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Effect of thiol-functionalisation on chitosan antibacterial activity: Interaction with a bacterial membrane model

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ABSTRACT

The antibacterial activity of chitosan modified with the thiol-containing 2-iminothiolane HCl (TC-IMI) and N-acetyl-L-cysteine (TC-NAC) was studied by Langmuir film balance technique using a dipalmitoylphosphatidylglycerol (DPPG) monolayer bacterial membrane model. The interactions of the biopolymer with the membrane model were assessed by monitoring the differences in the shape of the compression isotherms recorded in the absence and presence of chitosan and thiolated conjugates in the subphase. A low molecular weight chitosan (15 kDa) shifted the compression isotherms of DPPG monolayers towards larger areas ($A_{0,CS}$ = 145 Å^2), confirming its membrane disturbance capacity. Further thiolation induced higher yield of expansion, more pronounced in the case of TC-IMI. The expansion of the monolayer increased significantly ($A_{0,TC\text{-}NAC}$ = 150 Å^2 vs $A_{0,TC\text{-}IMI}$ = 175 Å^2) and the elasticity at a surface pressure of 30 mN/m, typical for bio-membranes decreased to a greater extent ($C^{-1}_{s,30\ TC-NAC}$ = 120 mN/m vs $C^{-1}_{s,30\ TC-IMI}$ = 87 mN/m) in presence of TC-IMI. Antibacterial tests against a Gram-negative *Escherichia coli* and a Gram-positive *Staphylococcus aureus* were in good agreement with these findings, suggesting that chitosan thiolated with 2-iminothiolane HCl acts as a bactericide disrupting the integrity of the bacterial cell membrane.

1. Introduction

Chitosan is a cationic polysaccharide with recognised antimicrobial properties against a wide range of microorganisms including algae, bacteria, yeasts and fungi [1,2]. The electrostatic interactions between the protonated amino groups of chitosan ($-NH_3^+$) and the negatively charged microbial cell membranes are believed to be essential for its antimicrobial activity. According to the widely accepted mechanism, the polymer binds to a microorganism and triggers a double interference with its cell membrane properties: (i) changes in the membrane wall permeability provoke internal cell imbalances and consequently inhibition of bacterial growth [3,4], whereas the (ii) hydrolysis of the membrane constituents induces the release of cell contents and subsequent cell death [5,6].

Intrinsic antimicrobial properties, together with a large-scale availability, biocompatibility and biodegradability of chitosan, account for its extensive pharmaceutical and medical applications as drug delivery systems [7] and scaffolds for tissue engineering [8]. However, the limited solubility in neutral aqueous solutions makes chitosan rather difficult to process in the desired design. In order to improve its solubility and reactivity various derivatives based on chemical modification of the available amino groups have been prepared [9–12]. Such modifications, on one side, preserve the fundamental skeleton of the polymer and, on the other, improve its solubility and bring new functionalities ascribed to the character of the introduced moieties. Likewise, the incorporation of thiol bearing compounds has been commonly carried out to enhance the polymer mucoadhesion [13,14], targeted tissue permeation [15] and enzyme inhibition properties [16]. Moreover, solutions of thiolated chitosan (TC) display in situ gelling properties at physiological pH due to the oxidation of free thiol groups to form inter- and intra-molecular disulphide bonds [17,18]. Despite the numerous references on improved functional characteristics and application, the antimicrobial properties of TC derivatives have been sparsely reported.

This study aims at evaluating the effect of chitosan functionalisation with the thiol compounds 2-iminothiolane HCl and N-acetyl-L-cysteine on the antimicrobial activity of the biopolymer, giving further insight on the mechanism of their action using a bacterial membrane model. The understanding that microbial membranes represent two weakly coupled monolayers prompted the use of Langmuir monolayers as a membrane model systems [19]. The Langmuir film balance technique measures the surface pressure (π) as a function of the area (A) on the monolayer occupied by the molecules spread at the air-aqueous interface. The resulting isotherms (π–A) demonstrate the phase behaviour of the monolayer in the course of compression, when the molecular packing gradually increases [20,21]. This technique has been used to elucidate the interactions of chitosan with cell membrane models at molecular level [22,23], thus indirectly assessing its antimicrobial

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activity. The latter is estimated by the capacity of chitosan to disrupt membranes comprising negatively charged dipalmitoylphosphatidylglycerol (DPPG) – a phospholipid present at the inner leaflets of Gram-negative and Gram-positive bacteria [24].

It has been established that chitosan induces an expansion in the membrane model isotherms through multiple interactions, including electrostatic and van der Waals forces, and hydrogen bonding with the phospholipid in the monolayer [22,25]. Nevertheless, the effect of thiolation on chitosan interaction with Langmuir monolayers has not been investigated so far. The objective of this work was to obtain molecular-level evidences for the antimicrobial action of chitosan and its thiolated conjugates using a suitable bacterial membrane model. These were further coupled to experimental data for the antimicrobial efficiency of the materials. Two different TC conjugates were synthesised and analysed for their ability to inhibit the bacterial growth of Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*.

2. Experimental

2.1. Materials

Medical grade chitosan (~15 kDa, DDA 87%) was obtained from Kitozyme (Belgium). 2-iminothiolane HCl, N-acetyl-L-cysteine (NAC), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC), 5,5′- dithiobis(2-nitrobenzoic acid) (Ellman's reagent), L-cysteine, highly purified dipalmitoylphosphatidyl glycerol (DPPG, Mw = 734.01 g/mol), HPLC grade chloroform and all other reagents were purchased from Sigma–Aldrich. Ultra-pure water obtained with a Simplicity UV Millipore equipment was used for all solutions.

2.2. Methods

2.2.1. Chitosan thiolation

Two different approaches were used to immobilise thiol-containing compounds onto chitosan (1% w/v) dissolved in CH_3COOH (1%, v/v) to reach the final concentration of. The first thiolation protocol was carried out in a one-step coupling reaction between 2-iminothiolane HCl and primary amino groups of chitosan [26]. Briefly, the pH of the chitosan solution was adjusted to 6 with NaOH (5 M), 2-iminothiolane HCl was added and left to dissolve at stirring for 3 h at room temperature. The resulting mixture was dialysed twice for 24 h against 5 mM HCl containing NaCl (1% w/v) and finally for 72 h in 1 mM HCl.

The second thiolation protocol involved a carbodiimide coupling reagent (EDAC) for the immobilisation of NAC. The pH of the chitosan solution was adjusted to 5 and then 400 mM of NAC and 50 mM of EDAC were added. The resulting mixture was stirred for 3 h at room temperature and dialysed as described above. The dialysed thiolated chitosan conjugates were freeze-dried and stored at +4 °C until further use.

The thiolation degree of the conjugates was determined spectrophotometrically using Ellman's reagent. One mg of the freeze-dried sample was hydrated in 500 μL of 0.5 M phosphate buffer (PB, pH 8) for 20 min. Then, 500 μL of Ellman's reagent (3 mg in 10 mL of PB) were added and the mixtures incubated for 3 h at room temperature. The mixtures were centrifuged (5000 rpm, 5 min) and the supernatants transferred to a microplate reader (Infinite M200, Tecan, Austria) where the absorbance was recorded at 450 nm. L-Cysteine standards were used to calculate the amount of thiol groups immobilised on chitosan.

2.2.2. Langmuir monolayer measurements

The subphase solutions in the Langmuir monolayer measurements were prepared by dissolving 1 mg/mL freeze dried chitosan and thiolated chitosan conjugates in a 0.3 mM HCl solution, pH 3.5. The measurements were performed with a Langmuir trough (NIMA, model 1232D1D2) mounted on an antivibration table and housed in an isolated box. The Teflon trough of the balance (total area 1200 cm^2) was filled with a subphase solution and surface pressure-area isotherms of DPPG monolayers were recorded at 21 ± 1 °C and barrier velocity of 10.2 $Å^2$ $molecule^{-1}$ min^{-1} (50 cm^2 min^{-1}; 2.5 cm min^{-1}). 120 μL (spreading volume) of 0.5 mg/mL DPPG in chloroform were deposited on the subphase. The control sample without polymer was prepared in the same way. During the film compression at the air–water interface, the surface pressure (π) was recorded against the area (A), where the surface pressure was measured with a Wilhelmy plate made of a filter paper (Whatman Ch1) connected to an electrobalance. The isotherms provide information about the film states, phases and phase transitions, where π is the difference between the surface tension of pure water (γ_0) and that of the film (γ), i.e. $\pi = \gamma_0 - \gamma$ [20,21]. The compression modulus (in-plane elasticity) C_s^{-1} was calculated from the slope of the π vs A isotherm:

$$C_s^{-1} = -A\left(\frac{d\pi}{dA}\right) \quad (1)$$

2.2.3. Test for antimicrobial activity

The antimicrobial activity of chitosan and thiolated chitosan was determined using a turbidity-based microdilution assay in Muller Hinton broth (MH), according to the procedure approved by The National Committee for Clinical Laboratory Standards (NCCLSs) with the modifications proposed by Weigand et al. [27]. A Gram-negative *E. coli* (CECT 101) and a Gram-positive *S. aureus* (CECT 86) from the Spanish Type Culture Collection were tested. The results were expressed in bacterial growth inhibition and minimum inhibitory concentrations (MIC) – the lowest concentration of biopolymers required to completely inhibit bacterial growth. Overnight bacterial strains cultures were regrown to exponential phases (OD_{600} of 0.5–0.6) and diluted to OD_{600} = 0.01 in MH broth to obtain bacteria stock solutions. Stock solutions of chitosan and TCs were thereafter prepared by dissolving the polymer and conjugates in a 0.3 mM HCl solution (pH 3.5) and filtering under sterile conditions with a 0.22 μm syringe filter. Ampicillin (50 μg mL^{-1}) prepared in the same solution and the solution itself was used as positive and negative control, respectively. The working solutions of the polymers and controls were prepared by serial dilutions of the stock solutions in MH broth. The working solutions (75 μL) were then mixed with the bacterial stock solutions in a 96-well polypropylene microplate (Corning #3359), giving the final concentration of bacteria of ~5×10^5 CFU/mL. The growth of bacteria was monitored by recording the optical density at 600 nm (corrected for the background absorbance of each blank) every 10 min during 10 h using a microplate reader (Infinite M200, Tecan, Austria). The specific growth rates (min^{-1}) were obtained from the exponential phases of the individual growth curves and related to the specific growth rate of the negative control. The MIC was defined as the lowest polymer concentration at which turbidity increase was not observed relative to the negative control. The reported MIC and inhibition growth rates are mean values of five independent experiments.

3. Results and discussion

3.1. Thiolated chitosan conjugates

Two different protocols were adopted to immobilise free thiol moieties onto chitosan through reactions with its amino groups: (i) the direct coupling of 2-iminothiolane HCl (Scheme 1A), and