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# On the role of alginate structure in complexing with lysozyme and application for enzyme delivery

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ABSTRACT

This study addresses the physicochemical properties and potential application of colloidal stable particles prepared by electrostatic self-assembly of alginate (Alg) and lysozyme (Lyz), here referred to as Alg-Lyz nanocomplexes (Alg-Lyz NCXs). The M/G ratio, molecular weight (Mw) or the addition of $Ca^{2+}$, all influence the capacity of Alg to associate Lyz as well as the size and zeta potential of Alg-Lyz NCXs. Systems comprising low-Mw Alg (Alg A Mw ~4000 g $mol^{-1}$ and M/G ~1.42 or Alg B Mw ~7000 g $mol^{-1}$ and M/G ~5.00) allow to glean further understanding of the influence of Alg block composition on the thermodynamic properties and on the underlying interactions between Alg and Lyz by ITC. Alg B is thought to exhibit a more extended structure leading to higher cross-linking with Lyz. Alg-Lyz NCXs, though retain the activity of Lyz, it is lower than that of the free enzyme. However, they are effective to co-associate a second enzyme, β-lactamase (BLA), and its activity is sensitive to the ionic strength.



## 1. Introduction

Polyelectrolyte-protein electrostatic complex have been the subject of extensive studies. Since the pioneering works of Bungerberg and Kruyt in 1929 (Bungenberg de Jong and Kruyt, 1929) on gum arabic-gelatin many subsequent studies have continued to address this and other systems. The more recent research in these systems makes use of advanced biophysical techniques, like small angle neutron scattering (SANS) (Morfin, Buhler, Cousin, Grillo, & Boué, 2011). Comprehensive reviews on this subject have also been published (Cooper, Dubin, Kayitmazer, & Turksen, 2005; Turgeon, Schmitt, & Sanchez, 2007). These systems are attractive from the theoretical physicochemical viewpoint, due to their significance in living organisms and due to their applications in the food, pharmaceutics, medicine, cosmetics, biotechnology and other fields (Mizrahy & Peer, 2012). Moreover, when the constituent polyelectrolytes are polysaccharides, of inherently high polydispersity and heterogeneous molecular composition, the major challenge is to establish structure-function relationships. These would enable a rational understanding and a better design and control of the properties of this type of systems.

Self-assembly is a physicochemical phenomenon whereby the components of a given system in solution (e.g. proteins, polysaccharides or small molecules) organize themselves into ordered macromolecular structures, thus reflecting the interplay of different non-covalent interaction forces (e.g. hydrogen bonding, electrostatic and hydrophobic interactions) (Grzybowski, Wilmer, Kim, Browne, & Bishop, 2009; Whitesides & Grzybowski, 2002). The type of interaction depends on the characteristics of the individual components such as electrical charge, molecular weight, solubility, hydrophobicity, electronegativity and flexibility, as well as environmental properties such as solvent type, temperature, pH and ionic strength (Grzybowski et al., 2009; Whitesides et al., 2002). Indeed, the properties of the individual interacting components encode the characteristics of self-assembled structures (Grzybowski et al., 2009; Whitesides et al., 2002).

In the present study we investigated the physicochemical aspects of the electrostatic self-assembling process of the negatively

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charged alginate (Alg) and positively charged lysozyme (Lyz) to form colloidal complexes that remain stable in liquid phase and that attain size dimensions in the sub-micron range, here regarded as nanocomplexes (NCXs). We particularly studied the role of the structural properties of alginate on the overall NCXs formation and physicochemical properties. The goal was to develop a novel type of self-assembled system for potential application in modulating the activity of enzymes.

Lyz (EC 3.2.1.17) is a ubiquitous enzyme and its structure is highly conserved (Sagermann & Matthews, 2002). It hydrolyses β(1 → 4) glycosidic linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan and between N-acetyl-D-glucosamine residues in chitin and chitosan. Lyz has a low molecular weight (14 300 g $mol^{-1}$), a high isoelectric point (pI = 11.16) (Kuehner et al., 1999) and shows the ability to bind some drugs (Qin, Su, & Liu, 2012). Lyz contains five α-helical regions and five regions containing β-sheets, linked by β-turns and random coils (Prilusky et al., 2011). In humans, Lyz is found in secretory cells, and in mononuclear and polymorphonuclear cells of the placenta, lung, lamina propria of the small intestine, lymph nodes and spleen (Klockars & Reitamo, 1975). Its main role is to provide defense particularly against Gram-positive bacteria (Prilusky et al., 2011). Alginate–protein complex systems have been applied in the development of drug delivery biocompatible carriers (Chen & Subirade, 2006). However, Lyz-alginate complexes have only been studied mostly in the context of Lyz enzymatic activity (Takahashi, Uchida, & Izumi, 2011), but not as colloidal nanocarriers of macromolecular drugs. Furthermore, no previous studies have systematically investigated the composition and molecular weight (Mw) of the Alg component and their impact on the molecular interactions governing the behavior of this system.

Alginates are polysaccharides found in certain bacteria (e.g. *Azotobacter vinelandii* and *Pseudomonas aeuruginosa*) and brown seaweeds, the latter used as a commercial source (Pawar & Edgar, 2012). Chemically, seaweed alginates are block-co-polymer polysaccharides comprising alternating blocks of homopolymeric (1→4)-linked poly–L–α-guluronate (poly-G), poly–D–β-mannuronate (poly-M) and hetero polymeric section of poly-MG. The overall composition is commonly expressed as the molar M/G ratio, which together with the Mw determines the physicochemical, functional and bioactive properties of Alg. The poly-G blocks provide gel-forming capacity, whereas the poly-M and poly-MG blocks determine flexibility. Standard grades of Alg form gels under acidic conditions. The $pK_a$ values for the M and G residues are 3.38 and 3.65, respectively. The gel-forming properties of Alg are determined by the proportion and length of poly-G blocks. The gelling capacity of Alg in the presence of specific bivalent cations ($Ca^{2+}$, $Ba^{2+}$ and $Sr^{2+}$ but not $Mg^{2+}$) reflects cooperative "egg box" coordination between poly-G blocks of more than six residues in length, in which every two carboxylate groups form a coordination complex with the cation, and positive or neutral patches in the Alg structure are generated leading to the formation of locally-ordered dimeric structures (Grant, Morris, Rees, Smith, & Thom, 1973). This phenomenon involves a two-step thermodynamic binding process involving an initial moderate release of heat when calcium associates with the carboxylate groups of G residues followed by a much more exothermic chain association, which is entropically favorable suggesting that the interaction is not simply electrostatically driven (Fang et al., 2007). Alginates have been investigated for example as biomaterials for encapsulation of bacterial or mammalian cells (DeVolder & Kong, 2012; Garate, Murua, Orive, Hernandez, & Pedraz, 2012). In the nanobiotechnology field Alg has been associated with chitosan to design novel materials for the transmucosal delivery of therapeutic peptides and proteins, nucleic acids, growth factors and vaccines (Azizi et al., 2010; Goycoolea, Lollo, Remuñán-López, Quaglia, & Alonso, 2009).

The thermodynamic aspects of self-assembly in polymeric systems are challenging to deal with, particularly for polydisperse materials (Alonso, Irigoyen, Iturri, Iarena, & Moya, 2013; Fang et al., 2007; Huang & Lapitsky, 2011; Ma, Lavertu, Winnik, & Buschmann, 2009). This lack of systematic understanding is more significant in systems comprising natural polymers (e.g. polysaccharides, DNA or gelatin) than synthetic ones. This is the consequence of their variable composition and high polydispersity associated with their biological source and extraction methods, among other factors. We therefore investigated the electrostatic self-assembly of lysozyme and different types of alginates varying in composition and Mw. We established a relationship between the structural characteristics of Alg and both the physical (size and ζ-potential) and thermodynamic properties of the resulting NCXs. We also investigated the potential use of these systems to bind and release the model bioactive protein β-lactamase.

## 2. Material and methods

### 2.1. Materials

Seaweed sodium alginate samples were obtained from Danisco© (Denmark), and their characteristics are summarized in Table 1. Lysozyme, β-lactamase and other high-purity reagents were purchased from Sigma–Aldrich (Munich, Germany). The lysozyme activity test was purchased from Molecular Probes (Eugene, OR, U.S.A.). Chromacef was purchased from Sopharmia Inc. MilliQ water was used for all experiments.

### 2.2. Methods

#### 2.2.1. Self-assembly of NCXs

The Alg solutions were mixed with Lyz solutions at room temperature after adjusting the pH of both components to 4.5 with HCl or NaOH as required. In a typical experiment, polyelectrolyte complexes were formed by adding 600 μL of Alg to 900 μL of lysozyme solution in a test tube using a calibrated Eppendorf micropipette with constant stirring. The concentration of both polyelectrolytes was calculated to achieve different molar charge mixing ratios ($[n^+]/[n^-]$) between cationic and anionic units, and the total concentration of charge was fixed in all mixtures to 2.0 mM. Lyz carries 11 positive charges at pH 4.5, so dividing the Mw by the number of charges, the equivalent charge Mw used for Lyz was 1302 g $mol^{-1}$. For Alg the Mw of one condensed monomer represents one charge (193 g $mol^{-1}$).

#### 2.2.2. Physical characterization

The particle size distribution was analyzed by dynamic light scattering with non-invasive back scattering (DLS-NIBS) at an angle

**Table 1**
Characteristics of the alginate and lysozyme samples.

| Macromolecule | *Mw* (g $mol^{-1}$)[a] | M/G[b] |
|---|---|---|
| Lyz | 14 300 | – |
| Alg A | 4000 | 1.42 |
| Alg B | 7000 | 5.00 |
| Alg 32 | 32 000 | 1.22 |
| Alg 74 | 74 000 | 1.26 |
| Alg H | 198 000 | 1.11 |

[a] Mw values according to manufacturers' specifications.
[b] M/G = molar ratio of mannuronic and gluluronic acids for alginates according to manufacturers' specifications.

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