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# ELECTROKINETIC BEHAVOIR OF CHITOSAN ADSORBED ON O/W NANOEMULSION DROPLETS

Francisco M. Goycoolea [1], Viktoria Milkova [1,2, *]

[1] *Institut für Biology und Biotechnologie der Pflanzen, Westfälische Wilhems-Universität Münster*
*Schlossgarten 3, Münster, 48149, Germany*
[2] *Institute of Physical Chemistry, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*

## ABSTRACT

Chitosans with different molecular weight and degree of acetylation (DA) were adsorbed onto lecithin stabilized nanoemulsion droplets at pH~4.7. The electrokinetic properties of the nanoemulsion were investigated by using dynamic light scattering. According to the experimental data, the isoelectric point (IEP) of the droplets in the suspension at the presence of oppositely charged chitosan, does not depend on the polyelectrolyte molecular weight (when DA of chitosan is lower than 32 %). Due to the high charge density of these polymer chains and low surface charge density of the lecithin stabilized droplets, the chitosan molecules retain part of the condensed counterions upon the adsorption process. Thus, the registered electrophoretic mobility corresponding to the "effective", and not "real", the electrokinetic charge of the droplets covered by a polymer layer. The stability behavior of the nanoemulsion in the presence of chitosan with lower charge density is different, and the IEP and stabilization of the system is achieved at higher polymer concentration. The coincidence in the polyelectrolyte concentration corresponding to IEP and minimum stability of the nanoemulsion against coagulation agrees with the notion that the electrostatic interactions are predominant in the coating layer formation. In spite of the similar electrokinetic behavior of the droplets covered with chitosan with low DA and different molecular weight, the polymers have different biological activity which might be results from different charge density of the molecules. In the present study, we show that the electrical polarizability is a complex parameter that can allow to more detailed characterization of the electrical properties of the chitosan.

*Keywords: Chitosan; Nanoemulsion; Counterion condensation; Electrokinetics,*

e-mail: vmilkova@ipc.bas.bg

## 1. Introduction

Chitosan is a copolymer of *β-(1-4)-2-acetamido-2-deoxy-β-D-glucopyranose* and *2-amino-2-deoxy- β-D-glucopyranose*, that is obtained by deacetylation of its parent polymer chitin, a polysaccharide widely distributed in nature (exoskeleton of insects, crustaceans and certain fungi) (1). Chitosan is a weak polyelectrolyte with reactive functional groups, gel-forming capability, and high adsorption capacity (2). The cationic nature of this polyelectrolyte enables the formation of polyelectrolyte complexes with negatively charged biomolecules, the interaction with cell membranes, and with other oppositely charged particles or enzymes. Chitosan charge density is pH dependent, which is beneficial for various therapeutic applications. The high charge density of chitosan (at pH < pKa~ 6.5) favors polyelectrolyte formation, whereas a low charge density at neutral pH contributes to its low cytotoxicity and facilitates the intracellular release of biomolecules. The low charge density, however, leads to low solubility, aggregation and the poor stability of chitosan-based formulations, depending on the type of chitosan applied. Previously, it has been shown that chitosan can stabilize emulsions via electrosteric interactions (3). At high enough chitosan concentration, polyelectrolyte molecules adsorb on the surface of oppositely charged surfactant-coated droplets, to form an interfacial layer consisting of a surfactant-chitosan membrane. Previously, we have shown that lecithin-chitosan membranes have better stability against flocculation and coalescence that droplets covered with lecithin alone (4). Recently, McClements et al. (5, 6) have shown the detailed investigation of factors affecting the formation and stability of O/W emulsions stabilized with chitosan. The authors have studied the influence of chitosan concentration and charge density, concentration and initial diameter of the droplets to find the optimum conditions for preparation of stable emulsions. The ability of to stabilize the dispersion from nanoparticles or emulsions and its unique properties (low toxicity to living tissues, biocompatibility biodegradability, antibacterial, antifungal, anti-tumor activity and immune enhancing effect) is a premise for usage of the polymer in the design of chitosan-based composite systems suitable for various bio-nano-technology applications. However, its biological activity depends on various factors such as molecular weight, degree of deacetylation, pH, pKa, ionic strength, the concentration of chitosan, biological source (e.g., crustacea or fungi) impurities of lipids and proteins as interferences, surface charge, reaction time and chelating capacity (7-9).

The relationship between the adsorption of chitosan on oil-core droplets, molecular characteristics of chitosan (Mw and charge density) and adsorption conditions (pH, oil concentration) have been studied in detail by McClements et al. (10). This study shows that the $\zeta$-potential of the droplets covered by chitosan does not depend on the molecular characteristics of the polyelectrolyte and decreases with increasing pH of the dispersion. Similar electrokinetic and stability behavior of oil-core nanocapsules coated by chitosan derivatives at different pH and salt concentration have also been presented in our previous study (11). Recently, we have investigated the influence electrical properties of chitosan on the formation of polyelectrolyte multilayer films on oxide nanoparticles (12-14). The results demonstrate that the charge density of the chitosan deposited on top of the film defines the electrical properties of the entire polymer-coated particle.

Variations in the molecular structure of chitosan may entail that the molecules with close Mw, but different DA (or *vice versa*), can lead to different charge distribution and electrical properties of the coated nanoemulsion particles. The present study addressed the role of the electrical properties of nanoemulsion in the presence of chitosans with different molecular weight and degree of deacetylation. We calculated the fraction of condensed counterions near to the polyion surface in the salt-free dispersion. In spite of the similar electrokinetic behavior of the dispersion in the presence of different chitosans, in the present study, we show that electrical polarizability, as a complex parameter that depends on the balance between Mw and DA can be used to estimate the electrical characteristics of chitosans.

## 2. Materials and Methods

### 2.1. Materials

Ultrapure biomedical grade chitosans (CHI) was from Heppe Medical Chitosan HMC+ GmbH (Halle, Germany), with different molecular weight (Mw), degree of polymerization (DP) and degree of acetylation (DA) were chosen for this study. The stock solutions of polyelectrolytes were prepared with concentration 1 mg/ml in 5 % stoichiometric excess of HCl.

### 2.2. Methods

**2.2.1. Dynamic Light Scattering.** The colloidal stability of the dispersion was evaluated regarding the evolution of the size (diameter) and electrophoretic mobility of the emulsion droplets as a function of the concentration of chitosan added to the system. The size distribution was determined by dynamic light scattering with non-invasive back scattering (DLS-NIBS) at an angle of 173° with automatic attenuator setting. The electrophoretic mobility ($U_{ef}$) was determined by mixed-laser Doppler electrophoresis and phase analysis light scattering (M3-PALS). Both types of measurements were conducted using a Malvern Zetasizer NANO-ZS (Malvern Instruments, Worcestershire, UK) equipped with a 4 mW He/Ne laser beam ($\lambda = 633$ nm). DLS-NIBS.

### 2.3. Preparation of the nanoemulsion/chitosan complexes

The nanoemulsion was produced according to the general procedure originally described by Calvo at al. (15). Briefly, the oil phase was prepared from 0.5 ml of a 40 mg/ml ethanolic lecithin solution (Epikuron 145V, Cargill texturing solutions Deutschland GmbH & Co. KG, Hamburg, Germany) mixed with 0.125 ml Miglyol 812 N (Sasol GmbH, Witten, Germany) and 9.5 ml ethanol. Lecithin was chosen because it is a negatively charged food grade emulsifier that can produce small oil droplets during the homogenization. The aqueous phase was a hydrochloric acid dilute aqueous solution (pH~4.7). After mixing, the organic solvent was evaporated in rotavapor at 40°C. The produced nanoemulsion was very stable against flocculation. The size (diameter) of the droplets after preparation (ca. 133±1 nm) and four months later (ca. 150±4 nm) were determined through DLS. The approximate droplets concentration (ca. $1.66\times10^{10}$ droplets/ml) was determined from the volume distribution of the nanoemulsion and the volume fraction of the Miglyol 812 N. The thickness of the lecithin layer was neglected in the calculations. For stability experiments, the samples were diluted 1:100 times in hydrochloric acid solution with pH~4.7 ($C^{*}\sim4.25\times10^{17}$ particles/ml)). The deposition of oppositely charged chitosan onto the emulsion droplets was performed by adding the nanoemulsion to the polyelectrolyte solution with the required concentration and stirring for 20 min at room temperature.

## 3. Results and Discussion

### 3.1. Stability of o/w emulsion in the presence of chitosan

Fig. 1A shows the dependence of the electrophoretic mobility of the droplets as a function of the concentration of chitosan added to the nanoemulsion. The presented results indicate that the isoelectric point (IEP) and the droplet overcharging do not depend on the molecular weight of chitosan with low DA (DA<32 %). The charge neutralization occurs at a mass ratio of about $3.77\times10^{-4}$ or $1.51\times10^{-3}$ gram of chitosan per gram of lecithin for chitosan with DA<32 % or DA 60 %, respectively. The stability behavior of chitosan with lower charge density (DA 60 %) is different, and the IEP and re-stabilization of the system are achieved at higher polymer concentration. However, the overcharging almost does not depend on the Mw and DA if the experimental data are presented as a function of the number of amine moles per one polyelectrolyte molecule, $n(NH_2)$ (Fig. 1C). This is probably because the electrophoretic mobility depends on the number of the charges per one monomer, but does not depend on the Mw (or contour length) of the molecule (16,17). The molar concentration $n(NH_2)$ in chitosan solution, can be estimated by using the relation

$$n(NH_2) = \frac{C_{Chitosan}(1-DA/100)}{M_W(D)(1-DA/100)+M_W(A)(DA/100)} \quad (1)$$

Where $M_W(D)$ is molar mass of deacetylated residues (162.16 g/mol), $M_W(A)$ is molar mass of acetylated residues (204.09 g/mol), $C_{Chitosan}$ is the chitosan concentration added to the dispersion (in mg/ml).

The hydrodynamic thickness of the adsorbed polymer layer can be calculated from the difference in the diameter of the nanoemulsion droplets in the stabilized dispersion before and after adsorption of chitosan. To this end, we considered the size of the droplets in the presence of $10^{-2}$ mg/ml chitosan into dispersion (ca. 145 nm). According to the experimental results, the thickness of the adsorbed layer (ca. 6 nm) does not depend on the $M_w$ and DA of chitosan. The calculated mass ratio in the stabilized suspension is $7.5\times10^{-3}$ gram of chitosan per gram of lecithin. The amount of chitosan bound per unit surface area of nanoemulsion droplets can be derived from the following relationship:

$$\Gamma = \frac{\rho_{Miglyol}\, d_{32}}{6\Phi}$$

(2)

where $\rho_{Miglyol}$ is the density of the oil phase (0.95 g/cm$^3$), $d_{32}$ is the surface-weighted mean diameter ($d_{32} = \sum n_i d_i^3 / \sum n_i d_i^2$=188 nm), $\Phi$ is the mass of Miglyol oil covered by chitosan per unit mass of chitosan (133 g) (10). The thus calculated adsorbed amount of chitosan ($\Gamma \approx 0.22$ mg/m$^2$) almost does not depend on the molecular weight and DA of the chitosan and is within the same range found for other biopolymers adsorbed on oil-water interfaces ($\Gamma \approx 0.4$ mg/m$^2$, (10)).

The comparison between the stability behavior of the nanoemulsions in the presence of different chitosans shown in Fig. 1 is possible only at polyelectrolyte concentrations below $2\times10^{-2}$ mg/ml, where the ionic strength of all solutions is almost constant ($3\text{-}5\times10^{-4}$ M). At higher chitosan concentration, though, the conductivity of the samples significantly increases (Fig. 2.). The higher ionic strength of the solution results in decreasing of the surface charge density of the nanoemulsion droplets (Table 2). The charge of the nanoemulsion droplets depends on the charge density of lecithin molecules exposed at its surface. Due to the presence of anionic phosphate groups of the molecules (pKa~1.5), the effective charge density of lecithin is negative in acid conditions and decreases with increasing the ionic strength of solution due to the electrostatic screening effects. The charge compensation point is reached at pH~3.8 (18).

The decreasing of the surface charge density of the droplets and increasing of the polyelectrolyte concentration results in increasing of the excess of no adsorbed chitosan molecules in solution. This can provoke bridging and aggregation in the system. The results indicate that increasing of the size of the polyelectrolyte/droplets complexes at high chitosan concentration correlates closely with the molecular weight (or the contour length of the molecules). Therefore, the dependency of the electrophoretic mobility and particle diameter does not show the saturation at high polyelectrolyte concentrations.

The presented results show a similar electrokinetic behavior of nanoemulsion in the present of chitosan with different Mw and DA (9-32 %). However, we suppose that the electrokinetic behavior of the droplets with adsorbed chitosan depends on the balance between the effect of Mw and DA. To distinguish the electrical properties of the different chitosans, we have to consider another complex parameter that depends on both characteristics of the polymers, as shown below.

### 3.2. Electrical polarizability of chitosan molecules in solution

It is known that the chitosan is a weak polyelectrolyte (pKa~6.5) (19), positively charged under acidic conditions. At the experimental conditions (pH~4.7) chitosan is regarded as an almost fully charged cationic polyelectrolyte whose charge density increases with decreasing DA. Since chitosans are dissolved in hydrochloric acid, $Cl^-$ are the predominant counterions of the chitosan molecules.

It is known that in solution of fully charged polyelectrolytes a part of the counterions undergo counterion condensation (20). This phenomenon is expected when the average distance between two charges along the polymer chain, *b,* is smaller than the Bjerrum length, $l_b$, ($l_b$=0.714 nm in water at room temperature). The Manning parameter, related to the charge density of polyelectrolyte is defined as $\xi=l_b/b$. In the case when $\xi \geq |z_i z_p|^{-1}$, where $z_i$ and $z_p$ are the valence of the counterions and of charge groups of the chain, part of the counterions undergo counterion condensation and have lower mobility than the diffuse ones. A fraction of condensed counterions decrease "real" the charge density of the polyelectrolyte to an "effective" one.

The distance between the charges along the chitosan molecule is related to the DA by *b=0.515/(1-DA),* where 0.515 nm is the length per monosaccharide unit (21). According to the Manning approximation ($\beta = 1/\xi$), a value of the fraction $\beta$ of free $Cl^-$ counterions in chitosan solution can be calculated. Table 3 shows the estimations for the used chitosan samples. The results indicate that $\xi > 1$ for the chitosans with low DA and part of the counterions are condensed because the strong electric field near to the polyion surface.

According to the Poisson-Boltzmann approach of Park *et al.* (22), the polyelectrolyte adsorption on oppositely charged hard surface is driven by a favorable entropic advantage, as the small counterions are released. However, taking into consideration the lateral correlation between the adsorbed molecules and charge-image effect, the full release of the condensed counterions onto highly charged polyelectrolyte during adsorption on oppositely charged surface is not always observed. Cheng and Olvera de la Cruz (23) have studied the adsorption of strongly charged rods on weakly charged surfaces by including the lateral correlations and equating the chemical potentials of the free and adsorbed chains and counterions, to calculate the fraction of monomers with condensed counterions on the adsorbed rods. By self-consistent analysis, the authors have shown that the fraction of released counterions is increasing with the charge density of the surface.

The fraction of free counterions, $\beta$, can be calculated by using the equation:

$$log[8\xi(1-\beta)] = log[(\kappa r)^2] - 2\xi\beta log\left[\frac{rh}{L_k(h)^2}\right] + 2log\left[\frac{\kappa(h+\lambda)}{2}\right] \qquad (3)$$

where $\kappa^{-1}$ is the Debye screening length ($\kappa^{-1} = 3.06 \times 10^{-10}/\sqrt{I}$), $r$ is a chain radius, $h$ is a distance between the polymer and droplet surface (or the thickness of the adsorbed layer), $\lambda$ is a Gouy-Chapmann length ($\lambda = 1/(2\pi l_B \sigma)$, $\sigma$ is the surface charge density of lecithin and $L_k$ is a local screening length in the Gouy-Chapmann layer ($L_k = \lambda/\sqrt{2}$) (24). The values of the parameters which are used to estimate the fraction of free counterions are: $\kappa^{-1}$ ~ 17.7 nm, $r$ ~ 5 nm, $h$ ~ 6 nm, $\lambda$ ~ 8.9 nm, $L_k$ ~ 6.3 nm and $\sigma$ ~0.03e/nm$^2$ ($\sigma$ is calculated according to Ref. 25)). The value of $\xi$ depends on the DA (Table 3). The estimation yields the fraction of free counterions for the all chitosan molecules adsorbed on nanoemulsion droplets that are very close to one calculated according to the Manning approximation for rod-like chains in solution. The estimations indicate that 10-12 % of the condensed counterions (*i.e.,* 1-$\beta$) are not released upon the chitosan adsorption onto the lecithin surface. These results are in line with the previous experimental and theoretical studies on polyelectrolyte adsorption on solid particles surface which predict that in the electrokinetic measurements what is estimated is the "effective" and not the "real" charge of the particle or emulsion droplets covered by polyelectrolyte (26-27).

In the presence of a weak electric field (like in the electrophoretic measurements) the formed complex between a macroion (particle or emulsion droplet) and its counterions, drifts along the electric field in the direction implied by the inverted charge, and the charge inversion is not altered until the electric field exceeds a critical value (27). Therefore, it can be expected that the polarization of condensed counterions along the polymer chain will define the electrical properties of the chitosan molecules and also the properties of the emulsion droplets covered by a polyelectrolyte layer (20, 28-30). Further, this agreement allows us to calculate the electrical polarizability of one polyelectrolyte molecule in solution that is due to the polarization of the layer of condensed counterions along the polyion contour. This polarizability is calculated using the Manning's equation (20, 29):

$$\gamma_M = \frac{e^2 L_c^{\,3}}{12kTb} \cdot \frac{1-\xi^{-1}}{1-2(\xi-1)\ln \kappa b} \qquad (4)$$

where $L_c$ is the contour length of the chitosan molecule that is calculated using relation:

$$L_c \sim b\frac{M_w}{M_W\,(D)(1-DA/100)+M_W\,(DA/100)} \qquad (5)$$

In spite of the very similar electrokinetic behavior of droplets with adsorbed layer from different chitosan, the values of the polarizability presented in Table 3 show that the electrical characteristics of the polyelectrolytes are different, and we can thus expect different adsorption behavior when they adsorb on the low charged hard or soft particle surface.

According to the Eq. (4) and the estimated values of $\xi$ (Table 3), hence,

$$\frac{e^2}{12kTb}\cdot\frac{1-\xi^{-1}}{1-2(\xi-1)\ln\kappa b}\sim \text{const}, \qquad (6)$$

$$\gamma_M \sim L_c^{\;3} \qquad (7)$$

Therefore, the electrical properties of chitosan depend predominantly on the molecular weight of the molecules, and this is in agreement with the dependence of the bioactivity of polyelectrolytes on their Mw (31, 32).

Previously we have documented similar estimations of the fraction of $Na^+$ counterions which are released upon the adsorption of fully charged synthetic polyelectrolytes (poly(4-styrene sulfonate), NaPSS (28) and carboxymethylcellulose, NaCMC (30)) on the weakly charged oxide surface. The calculated values of $\beta$ are very close to the fraction free $Na^+$ counterions in a solution of these polyelectrolytes. This means that the condensed counterions are not released from the chains upon the adsorption on weakly charged hard surface. The presence of condensed counterions is also confirmed by electro-optical experiments which indicate that the polarization of counterions with lower mobility is responsible for the registered electro-optical effect and defines the electrical properties of particles covered by a polyelectrolyte layer.

The Manning theory for counterion condensation is valid for a rod-like geometry, which is not completely fulfill in the case of chitosan chains (i.e., the estimated contour length of the chitosan samples used in this study is in the range of 123-770 nm). Recently, Schatz et. (33) have determined the conformation of chitosan molecules in aqueous solution by calculating the persistence length ($L_p$). The contribution of the electrostatic persistence length ($L_{p,\ e}$) to the total persistence length ($L_p$) is neglected at very low ionic strength. The estimated values of $L_p$ (ca. 4.5-8.8 nm) are consistent with a semi-flexible wormlike chain model. Also, McClements et al. (10) did not account for the effect of the presence of counterions with lower mobility on the electrokinetic behavior of chitosan, but they have registered a decreaseing in the linear charge density of chitosan due to the intermolecular interaction between the amine groups along the polyion. This hypothesis evolves from the expected strong overlap of the electrostatic repulsive forces along the polyion because the charge space distance is lower than $\kappa^{-1}$ .

Yet another added complication related to the calculations presented here is that in our case the adsorption of the chitosan chains occurs on a pre-formed “soft” surface. In connection with this, it has been suggested that the adsorption of the large molecules such as proteins or long, semiflexible polyelectrolytes (as DNA), on “soft” membranes is not the same as on the solid “rigid” surfaces. The difference between the two cases is significant: Whereas a solid surface remains unchanged by the adsorption, the structure of a membrane or lipid bilayer is distorted (34-36). Therefore, the adsorption of the molecules on oppositely charged membrane results mainly of the electrostatic interaction between them, but also of membrane induced attractive or repulsive interactions (37). Moreover, the adsorption of highly charged polyelectrolytes onto fluid membranes, involves the movement of the surface charges as a response to the field created by the charged rods (21).

In spite of some complications, the presented experimental results and associated calculations indicate that the estimation of the fraction of condensed counterions of polyelectrolyte adsorbed on weakly charged soft phospholipidic surface can be performed. This conclusion is in line with existing theoretical predictions and experimental studies on the presence of condensed counterions and their participation in the electrical particles of hard or soft particles covered by layers of fully charged polyelectrolytes.

## CONLUSIONS

The present study is focused on investigation of the electrical properties of chitosans with different molecular weight and degree of acetylation. The polyelectrolytes are adsorbed onto nanoemulsion droplets at acidic conditions where the chitosan is fully charged. The experimental results revealed a similar electrokinetic behavior of the nanoemulsion droplets covered with chitosans with DA lower than 32%. The estimation of the fraction of free ions released upon the polyelectrolyte adsorption is approximately 10-12 % out of the total counterions retained near to the polyion upon the adsorption process. The presence of the condensed counterions cannot be registered by using electrokinetics. Thus, the measured electrophoretic mobility corresponds to the "effective" and not "real" electrokinetic charge of the droplets covered by polymer layer. Based on the assumption that only a fraction of condensed counterions occur after the adsorption process, it is possible to calculate the electrical polarizability of one polyelectrolyte molecule. The polarizability is a complex parameter that can allow to distinguish the electrical properties of the chitosans when the electrokinetic behavior of particles or emulsion droplets covered by polymer layer are very similar. The electrical polarizability is calculated using the Manning theory for polarization of layer from condensed counterions along the polyion chain.

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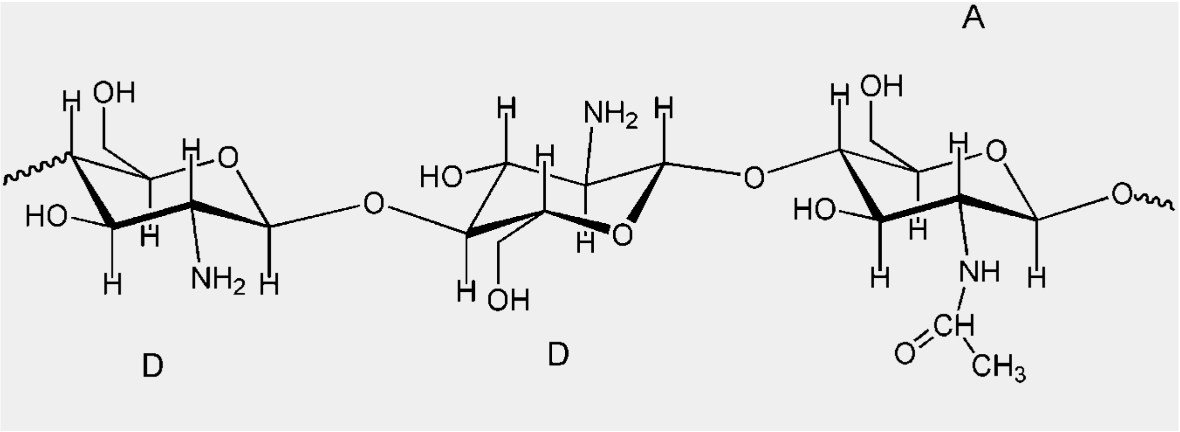
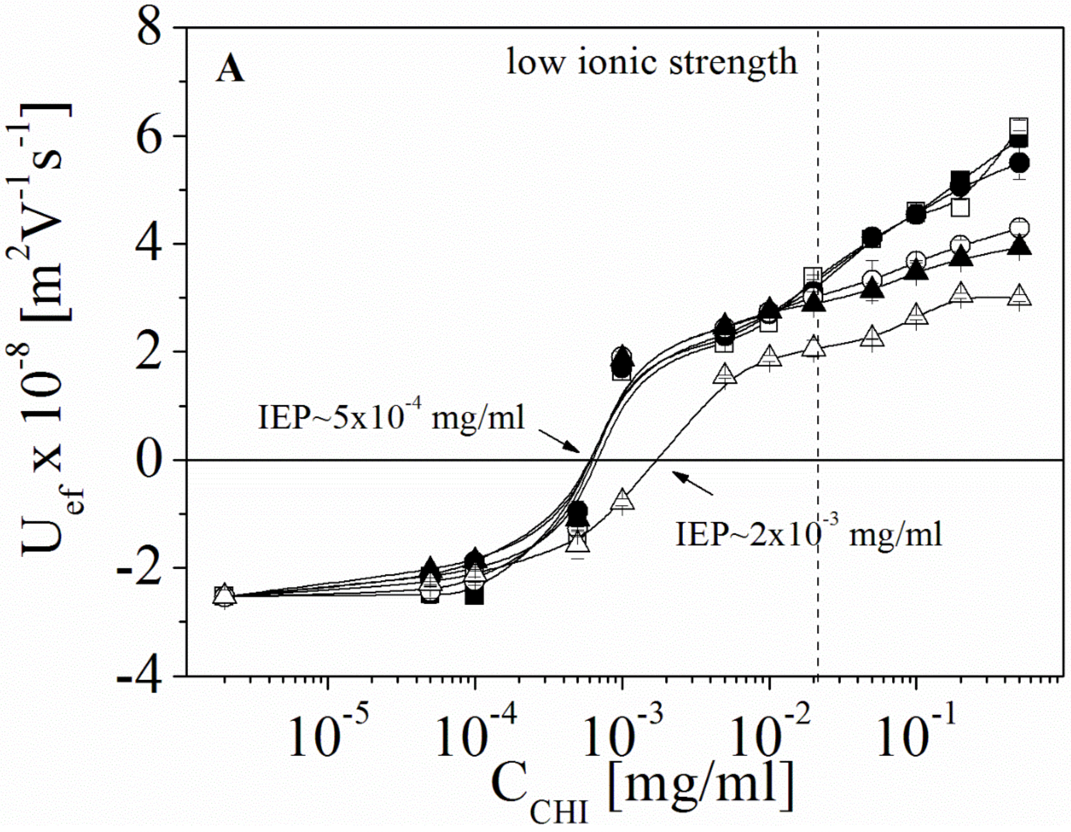
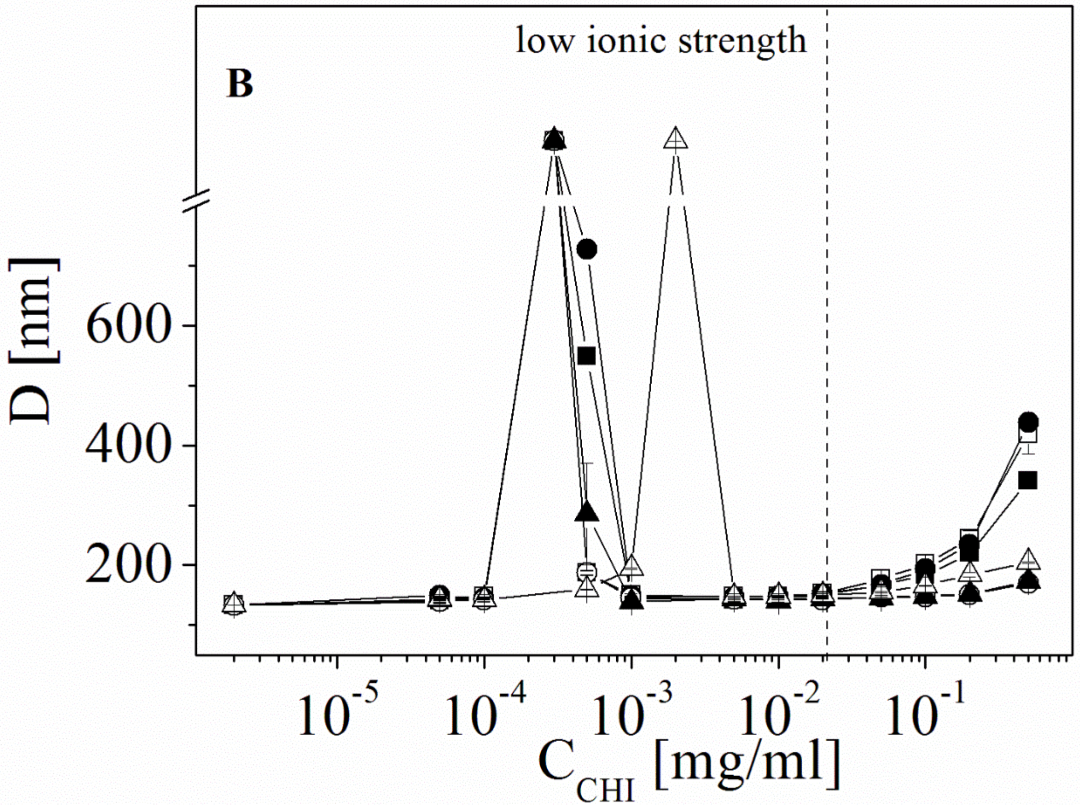
**Figure captions**



Scheme 1. Schematic chemical structure of chitosan: acetylated (A) and deacetylated (D) monomers.


A
low ionic strength
IEP~5x10$^{-4}$ mg/ml
IEP~2x10$^{-3}$ mg/ml
$U_{ef}$ x 10$^{-8}$ [m$^2$V$^{-1}$s$^{-1}$]
8
6
4
2
0
-2
-4
10$^{-5}$
10$^{-4}$
10$^{-3}$
10$^{-2}$
10$^{-1}$
$C_{CHI}$ [mg/ml]


B
low ionic strength
D [nm]
600
400
200
10$^{-5}$
10$^{-4}$
10$^{-3}$
10$^{-2}$
10$^{-1}$
$C_{CHI}$ [mg/ml]

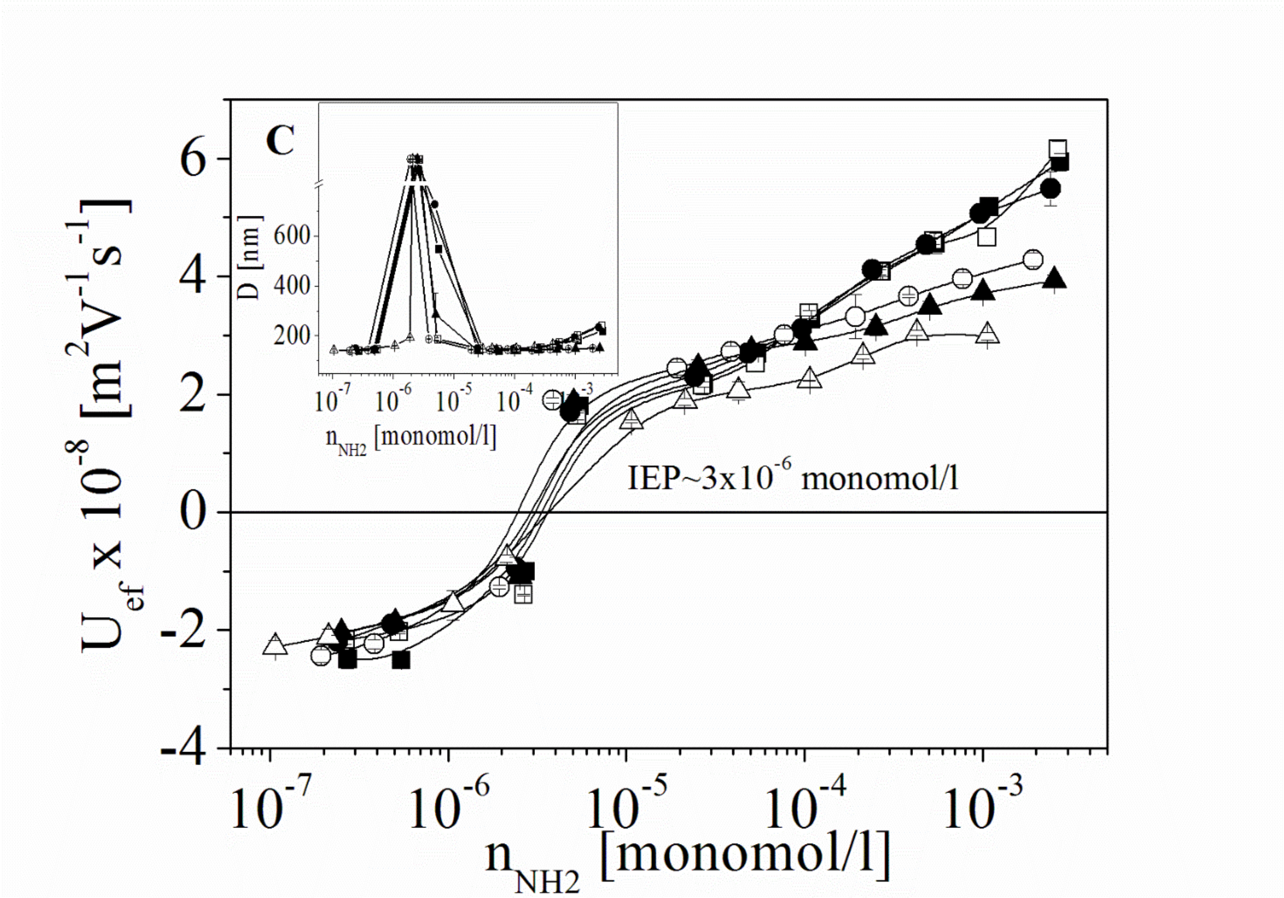


Fig. 1. Dependence of the electrophoretic mobility, $U_{ef}$, (A) and diameter (B) of nanoemulsion droplets at the presence of different concentration of chitosan: (■) CHI-A, (□) CHI-B, (●) CHI-C, (○), CHI-D, (▲) CHI-E, (△) CHI-F; (C). Dependence of the $U_{ef}$ and $D$ as function of $n(NH_2)$ in solution. The dot line indicates the range of the chitosan concentrations where the ionic strength of the suspensions is almost constant.

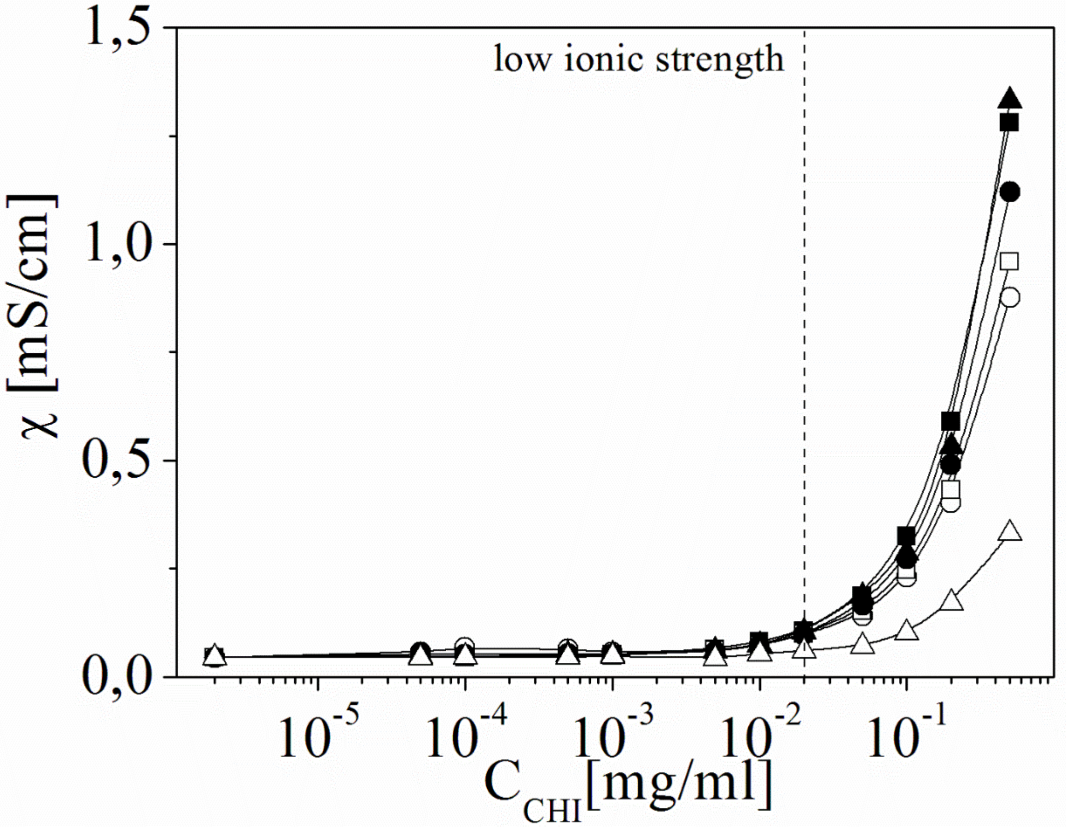


Fig. 2. Conductivity, $\chi$, of the dispersion at the presence of different concentration of chitosan: (■) CHI-A, (□) CHI-B, (●) SHI-C, (○), CHI-D, (▲) CHI-E, (△) CHI-F. The dotted line indicates the range of the chitosan concentrations where the ionic strength of the suspensions is almost constant.