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Antibacterial action mode of quaternized carboxymethyl chitosan/poly(amidoamine) dendrimer core–shell nanoparticles against *Escherichia coli* correlated with molecular chain conformation

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ABSTRACT

The action mode of quaternized carboxymethyl chitosan/poly(amidoamine) dendrimer core–shell nanoparticles (CM-HTCC/PAMAM) against *Escherichia coli* (*E. coli*) was investigated via a combination of approaches including measurements of cell membrane integrity, outer membrane (OM) and inner membrane (IM) permeability, and scanning electron microscopy (SEM). CM-HTCC/PAMAM dendrimer nanoparticles likely acted in a sequent event-driven mechanism, beginning with the binding of positively charged groups from nanoparticle surface with negative cell surface, thereby causing the disorganization of cell membrane, and subsequent leakage of intracellular components which might ultimately lead to cell death. Moreover, the chain conformation of polymers was taken into account for a better understanding of the antibacterial action mode by means of viscosity and GPC measurements. High utilization ratio of positive charge and large specific surface area generated from a compacted conformation of CM-HTCC/PAMAM, significantly different from the extended conformation of HTCC, were proposed to be involved in the antibacterial action.

1. Introduction

Chitosan, being a natural nontoxic biopolymer, has been studied extensively for its role as an antimicrobial agent with a broad spectrum of antibacterial activity against both Gram-positive and Gram-negative bacteria [1–3]. It is well known that the antibacterial activity of chitosan involves its polycationic nature, and chitosan exerts its growth inhibition effect only upon the pH being below its pKa (~6.5), where amino groups at the C-2 of chitosan are protonated. Introduction of quaternary ammonium groups [4–7], carboxymethyl groups [8] or dendrimer [9–11] to chitosan chains has been demonstrated as one of the effective approaches to obtain water-soluble chitosan derivatives with improved antibacterial activity over a broader pH range.

Despite the fact that the antibacterial activity of chitosan and chitosan derivatives in the form of solution is well documented, the exact action mode of them, particularly of dendronized chitosan derivatives, has remained not fully understood hitherto. Regarding how chitosan or chitosan derivatives act upon the bacteria, one prevailing mechanism assumes that the electrostatic interaction between the positively charged chitosan backbone and the negatively charged microbial cell surface alters the cell permeability and subsequently leads to the leakage of proteinaceous and other intracellular constituents, ultimately causing the death of the cell [3,12,13].

Recently chitosan or chitosan derivatives in the form of nanoparticles have been shown to be more active than the corresponding solution with respect to antibacterial activity [11,14–16]. In general, it is proposed that both their large specific surface area and the positive charge density of the nanoparticles, are together responsible for their remarkably improved growth inhibition effect as compared to chitosan solution [15,16]. The unique specific surface area will result in a strong adsorption among bacteria and nanoparticles, whereas the positive charge density will govern the interaction between the polymers and the negatively charged surface of bacterial cells, both of which eventually kill the cell. Unfortunately, few studies on the correlations between antibacterial activity and molecular conformation of chitosan and chitosan derivatives have been performed to the best of our knowledge, although polymers' chain conformation is known to considerably affect their physical and biological properties.

Novel water-soluble chitosan-based nanoparticles consisting of a poly(amidoamine) dendrimer (PAMAM) core and a quaternized carboxymethyl chitosan (CM-HTCC) shell were recently reported by our group [11]. These nanoparticles were found to exert stronger antibacterial activity against Gram-negative bacteria *Escherichia coli* (*E. coli*) than N-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride (HTCC) [11]. In the present study, the potential antibacterial action mode of CM-HTCC/PAMAM nanoparticles was further investigated

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using a combination of approaches, including measurement of cell membrane integrity, outer membrane (OM) permeability, inner membrane (IM) permeability, as well as scanning electron microscopy. Additionally, molecular chain conformation of CM-HTCC/PAMAM was taken into account for a better understanding of the antibacterial action mode by means of viscosity and GPC measurement.

2. Experimental

2.1. Materials

Chitosan (CS) with a degree of deacetylation about 80% (mean molecular weight 2.0×10^5 Da) was purchased from Qingdao Hecreat Bio-tech Co., Ltd. (Shandong Province, China). Methyl acrylate (MA) and ethylenediamine (EDA) were redistilled just before use. 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Shanghai Medpep Co., Ltd. (Shanghai, China). 3-Chloro-2-hydroxypropyltrimethyl ammonium chloride aqueous solution (CTA, 69% w/v) was obtained from Dongying Guofeng Fine Chemical Co., Ltd. (Shandong Province, China). 1-*N*-phenylnaphthylamine (NPN) was purchased from TCI Development Co., Ltd. (Shanghai, China). *O*-Nitrophenyl-β-D-galactoside (ONPG) was obtained from Bio. Basic Inc. (Canada). All other chemicals were of analytical grade and used without further purification.

2.2. Preparation of CM-HTCC/PAMAM dendrimer nanoparticles

The CM-HTCC/PAMAM dendrimer nanoparticles were prepared according to our method previously reported [11]. CM-HTCC was synthesized via quaternization of chitosan at C-2 with 3-Chloro-2-hydroxypropyltrimethyl ammonium chloride aqueous to yield HTCC with a degree of quaternization (DQ) of 74%, followed by carboxymethylation of HTCC at C-6 with chloroacetic acid to yield CM-HTCC with a degree of carboxymethylation (DC) of 76%. The preparation of CM-HTCC/PAMAM dendrimer involved a two-step reaction: the initial activation of carboxylic groups in CM-HTCC chains with EDC/NHS and then the subsequent condensation reaction of the amino groups from full generation amino terminated PAMAM (G1.0 and G2.0) with the activated carboxylic groups of CM-HTCC at the molar ratio of carboxylic groups to PAMAM 1:4. The structure of CM-HTCC/PAMAM dendrimers is proposed as depicted in Scheme 1. The formation of CM-HTCC/PAMAM core–shell nanoparticles with PAMAM cores and CM-HTCC shells were obtained via self-aggregation in aqueous solution, driven by the combination of hydrophobic interaction, hydrogen bonding and electrostatic interaction.

2.3. Viscosity measurements

Viscosity measurements were carried out following gradient dilution method through an Ubbelohde capillary viscometer in a series of HAc–NaAC buffer solutions (pH ~ 3.7) at different ionic strength at 30 ± 0.1 °C. Concentrations of all the sample solutions containing HTCC, CM-HTCC and CM-HTCC/PAMAM respectively varied from 0.4 to 3.0 mg/ml.

2.4. Gel permeation chromatography (GPC) measurements

Weight average molecular weight (M_w), intrinsic viscosity ([η]), hydrodynamic volume (R_h) and molecular weight distribution in terms of polydispersity index (M_w/M_n) of chitosan derivatives were determined using GPC equipped with multi-detector including viscosity, UV, differential refractive index and multi angle static lighting scattering detectors (TDA305, Malvern Instruments, England) at 30 °C. A 6000 M chromatographic column (300 × 7.8 mm) was used. 0.2 mol/l HAc–0.1 mol/l NaAC buffer solution was selected as eluant at a flow rate of 0.5 ml/min. Sample solutions were filtered through 0.45 μm of filter prior to GPC measurement. The injection volume was 100 μl.

2.5. Cultivation of the bacteria

Gram-negative bacteria *E. coli* provided by the College of Biotechnology of Tianjin University of Science & Technology, were cultivated in nutrient broth (peptone 1%, beef extract 0.5%, NaCl 0.5%, pH 7.4) at 37 °C with shaking overnight. The cultures were diluted with sterile nutrient broth to obtain cell suspensions containing ~10^7 CFU/ml used for the further study.

2.6. Morphology of the bacteria

Scanning electron microscopy (SEM) analyses were performed to observe the morphological changes of *E. coli* following treatment with HTCC or CM-HTCC/PAMAM dendrimer nanoparticles at the concentration of 200 mg/l based on bacterial growth inhibition assays [11]. HTCC was directly dissolved in sterile water to reach final solution, whose pH was adjusted to about 7.4 with 10% NaOH solution, whereas CM-HTCC/PAMAM dendrimer nanoparticle powders were dissolved in diluted hydrochloric acid (pH 5), yielding the resultant solutions with the same pH to that of HTCC following the same method. The above solutions were sterilized by filtration with 0.22 μm of filter membrane just before use.

Overnight cultured *E. coli* was centrifuged at 1000 × *g* for 10 min. The resulting pellets were washed once with 0.85% NaCl solution, then re-suspended in 0.1 mol/l sodium phosphate buffer solution (PBS) with pH 7.2 to $OD_{630\ nm}$ ~ 0.4. 3 ml of bacterial suspensions was re-centrifuged and mixed with the same volume of HTCC or CM-HTCC/PAMAM nanoparticle solutions. After 0.5 h or 1.5 h of treatment with sample solutions at 37 °C, the cells were washed twice with 0.1 mol/l PBS and then were fixed with 2.5% glutaraldehyde in 0.1 mol/l PBS at 4 °C for over 4 h, followed by washing twice with the same buffer solution. The samples were dehydrated with a series of ethanol solutions (30%, 50%, 70%, 80%, 90% and 100%), air dried, and then were coated with gold and observed under SEM (Hitachi S-4800 Scanning Electron Microscope, Japan) at 5 kV. The bacteria without treatment with sample solutions served as controls.

2.7. Cell membrane integrity assays

If the bacterial membrane is compromised, release of cytoplasmic constituents of the cell mainly including DNA and RNA can be monitored by determination of their characteristic absorption at 260 nm [3,17,18]. Overnight cultured *E. coli* was harvested, washed and re-suspended in 0.5% NaCl solution to $OD_{420\ nm}$ ~ 0.7. 1.5 ml of bacterial cell suspensions was treated with HTCC or CM-HTCC/PAMAM dendrimer nanoparticle solutions (pH 7.4) at required concentrations. The absorbance of cell-free supernatants was measured at 260 nm until no further increase in absorbance was observed with a spectrophotometer (UV1102, Shanghai Tian Mei Scientific Instrument Co., Ltd., China). The bacteria without treatment with sample solutions served as controls.

2.8. Outer membrane (OM) permeabilization assays

The OM permeabilization of *E. coli* treated with solutions containing HTCC or CM-HTCC/PAMAM dendrimer nanoparticles was evaluated using the hydrophobic 1-N-phenylnaphthylamine (NPN) fluorescent probe [3,13,18]. Overnight cultured *E. coli* was harvested, washed and re-suspended in 0.5% NaCl solution with an absorbance of 1.0 at 420 nm. 1.5 ml of HTCC or CM-HTCC/PAMAM dendrimer nanoparticle solutions (pH 7.4) at required concentrations and 1 ml of *E. coli* suspensions were mixed with 20 μl of 1 mM NPN, whereas the controls were carried out with sterile water alone. Fluorescence, with an excitation