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Characterization and antibacterial mechanism of poly(aminoethyl) modified chitin synthesized via a facile one-step pathway

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Chitin (PubChem CID: 6857375)
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2-Chloroethylamine hydrochloride (PubChem CID: 9793737)
Propidium iodide (PubChem CID: 104981)
o-Nitrophenyl-β-D-galactopyranoside (PubChem CID: 219973)
Coomassie brilliant blue G-250 (PubChem CID: 61363)

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ABSTRACT

This work aims to synthesize poly(aminoethyl) modified chitin (PAEMC) and ascertain its antibacterial activity and mechanism. FTIR and ^{1}H NMR results proved aminoethyl moieties were grafted to C6−OH and C3−OH on chitin backbone in the form of polymerization. XRD and TG/DTG analyses manifested its well-defined crystallinity and thermostability. PAEMC, with average molecular weight (MW) of 851.0 kDa, degree of deacetylation (DD) of 27.95%, and degree of substitution (DS) of 1.77, had good solubility in aqueous solutions over the pH range of 3–12, and also possessed high antimicrobial activity against *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus proteus*, and *Klebsiella pneumoniae*, commonly causing chronic wound infections. Nucleic acid release, protein leakage, increased inner membrane permeability, and decreased cell surface hydrophobicity implied bacterial cytomembranes were substantially compromised in the presence of PAEMC. Microscopically, PAEMC visually perturbed bacteria, illustrating deformed and even collapsed morphologies. Overall, PAEMC possessed good solubility, effectively destroyed bacteria via a membrane damage mechanism, and might serve as an antibacterial agent for treatments of chronic wound infections.

1. Introduction

Chronic wounds, usually related to neuropathy (*e.g.*, diabetic foot or pressure ulcers), vasculopathy (*e.g.*, venous stasis or arterial insufficiency ulcers), or trauma (Lipsky & Hoey, 2009), are increasingly receiving a great deal of attention, due to serious harmfulness to patients and large expenditures on treatments and therapies (Han & Ceilley, 2017). Unlike normal wound healing that inevitably undergoes four phases, *i.e.*, hemostasis, inflammation, proliferation, and remodeling (Frykberg & Banks, 2015), chronic wounds tend to be trapped in the inflammatory phase for a protracted period and during such a long period, microorganisms prefer colonizing at/near wound sites and further spread infections to deep tissue when reaching critical colonization, eventually resulting in failure of wound healing (Powers, Higham, Broussard, & Phillips, 2016). To prevent and/or control chronic wound infections, antimicrobial agents, such as antibiotics and antiseptics (*e.g.*, metronidazole, silver-based composites, and iodine-containing compounds), have long been used empirically to incorporate with wound dressings for topical administration (Gulati et al., 2014; Moon & Crabtree, 2003; Paul & Pieper, 2008; Politano, Campbell, Rosenberger, & Sawyer, 2013). Although some of them are effective against pathogenic microorganisms, the increasing undesirable outcomes, including antibiotic resistance (Tzaneva, Mladenova, Todorova, & Petkov, 2016), silver resistance (Randall, Gupta, Jackson, Busse, & O'Neill, 2015), contact dermatitis (Calow, Oberle, Bruckner-Tuderman, Jakob, & Schumann, 2009), and toxicity (Hirsch et al., 2010), are depriving surgeons of interest in clinical practice. It is thus crucial to develop new antimicrobial agents as alternatives to antibiotics and antiseptics currently used for preventing and/or controlling chronic wound infections.

Over the last decades, scientists have always been committed to developing new antimicrobial agents within their respective research areas. Numerous studies in the biomaterial field focus on chitin/chitosan (it is here termed chitosan (CS) when the degree of deacetylation

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(DD) is higher than 60%; conversely, the term chitin is used when DD is lower than 40%.) and their derivates, owing to excellent biocompatibility, high hemostatic activity, low immunogenicity, acceptable toxicity or non-toxicity, tunable biodegradability, and desirable antibacterial activity (Anitha et al., 2014). To improve the antibacterial activity and/or solubility of chitin/CS, some strategies have been deployed via the introduction of functional moieties to active sites (*i.e.*, C6−OH, C2−NH_2, or C3−OH) on polysaccharide chains (Pillai, Paul, & Sharma, 2009). Varieties of chitin/CS-based derivatives, such as diethylaminoethyl chitin (Kim, Kim, & Choi, 1997), quaternary ammonium CS (Jia, Shen, & Xu, 2001; Kim, Choi, Chun, & Choi, 1997; Xu et al., 2011), aromatic CS Schiff base derivative (Tamer et al., 2016), dicyandiamide CS (Khalil, Saad, Negim, & Saleh, 2015), and CS-thioglycolic acid (Geisberger et al., 2013), have been prepared, and most of them are of good solubility and high antibacterial activity against Gram-positive and Gram-negative bacteria. For instance, CS-thioglycolic acid soluble in normal saline (NS) kills 99.9–100% of each tested microorganism within 30 min; aromatic CS Schiff base derivative (II) shows better inhibitory activity against *E. coli* and *S. aureus* than CS at the concentration of 50 μg/mL. Despite these benefits, some of the synthetic procedures are multi-step and time-consuming, leading to high manufacturing costs and uncontrollable quality of products. Besides, the extract of hydroxypropyltrimethyl ammonium chloride CS with a higher degree of substitution exhibits toxicities towards L929 under the experimental conditions (Peng et al., 2010). Accordingly, developing novel chitin/CS derivatives for antibacterial applications and adopting facile synthesis approaches remain very interesting and significant.

The antibacterial mechanism of CS is not well understood so far, though several hypotheses have been tentatively proposed: (1) CS selectively chelates with trace metals to inhibit bacterial growth (Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003); (2) CS binds nucleic acids through penetration to nuclei of microorganisms to impede RNA and protein syntheses (Goy, de Britto, & Assis, 2009); (3) CS alters bacterial cytomembrane permeability via binding to cytomembranes by electrostatic interactions, causing the death of cells (Tao, Qian, & Xie, 2011). Among them, the most approved one is the third, based on which, some multi-amino derivatives have been synthesized recently, like 6-aminoethylamino-6-deoxy CS (Hu et al., 2016), aminoethyl modified CS (Meng et al., 2012), 3,6-*O*-[*N*-(2-aminoethyl)-acetamidetyl]-chitosan (AACS) (Yan et al., 2016), poly(aminoethyl) modified CS (Zhang et al., 2017), and 3,6-*O*-*N*-acetylethylenediamine modified CS (Dang et al., 2018). Interestingly, these CS-based derivatives possess improved solubility and high antibacterial activity against the bacteria tested.

If the solubility of chitin derivatives is related to crystallinity and/or hydrophilic groups, then decreasing the crystallinity via chitin alkalization and grafting aminoethyl moieties onto chitin skeleton will enhance the solubility. Also, if the antibacterial activity of chitin derivatives is related to amino groups, then the introduction of aminoethyl moieties to chitin chains will improve the antibacterial activity. A new strategy to synthesize a multi-amino chitin derivative using a one-step method was here proposed, *i.e.*, poly(aminoethyl) chitin (PAEMC) is prepared via grafting aminoethyl moieties onto alkaline chitin chains. If this protocol is available, PAEMC is very likely to possess not only outstanding solubility but also high antimicrobial activity.

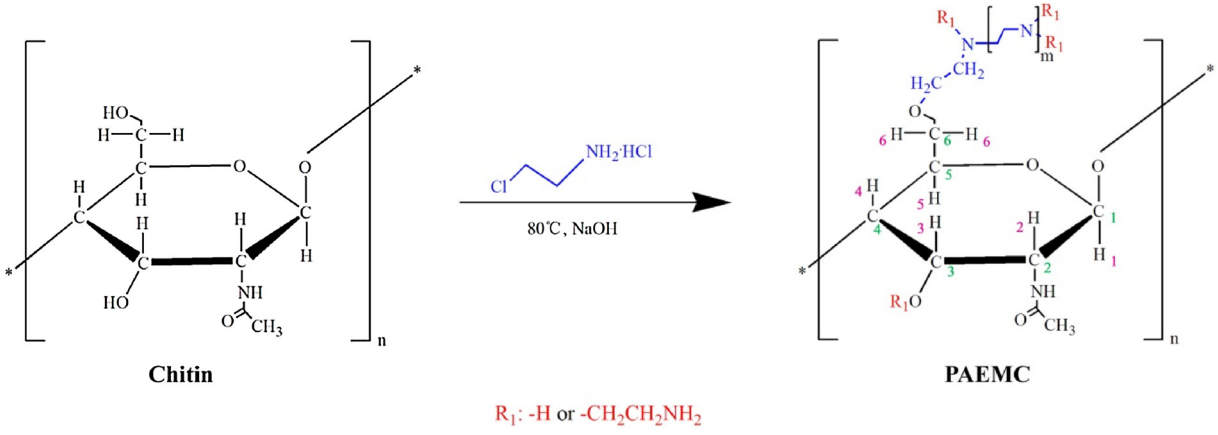
Work presented here demonstrated that novel PAEMC was successfully synthesized through a one-step pathway, which was confirmed by FTIR and ^{1}H NMR techniques. Its crystallinity and thermostability were characterized by XRD and TG/DTG analyses. The solubility of PAEMC was investigated in different solvents and solutions at pH values ranging from 3 to 12. Its average molecular weight (MW), DD, and degree of substitution (DS) were calculated on the basis of NH_2 (%) measurements. Six bacteria commonly causing chronic wound infections were chosen as test strains, and a series of tests and observations, including minimum inhibitory concentration, fluorescence microscopy, cell membrane integrity, protein leakage, inner membrane permeability, cell surface hydrophobicity, and scanning electron microscopy, was respectively conducted to ascertain the antibacterial activity and mechanism of PAEMC, and preliminarily verify its feasibility to use as an antibacterial agent.

2. Materials and methods

2.1. Materials and strains

Chitin (MW ≥ 638.00 kDa; DD 6.00%) derived from *Metapenaeus ensis* shells was purchased from Qingdao Baicheng Marine Biological Resources Development Co., Ltd. (China). Coomassie brilliant blue (CBB) G-250, 2-chloroethylamine hydrochloride, propidium iodide (PI), o-nitrophenyl-β-D-galactopyranoside (ONPG), hexadecane, and nutrient broth medium were commercially procured from Sigma Chemical Co. (St. Loius, MO, USA). All the other chemical reagents used were of analytical grade, and were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China).

Staphylococcus epidermidis (*S. epidermidis*, ATCC 12228), *Staphylococcus aureus* (*S. aureus*, ATCC 25923), *Pseudomonas aeruginosa* (*P. aeruginosa*, ATCC 27853), *Escherichia coli* (*E. coli*, ATCC 25922), *Bacillus proteus* (*B. proteus*, ATCC 22929), and *Klebsiella pneumoniae* (*K. pneumoniae*, ATCC 700603) were kindly supplied by the Central Laboratory of Qingdao University.



Scheme 1. Schematic synthetic procedure of PAEMC. PAEMC, poly(aminoethyl) modified chitin.