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Chondroitin sulfate interacts mainly with headgroups in phospholipid monolayers

Lucinéia F. Ceridório[a,*], Luciano Caseli[a], Osvaldo N. Oliveira Jr.[b]

[a] Institute of Environmental, Chemical and Pharmaceutical Sciences, Federal University of São Paulo, UNIFESP, Diadema, SP, Brazil
[b] Institute of Physics of São Carlos, University of São Paulo, USP, São Carlos, SP, Brazil

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ABSTRACT

Sulfated glycosaminoglycans are precursors of the extracellular matrix used to treat diseases related to blood clotting and degenerative joint diseases. These medical applications have been well established, but the mode of action at the molecular level, which depends on the interaction with cell membranes, is not known in detail. In this study, we investigated the interaction between chondroitin sulfate (CS) and phospholipid monolayers that mimic cell membranes. From surface pressure isotherms and polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS), CS was found to interact mainly with the polar groups of dipalmitoyl phosphatidylcholine (DPPC) and dipalmitoyl phosphatidylglycerol (DPPG), with negligible penetration into the hydrophobic tails and only small changes in monolayer elasticity for the packing corresponding to a real cell membrane. The changes in surface pressure and surface potential isotherms depended on CS concentration and on the time allowed for its adsorption onto the monolayer, which points to a dynamic adsorption-desorption process. The charge of the phospholipid was also relevant, since CS induced order into DPPC monolayers while the opposite occurred for DPPG, according to the PM-IRRAS spectra. In summary, interaction with polar groups is responsible for the CS effects on model cell membranes.

1. Introduction

Glycosaminoglycans (GAGs) are long unbranched anionic polysaccharides found in the extracellular matrix at the cell surface combined with proteins and forming a structure known as proteoglycan (PG) [1,2]. Its chemical diversity arises from the relative quantity of sulfate groups and their position, in addition to the molecular weight. They comprise hyaluronanic acid, chondroitin sulfate (CS), heparan sulfate, keratan sulfate, and dermatan sulfate. These molecules differ in the kinds of the charged groups, the charge density and positions. Chondroitin sulfate, in particular, contains alternated sulfated units of *N*-acetyl-galactosamine (*o*-sulphated on C-6 or/and C-4) and glucuronic acid (*O*-sulphated on C-3 or C-2). Among the GAGS, CS is particularly important because this kind of molecule participates in various biological functions in the central nervous system, wound repair, cell division, differentiation, adhesion, migration and response to growth factors [3–5]. With these several functions, CS is of interest for pharmaceutical sciences and used as therapeutic agents in atherosclerosis/thrombosis [6–9], osteoarthritis and osteoarthrosis [10–13] and infections [14–16]. These potential physiological and pathological roles are likely dependent on the interaction with cell membranes, whose framework is made of a phospholipid bilayer that also contains embedded proteins and polysaccharides [17]. These phospholipids normally possess a double alkyl chain and typical headgroups are choline, ethanolamine, serine, glycerol, glucose and inositol [18].

Additionally, many researchers have been studying CS due to its role in cartilage and bone tissues and its interactions with growth factors and other proteins. Therefore, the characterization of the CS behavior with cell membrane molecules at molecular level is necessary. Thus, in this work, we investigated the interaction of CS with Langmuir monolayers of phospholipids with the same chain length and saturation level, but with different polar heads: dipalmitoyl phosphatidylcholine (DPPC), a major lipid found in outer leaflet of the membrane [18], and dipalmitoyl phosphatidylglycerol (DPPG), a negatively charged phospholipid. This methodology was chosen because Langmuir monolayers mimic half a membrane, with ease control over lateral packing and composition [19,20]. Furthermore, even though extensive studies exist for CS in the medical literature, little is known about molecular-level interactions in such uses [21–23].

* Corresponding author.
E-mail addresses: lceridorio@yahoo.com, lceridorio@unifesp.br (L.F. Ceridório).

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2. Materials and methods

Dipalmitoyl phosphatidylcholine (DPPC) and dipalmitoyl phosphatidylglycerol (DPPG) sodium salt were purchased from Avanti Polar Lipids. Chondroitin sulfate sodium salt (CS) from shark cartilage (CAS number 9007-28-7) acquired from Sigma Aldrich is a mixture of derivatives of chondroitin, mainly Chondroitin 4-Sulfate and Chondroitin 6-Sulfate. For CS from shark cartilage ca. 70% of 6-*O* sulfatation of *N*-acetylGalactosamine and 2-*O* sulfatation of glucuronic acid were found [24]. CS was dissolved in water to obtain a concentration of 10 mg/mL and then used as stock solution. DPPC was dissolved in chloroform while DPPG was dissolved in a mixture of chloroform/methanol (3:1 in volume) at a concentration of 0.5 mg mL^{-1}. Chloroform and methanol of analytical grade were purchased from Merck. Ultrapure water from a Milli-Q Plus system with resistivity of 18.2 MΩ cm and pH 6.2 was used as subphase for the Langmuir monolayers for all CS solutions. It is important to emphasize that pure water was employed as subphase instead of phosphate buffer because in previous experiments we observed that the salts present in the buffer affected the interfacial properties of CS-lipid monolayer, especially for the surface potential measurements, leading us to inconclusive results. Because of that, we avoided the use of buffer in the aqueous subphase and pure water was employed for all cases. The structures of the phospholipids and CS are shown in Fig. 1.

Surface activity of CS aqueous solutions was monitored with a tensiometer. Adsorption kinetics of CS in the presence of DPPC and DPPG was carried out for four solutions of CS; two of which were relatively highly concentrated (1.8 and 0.6 mg/mL), while the other two were diluted (1.8×10^{-3} and 0.6×10^{-3} mg/mL). The measurements were carried out in a Kibron tensiometer for the most concentrated solutions and in a KSV mini-though for the most diluted solutions. Since the results indicated no surface tension for CS aqueous solutions below 2.0 mg/mL, the concentrations of 6×10^{-3} mg/mL and 18×10^{-3} mg/mL were chosen for measurements in Langmuir monolayers.

The Langmuir monolayers were obtained with a computer controlled KSV mini-though (KSV-Instruments, Finland) housed in a class 10,000 clean room. Phospholipid solutions were carefully spread on the aqueous surface with a Hamilton microsyringe and left for 15 min for solvent evaporation before the measurements. Then the monolayer was symmetrically compressed using movable barriers at the rate of 3.65 A^2/mol min. The surface pressure and surface potential were measured during compression using a Wilhelmy plate made of filter paper connected to a balance and a vibrating plate with a Kelvin probe, respectively. For mixed monolayers, the subphase was prepared with CS aqueous solutions in 250 mL of ultrapure water at 6×10^{-3} and 18×10^{-3} mg/mL. In the presence of CS, π-A isotherms were measured after 1 h or 4 h of phospholipid spreading to ensure the adsorption *n* of CS on the monolayer. The mean molecular area was obtained without considering the CS molecules adsorbed at the monolayer. Measurements were repeated at least three times, showing reproducibility, with standard deviation of the points of the isotherms lower than 2%. In order to verify reversibility of the mixed monolayers, hysteresis experiments were conducted by compressing and subsequently decompressing the monolayer ten times. CS/phospholipid mixed monolayers were cycled to a maximum pressure of 40 mN/m and expanded at high areas per molecule, $\pi = 0$ mN/m. The limit of compression should be below collapse to avoid irreversible changes with formation of lipid aggregates and multilayers.

The morphology of DPPC and DPPG monolayers on subphases containing CS was investigated by Brewster Angle Microscopy (BAM) using an ULTRABAM instruments (Acccurion GMbH Gottingen, Germany) equipped with a 50 mW laser emitting *p*-polarized light at 658 nm, a 10× magnification objective polarizer, an analyzer and a CCD camera. The spatial resolution was 2 μm.

The molecular-level interactions in Langmuir monolayers were investigated with polarization modulation infrared reflection-absorption spectroscopy (PM-IRRAS) with a KSV instrument. The incidence angle to the normal was 80°, where intensity is maximum and noise level is lowest. The setup allows one to measure *p* and *s* polarization reflectivities (Rp and Rs respectively) where *p* and *s* correspond to the fraction of radiation polarized parallel and perpendicular to the plane of incidence, respectively. From the relation $(Rp - Rs)/(Rp + Rs)$, a spectrum is obtained, with surface specific absorption bands. For a PM-IRRAS spectrum, a negative band indicates a transition moment oriented preferentially perpendicular to the surface, whereas a positive reflection absorption band indicates a transition moment oriented preferentially along the surface plane. The temperature in all experiments was kept at 20 ± 1 °C.

3. Results and discussion

The surface tension measurements of CS aqueous solutions showed no relevant decrease in water surface tension for concentrations below 2.0 mg/mL, which reveal a negligible surface activity for CS. CS was then inserted below phospholipid monolayers at 30 mN/m in several points of injection, and the surface pressure was monitored along time as shown in Fig. 2. To minimize problems with lateral diffusion this experiments were carried out in miniaturized wells with aqueous subphase of few milliliters. For neat phospholipids the surface pressure fall due to aggregation considering that this surface pressure is attained out-of-equilibrium. Particularly, the decrease of surface pressure for DPPG from 30 mN/m to 20 mN/m is related to the fact that this lipid is in a condition above its equilibrium surface pressure, which is a few mN/m. Ordered monolayers are, consequently, generally metastable with highs surface pressures attained because molecular rearrangements is not fast enough to follow the conditions established to attain initially the high values of surface pressure. For mixed monolayers, it is observed a considerable decrease in surface pressure for DPPC within 3 h, thus pointing to either removal of lipids from the interface by CS or condensation of the DPPC monolayer. In contrast, the surface pressure increased by 2 mN/m for the DPPG/CS monolayer for the same interval. This opposite effect suggests that the interaction with CS depends on the charge of the phospholipid head group. Investigation on other initial surface pressures was carried out and we observed or constant values of surface pressure either a low decrease of the surface pressure upon injection of CS. Furthermore, as the surface pressure of 30 mN/m is those related to natural membranes, we focused the discussion on this value.

The incorporation of CS into DPPC and DPPG monolayers affected the surface pressure-area isotherms, depending on both the CS concentration and the adsorption times, as shown in Fig. 3. The adsorption time corresponds to the time elapsed between the spreading of the lipid on the CS solutions and the beginning of the compression. For DPPC, CS had almost no effect at large areas per molecule and the liquid-expanded to liquid-condensed phase transition was little affected. At high pressures, there was little effect with two exceptions: (i) large condensation for 1 h waiting time, especially for the highest concentration (18×10^{-3} mg/mL); (ii) higher collapse pressure for 4 h with 6×10^{-3} mg/mL. In contrast, for DPPG an expansion on the monolayer was observed at large areas (low surface pressures) in all cases. No significant effect occurred at high pressures, with the exception of a condensation for 1 h and 18×10^{-3} mg/mL. It is important to emphasize that for small concentrations of CS, at an adsorption controlled by diffusion, CS will take more time than for the higher concentrations. As