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Structural effect of quaternary ammonium chitin derivatives on their bactericidal activity and specificity

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ABSTRACT

The effect of the quaternary ammonium chitin structure on the bactericidal activity and specificity against *Escherichia coli* and *Staphylococcus aureus* was investigated. Quaternary ammonium chitins were synthesized by the separate acylation of chitin (CT) with carboxymethyl trimethylammonium chloride (CMA), 3-carboxypropyl trimethylammonium chloride (CPA) and *N*-dodecyl-*N*,*N*-(dimethylammonio)butyrate (DDMAB). The successful acylation was confirmed by newly formed ester linkage. All three derivatives had a higher surface charge than chitin due to the additional positively charged quaternary ammonium groups. The *N*-short alkyl substituent (methyl) of CTCMA and CTCPA increased the hydrophilicity whilst the *N*-long alkyl substituent (dodecyl) of CTDDMAB increased the hydrophobicity compared to chitin. Chitin did not exhibit any bactericidal activity, while CTCMA and CTCPA completely killed *E. coli* and *S. aureus* in 30 and 60 min, respectively, and CTDDMAB completely killed *S. aureus* in 10 min but did not kill *E. coli* after a 2-h exposure. Therefore, the *N*-short alkyl substituent was more effective for killing *E. coli* and the *N*-long alkyl substituent conferred specific bactericidal activity against *S. aureus*.

1. Introduction

Microorganisms, including bacteria, play an important role in the ecosystem as decomposers, and are utilized in the food, pharmaceutical and biotechnology industries [1]. Moreover, some symbiotic or commensal bacteria that inhabit animal bodies (including humans) can synthesize essential vitamins or other nutrients [2], or act as probiotics. In contrast, other bacteria can be pathogenic and cause diseases or wound infections. Several kinds of antibacterial agents, for example, silver nanoparticles [3,4], titanium dioxide [5] and zinc oxide [6] have been widely used for killing bacteria. However, these antibacterial agents are inorganic substances and cytotoxic when they are applied in the body [7,8].

Antibacterial agents used for application locally or systemically as nanomedicines, such as those administered orally or injected into the circulating system, should preferentially be considered on the basis of being non-cytotoxic and biocompatible. This has led to an increased interest in biomolecular antibacterial agents. Chitin (CT; 2-acetamido-2-deoxy-(1–4)-β-D-glucopyranose) is a naturally occurring, biodegradable and essentially non-toxic polymer that can be degraded by lysozymes [9,10] which exists in the human body [11]. In addition, chitin can be easily formed into a hydrogel [12] and so it can be applied or modified for many applications. Although the discovery of chitin-based antibacterial agents is of interest, chitin itself exhibits no significant antibacterial activity when tested as chitin powder suspended in bacterial broth under ASTM E2149-10 standard method [13]. In contrast, its deacetylated derivative chitosan (2-amino-2-deoxy-(1–4)-β-D-glucopyranose) contains positively charged (at acidic pH) amino groups instead of acetamido groups and exhibits antibacterial activity. But this positive charge is markedly reduced near neutral pH at the physiological condition [14]. The chemical modification of chitin to introduce positive charges, such as the addition of quaternary ammonium groups, improves the antibacterial activities [13]. With respect to antibacterial activity, several studies have evaluated on chitosan derivatives such as quaternary ammonium chitosan derivatives [15–19], *N*-quaternary ammonium-*O*-sulfobetaine chitosan [20], and quaternary phophonium chitosan [21,22], but there is little information on chitin derivatives such as aminoethyl-chitin [23] and quaternary ammonium chitin derivative of chitin betainate which was water insoluble [13]. Besides, some water soluble 2-hydroxypropyltrimethylammonium chitin was homogeneously synthesized in sodium hydroxide (NaOH)/urea aqueous solution [24,25]. However, these include only limited information on the effect of different quaternary ammonium chitin/chitosan structures on the resultant antibacterial activity, where the effect of

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the quaternary ammonium side chain length upon the bactericidal activity or specificity is unknown.

The bactericidal specificities overcome the problems of eliminating various kinds of bacteria for both beneficial and infectious bacteria or both Gram-positive and Gram-negative bacteria, causing some medicinal side effects. In order to reduce the bactericidal effect upon commensal bacteria during treatment of specific pathogen infections and also to reduce side effects of antibacterial agents, the discovery of new chitin-based antibacterial agents that target either Gram-positive or Gram-negative bacteria may be of interest. These discoveries must take into account the cell wall composition of Gram-positive and Gram-negative bacteria. The cell wall of Gram-positive bacteria consists of a thick peptidoglycan layer, whilst Gram-negative bacteria have a thinner two layered membranes with the inner membrane being the periplasmic space and a thin peptidoglycan layer, while the outer membrane consists of lipopolysaccharide (LPS).

This research investigated the structural effect of quaternary ammonium chitin on the bactericidal activity against a model Gram-positive *Staphylococcus aureus* (*S. aureus*) and Gram-negative *Escherichia coli (E. coli*) on the assumption that the positive charge would enhance the disruption of the LPS rich outer membrane of Gram-negative bacteria, while the long length of hydrophobic substituent chain might enhance penetration though the peptidoglycan membrane of Gram-positive bacteria. Accordingly, chitin was modified with three quaternary ammoniums having different structures, namely (i) carboxymethyl trimethyl ammonium chloride (CMA) to yield CTCMA, a quaternary ammonium chitin consisting of a C1 (methyl) spacer and *N,N,N*-trimethyl substituent, (ii) 3-carboxypropyl trimethyl ammonium chloride (CPA) to give CTCPA, a quaternary ammonium chitin with a C3 (propyl) spacer and *N,N,N*-trimethyl substituent and (iii) 3-carboxypropyl-*N*-dodecyl-*N,N*-dimethylammonium chloride (DDMAB) to yield CTDDMAB, a quaternary ammonium chitin with a C3 spacer and *N*-dodecyl-*N,N*-dimethyl substituent. Thus, the CTCPA had the same structure of trimethyl ammonium groups as CTCMA but with a longer spacer (C3) between chitin and trimethyl ammonium groups, whilst CTDDMAB had the same C3 spacer as CTCPA but with a *N*- long alkyl substituent.

In addition, the mechanism of action was investigated via the observation of morphological changes in the bacterial cells using field emission scanning electron microscope (FE-SEM).

2. Materials and methods

2.1. Materials

Chitin with a degree of acetylation (DA) of 71, as determined by Fourier transform infrared spectroscopy (FT-IR) or DA of 95 determined by solid state carbon-13 Nuclear magnetic resonance (^{13}C NMR), was supplied from A.N. Lab (Bangkok, Thailand). Chitin viscosity average molecular weight (M*v*) was approximately 700 kDa, determined after dissolving it in dimethylacetamide/5%(w/v) lithium chloride with the concentration range of 0.03–0.05 g/dL at 30 °C and calculated based on intrinsic viscosity [η] value utilizing the Mark-Houwink-Sakurada equation (Eq. (1)).

$$[\eta] = KM_v^a \quad (1)$$

where K = 7.6 × 10^{-5} dL/g and a = 0.95 are constants that depend on the solvent-polymer system [26].

N,N-dicyclohexyl carbodiimide (DCC), CMA, CPA and DDMAB were purchased from Sigma-Aldrich (Munich, Germany). Dimethyl acetamide (DMAc) and acetone were obtained from RCI Lab Scan Ltd. (Samutsakorn, Thailand). Lithium chloride (LiCl) was purchased from Ajax Finechem Co., Ltd. (Auckland, New Zealand). The *S. aureus* (ATCC 6538) and *E. coli* (ATCC 25922) strains were gifted from the Department of Microbiology, Chulalongkorn University. Difco™ Trypticase soy broth (TSB) was supplied from Becton, Dickinson and Co. (Franklin Lakes, New Jersey, USA).

2.2. Preparation of CTCMA and CTCPA

Chitin was first purified by dissolving in DMAc/5% (w/v) LiCl and precipitating in acetone. The purified chitin (2 g; 0.01 equivalent mole to pyranose) was then dissolved in 150 mL DMAc/5% (w/v) LiCl and then CMA (3.07 g; 0.02 mol) was added and stirred until completely dissolved. The acylation of chitin with CMA was performed using DCC (8.24 g; 0.04 mol) as the coupling agent at room temperature (~25–28 °C) for 24 h. The mixture was then centrifuged to remove the *N,N*'-dicyclohexylurea (DCUrea) by-product. The supernatant was precipitated in acetone, rinsed with distilled water, washed with acetone and dried under vacuum to yield the CTCMA. The same procedure was used to synthesize CTCPA except using CPA (3.63 g; 0.02 mol) in place of CMA. The chemical structure of CTCMA and CTCPA are shown in Scheme 1.

2.3. Synthesis of CTDDMAB

The preparation of CTDDMAB was slightly different from that of CTCMA and CTCPA since DDMAB has a carboxylate ion. Therefore, the carboxylate ion (COO^-) of DDMAB was first transformed to a carboxyl group (COOH) by the addition of hydrogen chloride (HCl) gas to a DDMAB (5.99 g; 0.02 mole) solution (10 mL DMAc/5% (w/v) LiCl) and the excess HCl removed by evaporation. The resulting DDMAB-HCl solution was then mixed with chitin solution (2 g; 0.01 equivalent mole to pyranose), and the acylation was performed as in Section 2.2 to obtain CTDDMAB. The chemical structure and synthesis of CTDDMAB is summarized in Scheme 1.

2.4. Structural characterization of quaternary ammonium chitin derivatives (CTCMA, CTCPA and CTDDMAB) and their degree of substitution (DS)

The chemical structure of each quaternary ammonium chitin was confirmed by FT-IR analysis (Nicolet 6700, Thermo Scientific, Madison, Wisconsin, USA). The obtained quaternary ammonium chitin was ground with potassium bromide (KBr) into a pellet. The IR spectra were scanned for 64 scans in the range of 4000–400 cm^{-1} at a resolution of 4.0 cm^{-1}.

The degree of substitution (DS) of quaternary ammonium on the chitin was estimated from the degree of acylation, calculated from the integral ratios between the carbonyl groups of the ester/amide I and C—O of the pyranose rings, as shown in Eq. (2);

$$DS(acylation) = (A_{1735} + A_{1650})/A_{1072} \quad (2)$$

where A_{1735}, A_{1650} and A_{1072} are the integral areas under the peak of the ester around 1735 cm^{-1}, amide I around 1650 cm^{-1} and the C—O of pyranose ring at 1072 cm^{-1}, respectively. However, the final DS should be subtracted from the original chitin.

The structure of the chitin and quaternary ammonium chitin derivatives were also characterized by solid state cross-polarization/magic angle spinning (CP/MAS) ^{13}C NMR (400 MHz) using an ASCEND 400WB Bruker spectrometer (Bremen, Germany). ^{13}C NMR spectrometer resonate at frequency of 100 MHz, 3600 scans. The contact and delay times were set at 2 ms and 3 s, respectively.