Colloids and Surfaces B: Biointerfaces 145 (2016) 201–207

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Experimental evidence for the mode of action based on electrostatic and hydrophobic forces to explain interaction between chitosans and phospholipid Langmuir monolayers

Adriana Pavinatto[a], Jorge A.M. Delezuk[b], Adriano L. Souza[b], Felippe J. Pavinatto[b], Diogo Volpati[b,c], Paulo B. Miranda[b], Sérgio P. Campana-Filho[a], Osvaldo N. Oliveira Jr.[b,*]

[a] Institute of Chemistry of São Carlos, University of São Paulo, P.O. Box 780, 13560-970, São Carlos, SP, Brazil
[b] São Carlos Institute of Physics, University of São Paulo, USP, P.O. Box 369, 13566-590, São Carlos, SP, Brazil
[c] Department of Natural Sciences, Mid Sweden University, SE-851 70, Sundsvall, Sweden

ARTICLE INFO

Article history:
Received 23 December 2015
Received in revised form 22 March 2016
Accepted 1 May 2016
Available online 3 May 2016

Keywords:
Chitosan
Langmuir monolayers
PM-IRRAS
SFG
Conformation

ABSTRACT

The interaction between chitosans and Langmuir monolayers mimicking cell membranes has been explained with an empirical scheme based on electrostatic and hydrophobic forces, but so far this has been tested only for dimyristoyl phosphatidic acid (DMPA). In this paper, we show that the mode of action in such a scheme is also valid for dipalmitoyl phosphatidyl choline (DPPC) and dipalmitoyl phosphatidyl glycerol (DPPG), whose monolayers were expanded and their compressibility modulus decreased by interacting with chitosans. In general, the effects were stronger for the negatively charged DPPG in comparison to DPPC, and for the low molecular weight chitosan (LMWChi) which was better able to penetrate into the hydrophobic chains than the high molecular weight chitosan (Chi). Penetration into the hydrophobic chains was confirmed with polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS) and sum frequency generation (SFG) spectroscopy. A slight reduction in conformational order of the lipid chains induced by the chitosans was quantitatively estimated by measuring the ratio between the intensities of the methyl (r^+) and methylene (d^+) peaks in the SFG spectra for DPPG. The ratio decreased from 35.6 for the closely packed DPPG monolayer to 7.0 and 6.6 for monolayers containing Chi and LMWChi, respectively. Since in both cases there was a significant phospholipid monolayer expansion, the incorporation of chitosans led to chitosan-rich and lipid-rich condensed domains, which mantained conformational order for their hydrophobic tails. The stronger effects from LMWChi are ascribed to an easier access to the hydrophobic tails, as corroborated by measuring aggregation in solution with dynamic light scattering, where the hydrodynamic radius for LMWChi was close to half of that for Chi. Taken together, the results presented here confirm that the same mode of action applies to different phospholipids that are important constituents of mammalian (DPPC) and bacterial (DPPG) cell membranes.

1. Introduction

Cell membrane models using phospholipid Langmuir monolayers [1] have been used to correlate molecular-level interactions with the biological actions of chitosans [2–4], especially those related to antimicrobial and bactericidal effects [5–7]. Establishing such correlations is extremely demanding owing to the many variables involved. For instance, chitosan action depends on its molecular weight [8–10], degree of acetylation [8,9] and functionalization [11]. The modes of action, moreover, are still not completely consolidated. In bactericidal and antimicrobial activity, chitosan is believed to interact with and damage the cell membrane [12]. Essential for this action are the electrostatic interactions since the positively charged chitosans are attracted to the negatively charged cell membranes, which also explains the strong activity of other positively charged polymers [13] and oligomers [14,15].

In terms of understanding the mechanisms at the molecular level, results from Langmuir monolayers are revealing [2–4,10,16–24]. Indeed, electrostatic interactions have been proven crucial, as indicated by specific tasks performed by chitosans that only take place on negatively charged phospholipids [4,8,20,22].

* Corresponding author.
E-mail addresses: adrianapavinatto@iqsc.usp.br (A. Pavinatto), chu@ifsc.usp.br (O.N. Oliveira Jr.).

http://dx.doi.org/10.1016/j.colsurfb.2016.05.001
0927-7765/

An important example in this context was the ability of chitosan to remove proteins attached to phospholipid monolayers from the air/water interface [18]. Despite the ubiquity and importance of electrostatic interactions, they are not the sole relevant ones, and this has been amply demonstrated in various studies [2,3,21]. Hydrophobic interactions seem equally important in governing the ability of chitosan in penetrating into membranes, in addition to affecting their elasticity [4,17]. Direct proof came from studies with acylated chitosans whose effect on Langmuir monolayers of dimyristoyl phosphatidic acid (DMPA) increased with increasing hydrophobicity of the derivative [20]. Hydrophobic interactions are also central for the larger effects from low molecular weight chitosans [22]. H-bonding, on the other hand, appears not to be essential for the action of chitosans on model membranes since blocking the groups capable of H-bonding in functionalized chitosans had negligible effect [20].

This body of experimental evidence has permitted the proposal of a pictorial model that accounts for all the findings published to date [4,17,20–22]. According to this scheme for the mode of action, electrostatic attraction is key to making chitosan, which is otherwise not surface active, to couple to the membrane represented by a Langmuir monolayer. Chitosan chains then penetrate and expand the monolayer, increasing its elasticity, to an extent that depends on the chitosan hydrophobicity. The stronger effects from lower molecular weight chitosans are then associated with their larger ability to penetrate into the membrane, in comparison with higher molecular weight chitosans. Significantly, increased effects on the model membranes are well correlated with stronger biological activity [15].

In spite of the success of the mode of action proposed in [22] we cannot generalize its application to all types of cell membranes because experiments have been performed basically with one single phospholipid, namely DMPA. DMPA is a synthetic phospholipid which is not found in real cell membranes. Moreover, the assumption that smaller chitosan chains would be better able to penetrate into the membrane still requires confirmation from results of lipid and polysaccharide conformation.

In this study we set out to fill in the gaps mentioned in the last paragraph, for which a variety of experiments were designed. First, in order to prove that the effects explained by the mode of action in [22] are not restricted to a single negatively charged phospholipid (DMPA), we carried out a systematic study with the zwitterionic dipalmitoyl phosphatidylcholine (DPPC) and the negatively charged dipalmitoyl phosphatidylglycerol (DPPG), interacting with two samples of chitosan with very distinct molecular weights. DPPC (and other lipids containing the PC head-group) are phospholipids prominently found in mammalian cell membranes, while DPPG (and other lipids containing the PG head-group) are prominent components of bacterial membranes. The experiments included surface pressure isotherms and vibrational spectroscopic measurements using the surface-specific techniques polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS) [25,26] and sum-frequency generation spectroscopy (SFG) [27,28]. While the isotherms served to establish the level of monolayer expansion and change in elasticity, the spectroscopic measurements allowed us to infer the molecular-level interactions taking place between chitosan and the phospholipids. We also performed dynamic light scattering (DLS) measurements with chitosan solutions to probe the distinct conformations of the samples with different molecular weights, which indeed confirmed the expectation of better ability of the low molecular weight sample to penetrate the membrane. The analysis of these new set experiments, in comparison with data available in the literature, allows us to generalize the mode of action from [22] for all the biological effects that depend on interaction between chitosans and cell membranes.

2. Experimental details

2.1. Preparation of chitosans and Langmuir films

Commercial chitosan (Chi) obtained from Galena (Brazil), previously purified, had average degree of acetylation DA = 22%, average molecular weight Mw = 479,000 g mol^{-1} and polydispersity index PDI 4.2. The lower molecular weight chitosan (LMWChi) was produced from Chi depolymerization using $NaNO_2$, leading to a sample with DA = 10%, Mw = 23,500 g mol^{-1} and PDI 2.7. The molecular weight (Mw) and polydispersity index (PDI) were determined by size exclusion chromatography (SEC) measurements using an Agilent Series 1100 equipment with detection based on the refractive index, Shodex Ohpak SB-G (50 mm × 6 mm – pre column) + Shodex Ohpak SB-803-HQ (8 mm DI × 300 mm) + Shodex Ohpak SB-805-HQ (8 mm DI × 300 mm) columns and acetic acid buffer as solvent. Details of the LMWChi preparation and characterization have been described previously [20].

DPPC and DPPG were purchased from Sigma Chemical Co. and used as received. The Langmuir films were produced in a mini KSV Langmuir trough (KSV Instruments) placed in a clean room class 10,000 at 20 ± 1 °C. The trough is provided with a Wilhelmy pressure sensor. The monolayers were compressed at a 750 mm^2 min^{-1} rate; the trough surface area is 24,300 mm^2 with a volume of 250 mL. The films were made by spreading 30 μL of a 0.80 mg mL^{-1} chloroform (HPLC grade, ≥99.9% purity) solution of DPPC or DPPG on a Theorell-Stenhagen (TS) subphase, pH 3.0 (μ = 0.03 mol L^{-1}). Some TS buffer solutions also contained 0.2 mg mL^{-1} of Chi or LMWChi under a phospholipid monolayer. The compressional modulus (Cs^{-1}) was calculated from the surface pressure isotherm using $Cs^{-1} = -A(\partial\pi/\partial A)$, where π is the surface pressure and A is the area per molecule [29,30]. The isotherms for pure phospholipids were made in triplicate whereas those containing chitosan in the subphase were carried out in duplicate. The error values correspond to the standard deviation of all points, being ± 1 Å^2 in area per molecule, ± 0.5 and ± 0.6 mN m^{-1} for surface pressure in neat monolayers of DPPC and DPPG, respectively, and ±1.3 and ±0.7 mN m^{-1} for surface pressure in monolayers containing Chi and LMWChi, respectively. In Cs^{-1} calculations the error values were ±17 mN m^{-1}.

2.2. Polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS)

PM-IRRAS measurements were performed using a KSV PMI550 instrument (KSV, Finland) coupled to the mini Langmuir trough. The light beam reached the monolayer at a fixed incidence angle of 80°, being continuously modulated between *s*- and *p*-polarizations at a high frequency. This allows for the simultaneous measurement of the spectra for the two polarizations. The difference spectrum provides surface-specific information on oriented moieties, while the sum gives the reference spectrum. In addition, with the simultaneous measurements, the effect of water vapor is reduced. The resolution of the spectra was 8 cm^{-1}. The spectra were acquired for DPPC and DPPG monolayers on a TS buffer and in subphases containing 0.2 mg mL^{-1} of Chi or LMWChi at surface pressures ranging from 0 to 30 mN m^{-1}.

2.3. Sum frequency generation spectroscopy (SFG)

Sum-frequency generation (SFG) measurements were made to assess the molecular-level interactions. SFG is a nonlinear optical technique specific to interfaces, where the signal is vanished