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Controlled release and antibacterial activity chlorhexidine acetate (CA) intercalated in montmorillonite

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ABSTRACT

The aim of this study was to prepare chlorhexidine acetate (CA)/montmorillonite intercalation composites and its antibacterial potential was evaluated with pathogenic bacteria, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The CA/MMT was characterized by X-ray diffraction (XRD), Fourier transformed infrared (FT-IR), and thermogravimetric analysis (TGA). CA was successfully intercalated into the interlayer of MMT and in vitro release properties of the intercalated CA have been investigated in phosphate buffered saline media (pH 7.4) at 37 ± 0.5 °C. At drug release study, CA showed initial burst effect for 24 h and then continuously released for 72 h. Their antibacterial activity was assayed by the inhibitory zone method. The CA/MMT was tested for antimicrobial activity against *S. aureus* and *P. aeruginosa*. The CA/MMT strongly inhibited the growth of a wide variety of microorganisms, including Gram-positive bacteria, Gram-negative bacteria.

1. Introduction

Chlorhexidine acetate which has broad-spectrum antibacterial activity against Gram-positive and Gram-negative bacteria (Aly and Maibach, 1979), has been considered the most acceptable for reducing nosocomial transmission of infections in intensive care units (Frantz et al., 1997). The most common nosocomial pathogens are Gram-positive bacteria such as *Staphylococcus aureus* and Gram-negative pathogens such as enterobacteriacae and *Pseudomonas aeruginosa*. The chlorhexidine-loaded nanocapsules, exhibiting an immediate and long-lasting antimicrobial activity against (i) several bacterial strains in vitro, and (ii) *Staphylococcus epidermidis* inoculated artificially onto skin surface for 8 h, might constitute a promising strategy for lowering the frequency of disinfection (Labadie et al., 2002). The sustained antiseptic effect was attributed to the severe adsorption of positively charged nanocapsules to the cell wall of bacteria, and the subsequent chlorhexidine diffusion through the polymer and the bacterial membrane (Lboutounne et al., 2004).

Montmorillonite (MMT) clay is one of the smectite group, composed of silica tetrahedral sheets layered between an alumina octahedral sheets. The imperfection of the crystal lattice and the isomorphous substitution induce a net negative charge that leads to the adsorption of alkaline earth metal ions in the interlayer space. Such imperfection is responsible for the activity and exchange reactions with organic compounds. MMT also contains dangling hydroxyl end-groups on the surfaces (Khalil et al., 2005). MMT has large specific surface area; exhibits good adsorb ability, cation exchange capacity, standout adhesive ability, and drug-carrying capability. Thus, MMT is a common ingredient as both the excipient and active substance in pharmaceutical products (Wang et al., 2008). The intercalation of organic species into layered inorganic solids provides a useful and convenient route to prepare organic–inorganic hybrids that contain properties of both the inorganic host and organic guest in a single material (Mohanambe and Vasudevan, 2005).

In recent years, smectite clays intercalated by drug molecules have attracted great interest from researchers since they exhibit novel physical and chemical properties. Zheng et al. (2007) have investigated the intercalation of ibuprofen into MMT as a sustained release drug carrier. Lin et al. (2002) studied the intercalation of 5-fluorouracil with MMT as drug carrier. Fejer et al. (2001) reported intercalation and release behavior of promethazine chloride and buformin hydrochloride from MMT. Dong and Feng (2005) synthesized the poly(D,L-lactide-co-glycolide)-MMT nanoparticles by the emulsion/solvent evaporation method for oral delivery of

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paclitaxel. Park et al. (2008) studied the intercalation of donepezil intercalated in smectite clays as drug carrier Joshi et al. (2009) studied montmorillonite as a drug delivery system in vitro release of timolol maleate. Pharmacology studies have revealed that montmorillonite (MMT) adsorbed bacteria such as *Escherichia coli*, *S. aureus* and immobilize cell toxins (Hu and Xia, 2006; Zhou et al., 2004). Some researchers found that natural clay minerals showed no antibacterial effect, but could adsorb and kill bacteria when materials with antimicrobial activity were intercalated. There are a certain number of reports about modified MMT with antibacterial activity, such as MMT-carrying copper and silver ions as effective bacteriostasis materials (Jantova et al., 1997; Wang et al., 2007). There are relatively few publications pertaining to organically modified clay minerals showing antibacterial activity. The previous work that exists has focused on quaternary ammonium compound modified clays and their uses in water purification and cosmetics (Guilbeaux, 1988; Alther, 2000; Herrera et al., 2000, 2004).

The present paper focused on the intercalation of CA into the interlayer of MMT under different reaction conditions. The CA/MMT was characterized by XRD, FT-IR, and TG. To examine the possibility of applying the MMT/CA in drug delivery and controlled release systems, Release profile of CA from CA/MMT was carried out in the dialysis membrane. The CA/MMT composites were tested for antimicrobial activities against *P. aeruginosa* and *S. aureus*. We hope the composite of CA/MMT will reach a goal of releasing CA in situ to eliminate bacterial infection of implanted devices in the future clinical application.

2. Experiment

2.1. Materials

MMT (Na^+-MMT) with a cation exchange capacity (CEC) of 0.90 mequiv./g was supplied by ZheJiang Clay Minerals Co. Chlorhexidine acetate ($C_{22}H_{30}N_{10}Cl_2{\cdot}2C_2H_4O_2$) was supplied by Jintan Pharmaceutical Co., China, with a purity of 99%. *P. aeruginosa* (ATCC27853) and *S. aureus* (ATCC25923) were obtained from JiangSu Center for Disease Prevention and Control, China.

2.2. Preparation of the CA/MMT

In the preparation of the CA/MMT, 1 g of MMT was dispersed in 30 ml of distilled water with vigorous stirring for 0.5 h at a room temperature. Various amounts (0.33 g, 1 g, 2 g, 3 g) of CA were dissolved in 150 ml of distilled water (pH value adjusted to 4.1 with H_3PO_4). Then, the two solutions were mixed together and kept at 80 °C with vigorous stirring for 3 h. After centrifugation, the sediment was washed with deionized water, and then further dried in a vacuum oven at 80 °C for 24 h.

2.3. Characterization

Powder X-ray diffraction (XRD) patterns were recorded between 1.5° and 20° (2θ) at a scanning speed of 2° min^{-1} (D/max 2500VL/PC diffractometer, Cu Kα). The thermogravimetric measurements were performed on a PerkinElmer TG 7 instrument using a heating rate of 20 °C/min up to 800 °C in nitrogen atmosphere. An infrared spectrum is recorded within the range of 4000–400 cm^{-1} by a German Nicolet FT-IR Nexus-670 IR spectrophotometer in KBr discs.

2.4. Release of CA from CA/MMT

In vitro release studies were carried out in phosphate buffered saline media of pH 7.4 using the dialysis bag technique. Dialysis sacs were equilibrated with the dissolution medium for a few hours prior to experiments. 100 mg of CA/MMT in 5 ml of buffer solution was taken in the dialysis bag. Dialysis bag was dipped into receptor compartment containing 100 ml dissolution medium, which was shaked at 37 ± 0.5 °C.

The receptor compartment was closed to prevent the evaporation losses from the dissolution medium. The shaking frequency was kept at 100 rpm. 5 ml of sample was withdrawn at regular time intervals and the same volume was replaced with a fresh dissolution medium. Samples were analyzed for CA content by UV spectrophotometer at 260 nm. These studies were performed in triplicate for each sample and the average values were used in data analysis.

2.5. The inhibitory zone tests

The bacteria (*P. aeruginosa* ATCC27853 or *S. aureus* ATCC25923) were subcultured to nutrient agar and incubated overnight at 37 °C. Then, the cells were dispersed in the same medium to reach 10^6 CFU/ml. Agar plates were streaked with a sterile swab moistened with the bacterial suspension. The MMT and CA/MMT (20 mg) were pressed into pellets (13 mm diameter) and placed over the surface of the agar plates and then incubated. All the test plates were incubated overnight at 37 °C. The reaction of the microorganisms to the CA/MMT was determined by the size of the inhibitory zone. When the materials have an excellent antibacterial activity, the inhibitory zones are very large.

3. Results

3.1. XRD analysis

The XRD patterns of MMT and CA/MMT are shown in Fig. 1. The molecular configuration of chlorhexidine acetate is shown in Fig. 2. The basal spacing (d_{001}) of MMT was 1.53 nm, a characteristic d value for MMT (Gao et al., 2008). When the ratio of CA to MMT reached 1:3, the d-spacing of the CA/MMT was 1.51 nm, similar to that of MMT. The results indicated that with increasing content of CA, the d-spacing of MMT increased from 1.51 nm to 1.94 nm. Their basal spacings were similar and located at 1.93–1.94 nm. This d value was obviously lager than that of MMT, indicating that CA had entered into the MMT interlayer. Therefore, the ratio of CA to MMT reached 2:1 was selected as the representative material, and an in-depth feasibility study of the CA/MMT was carried out using this composition.

As is well known, the layer thickness of montmorillonite is 0.97 nm (Akelah, 1996). When the ratio of CA to MMT reached

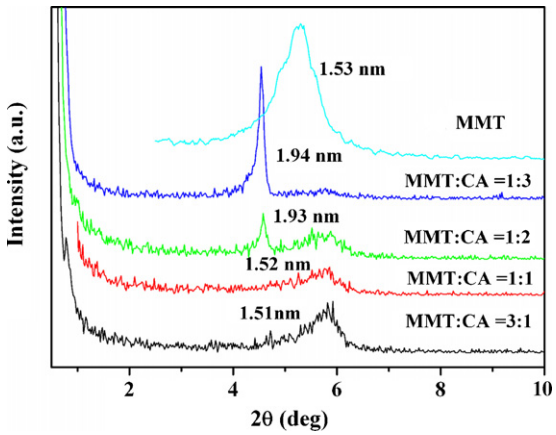

Fig. 1. X-ray powder diffraction pattern of CA/MMT composite prepared at different mass ratios (MMT:CA) and MMT.

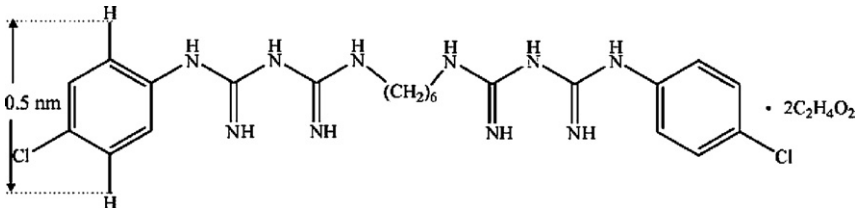

Fig. 2. The molecular configuration of chlorhexidine acetate.

1:3, the interlayer height of CA/MMT should be 0.54 nm. This value almost equals to the height of a phenol ring in CA (0.5 nm) as shown in Fig. 2 (both the heights of –CH_2–and–NH– groups are less than 0.5 nm). Hence, a lateral-monolayer arrangement is supposed for the intercalated CA in the montmorillonite interlayer. When the ratio of CA to MMT reached 2:1or 3:1, the interlayer height of CA/MMT was about be ca. 0.96 nm. This interlayer distance is bigger than that when CA adopts a lateral-monolayer arrangement (0.5 nm), but smaller than that of a lateral bilayer arrangement (ca. 1 nm). Our molecular modeling of organoclays demonstrates a transition structure from lateral-monolayer to lateral-bilayer, in which a partial overlapping of organic molecules was observed.

3.2. The FT-IR spectra analysis

Fig. 3 shows the FT-IR spectra for MMT and CA/MMT. MMT shows the characteristic absorption bands at 3400 cm^{-1} due to –OH stretching band for adsorbed water. The bands at 3620 and 3698 cm^{-1} are due to –OH band stretch for Al–OH and Si–OH. The shoulders and broadness of the structural –OH band are mainly due to contributions of several structural –OH groups occurring in the MMT. The characteristic peak at 1115 and 1035 cm^{-1} is due to Si–O stretching (out-of-plane) and Si–O stretching (in-plane) vibration for layered silicates, respectively. Peaks at 915, 875, and 836 cm^{-1} are attributed to AlAlOH, AlFeOH, and AlMgOH bending vibrations, respectively (Patel et al., 2007b). The stretching vibrations of sp^3 C–H were also seen at 2926 and 2856 cm^{-1} (Pavia et al., 1996; Singh et al., 2002). These characteristic bands were also clearly shown in the spectra of the CA/MMT, indicating that CA molecules were well stabilized in the interlayer space of clay without any chemical deterioration of functional groups.

3.3. Thermal analysis

As indicated by the TGA curves of MMT (Fig. 4), MMT is thermally stable in the temperature range of 200–600 °C. Hence, the weight loss in this temperature range should be attributed to the decomposition of CA. The TGA curves of CA/MMT showed two distinct steps. The first one of about 3.9% at 40–140 °C is due to the free water evaporation. The second step corresponded to decomposition of the organic matter present in the CA/MMT (CA-loaded amount 32.6 wt.%). The TGA curve of CA/MMT showed a sharp weight loss at around 218 °C due to the decomposition of intercalated CA. The maximum weight loss is found to vary with the type of organic modifier.

3.4. In vitro drug release studies

Intercalated species in interlayer space of smectite is typically released by ion-exchange reaction with other cations. However, bulky and hydrophobic organic cations in interlayer space cannot be easily deintercalated by ion-exchange reaction with the simple cations such as Na^+, Ca^{2+}, etc. (Jung et al., 2008). As shown in Fig. 5, CA was continuously released over 72 h and burst release of CA was observed for initial 24 h. Fig. 4 shows that only 24% of intercalated CA was released during the first 24 h. The initial burst of CA may be attributed to CA existing at or near the surface of MMT. The maximum release of CA from CA/MMT was observed to be in the range 31%. Based on the release profiles at pH 7.4, the equilibrium percentage released of CA was not up to 100%. This is probably due to the characteristic of ion-exchange reaction, i.e. this is an equilibrium process, and the interlayer cations cannot be exchanged completely.

3.5. The inhibitory zone tests of CA/MMT

The inhibitory zone results of MMT and the CA/MMT are shown in Figs. 6 and 7. The MMT showed no inhibitory zones for *P. aeruginosa* and *S. aureus*, reflecting no antibacterial activity for these materials. However, the CA/MMT composites showed very clear inhibitory zones around the specimen. The results

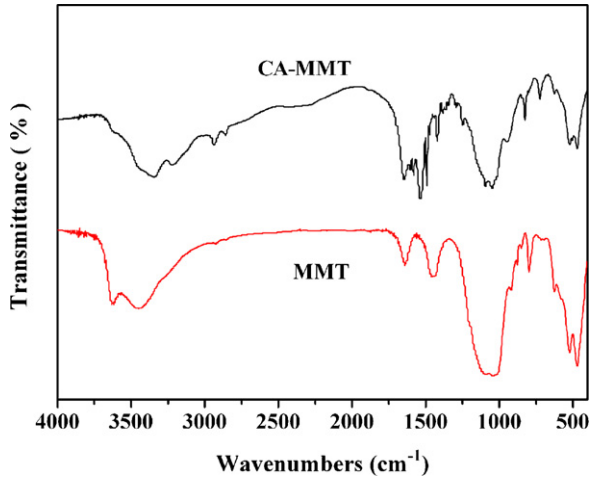

Fig. 3. The FT-IR spectra of CA/MMT (MMT:CA = 1:2) and MMT.

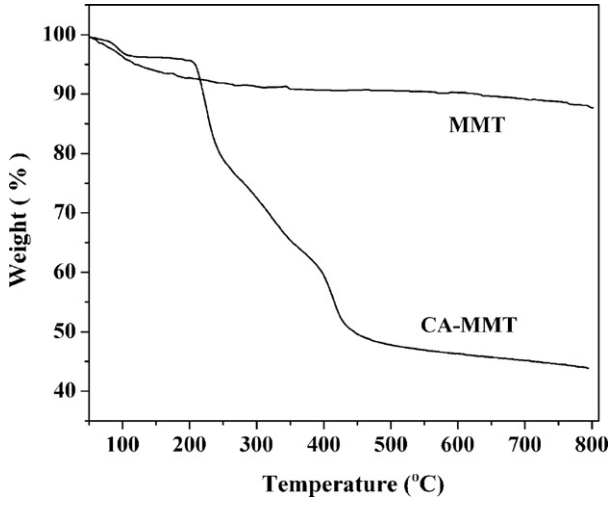

Fig. 4. TGA curves of MMT and CA/MMT (MMT:CA = 1:2).

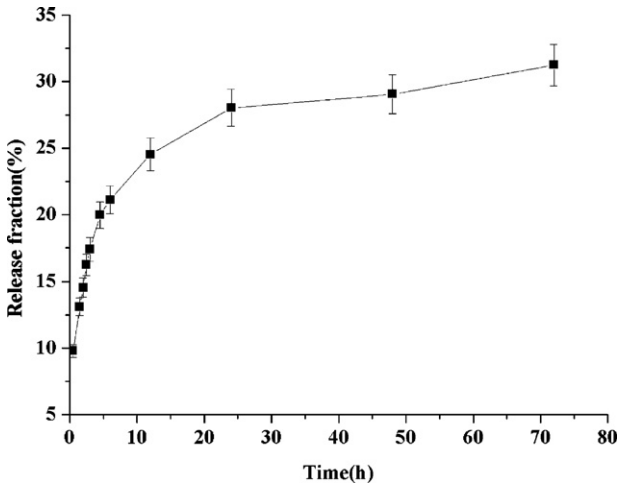

Fig. 5. Release profile of CA from the CA/MMT (MMT:CA = 1:2) in at 37 ± 0.5 °C.

Table 1
The inhibition zone (diameter in mm) of MMT and CA/MMT against *S. aureus*, *P. aeruginosa*.

Testing bacteria	The inhibition zone	
	MMT (mm)	CA/MMT (mm)
S. aureus	–	24.5
P. aeruginosa	–	21.4

Initial diameter: 13 mm; –: no inhibition.

from the zone of inhibition tests are given in Table 1. The diameter of the zone of inhibition and the amount of diffusion from the edge of each hole in the agar plate are given in mm. The inhibition zone of CA/MMT on *S. aureus* and *P. aeruginosa* were 24.5 and 21.4 mm. The results indicated that CA/MMT had stronger activity against Gram-positive than Gram-negative bacteria.

4. Discussion

Two possible mechanisms can be responsible for the observed antibacterial activity. The first mechanism involves the immobilization of the bacteria on the surface of the MMT. The surface property transformation from hydrophilic (MMT) to hydrophobic (organomontmorillonite) while the latter results from the replacement of the interlayer hydrated cations by cationic surfactant. These hydrophobic moieties on the MMT surface can interact with the lipophilic components of the bacterial cell walls, such as lipoproteins, liposaccharides, and phospholipids. Bacterial cell surfaces are negatively charged. However, the exchange of MMT with positively charged antibacterial agents produces a material that is positively charged. The charge of these exchanged MMT enhances their ability to attract bacteria through electrostatic interactions. As a result of these two types of interactions, the bacteria are brought in close association to the surface of the exchanged MMT (Dizman et al., 2007).

The second mechanism involves the release of the antibacterial agents from the MMT and exertion of their antibacterial effects on bacteria in suspension. The zone of inhibition studies indicate that there are inhibition zones for all treated MMT. This is a sign of the diffusion of the antibacterial agents from treated MMT into the agar. The mechanism of the antibacterial activity of CA: (1) adsorption onto the bacterial cell surface; (2) diffusion through the cell wall; (3) binding to the cytoplasmic membrane; (4) disruption of the cytoplasmic membrane; (5) release of the cytoplasmic constituents; (6) death of the cell.

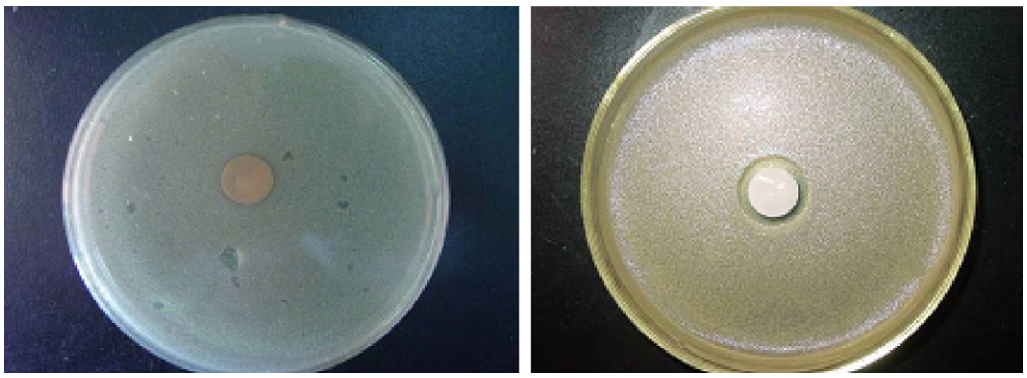

Fig. 6. Photograph of antimicrobial test of MMT and CA/MMT against *P. aeruginosa*.

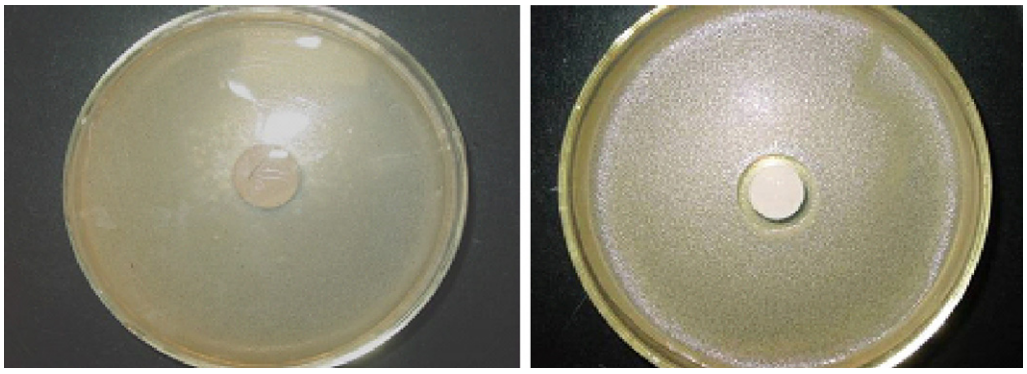

Fig. 7. Photograph of antimicrobial test of MMT and CA/MMT against *S. aureus*.

5. Conclusions

In this study, organic antibacterial composites were synthesized using montmorillonite and chlorhexidine acetate. XRD patterns of CA/MMT show an increase in the *d*-spacing, conforming the intercalation of CA into the interlayer of MMT.

In vitro release study showed that CA/MMT was burst release of CA for initial 24 h and continuously released over 72 h. Therefore, the MMT clay materials could be suggested as an advanced drug delivery carrier with controlled release characteristics.

The antibacterial activity of CA/MMT and MMT was evaluated by the inhibitory zone tests. The results showed that CA/MMT composites had broad-spectrum antibacterial capability.

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