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Antibacterial action of a novel functionalized chitosan-arginine against Gram-negative bacteria

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ABSTRACT

The antimicrobial activity of chitosan and chitosan derivatives has been well established. However, although several mechanisms have been proposed, the exact mode of action is still unclear. Here we report on the investigation of antibacterial activity and the antibacterial mode of action of a novel water-soluble chitosan derivative, arginine-functionalized chitosan, on the Gram-negative bacteria *Pseudomonas fluorescens* and *Escherichia coli*. Two different arginine-functionalized chitosans (6% arginine-substituted and 30% arginine-substituted) each strongly inhibited *P. fluorescens* and *E. coli* growth. Time-dependent killing efficacy experiments showed that 5000 mg l^{-1} of 6%- and 30%-substituted chitosan-arginine killed 2.7 logs and 4.5 logs of *P. fluorescens*, and 4.8 logs and 4.6 logs of *E. coli* in 4 h, respectively. At low concentrations, the 6%-substituted chitosan-arginine was more effective in inhibiting cell growth even though the 30%-substituted chitosan-arginine appeared to be more effective in permeabilizing the cell membranes of both *P. fluorescens* and *E. coli*. Studies using fluorescent probes, 1-*N*-phenylnaphthylamine (NPN), nile red (NR) and propidium iodide (PI), and field emission scanning electron microscopy (FESEM) suggest that chitosan-arginine's antibacterial activity is, at least in part, due to its interaction with the cell membrane, in which it increases membrane permeability.

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1. Introduction

Chitosan is a carbohydrate biopolymer derived from deacetylation of chitin, the main component of crustacean (e.g., shrimp, crab, lobster) exoskeletons. Chitin's abundance is second only to cellulose among polysaccharides found on Earth [1]. Chitosan is medically important due to its biological properties, such as antimicrobial activity, haemostatic activity, anti-tumor activity, acceleration of wound healing, tissue-engineering scaffolds, and promise for drug delivery [2]. It is also biodegradable and biocompatible, with low toxicity to mammalian cells [1]. Bacteria are not known to develop chitosan resistance [3].

Chitosan's antimicrobial activity has been well documented. It displays a broad spectrum of antibacterial activity against both Gram-positive and Gram-negative bacteria, with minimum inhibitory concentrations (MICs) reported to range from 100 to 10,000 mg l^{-1} against Gram-negative bacteria [4], and from 100 to 1250 mg l^{-1} against Gram-positive bacteria [5–8].

Chitosan's antimicrobial activities are thought to be affected by chemical, physical and biological factors that include chitosan concentration, molecular weight, degree of deacetylation, pH, temperature, salinity, divalent cations, chitosan solvent, suspending medium, and bacterial growth phase [1,8–20]. Because chitosan and its derivatives have been tested under widely varied conditions, it is hard to compare chitosan's antibacterial effect among results obtained by different researchers.

The exact mode of action of chitosan is still not fully understood, although several mechanisms have been proposed for its antimicrobial activity [1,21]. The key feature of chitosan is thought to be its positive charge of the amino groups ($-NH_3^+$) at the C-2 positions in the glucose monomer when the pH is lower than its pK_a (~6.3). This forms a polycationic structure that can interact with the anionic compounds and macromolecular structures of bacteria [4,22]. This charge interaction can alter bacterial surface morphology, which either increases membrane permeability, causing leakage of intracellular substances (e.g., proteins including lactate dehydrogenase, nucleic acids and glucose), or decreases membrane permeability, preventing nutrient transport [14,15,17,23,24]. The bulk of evidence supports increased membrane permeability and disruption of cell membranes [3,4,12,14,15,24]. It has

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also been postulated that positively charged chitosan binds with cellular DNA following chitosan penetration into the cells, thereby inhibiting transcription [1,17].

Researchers have applied multiple techniques to investigate chitosan's antibacterial mode of action, including outer membrane (OM) permeability assays (1-*N*-phenylnaphthylamine (NPN) uptake, SDS-promoted cell lysis), an inner membrane (IM) permeability assay (β-galactosidase activity), lipopolysaccharide (LPS) release, intracellular constituents leakage ($OD_{260\ nm}$, $OD_{280\ nm}$, SDS–PAGE), scanning electron microscopy, transmission electron microscopy and atomic force microscopy [3,4,12,14,15,23–31].

The use of chitosan is limited because of its insolubility at neutral pH. Therefore, much effort has been made to prepare functionalized chitosan derivatives that are soluble in water at physiological pH. Chemically modified chitosans include a maltose–chitosan derivative [19] and the proprietary arginine-functionalized chitosan that we tested in this study [32]. Arginine-functionalized chitosan is a chitosan derivative modified with arginine groups to different degrees of substitution. It is highly soluble in water owing to the high pK_a of the guanidinium side chain of arginine ($pK_a = 12.48$), rendering it positively charged in neutral pH environments.

Here we report on the antibacterial activity and the antibacterial mode of action of arginine-functionalized chitosan on model Gram-negative bacteria, *Pseudomonas fluorescens* and *Escherichia coli*. Fluorescence spectroscopy and electron microscopy were used to evaluate cellular effects in the target bacteria. Three fluorescence probes, 1-*N*-phenylnaphthylamine (NPN), nile red (NR), and propidium iodide (PI), were used in the study. NPN is a hydrophobic probe widely used to assess cell membrane permeability. NR stains, and is sensitive to, neutral lipids in cells. PI, a DNA intercalator, is used to indicate cell death. The fluorescence spectra of these probe molecules are sensitive to their surroundings, reflecting the slight change of their excited state under different environment, as detailed in the Section 4.

2. Materials and methods

2.1. Chemicals and bacteria

Proprietary arginine-functionalized chitosans (6% arginine-substituted and 30% arginine -substituted) were provided by Bio-Star West, Claremont, CA. Trypticase Soy Broth (TSB) (Difco™, BD Company), Luria–Bertani Broth (LB) (Difco™, BD Company), Cation-Adjusted Muller Hinton Broth (MH II broth) (BBL™, BD Company) and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) were purchased from Fisher Scientific; NPN, NR, PI, tannic acid and poly-L-lysine from Sigma; ethanol, glutaraldehyde, paraformaldehyde, osmium tetroxide and hexamethyldisilazane (HMDS) from Electron Microscopy Science; PBS buffer from Invitrogen. Both bacteria were from ATCC.

2.2. Bacteria overnight culture growth

E. coli (ATCC #25922) and *P. fluorescens* (ATCC #700830) were routinely cultivated in LB or TSB at 37 °C with shaking overnight.

2.3. Evaluation of chitosan-arginine's antibacterial activity

2.3.1. Bacterial growth inhibition

Overnight cultures of *E. coli* and *P. fluorescens* were inoculated into fresh LB to $\sim 10^6$ cells ml^{-1} and were mixed with a series of concentrations of chitosan-arginine (0–512 mg l^{-1}). Optical density (OD) at 595 nm was monitored at 37 °C using a Thermomax Microplate Reader for 24 h. The value of $OD_{595\ nm}$ reported is the average of triplicate samples. Statistical analysis was performed for data points collected at 24 h by two-way ANOVAs using Minitab-15 software.

2.3.2. MICs and minimum bactericidal concentrations (MBCs) determination

MICs were determined by microtiter broth dilution method, following the guidelines in the literature [33]. Briefly, inocula of *P. fluorescens* and *E. coli* were prepared by adjusting overnight culture to containing 2×10^5 cells ml^{-1} in MH II broth. Aliquots of 50 µl inoculum were mixed with 50 µl of serial twofold dilutions of 6%-substituted and 30%-substituted chitosan-arginine in MH II broth in a 96-well plate. The plate was incubated with shaking at 37 °C for 18 h. MIC was defined as the lowest concentration of chitosan-arginine where no growth was observed by microscopic examination. After 18 h incubation, 10 µl mixtures from wells with no growth were spread on agar plates for MBC determination. MBC was defined as the lowest concentration of chitosan-arginine where no colony growth was observed on agar plates after 48 h incubation at 37 °C. The MIC/MBC determinations were carried out in triplicates, with two independent experiments performed.

2.3.3. Time-dependent killing efficacy

Briefly, overnight cultures of *P. fluorescens* and *E. coli* were adjusted in MH II broth to contain 10^7 cells ml^{-1} and mixed with 5000 mg l^{-1} of 6%- and 30%-substituted chitosan-arginine. The mixtures were incubated at 37 °C with shaking and aliquots were withdrawn to perform colony count every 30 min for 4 h.

2.4. Fluorescent probe-permeability assays

Overnight cultures of *E. coli* and *P. fluorescens* were centrifuged at 1000*g* for 10 min. The supernatant was discarded. Bacterial pellets were re-suspended in 5 mM HEPES (pH 5.3 ± 0.1) to $OD_{600\ nm} \sim 0.2$ for fluorescence probe assays. All assays were performed at room temperature.

All fluorescence measurements were done on a spectrofluorometer (Photon Technology International) with a xenon lamp as the excitation source. The slit widths were set to 4 nm for both the excitation and the emission monochromators.

For each assay with respective probes, 3 ml of bacterial suspension was first mixed with either NPN (1.1 mg ml^{-1} in acetone), NR (1 mg ml^{-1} in methanol) or PI (2.5 mg ml^{-1} in water) to a final probe concentrations of 2.2 µg ml^{-1} for NPN, 10 µg ml^{-1} for NR and 17 µg ml^{-1} for PI. Fluorescence measurements were then taken. Next, nanopure water (for controls) or chitosan-arginine (final concentration of 50 mg l^{-1}) was added to the mixture. The mixture was thoroughly stirred before fluorescence measurements were taken again. There was typically a 3-min lapse between the addition of chitosan-arginine and the following fluorescence measurement for NPN and NR. Fluorescence intensity of both excitation and emission peaks was monitored over time. For PI, fluorescence intensity was taken every 10 min for up to 2 h, or hourly for up to 8 h and at 24 h.

2.5. Electron microscopy

Overnight *E. coli* and *P. fluorescens* cultures were washed once with 0.85% NaCl solution, then re-suspended in 5 mM HEPES (pH 5.3 ± 0.1) to $OD_{600\ nm} \sim 0.4$. After 3-h incubation with or without 100 mg l^{-1} chitosan-arginine at 37 °C, *E. coli* and *P. fluorescens* cells were washed once with PBS and fixed (2% paraformaldehyde and 1% glutaraldehyde in pH 7.3 ± 0.1 PBS). Cells were then spread onto glass cover-slips pre-treated with poly-L-lysine, post-fixed in 1% osmium tetroxide, post-stained in 1% tannic acid, dehydrated in a series of increasing ethanol concentrations (70–100%), and air-dried in HMDS. Samples were sputter-coated with platinum using

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