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Silver deposited carboxymethyl chitosan-grafted magnetic nanoparticles as dual action deliverable antimicrobial materials

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ABSTRACT

Carboxymethyl chitosan (CMCS) was known to have a much better antimicrobial activity than chitosan due to the increased cationic $-NH_3^+$ groups resulted from the intra- and intermolecular interactions between the carboxyl and amino groups. CMCS was grafted onto the surface of silica coated magnetic nanoparticles (MNPs) to obtain magnetically retrievable and deliverable antimicrobial nanoparticles (MNPs@CMCS). The presence of carboxylate groups in CMCS not only enhanced antimicrobial activity but also enabled Ag ions chelating ability to induce the *in situ* formation of Ag nanoparticles (AgNPs). The deposition of AgNPs on the surface of MNPs@CMCS could significantly increase its antimicrobial activity against planktonic cells due to the dual action of CMCS and AgNPs. Due to its high magnetism, the as-prepared MNPs@CMCS-Ag could be efficiently delivered into an existing biofilm under the guidance of an applied magnetic field. Without direct contact, the Ag ions and/or radical oxygen species (ROS) released from the deposited Ag nanoparticles could effectively kill the bacteria embedded in the extracellular polymeric substances (EPS) matrix of biofilm.

1. Introduction

Chitosan, a biopolymer, has been widely studied for its pharmaceutical and medical applications due to its valuable characteristics such as biorenewability, biodegradability, biocompatibility, bioadhesivity and nontoxicity. Chitosan and its derivatives are used in various fields such as biomedicine, water treatment, cosmetics, agriculture and food industry [1]. Over the past few decades, the antibacterial and antifungal activities of chitosan have been studied with a great interest [2–5]. However, chitosan exhibits its antibacterial activity only in an acidic medium due to its poor solubility at pH above 6.5 [6,7]. Among many chitosan derivatives prepared to enhance its water solubility [8–10], carboxymethyl chitosan (CMCS) is the one has been widely studied because of its easy synthesis, amphoteric character, and enhanced antimicrobial activity [11]. The enhanced activity can be explained based on the interaction between carboxyl and amino groups in CMCS structure that favors the formation of cationic $-NH_3^+$ groups that leads to a stronger electrostatic interaction to the negatively charged bacterial cell membrane. Through this interaction process, CMCS can disrupt the normal functions of cell membrane by promoting cell lysis and inhibiting nutrients transport [12]. Moreover, CMCS is soluble in a wider pH range from 3 to 11 that makes it has broader applications as an antibacterial agent [7,11]. CMCS coating has been demonstrated to reduce the adhesion of *Escherichia coli* and *Proteus mirabilis* to the surface of medical materials by 90% [13]. Its antibacterial activity against *Escherichia coli* and *Micrococcus luteus* has also been demonstrated by its coating on cotton fabrics [14]. CMCS grafted on magnetic nanoparticles has shown its ability to disrupt biofilms of *Staphylococcus aureus* and *Escherichia coli* under the influence of applied magnetic field [12].

Although Chen et al. [12] has demonstrated that CMCS functionalized magnetic nanoparticles (MNPs@CMCS) not only has strong bactericidal activities against both Gram-positive *S. aureus* and Gram-negative *E. coli* planktonic cells but also the biofilms disruption ability when a magnetic field is applied. However, the bactericidal activity of MNPs@CMCS is basically resulted from its direct contact with bacterial cells that facilitated the membrane-disruptive effect of CMCS. In other words, only the bacterial cells in contact with CMCS can be killed by MNPs@CMCS. For killing the planktonic cells, the contact killing property of CMCS on MNPs is acceptable because the gentle shaking can provide enough contact between MNPs@CMCS and cells. In contrast, killing the bacterial cells embedded in the extracellular polymeric substances (EPS) matrix of biofilm by CMCS on MNPs will be very ineffective because the direct contact with cells will be significantly blocked by EPS.

Silver nanoparticles (AgNPs) are one of the popular antibacterial and antifungal agents currently employed in cosmetics, fabrics, and medical uses [15]. The antibacterial mechanisms of AgNPs are believed to involve: (i) adsorb onto the cell membrane surface leading thus to its permeability and respiration malfunctions, (ii) induce the formation of free radicals which cause membrane damage, or (iii) penetrate inside the bacteria and subsequently release silver ions. If silver ions were

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released, they can inactivate proteins and/or intercalate between the purine and pyrimidine bases of DNA [16–17]. *Via* ion exchange of Ag ions to the carboxyl groups, AgNPs can be easily *in situ* synthesized and immobilized on various carboxylated substrates such as carboxylated bacterial cellulose or other carriers to prevent the AgNPs from aggregation [18–20]. CMCS grafted on MNPs, therefore, can be a good matrix to prevent aggregation of the *in situ* synthesized AgNPs due to its high metal ions chelation capacity [19,21,22]. Tollen's reagent, $[Ag(NH_3)_2]OH$ has been confirmed to be an effective reagent to synthesize AgNPs in CMCS matrix [19,23] and on the surface of cellulose nanofibers [24] and electrospun PVA nanofibers [24]. Therefore, in this work Tollen's reagent was employed to provide Ag ions for *in situ* formation of AgNPs within the CMCS grafted on MNPs (MNPs@CMCS). The AgNPs deposited will confer MNPs@CMCS an additional non-contact killing activity since Ag ions released and/or the radical oxygen species (ROS) generated from AgNPs has known to kill bacteria very effectively [17]. The retrievable and deliverable MNPs@CMCS-Ag prepared in this work was demonstrated to have contact and non-contact dual action on killing bacteria. Not only the planktonic cells could be killed more effectively due to the newly conferred non-contact killing ability from AgNPs. Also the bacterial cells embedded in biofilm were more effectively killed by delivering MNPs@CMCS-Ag to the vicinity of bacterial cells embedded in the EPS matrix with the guidance of an applied magnetic field to release enough dose of Ag ions and ROS.

2. Experimental section

2.1. Materials

Low molecular weight of chitosan (M_v ~ 58 kDa, calculated by measuring intrinsic viscosity and using the Mark–Houwink–Sakurada equation) [26] with degree of deacetylation approximately 90% was purchased from Shin Era Technology Co., Ltd., Taiwan. Magnetic nanoparticles (Bayoxide E 8706) were obtained from Lanxess Energizing Chemistry, Germany. Monochloroacetic ($ClCH_2COOH$, 99 +%), tetraethoxysilane (TEOS, 98%), 3-aminopropyltriethoxysilane (APTES, 99%), sodium cyanoborohydride ($NaCNBH_3$, 95%), Ninhydrin (reagent ACS), sodium metasilicate nonahydrate ($Na_2SiO_3.9H_2O$) were purchased from Acros. Sodium hydroxide (NaOH, Laboratory Reagent Grade), Glutaraldehyde (GA, 25% aqueous solution) was purchased from Alfe Aesar. Silver nitrate ($AgNO_3$, 99%) was purchased from Sigma-Aldrich. *E. coli* BL21 (Novagen, Madison, WI, USA) and *S. aureus* ATCC6538P (Taiwan Textile Research Institute, Taiwan).

2.2. Carboxymethyl chitosan (CMCS) preparation

In brief, 15 g chitosan and 9 g monochloroacetic acid were suspended in 150 mL, 42% (*w*/*v*) sodium hydroxide solution. The mixture was reacted at −20 °C for 48 h then the pH was adjusted to 1.0 using hydrochloric acid solution. The solid in the solution was considered as CMCS product. After filtered out on filter paper, the CMCS solid was washed twice with methanol and dried in an oven at 60 °C [7].

2.3. CS/CMCS grafted magnetic nanoparticles (MNPs@CS, MNPs@CMCS)

The procedure for grafting CS or CMCS on MNPs was based on the report for the preparation of aldehyde surface-functionalized particles [27]. MNPs of 2 g were dispersed in 120 mL aqueous solution containing 4.6 g $Na_2SiO_3.9H_2O$ and 0.4 g NaOH. After ultrasonication for 5 min in an ultrasonic bath (Branson 5800), the pH of the MNPs suspension was adjusted to 6.0 by slow addition of 2 M HCl. The MNPs were then collected by a magnet and washed thoroughly with deionized water and designated as MNPs@SiO_2. MNPs@SiO_2 were further coated with a porous silica layer by using a sol–gel process based on the hydrolysis of TEOS in ethanol/ammonia solution. In brief, the collected MNPs@SiO_2 were dispersed by ultrasonication in a solution consisted of 50 mL ethanol, 50 mL deionized water and 1 mL, 28 wt% ammonia solution. TEOS solution (0.2 mL TEOS in 10 mL of ethanol) was then added at a rate of 1 mL/min under mechanical stirring and the reaction was allowed to proceed at 85 °C for 0.5 h. Thus MNPs@SiO_2@TEOS were obtained after washing several times with deionized water. The MNPs@SiO_2@TEOS were again dispersed in 50 mL ethanol and 1 mL APTES was added with mechanical stirring at room temperature for 1 h to obtain surface-aminated magnetic nanoparticles (MNPs@APTES). MNPs@APTES were activated with 10 mL, 25% glutaraldehyde in 50 mL 0.1 M, pH 7.4 phosphate buffer containing 1.0 mL, 0.1 mg/mL sodium cyanoborohydride at room temperature for 6 h. CS or CMCS was then grafted onto the glutaraldehyde activated MNPs (MNPs@GA) *via* Schiff base reaction. MNPs@GA were stirred for 24 h at room temperature with 50 mL, 10 mg/mL CS or CMCS in 0.2 M acetic acid containing 0.3 g $NaCNBH_3$. The obtained CS or CMCS grafted MNPs were washed, collected and dried under vacuum at room temperature until further use. The reaction scheme for the surface modification of MNPs is shown (Scheme 1).

2.4. In situ deposition of silver on MNPs and MNPs@CMCS (MNPs@Ag and MNPs@CMCS-Ag)

The ammoniacal silver nitrate solution (Tollen's reagent) was prepared by dissolving 10.0 mg/mL silver nitrate in distilled water with slowly adding ammoniac solution until the solution became transparent [25]. MNPs@CMCS of 25 mg were added into 5 mL of the Tollen's reagent with different dilutions and shaken at 200 rpm for 30 min. Five mL glucose solution (at the concentration of twice high as the added Tollen's reagent) was added slowly into the above mixture for the reduction of Ag ion. The reaction was allowed to continue for 2 h under shaking at room temperature. The modified particles were collected by a magnet and washed twice with deionized water followed by ethanol. The washed samples were then vacuum dried at room temperature overnight and designed as MNPs@CMCS-Ag-0.1, MNPs@CMCS-Ag-0.5, MNPs@CMCS-Ag-1.0, MNPs@CMCS-Ag-5.0 and MNPs@CMCS-Ag-10.0 for samples prepared from 0.1, 0.5, 1.0, 5.0, and 10.0 mg/mL of ammoniacal silver nitrate, respectively [28]. The MNPs@Ag-0.1 were also prepared based on the same procedure using 25 mg bare MNPs in 5 mL of 0.1 mg/mL Tollen's reagent.

2.5. Antibacterial effect

E. coli and *S. aureus* cells were cultured at 37 °C overnight in Luria broth (LB) and Tryptic soy broth (TSB), respectively. The inhibition zone test of antimicrobial activity of CS suspension, CMCS solution (10 mg/mL in water) and as-prepared MNPs slurry of 10 mg dry weight in 10 mM, pH 7.4 PBS were carried out by dropping aliquot of suspensions on bacteria plated agar plates and incubated at 37 °C for 12 h. To quantify the antibacterial effect of the as-prepared samples against *E. coli*, the bacterial suspension was centrifuged at 8000 rpm for 3 min to collect the cell pellet. After washing twice with 10 mM, pH 7.4 PBS buffer, the cell pellet was then re-suspended in PBS and adjusted concentration to 0.2 OD (optical density at 600 nm) which is about ~2×10^8 CFU/mL [13,29]. MNPs suspensions of 4 and 10 mg/mL were prepared by dispersed particle samples into 5 mL, 10 mM pH 7.4 PBS buffer by ultrasonication for 5 min in an ultrasonic bath (Branson 5800). *E. coli* suspension (OD 0.2) of 5 mL was then mixed with the 5 mL MNPs suspension under rotary shaking of 200 rpm at 37 °C. During shaking incubation, the colony-forming unit (CFU) in the cell suspension was measured from time to time by decanting MNPs using an applied external magnetic field.

2.6. Minimum inhibitory concentration (MIC)

Various concentrations of MNPs@CMCS-Ag (0– 2.0 mg/mL) were added into LB medium containing *E. coli* of ~10^5 CFU/mL. The growths of these *E. coli* cultures at 37 °C were measured at 600 nm using an