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Polarization Modulation Reflection-Absorption Spectroscopy applied in ultrathin films of algal biomacromolecules may explain the mechanism associated to the removal of pollutant metals

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ABSTRACT

The current work investigates the action of extracts of exopolysaccharides and proteins (EPS) from the microalgae *Cryptomonas tetrapirenoidosa* immobilized on Langmuir-Blodgett films (LB) of the lipid dioctadecyldimethylammonium bromide (DODAB). The main objective of this paper was to evaluate the changes provided in the structure of the lipid-algal biomolecules composite film by Polarization Modulation Infrared Reflection-Absorption Spectroscopy (PM-IRRAS) when these supramolecular structures are immersed in solutions with metallic ions of environmental interest. EPS was incorporated at DODAB monolayer as analyzed with surface pressure-area isotherms, PM-IRRAS and Brewster Angle Microscopy. The mixed films were transferred to solid supports as LB films and amide bands related to proteins present in the extract were analyzed since they are highly sensitive to molecular conformation. The films were put in contact with selected ions of environmental interest, such as lead, cooper, iron, and mercury, and changes in the protein structure were investigated with PM-IRRAS. The results showed a specific interaction of each ion with the LB films, which may be promising for building biosensors with specificity for metallic ions.

1. Introduction

Extracts containing extracellular polysaccharides and proteins (EPS) are organic extracellular materials that are excreted by some microorganisms such as bacteria, fungi and algae. Algal EPS have been studied by different areas such as food [1] and pharmaceutical industries [2,3]. Some algal species can produce EPS with the property of adsorbing metals dissolved in water, which make them useful to construct devices able to retain metals as a method of depollution of aqueous environments [4–6]. The Langmuir technique has been employed as a strategy to preserve the structure of some biomolecules as proteins and polysaccharides [7]. Based on these facts, possible interactions between algal polysaccharides and lipidic interfaces can be useful to maintain the stability and conservation of the involved biomolecules.

Since many aquatic environments have been polluted by metallic ions, it is interesting to find new techniques able to identify at the molecular level how EPS interact with such ions. Knowledge that EPS are able to remove metals ions is well stablished [8]. Some techniques such as Energy-dispersive X-ray Spectroscopy and x-ray photoelectron spectroscopy have been useful to measure the presence of metals in several samples [9,10], but details on how biomacromolecules change their conformation upon metal ions contact can be better understood using vibrational spectroscopy able to investigate changes in the surface of the sample [11,12]. For that, it is relevant the construction of structures organized at the monomolecular scale such as Langmuir and Langmuir-Blodgett (LB) films. Therefore, the present study was carried out taking some advantages of this technique. Previous papers from our group have proved that EPS adsorbs on DODAB Langmuir monolayers [13], and can interact with silver ions [14]. However, little is now about how ions can interact with lipids and the biomacromolecules and if these properties are extensive to other ions. Since the lipids are probably blinded by the charges of the biomolecules, the interaction of the lipid-EPS structure with ions may be driven by ion-dipole or ion–ion interactions. However, little is known about how these interactions affect the conformation of polysaccharides and proteins since potential binding sites are not yet completely known. As a result, in order to exploit the potential of algal materials to adsorb other metal ions of environmental importance, this paper was carried out using algal EPS from *Cryptomonas tetrapyrenoidosa* and the lipid DODAB. The

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algal EPS and the lipid were co-immobilized as a mixed ultrathin film using the Langmuir-Blodgett technique in order to provide favorable conditions for the construction of a device able to detect and to remove pollutant metals from water environments.

2. Materials and methods

Water employed in this work was purified from Milli-Q® system from Millipore (pH of 6.2 and resistivity of 18.2 MΩ cm).

2.1. Obtaining the EPS from Cryptomonas tetrapyrenoidosa

The *Cryptomonas tetrapyrenoidosa* inoculum was obtained from the culture collection of the Microalgal Laboratory of Federal University of São Carlos (São Carlos, Brazil) and registered as strain BB021. Bath cultures were grown axenically in WC medium [15] under controlled conditions. After 40 days, the culture was finished and the EPS was obtained as described previously [8]. The *Cryptomonas tetrapirenoidosa* extract was composed of two fractions [16]: Fraction 1 and Fraction 2. Fraction 1, represents only 48% of Fraction 2, and is a complex heteropolysaccharide composed mainly of 24,3% of fucose, *N*-acetyl glucosamine, 15,4% of mannose, 13.7% of galactose and also small amounts of rhamnose (9,0%), xylose (4,7%), glucose (3,5%), glucuronic acid (4,1%) and *N*-acetyl galactosamine (8,6%). Fraction 2 was quantitatively more significant than Fraction 1 and showed an uncommon composition for phytoplanktonic EPS, with 47% of glucuronic acid and 36% of galactose, besides smaller proportions of fucose (8,6%), rhamnose (0,8%), xylose (0,4%), mannose (0,8%), glucose (0,5%), galacturonic acid (4.5%), N acetyl-glucosamine (1,1%) and galactosamine (0,27%). Proteins accounted for 9.8% of the EPS dry weight for Fraction1 and 4% for Fraction 2. EPS present net negative charge at pH around 6.0 and is highly soluble in pure water.

2.2. The construction of Langmuir and Langmuir-Blodgett films

The interactions of EPS with Langmuir films were investigated using the positively charged lipid dioctadecyldimethylamonium bromide (DODAB, Sigma Aldrich). The lipid was chosen because EPS are composed of proteins and heteropolysaccharides with acidic sugars, which gives the EPS a net negative charge at the pH of the purified water employed in this word (6.2).

The construction of the mixed EPS/DODAB LB film is described with detail in [13,14]. Briefly, a solution of DODAB in chloroform (0.5 mg/mL) was spread on the air-water interface of an aqueous solution of EPS (0.5 g/L) contained in a Langmuir trough (KSV-model mini). After solvent evaporation, the film formed at interface was compressed using mobile barriers. The floating monolayer was characterized using surface pressure-area isotherms, Polarization Modulation Reflection-Absorption Spectroscopy (PM-IRRAS) (KSV-Nima) at a fixed incidence angle to the normal of 80°. At this angle, the intensity is maximum and the noise level is minimum. The setup generates p- and s- polarization reflectivity beams (R_p and R_s respectively), where p and s corresponds to the fraction of radiation polarized parallel and perpendicular to the plane of incidence respectively. From the relation $(R_p - R_s)/(R_p + R_s)$, a spectrum is obtained, with surface-specific absorption bands [17]. Background correction for the floating monolayers was taken using a clean air-water interface, and for the solid supports, we used the glass solid substrates without the film cover as reference. Selected parts of the spectra were selected and baseline was employed using Origin® program (version 8.0).

Brewster Angle Microscopy (BAM-model mini KSV) was employed to analyze the floating monolayers. A p-polarized beam is reflected on the air-water interface at the Brewster angle, and a detector is able to catch the reflecting light due to changes in the optical parameters of the surface because of the presence of the monolayer.

In order to form the LB films, the mixed DODAB-EPS monolayers were previously compressed until the surface pressure of 30 mN/m. Glass or solid substrates, previously cleaned with KOH dissolved in ethanol, were immersed into the aqueous subphase before the lipid spreading and were removed vertically from the subphase at a rate of 10 mm/s and a constant surface pressure of 30 mN/m. The quality of the transfer of the film from the air-water interface to the solid support was attested by the transfer ratio, which is the ratio between the trough area swept by the barriers to keep the surface pressure constant and the area of the solid support that was in contact with the air-water interface during the procedure. For a successful transfer, unity as transfer ratio is desirable. Sequential immersions and removals of the support could be carried out until 7 deposited layers as a Y-type LB film. X- or Z-type LB films are not possible for DODAB-EPS mixed films since multilayer deposition does not result in positive transfer ratios. It is important to mention that control analyses of the LB films inserted in pure water were carried out and no variation in the vibrational spectra was observed after the removal of the films. Also, control experiments were carried out in order to check the influence of water retained in the transferred film. The vibrational spectra of wet films were obtained and compared to the LB films after they were sufficiently dried until water molecules retained on the surface of the LB film structure were low enough in order to not influence the spectra of the other components of the films.

2.3. Identifying metal-film interactions

The metals ions of environmental importance that were tested were cupper (Cu^{2+}), mercury (Hg^{2+}), iron (Fe^{3+}) and lead (Pb^{2+}), obtained from dissolving in water $Cu(NO)_2{\cdot}3H_2O$, $Hg(NO_3)_2$, and $Pb(NO_3)_2$ purchased from Sigma-Aldrich (purity higher than 99%). The mixed LB films were characterized with Polarization Modulation Reflection-Absorption Spectroscopy (PM-IRRAS) (KSV-Nima) at a fixed incidence angle to the normal of 80°. The films were then immersed in solutions with different metals during 30 min and analyzed again with PM-IRRAS. Ion concentrations of 0.001 M and 0.1 M were essayed.

3. Results and discussion

Fig. 1 (panel A) shows the surface pressure-area isotherms for DODAB and EPS-DODAB. DODAB presents a typical curve [9] featuring a liquid-like nature during the compression, going to the gaseous state at high molecular areas, where the surface pressure is close to zero, until collapse, where the monolayer structure is destroyed. With EPS the isotherm is shifted to lower molecular areas due to an effect of condensation of the lipid monolayer [14]. EPS contains negatively charged groups at the pH of 6.2 and DODAB is positively charged. Ionic electrostatic interactions may minimize lateral repulsion and stabilize the lipid monolayer. The inset in the Figure shows the BAM image for DODAB-EPS at the surface pressure of LB transfer (30 mN/m). It is observed a homogeneous pattern, indicating that EPS does not provoke relevant aggregation in the floating monolayer prior to the LB transfer.

Panel B for Fig. 1 shows the PM-IRRAS spectra for mixed EPS-DODAB monolayers. DODAB does not present relevant bands in this region, so that it is an interesting region to confirm the presence of EPS at the interface. Generally, infrared spectra obtained for polysaccharides show a complex region of absorption between 1000 and 1300 cm^{-1}, which is assigned to vibrations of glucosydic ring, and attributed do C—O—C stretches in polysaccharides. The small bands centered at 1523 and 1570 cm^{-1} may