Lab}</i>	nd	nd	21	4.3
<i>NP- CS_{Com}</i>	nd	nd	31	12.2
<i>NP-P</i>	nd	nd	4	1.0
<i>NP-CS_{Lab}P</i>	$0.6 \pm 0.2 \cdot 10^6$	$1.7 \pm 0.4 \cdot 10^{-6}$	72	0.3
<i>NP- CS_{Com}P</i>	$0.7 \pm 0.1 \cdot 10^6$	$1.5 \pm 0.3 \cdot 10^{-6}$	280	0.9
<i>NP- CS_{Com}mod1P</i>	$0.60 \pm 0.05 \cdot 10^6$	$1.6 \pm 0.1 \cdot 10^{-6}$	170	0.6
<i>NP- CS_{Com}mod2P</i>	$0.64 \pm 0.05 \cdot 10^6$	$1.6 \pm 0.1 \cdot 10^{-6}$	71	0.8

¹ from table 1, * K_a Binding constants were deduced from the fit of the endothermic part (second part) of the curves giving the enthalpy of interaction as a function of the molar ratios of the reactants using the ITC one binding site model. ** K_d dissociation constant calculated from the inverse of K_a . (nd curves could not be fitted)

In general, the interaction enthalpy, ΔH , contribution of the free energy of interaction, were the highest for samples prepared with chitosan only, i.e. chitosan solutions and NP-CS_{Lab} to NP- CS_{Com}-mod2 nanoparticles. It varied from 100 to more than 1000 kJ.mol⁻¹. Interaction enthalpies were much higher considering samples containing also poloxamer 188, i.e. siRNA carriers including NP- CS_{Lab}P to NP- CS_{Com}-mod2P nanoparticles. Calculated values of the standard Gibbs free energy of the reaction deduced from the Van't Hoff equation (Equation 5) were negative, indicating spontaneous interactions between siRNA and components of the tested samples. The positive value of the entropy acknowledged entropic driven phenomena that can be attributed to dehydration and chain rearrangement phenomena. Values of the entropic term, $T\Delta S$, and of the enthalpy term, ΔH_{fit} , of the reaction appeared similar in absolute values, indicating that it involved the formation of electrostatic bonds and acknowledged the occurrence of a dehydration process as well. The normalized interaction heat change per injection, ΔH , monitored during titration was also expressed in μJ per surface unit of nanoparticle available for the interaction with siRNA (figure 5 Ca, Cb, Cc) to better highlight the influence of the nanoparticle surface on their interaction. The interaction enthalpy, $\Delta H'_{\text{graph}}$, was evaluated from the difference between the higher and lower ΔH recorded during titration. Lowest values were obtained considering the nanoparticles coated with both chitosan and poloxamer (NP-CS_{Lab}P, NP- CS_{mod}P, NP- CS_{Com}-mod1P, NP- CS_{Com}-mod2P) consistently with the other results.

Figure 6: Comparison of thermodynamic characteristics of interactions between siRNA and chitosan either in solution or on nanoparticles (NP-CSP). Interaction enthalpy (ΔH_{fit}) was given by the one binding site model fit of the integrated data shown on figure 5 (Hatched bars). Entropy term ($-T\Delta S$) (empty bars) and the standard Gibbs free energy (ΔG) (full bars) were calculated from ΔH_{fit} and of the binding constant given by the fit by applying the Van't Hoff equation (Equation 5).

Binding constants could be determined from isotherms monitoring interactions of siRNA with chitosan in solutions and with nanoparticles NP- CS_{Lab}P to NP- CS_{ConfMod2}P. Dissociation constants calculated from the binding constants ranged between 1.02 to $7.69 \cdot 10^{-6}$ M. They were of the same order of magnitude for the different samples indicating that the nature of interactions occurring with siRNA was the same whether chitosan was in solution or immobilized at the surface of nanoparticles together with poloxamer 188 (Table 3).

4 Discussion

Scaling up the preparation of a nanomedicine can be a bottleneck to its clinical translation [45-52]. Beside potential methodological difficulties to reproduce the nanomedicine with similar quality and efficiency than that produced at the lab scale, suitable amount of chemicals responding to the required quality criteria must be available. To achieve the development of a siRNA carrier based on the poly(alkylcyanoacrylate) nanoparticle technology, a commercial source of chitosan was needed to

replace the home-tediously prepared batches of chitosans used to carry on our research projects [43,44]. The new source of chitosan was selected based on structural parameters known to influence the formation of complex with siRNA and interfering with the siRNA activity obtained in vitro and in vivo. Consistently, quality criteria included a similarity of the degree of deacetylation and molecular weight as they were the most cited structural parameters of chitosan influencing performances of nucleic acid chitosan-based carriers [26,30,35,37,38,40,41,60]. Nanoparticles designed as siRNA carrier produced with the home-prepared chitosan, NP-CS_{Lab}P, and chitosan of the commercial source, NP-CS_{Com}P, displayed very similar quality based on size and zeta potential criteria. However, further investigations of the qualities of the siRNA carrier obtained with CS_{Com} revealed a lower capacity to associate siRNA accompanied by a modification of thermodynamic parameters of interactions with siRNA highlighted by ITC.

Based on the literature, association of siRNA with the carrier was assumed to take place thanks to electrostatic interactions with chitosan [26,34,35] whereas chitosan was stranded at the nanoparticle surface together with poly(ethylene glycol) (PEG) chains of poloxamer 188 [69]. The similarity of binding constants found considering association of siRNA with chitosan in solution or with siRNA carriers acknowledged interactions of similar nature attributed to interactions of the siRNA with chitosan found in both samples. Consistently, ITC experiments also confirmed that poloxamer 188, either on solution or alone at the surface of the nanoparticles NP-P, did not interact directly with the siRNA during association with the siRNA carrier as barely no interaction involving thermal exchange detectable by ITC were evidenced (Figure 5 Aa, Ba). The predominant role of chitosan while associating siRNA with the carrier was observed independently of the source of chitosan used to prepare the siRNA carrier. However, the difference in siRNA association capacity and thermodynamic conditions of the interactions observed between samples prepared with CS_{Lab} and CS_{Com} could not be explained by a difference in degree of deacetylation and molecular weight of chitosans as both were very similar based on these characteristics. Although not considered as an important parameter influencing siRNA association with chitosan [10,26,29,40,61], we have postulated that the difference in the type of salt revealed from our analysis of CS_{Lab} and CS_{Com} could explain the difference of siRNA association highlighted by gel electrophoresis (Figure 3). This hypothesis was supported from results of experiments performed with CS_{Com}-mod1 and CS_{Com}-mod2 that were prepared from CS_{Com} with the aim to exchange the chloride counterion by acetate, the counterion found in CS_{Lab}. Samples prepared with CS_{Com}-mod2, which contained the lower amount of chloride salt and the highest amount of acetate salt, associated the siRNA like those prepared with CS_{Lab}. The restoration of the capacity to better associate siRNA taking a chitosan acetate salt, CS_{Com}-mod2, prepared from a chitosan hydrochloride salt, CS_{Com}, was never reported before but supported our hypothesis. Investigations of thermodynamic parameters of interactions between siRNA and various samples prepared with different chitosans by ITC have provided more proofs on the influence of the type of salt of chitosan while associating siRNA with a chitosan-based carrier. In this set of experiments, thermodynamic parameters of the interactions of siRNA with samples prepared with CS_{Lab} and CS_{Com} differed (see figure 6). In contrast, consistent results were obtained considering samples prepared with either the two chitosan acetate salts, CS_{Lab} and CS_{Com}-mod2, or the two chitosan chloride salts, CS_{Com} and CS_{Com}-mod1. The fact that thermodynamic parameters of interactions of siRNA with samples prepared with CS_{Com} were changed into those found with samples prepared with CS_{Lab} exchanging the chloride counterion by acetate, i.e. preparing samples with CS_{Com}-mod2, demonstrated a clear influence of the nature of chitosan salt on interactions between siRNA and chitosan independently of its presentation, i.e. solution or

immobilized at the surface of nanoparticles. According to our results, the nature of the salt of chitosan influenced slightly the capacity of chitosan to associate siRNA with chitosan-based carriers, but it influenced the thermodynamic characteristics of the interactions hence their mechanisms. This may have consequences on stability of complexes and release of the siRNA hence activity of the nanocarriers and will need to be considered in future work. Thus, the nature of the salt of chitosan should be considered as another key attribute of chitosan to achieve siRNA-carrier quality while the only structural parameters including the degree of deacetylation and molecular weight were taken into account so far [26,30,35,37,38,40,41,62].

The aim of the present work was not to elucidate the role of the nature of salt of chitosan on the formation of complexes with siRNA but results from our ITC experiments provided some clues. Interactions between siRNA and chitosan or nanoparticles with chitosan on their surface occurred spontaneously being driven by entropic phenomena. Both electrostatic interactions and non-electrostatic interactions were involved during complex formation, the latter being attributed to dehydration phenomena and molecular rearrangements. Dehydration phenomena can be affected by the lyotropic character of ions due to their salting-out and salting-in effect produced on macromolecules [72-74]. In the Hofmeister series which gives a classification of anions according to their lyotropic character, acetate anions are more hydrated than chloride anions producing the strongest salting-out effect on macromolecules [72-78]. From our samples, it can be postulated that the lyotropic character of counterions of chitosan may influence the dehydration of chitosan chain influencing thermodynamic conditions of their interactions with siRNA. According to this hypothesis, the salting-out effect produced on chitosan chains by acetate counterions would be stronger than that produced by the chloride counterions hence reducing the entropy term of interaction, $T\Delta S$, assuming that the chitosan chain interacting with siRNA would already be more dehydrated. This was consistent with what was observed considering samples containing chitosan in solutions where the monitored entropy of interactions was reduced with solutions of chitosan acetate, CS_{Lab} and CS_{Com-mod2}, compared with corresponding samples prepared with chitosan hydrochloride (CS_{Com} and CS_{Com-mod1}) (figure 6). At the surface of our siRNA carrier, the salting-out effect produced by counterions on the chitosan chains may indirectly be reinforced by surrounding PEG chains of poloxamer 188. Indeed, PEG can behave like salting-out agent on biological macromolecules [79]. It was also reported to enhance the salting-out effect caused by anions on other polycations used to formulate delivery systems for nucleic acids by a crowding effect [73,80,81]. Although our results suggested that poloxamer 188 did not interact directly with siRNA, its PEG chains may indirectly interfere on the dehydration of the chitosan chains stranded on the surface of the siRNA carrier according to salting-out mechanisms. This could explain the entropic gain observed comparing entropic terms of interactions in samples containing chitosan solutions of CS_{Com} (-3430 kJ.mol⁻¹) and CS_{Com-mod1} (-1730 kJ.mol⁻¹) with those of the corresponding samples including siRNA carriers, i.e. NP- CS_{Com}P (-150 kJ.mol⁻¹) and NP- CS_{Com-mod1}P (-510 kJ.mol⁻¹). A lower influence was also observed investigating interactions of siRNA with siRNA carriers prepared with chitosan acetate, NP-CS_{Lab}P and NP- CS_{Com-mod2}P. Going further, it was recently pointed out that interactions between polyelectrolytes can be influenced when they take place in a confined environment [82]. Interactions of siRNA with our carrier occur in such conditions and the influence of the counterion on the thermodynamic aspects of interactions between siRNA and chitosan should also be considered regarding confinement found at the surface of nanoparticles.

5 Conclusion.

Here, we have demonstrated that the shift of chitosan source for the preparation of a siRNA carrier can be problematic altering the quality of the formulation. Thus, the degree of deacetylation and molecular weight of chitosan are insufficient parameters to consider obtaining chitosan-based siRNA carriers having similar quality in terms of siRNA association when they are prepared with chitosan from different sources. The nature of the counterion associated with chitosan previously described as having only a marginal effect was shown here to influence interactions between siRNA and chitosan on the thermodynamic standpoint. The effect was interpreted from the lyotropic character of the counterion. This relates to the application of a different salting-out effect on chitosan chains depending on the nature of the counterion that can interfere with dehydration phenomena involved during interactions of chitosan with siRNA. Thus, the main conclusion of this work was that the nature of the salt of chitosan is another structural parameter of chitosan that should be considered while formulating chitosan-based delivery systems for siRNA. With this recommendation, this work opens perspectives to improve the rate of success of translation for the fabrication of clinical batches of chitosan-based delivery systems for siRNA. Besides, results and assumptions drawn from this work can serve as starting point to elucidate in-depth mechanisms by which counterions of chitosan interfere during the formation of siRNA-chitosan complexes. In complement, it would be interested to elucidate whether the nature of the counterion of chitosan would also interfere on thermodynamic aspects of interactions of chitosan with other nucleic acids and what would be the influence on the stability of the complexes and the releasing properties of the nucleic acids.

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