Colloids and Surfaces B: Biointerfaces 104 (2013) 48–53

Contents lists available at SciVerse ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Low molecular-weight chitosans are stronger biomembrane model perturbants

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ARTICLE INFO

Article history:
Received 4 October 2012
Received in revised form
26 November 2012
Accepted 27 November 2012
Available online xxx

Keywords:
Chitosan
Langmuir films
DMPA
Membrane models
Molecular weight

ABSTRACT

The influence from the chitosan molecular weight on its interaction with cell membrane models has been studied. A low molecular weight chitosan (LMWChi) adsorbed from the subphase expanded the surface pressure–area and surface potential–area isotherms of dimyristoyl phosphatidic acid (DMPA) monolayers and decreased the compressional modulus. The expansion in the monolayers and the decrease in the compressional modulus were larger for LMWChi than for a high molecular weight chitosan (Chi). The polymeric nature is still essential for the interaction though, which was demonstrated by measuring negligible changes in the mechanical properties of the DMPA monolayer when the subphase contained glucosamine and acetyl-glucosamine. The results were rationalized in a model through which chitosan interacted with the membrane via electrostatic and hydrophobic interactions, with the smaller chains of LMWChi having less steric hindrance to be accommodated in the membrane. In summary, the activity based on membrane interactions depends on the distribution of molar mass, with lower molecular weight chitosan more likely to have stronger effects.

1. Introduction

Chitosan is a natural polysaccharide derived from chitin, used for biomedical applications [1] and in pharmaceutical and biotechnology industries [2], in which its properties such as biocompatibility, biodegradability and non-toxicity are exploited [3]. Owing to its cationic character in acidic solutions, chitosan may interact with the negatively charged surface of biomembranes, and therefore understanding such interactions at the molecular level is important for the use in humans. The mechanisms responsible for chitosan activity are still unknown in detail. Studies to elucidate interaction between chitosan and biomembranes have been made, generally using cell membrane models such as Langmuir films [4–9], and vesicles [10–13]. For its antimicrobial action, in particular, the bioactivity of chitosan depends on the molecular weight [14–19] and degree of acetylation [20], but the exact mechanism of action is still unknown. Some hypotheses have been made, as follows: (i) chitosan penetrates into the cell and induces cellular dysfunction (i.e. binding to DNA and preventing protein synthesis or acting as chelating agent) [21–23]; (ii) chitosan molecules form stacks over microorganisms, preventing the exchange of material with the environment (nutrient transport, metabolites excretion) [24] and (iii) chitosan causes disruption of the cell membrane [25,26].

The influence from the molecular weight on the antimicrobial function of chitosan has been studied [14–19], and the results are somewhat contradictory. For some *Bacillus cereus* and *Escherichia coli*, chitosan with low molecular weight ($10{,}000\,g/mol < M_w \leq 250{,}000\,g/mol$) or chitosan oligomers had a larger effect than high molecular weight chitosan ($M_w > 250{,}000\,g/mol$) [16,17,25]. In contrast, for some species of fungus, e.g. *Rhizopus stolonifer*, chitosan oligomers ($1000\,g/mol < M_w \leq 10{,}000\,g/mol$) could only inhibit one stage of growth [14,17]. Moreover, for *Candida albicans*, *Candida krusei* and *Candida glabrata*, the activity decreased with decreasing molecular mass [18]. Some controversy also exists for the effects from molecular weight on the interaction with model membranes made with Langmuir monolayers or bilayers of phospholipids, such as dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG). Fang et al. [27] did not observe significant differences in the changes in the membrane caused by chitosan with two molecular weights ($M_w = 113 \times 10^3$ g/mol or 213×10^3 g/mol), with both samples increasing the membrane fluidity. Here we state that a decrease in surface elasticity corresponds to an increased fluidity because the molecular movement of the membrane constituents as well as the penetration of guest molecules into the membrane is facilitated when the surface elasticity decreases. Quemeneur et al. [12] also observed similar effects regardless of the chitosan molecular weight ($M_w = 100 \times 10^3$ g/mol

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http://dx.doi.org/10.1016/j.colsurfb.2012.11.047

and 500×10^3 g/mol) at fixed pH and same acetylation degree. In contrast, stronger monolayer destabilization was observed for lower molecular weight chitosans by Krajewska et al. [19] for the lipids studied (DPPC, DPPG and cholesterol).

In this study we assessed the importance of the molecular weight focusing specifically on the interaction with simplified cell membrane models represented by phospholipid Langmuir monolayers. We compared two chitosan samples with distinctly different molecular weights, in addition to using a mixture of the monosaccharides GlcNAc/GlcN with the same proportion of acetyl groups as the polymeric samples. The low-molecular weight chitosan sample was synthesized, purified and characterized in order to obtain a pure product with a significant amount of oligomers in its composition. This feature turned out to be important, as the comparison with other chitosan samples was made. The monolayers were characterized using surface pressure and surface potential isotherms. In order to ensure that Chi, LMWChi and GlcNAc/GlcN were indeed adsorbed onto the monolayers, we performed polarization-modulation infrared reflection absorption spectroscopy measurements (PM-IRRAS) on the air–water interface and on Langmuir–Blodgett (LB) films.

2. Experimental details

2.1. Preparation of chitosan

DMPA was purchased from Sigma Chemical Co. and used as received. Both chitosan (Chi) and chitosan with low molecular weight (LMWChi) samples were prepared by the authors. Chi was obtained from the deacetylation of chitin extracted from squid pens, using the ultrasound-assisted deacetylation method (USAD) [28]. Sonication was performed in NaOH solution (40%) at 60 °C in three periods of 50 min. LMWChi was obtained by ultrasound-assisted depolymerization following the procedure of Baxter [29], with sonication in acetic acid solution (10%) at 33 °C for 4 h. Size exclusion chromatography (SEC) measurements were performed in a Shimadzu (CTO-10A), RID – 6A equipment, using 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, refractive index detector, and acetic acid buffer as solvent. This is the most important characterization to show the success of the low molecular-weight chitosan syntheses. However, ^{1}H NMR and FTIR measurements were also conducted to evaluate the chemical structure and degree of acetylation of the samples. The experimental conditions and results for this characterization are given in the Supplementary Information. The glucopyranose repeating units comprising chitosan chains, namely 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-D-glucopyranose (GlcN), were purchased from Sigma and used without further purification. They have molecular weight of 221 g/mol (M_w) and 215 g/mol (M_w), respectively.

2.2. Langmuir films characterization

Langmuir films were produced by spreading 50 μL of a 0.50 mg mL^{-1} chloroform solution of DMPA on a Theorell–Stenhagen (TS) buffered subphase at pH 3.0. The buffer was prepared using NaOH, citric acid, boric acid, phosphoric acid and ultrapure water with pH 6.0 and resistivity of 18.2 MΩ cm, provided by a Millipore system. The water surface tension was 73 mN m^{-1}. The final pH of the buffer was adjusted to 3.0 by adding HCl 2 mol L^{-1} and the ionic force was maintained at 0.03 mol L^{-1}. At this pH, the amine groups from chitosan samples are fully protonated since their pK_a is ca. 6.5 [30]. The films were fabricated in a Langmuir minitrough (KSV Instruments) housed in a class 10,000 clean room at a temperature of 22 ± 2 °C. The trough is equipped with a surface pressure sensor based on the Wilhelmy method and a Kelvin probe to measure surface potential. The monolayers were compressed at a 750 mm^2 min^{-1} rate, and the trough area is 24,300 mm^2. A buffer solution with Chi, GlcNAc/GlcN or LMWChi in different concentrations, ranging from 0.05 to 0.3 mg mL^{-1}, was used as subphase. The sample GlcNAc/GlcN is a mixture of the monosaccharides with the molar ratio of 0.06 GlcNAc + 0.94 GlcN, which was used to get a sample with 6% acetylated repeating units, analogously to Chi. Also, the total concentration of this sample was calculated to match the exact concentrations used for chitosan. The measurements were made in duplicate. The surface compressional modulus (Cs^{-1}), also known as the in-plane elasticity, was calculated from the surface pressure isotherms using the expression: $Cs^{-1} = -A(\partial\pi/\partial A)_T$, where π is surface pressure and A is the mean molecular area [31].

PM-IRRAS measurements were performed using a KSV PMI550 instrument (KSV, Finland). The light beam reached the monolayer at a fixed incidence angle of 80°, being continuously modulated between s- and p-polarization at a high frequency. This allows for the simultaneous measurement of the spectra for the two polarizations. The difference spectrum thus 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.

It should be emphasized that all isotherms were recorded 20 min after spreading the DMPA monolayer on the chitosan-containing subphase. We know from subsidiary experiments that the adsorption of chitosan onto the monolayer depends on a number of parameters, including the molecular weight of chitosan and the dimensions of the trough. For the present experiments, 20 min were not sufficient for saturation of the chitosan adsorption for all samples. Considering the duration of the experiment in compressing the barriers to record the isotherm (a few minutes), the possible change in the surface pressure owing to additional chitosan adsorption would be only a few mN m^{-1}, and therefore should not affect the analysis of the data.

2.3. LB deposition

Langmuir–Blodgett films were deposited from Langmuir monolayers of DMPA previously formed over buffer solutions containing 0.2 mg mL^{-1} of Chi, LMWChi and GlcNAc/GlcN. The substrates were glass slides coated with Au. LB films with one layer could be transferred for mixed films of DMPA-Chi, DMPA-LMWChi and DMPA-GlcNAc/GlcN, which rendered a transfer ratio of 0.9, 1.06 and 1.07, respectively. The transfer was performed at a surface pressure of 30 mN m^{-1} with a dipping speed of 7.0 mm min^{-1}.

3. Results

In order to concentrate on the effects from molecular weight, we tried to get chitosan samples with similar degrees of acetylation and polydispersity, as the latter factors can also influence the interaction with model membranes. We have therefore prepared the samples Chi and LMWChi. Chi can be considered a high molecular weight chitosan, with M_n = 280,000 g/mol and M_w = 730,000 g/mol (polydispersity index of 2.6), while LMWChi has M_n = 31,000 g/mol and M_w = 88,000 g/mol (polydispersity index of 2.8), i.e. it is a low molecular weight chitosan with considerable amount of oligomers, as can be inferred from the chromatogram shown in the Supplementary Material. Significantly, the degree of acetylation, as determined with ^{1}H NMR spectroscopy in D_2O/HCl (1:9), is 6% for both Chi and LMWChi, as indicated in the data in the Supplementary Material.