Lysozyme – Alginate Nanocomplex: The Role of Alginate Composition

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ABSTRACT

In the present work we investigated the electrostatic self-assembly of nanocomplexes (NCXs) made of lysozyme (Lyz) and different alginates (Alg A Mw ~4kD and M/G ~1.42 or Alg B Mw ~7kD and M/G~5.00) to establish a relationship between the alginate structure and both the physical (size and zeta potential, optical density) and the thermodynamic properties of the systems. It was found that the alginate rich in mannuronic unit (Alg B) had a greater binding stoichiometry and lower affinity than Alg A, a fact that correlated with a more extended structure that in turn affected the capacity to form hydrogen bonds associated with the differences in charge distribution. Both alginates show higher affinity and temperature stability than poly-acrylic acid (PAA), showing the importance of the polyasaccharide structure in the NCXs formation. For the first time we showed that the M/G ratio plays a role on the affinity of interaction of alginate and lysozyme. Finally, these NCXs seem promising systems for biomedical application due to their *in vitro* biocompatibility and macromolecules retention capacity.

Keywords: Nanocomplex, alginate, lyzozyme, interactions.

1. INTRODUCTION

Lysozyme (Lyz) is a defense enzyme with antibacterial activity characterized by having a low molecular weight, high isoelectric point (p^0I = 11.16)[1]and the ability to bind some drugs[2]. These characteristics make it an ideal candidate macromolecule to interact with negatively charged polysaccharides so as to harness self-assembled nanostructured polyelectrolyte complexes (NCXs). Lyz-polysaccharide complexes have been studied mostly from the viewpoint of the enzymatic activity[3]. Alginates (Alg) are linear polysaccharide block copolymers of (1 → 4) β-D-mannuronic acid (M) and (1 → 4) α-L-guluronic acid (G). Their overall composition is commonly expressed as the molar M/G ratio. In the present work, we investigated the electrostatic self-assembly of nanoparticles made of Lyz and different alginates (Alg A Mw ~4kD and M/G ~1.42 or Alg B Mw ~7kD and M/G~5.00) to establish a relationship between the alginate block structure and the physical (size and zeta potential, optical density) and the thermodynamic properties of the systems. Also to understand better the electrostatic self-assembly process Polyacrylic acid (PAA) was used as a model of a classical polyelectrolyte. Together with this the practical utility of a system comprised by alginate and Lyz is tested using a high molecular weight alginate (Alg H; Mw ~198kD; M/G ~1.11), to see the cytotoxicity and ability to bind low molecular weight molecules (voriconazol and naproxene) and macromolecules like β-lactamase.

2. MATERIALS

2.1 Materials

The alginate samples were obtained from Danisco©, Lyz, PAA and other reagents were of high purity were purchased from Sigma-Aldrich.

Macromolecule	M_w (kDa)[a]	M/G
Lyz	14.3	---
Alg A	4	1.42
Alg B	7	5.00
Alg H	198	1.11
PAA	5	---

[a] M_w values according with manufacturer´s specifications

Table 1: Macromolecules characteristics

3. RESULTS AND DISCUSSION

3.1 NCXs Formation and Characterization

Figure 1 shows the optical density data at varying $[n^+]/[n^-]$ for the NCXs. Both Alg A and B can form NCXs in excess of Alg ($[n+]/[n-]<0.6$), where turbidity is observed but precipitation is not, while in excess of Lyz ($[n^+]/[n^-]>1.0$) only NCXs that comprise Alg B do not precipitate. It is also possible to appreciate that PAA generates higher optical density that both alginates at $[n^+]/[n^-]$ <1.0, but is interesting to note the inversion of the tendency at higher ratio, where the turbidity is higher for the Alg A. From this technique it is not possible to estimate which polymer has a higher affinity for Lyz. At lower ratio it seems that the interaction is driven by charge density of the anionic polyelectrolyte specially for PAA that has the greater negative charge density followed by both alginates[4]. This tendency is inverted at higher charge ratios and there is a second maxima at $[n^+]/[n^-]$ ~1.8 in the three systems. This is attributed to particle-particle interactions. To further clarify these results, Figure 2 shows the percentage of Lyz retention at varying ratio for the three systems.

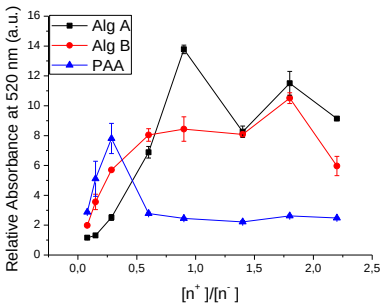

Figure 1, optical density (λ=520 nm) data at varying $[n^+]/[n^-]$ for the NCXs 1/3 dilution (pH 4.5 at 20°C)

At first glance, it is clear from Fig. 2 that at ratios lower than 1.0 Alg B and PAA are able to retain considerable more Lyz than do complexes at higher ratios ($[n^+]/[n^-]$ > 1). This is probably because the absorbance (Figure 1) is more related to the size of the complex than to the concentration of it[5]. Figure 2 the previous data, and allows to think that at lower ratios the low amount of Lyz, drives the arrangement of the polyanionic chain surrounding the protein and the formation of stable non aggregated NCXs of smaller size, hence of greater density and lower optical density. These NCXs can retain higher amounts of Lyz (Figure 2). At low ratios the greater charge density of PAA explains the greater retention efficiency of Lyz, but not the observed difference between both alginates.

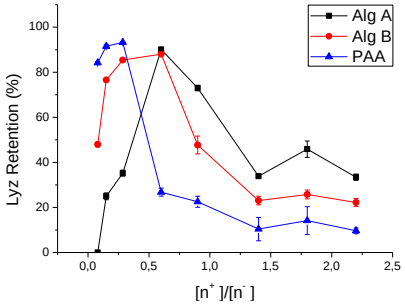

Figure 2, Lyz retention (%) data at varying $[n^+]/[n^-]$ for the NCXs (pH 4.5 at 20°C)

Alg A has the smaller distant between charges, hence the a greater charge density than Alg B[4], and yet Alg B retains greater amounts of Lyz. In the case of systems comprising alginate, the formation of the NCXs seems to be controlled by the differences in the Lyz retention efficiency and/or by the overall lower chain flexibility with respect to the highly flexible PAA. However at higher ratios it seems that Lyz associates to the polyanionic chain but with lower Lyz retention efficiency than at [n+]/[n-] <1.0. The lowest PAA retention could be affected by the Lyz repulsive behavior in solution[6] that could counteract the tendency of PAA to aggregate the protein in its surface, in line with previous reports on PAA-dye interactions[7].

Table 2 summarizes the characteristics of the NCXs comprising the two alginates at $[n^+]/[n^-]$ = 0.285. Notice that Alg B is able to retain ~3-fold more Lyz than Alg A, a result that is in line with the larger diameter size of NCXs formed by Alg B. This fact could be associated to the more extended structure of Alg B, which facilitates the greater Lyz association the consequence of the greater charge separation than in Alg A. Differences in chain flexibility between both alginates can be traced back to persistent leng (L_p) values documented for poly M, poly G and poly MG, that establishes the following sequence: GG > MM > MG[8-10] that reflects that the flexibility follows the inverse order GG < MM < MG. The fact that Alg B has a M/G ~ 5.0, may entail that it contains less amount of poly MG, and hence, it may be less flexible than Alg A. We are currently evaluating experimentally this suggestion. The lower polydispersity index (PdI) of Alg B NCXs, can be ascribed to a more controlled aggregation due to its lower flexibility, that also could explain why the aggregates at ratio higher than 1.0 retain more for Alg A than for Alg B. The lowest zeta potential (ζ) of the NCXs of Alg B is consistent with the fact that the greater Lyz retention for Alg B than for Alg A NCX leads to greater charge compensation and hence, greater neutralization.

System	[Lyz] (%)	Z-Ave (d.nm)	PdI	ζ Potential (mV)
Alg B	81.8±1.4	168±1	0.09±0.02	-27.9±2.3
Alg A	35.0±1.3	101±3	0.22±0.01	-42.9±1.7

Table 2, Physical characteristic of nanocomplexes formed by alginate and lysozyme ($[n^+]/[n^-]$ = 0.285; pH 4.5; 25° C)

In a different experiment, we also addressed the effect of temperature on the stability of the different NCXs at a ratio 0.285. Results in Figure 3 show that the decrease in temperature from 37 to 4°C leads invariably to an increase of the absorbance for all the NCXs. This could probably be due to a increase in size of the NCXs. It was interesting to notice that the NCXs comprising PAA were the more temperature sensitive in this respect, probably because its interaction is based on long range electrostatic forces an supported by hydrogen bond.[11] Additionally, the more densely charged Alg A shows slightly more temperature-sensitivity than does Alg B, thus reflecting subtle variation in the type of binding mechanisms driving the formation of the NCXs.

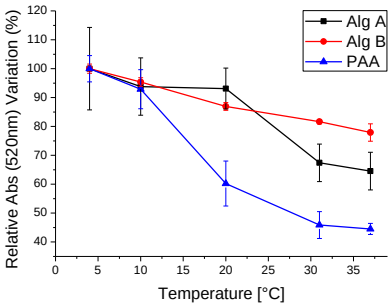

Figure 3, temperature dependence absorbance (λ=520nm) of the complex (pH 4.5)

It was also interesting to investigate the role of the Mw of alginate on the complexing behavior with lysozyme. To this end, other alginate samples of varying Mw were studied. Figure 4 represents the dependence between the molecular weight of the alginate and the percentage of Lyz retention for NCXs prepared at ratio 0.285. Notice that when for the alginates of M/G ratio ~1.0 (Alg of 32, 74 and 198 kD) a linear dependency is observed. In the same plot are represented Alg A and Alg B of low Mw and whose Lyz retention values that do not seem to fit into the linear trend observed for the rest of the samples, particularly for Alg B that in comparison shows an extremely high Lyz retention. These results reflect the importance of the alginate composition in determining its complexation behavior with Lyz.

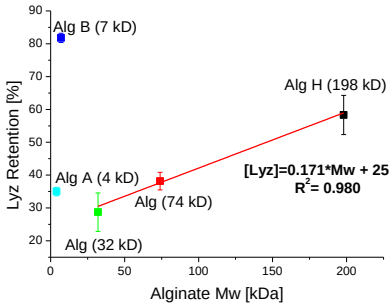

Figure 4: Lyz retention NCXs at ratio 0.285 for alginate of different Mw (pH 4.5 at 20°C)

3.2 Thermodynamic of Alg-Lyz Interaction

Table 4 shows the thermodynamic parameters obtained by ITC for the interaction of Alg-Lyz compared with PAA-Lyz. The ITC curves were perfectly fitted with one set of site model (results not shown). Notice that the self-assembly process is enthalpically driven in the three systems corresponding with an electrostatic interaction in which the negative entropy is due to the structural fixation of highly hydrated molecules[12]. Thus a higher Lyz retention may entail a greater entropy loss, as evidenced for PAA-Lyz system. A more flexible PAA chain and greater temperature sensitivity for PAA-Lyz (Figure 3) in which hydrophobic interactions seem not to be at play, may explain the greater loss of entropy in this system. Also notice that with decreasing enthalpy is compensated with a loss in entropy, this compensation is normally associated to a interaction with a preponderant participation of water molecule for the reorganization of the hydration shell of the macromolecules involved.[13] It is also possible to observe a relationship between increasing diameter and percentage of retained Lyz (Table 2) of the NCXs with the number of alginate monomers bound per Lyz (N). As previously mentioned PAA has high ability to aggregate molecules in its surface[7] and hence shows the highest N value. These results agree well with the hypothesis that a more extended structure of Alg B than that of Alg A also favors greater stoichiometry of Lyz. The difference in affinity found with the alginate retention (results not shown) and the sensitivity to the temperature are also reflected by ITC where Alg A has the higher affinity constant followed by Alg B and finally by PAA, thus reflecting the possible preponderant

role of hydrogen bonding capacity and also of the steric distribution of charge in the polyelectrolyte chain played in the affinity of interaction with Lyz.

System	N[c]	Log(K)	ΔH	TΔS	ΔG
PAA[a]	64.6±2.4	5.9±0.1	-46.4±3.3	-38.4± 3.4	-7.98
Alg B[b]	27.1±0.0	6.6±0.1	-23.0±0.6	-13.9±0.4	-9.08
Alg A[a]	14.5±0.0	6.9±0.1	-19.9±0.7	-10.5.3±0.1	-9.33

Table 4, Isothermal titration calorimetry data [a] Lyz (1000 μM) added to Alg A or PAA (500 μM);[b] Lyz (800 μM) added to Alg B 500 μM;[c] N its monomer of alginate per Lyz.;[d] ΔH, TΔS and ΔG in kcal/mol (acetate buffer 20mM pH4.5 at 25°C)

4. APPLICATION

NCX between the high molecular weight alginate (Alg H) and Lyz were prepared in essentially identical conditions as for the previously described systems but in presence of 40 mM NaCl, to favor the association between the two components. The characteristics of the NCXs were: Z-average diameter 274±10 nm; PDI =0.21±0.01; ζ = -52±3 mV; and the Lyz association efficacy of 77±1%. The cytotoxicity of the NCXs was studied by MTT test on MDCK-C7 cells and up to 567 μg/cm^2 the NCXs or the individual components does not decreased the cell viability. In other experiment, the activity of Lyz in the NCXs was found to be dramatically lower in comparison with the free enzyme at identical concentration (Figure 5), showing that Lyz is confined inside the NCXs, with low access to the substrate or/and the solvent. The experiment also reflected that the presence of the substrate does not destabilize the NCXs by sequestering Lyz. Probably the negative potential of the NCXs surface prevents any possible interactions with the negatively charged lipopolysaccharide substrate. The association efficiency for two low Mw drugs, voriconazole and naproxene showed that the Alg-Lyz NCx are unable to retain these drugs to almost any extent, in contrast, β-lactamase, a model high Mw protein was retained in 45±15%, thus being a promising result for therapeutic macromolecules delivery such as insulin, vaccines and growth factors.

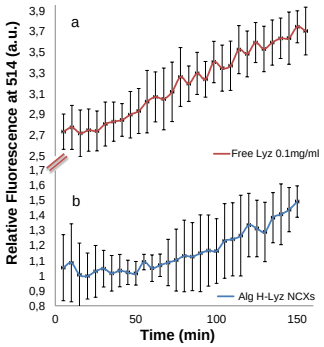

Figure 5, activity of 20% of (a) Alg04-Lyz NCXs (in 40mM NaCl) and (b)Lyz 0.1mg/ml (HEPES buffer pH 7.1 at 37 °C)

5. CONCLUSION

The interaction between Alg and Lyz has an important component of H-bonding formation. The differences in the complexation behavior can be attributed to Alg intrinsic flexibility that affects the capacity to accommodate Lyz molecules. Both Algs show higher affinity and temperature stability than the synthetic PAA, showing the importance of the sugar ring in the NCXs formation. For the first time we showed that the M/G ratio plays a role on the affinity of interaction of Alg and Lyz. This self-assembled nanocomplex looks as a promising nanostructured biomaterial for biomedical application due its *in vitro* biocompatibility and macromolecular retention capacity.

ACKNOWLEDGEMENT

Support from DFG (Germany; Project GRK 1549 International Research Training Group 'Molecular and Cellular GlycoSciences') and the Vredenburg Scolarhip from John Hopkins University (USA) to sponsor the visit of of SP to Müunster are both gratefully acknowledged. We are indebted to Susana M. Pereira for her valuable help in the cell culture studies.

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