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Marine sulfated polysaccharides as versatile polyelectrolytes for the development of drug delivery nanoplatforms: Complexation of ulvan with lysozyme

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ABSTRACT

Ulvan, a marine sulfated polysaccharide isolated from green algae, has been recently recognized as a natural biopolymer of biomedical interest. A series of lysozyme/ulvan complexes prepared under various charge ratios at physiological pH were studied. The resulting complexes were examined with light scattering techniques in order to characterize the size, the distribution and the ζ-potential of the nanocarriers, which were found to depend on the charge ratio employed. Increased complexation efficiency of lysozyme was observed for certain charge ratios, while ATR-FTIR data suggested that the protein structure after complexation was retained. Bacterial growth studies showed that lysozyme once complexed with ulvan not only retains its antibacterial activity against the Gram positive strain *Staphylococcus aureus*, but actually exhibits increased levels of activity. In this model study, the results highlight the potential of ulvan as a promising nanocarrier for positively charged bioactive molecules.

1. Introduction

Natural polymers have recently attracted attention as alternative materials in the biomedical sector due to their biocompatibility and biodegradability. The inherent unique properties of biopolymers are highly appreciated and are therefore widely exploited for biomedical applications [1, 2]. In particular, polysaccharides possessing an array of functionalities and exhibiting varying physicochemical properties and important biological activities are considered as attractive materials for the development of novel systems for bioapplications, such as drug delivery and tissue engineering [3, 4]. There is a constantly increasing number of natural carbohydrates that are utilized either in their native form or after processing, so as to deliver tailor made materials for specific applications [5]. Marine algae produce polysaccharides which are regarded as safer and non-immunogenic material, especially when compared to those of animal origin [6, 7]. Algal biomass, commonly harvested from marine eutrophicated coastal areas, can represent a renewable and sustainable source of biomaterials for exploitation.

Ulvan, a water soluble and complex anionic sulfated polysaccharide, represents a major constituent of the cell walls of green seaweeds of the order Ulvales (Chlorophyta) [8]. Its carbohydrate composition is characterized by great complexity and variability, being mainly composed of rhamnose, glucuronic and iduronic acids and xylose. The physical properties and pharmacological activity of ulvan have been systematically investigated [9–11] and a wide range of biological activities, including antibacterial [12, 13], antiviral [14, 15], anticoagulant [16], antioxidant [17, 18] and antihyperlipidemic [19], have been reported. However, the utilization of ulvan as a biomedical polymer has not yet been investigated in depth. Hitherto, ulvan-based scaffolds have been prepared for drug delivery or tissue engineering applications after crosslinking [20–23] or preparation of hybrid complex materials [24–27], 2D crosslinked membrane [28] or polymer-blended nanofibers [29, 30]. Moreover, ulvan has also been investigated as coating material for medical grade PVC to provide antibacterial activity to its surface [31].

The complexation of proteins with polyelectrolytes has been used in the development of functional and hybrid biomaterials for biomedical applications [32, 33]. Macromolecules carrying a relatively large number of functional groups charged under specific conditions are characterized as polyelectrolytes [34, 35]. Upon mixing them with oppositely charged macromolecules in solution, polyelectrolyte complexes are spontaneously formed without the need for any further covalent crosslinking. The major interactions governing the polyelectrolyte

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complex formation include the strong and reversible electrostatic and dipole–dipole interactions, as well as hydrogen and hydrophobic bonds. Polyelectrolyte complexes are generally considered as non-toxic, well-tolerated and biocompatible.

Recently, polyelectrolyte complexes of polysaccharides and proteins have been investigated [36] and applied in drug encapsulation and delivery, DNA-binding, enzyme immobilization, tissue engineering, etc. [34, 37–39]. Ulvan, as an anionic sulfated polysaccharide, is considered a polyelectrolyte negatively charged at neutral pH, which can potentially form a polyelectrolyte assembly with oppositely charged macromolecules. Lysozyme (LYZ) is a ubiquitous enzyme responsible for bacterial cell wall lysis, synthesized and secreted by glandular serous cells, surface epithelial cells and macrophages, having a positive charge at neutral pH. As such, LYZ could be used as a model protein for the formation of lysozyme/ulvan (L/U) polyelectrolyte complexes. Therefore, in this model study, the formation, characterization and evaluation of L/U complexes is reported. The negatively charged ulvan interacts with the positively charged LYZ affording complexes that were characterized by dynamic light scattering (DLS), ζ-potential and FTIR spectroscopy. The morphology of the polyelectrolyte complexes was also examined, while their antibacterial activity was evaluated against the Gram positive bacterial strain *Staphylococcus aureus*.

2. Materials and methods

2.1. Materials

Specimens of the green alga *Ulva rigida*, collected in Chalcis in the island of Euboea, Greece, were used for the isolation of ulvan. The extraction and chemical analysis of ulvan was carried out as previously described [30]. Subsequently, the isolated polysaccharide was deproteinized by proteinase digestion with proteinase K [9]. The crude ulvan was solubilized in 0.5% SDS, 25 mM Tris-HCl, pH 7.8, 1 mM $CaCl_2$ buffer at a final concentration equal to 40 $mg \cdot mL^{-1}$. Proteinase K was added to a final concentration of 100 $\mu g \cdot mL^{-1}$ and the solution was incubated at 37 °C for 1 h. Inactivation of the enzyme was succeeded with the addition of EDTA and heating of the solution at 70 °C for 10 min. The assay mixture was dialyzed against ultrapure water until complete removal of the buffer components, the resulting solution was further precipitated with EtOH (70% final concentration) and the obtained precipitate was lyophilized.

2.2. Chemicals

Tris(hydroxymethyl)aminomethane and $CaCl_2$ were obtained from Mallinckrodt (Dublin, Ireland), EDTA from Serva (Heidelberg, Germany), NaCl from Merck (Kenilworth, N.J. USA), agar bacteriological from Scharlau (Barcelona, Spain), yeast extract from Fluka (Bucharest, Romania), tryptone from BioChemica (Darmstadt, Germany), while chicken egg white lysozyme (~100,000 U/mg), lauryl sulfate (SDS) and MES (2-[N-Morpholino]ethanesulfonic acid) were purchased from Sigma (Darmstadt, Germany).

2.3. Characterization of ulvan

High pressure size exclusion chromatography (SEC) was used to determine the molecular weight distribution of ulvan. The analysis was performed on an Agilent 1100 HPLC Series system, composed of a liquid chromatography pump and an RI detector equipped with a BIO BASIC SEC-1000 (Thermo Scientific, 300 mm × 7.8 mm) column. Ultrapure water was used as the mobile phase at a flow rate of 1 mL/min [29]. The lyophilized ulvan samples were dissolved in the mobile phase solution (1 $mg \cdot mL^{-1}$), heated for 20 min at 70 °C and filtered through 0.45 μm membrane filter before analysis. Calibration was performed with pullulan standards with weight-average molecular weights in the range of 10,000 to 805,000 $g \cdot mol^{-1}$.

pHmetric titration of the proteinase K-treated ulvan was conducted on its acidic form (produced after initial dialysis against 0.2 M HCl and afterwards against ultrapure water before final freeze-drying) [40]. The carboxylate content was then determined by dissolving ulvan in 0.1 M $NaNO_3$ (417 $\mu g \cdot mL^{-1}$) and titration with 0.1 M NaOH.

2.4. Preparation of L/U complexes and determination of LYZ complexation efficiency

Initially, different concentrations of ulvan solutions in 10 mM Tris buffer, pH 7.4 were tested for their physicochemical characteristics and stability. It was found that at a concentration equal to 2.5 $mg \cdot mL^{-1}$ buffer, ulvan exhibits its most condensed conformation. Therefore, the performed complexation experiments were conducted at this concentration.

Afterwards, stock solutions of LYZ (1.24 $mg \cdot mL^{-1}$) and ulvan (2.5 $mg \cdot mL^{-1}$) in 10 mM Tris buffer, pH 7.4 were prepared. Complexes at different L/U charge (+/−) ratios were prepared by adding different amounts of the LYZ stock solution to a constant volume of the ulvan stock solution under rigorous stirring [41, 42]. LYZ was allowed to interact with ulvan for 20 min. Finally, appropriate volumes of buffer solution were added so as to reach a constant final volume for all prepared solutions. The as-prepared complexes were left for equilibration overnight. The above-described preparation protocol was used as it has been already proven as non-affective to the protein conformation and/or leading to aggregation [43, 44]. Afterwards, the formed L/U nanocomplexes were separated from the non-associated LYZ in the aqueous phase by centrifugation at 7000 RCF for 20 min. The obtained sediments were washed with ddH_2O and dried under vacuum. The supernatant was collected and the contained free LYZ was quantified by UV–Vis spectroscopy by monitoring LYZ absorbance at 281 nm on a PerkinElmer Lambda 40 spetrophotometer. Calculations were based on a standard calibration curve of LYZ ($R^2 = 0.9986$).

LYZ complexation efficiency was calculated for complexes obtained at different L/U molar ratios, according to the equation [32]:

$$\text{Complexation efficiency } (\%) = \frac{\text{Total amount of LYZ} - \text{Free LYZ}}{\text{Total amount of LYZ}} \times 100 \quad (1)$$

All samples were measured at least in triplicate.

2.5. Characterization of L/U complexes

2.5.1. Determination of mean particle size and ζ-potential

Dynamic and Electrophoretic Light Scattering (DLS and ELS) techniques were used for the physicochemical characterization of the prepared complexes. Buffer and sample solutions were filtered through 0.20 μm nylon Titan2 syringe filters (SUN-SRi) to remove any particles. The mean hydrodynamic diameter (D*h*), polydispersity index (PDI) and ζ-potential of the particles were used for the characterization of the ulvan dispersions immediately after preparation ($t = 0$ days), as well as for monitoring their physical stability over time ($t = 25$ days). Additionally, measurements were performed at the L/U complexes obtained after centrifugation, washing with ddH_2O and redispersion in buffer. Measurements were performed at a detection angle of 90° and at 25 °C in a photon correlation spectrometer (Zetasizer 3000 HSA, Malvern, UK) and analyzed by CONTIN method (MALVERN software). For each dispersion, three sets of ten light scattering measurements were collected, and the results were averaged. The ζ-potential of the as-prepared particles was measured using Zetasizer 3000HAS (Malvern Instruments, Malvern, UK). Actually, 1 mL of dispersion was diluted in 2 mL of HPLC-grade water and ζ-potential was measured at room temperature at 633 nm. The ζ- potential values were calculated from