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PLLA microcapsules combined with silver nanoparticles and chlorhexidine acetate showing improved antibacterial effect

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ABSTRACT

In this study, composite antibacterial microcapsules combining of two antibacterial agents: chlorhexidine acetate and silver nanoparticle were prepared. The chlorhexidine acetate was encapsulated inside of the microcapsules and nano-sized silver particles were modified on the surface of microcapsules by electrostatic adsorption methods. Results show that this method decreases the silver usage dramatically, and promises a sustained antibacterial effect >30 days. These microcapsules can also be modified on the surface of polymer films easily, which demonstrated the potential in functionalizing the implanted materials with antibacterial property.

1. Introduction

Researchers never stop to explore new and effective treatment against the disease-causing organisms, especially pathogenic bacterium. In recent years, researchers focus on discovering simple anti-microbial treatment of biomedical materials, and have developed some products, such as anti-microbial catheters etc. In this way, excogitating a simple antibacterial treatment, which can be effective both immediately and sustained, have drawn the attention of vast researches.

Incorporating silver nanoparticles into a wide range of medical products has become one of hotspots of application, because of its excellent antimicrobial properties [1–4]. However, if silver nanoparticles had been embedded inside the material (like polymer), their effectiveness of would be weakened by the reduced release of silver ions [5–7]. Even the particles are immobilized on the surface of materials, researchers still need to worry about their effectiveness in the initial stage, as the insufficient release of silver ions at the beginning. In other words, if combining with another tool, which could compensate for the initial shortage, the blade of silver nanoparticles would be sharpened.

Incorporating micro-encapsulation technology with an immediate-effective antibacterial agent, thus far, is a simple but efficacious method [8–13]. Chlorhexidine has been used for quite a long time since its discovery in 1986, displaying a broadly antibacterial activity and safety. Moreover, as a cationic surfactant it can take the place of other possible surfactants [14]. PLLA possesses excellent biodegradable and biocompatible characteristics, and its perfect mechanical property makes it to be ideal polymer materials for applications in medicine and pharmacy [15–19]. In this research, chlorhexidine was applied as core material and emulsifying agent of microcapsules, poly-L-lactic acid (PLLA) was used as wall material of microcapsules. Then silver nanoparticles were adhered on the surface of microcapsules to complete the composite microcapsules (Fig. 1). Releasing and antibacterial test demonstrated that a sustained antibacterial effect for a month. We believe that this composite microcapsule hold potential in different biomedical application for its sustained antibacterial effect and simplicity.

2. Materials and methods

2.1. Materials

Chlorhexidine acetate (CA, Aladdin), silver nitrate ($AgNO_3$, Aladdin), sodium borohydride ($NaBH_4$, Aladdin), sodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$, Aladdin), sodium chlorite ($NaClO_2$, Aladdin), sodium periodate ($INaO_4$, Aladdin), (3-Mercaptopropyl) trimethoxysilane ($C_6H_{16}O_3SSi$, Aladdin), poly-etherimide (PEI, M_w = 600, and 10,000, Aladdin), Agar (,Aladdin), beef extract (Aladdin), peptone (Aladdin), polyvinyl alcohol (PVA, M_w = 31,000, Aldrich), dichloromethane (Sinopharm), hydrochloric acid (HCl, Sinopharm), tetrahydrofuran (THF, Sinopharm),

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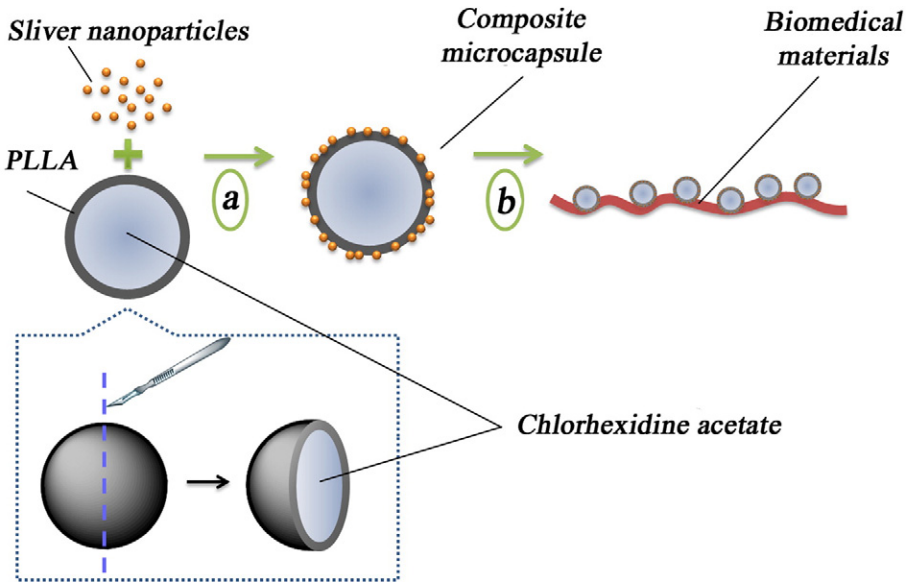

Fig. 1. Schematic diagram of preparation of microcapsules, (a) electrostatic adherence, (b) combination of mercapto-group and silver nanoparticle, dashed box shows a cross-sectional schematic view of microcapsules.

poly(L-lactide) COOH (PLLA-COOH, Mw = 30,000, Daigang), Standard strains (*Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC25922) were provided by pathology department of Jiangsu Province Hospital of TCM. Polyurethane (PU) master batch was a gift from Jiangsu Luyuan New Material Co., Ltd. All chemicals were used without further purification. The water phase was ultrapure water ($r = 18.2\ M\Omega$).

2.2. Preparation of silver nanoparticle and carboxylation of PVA

We referred to a classical chemical reduction method [23,26,27], using sodium borohydride and sodium citrate as reductants, to synthetize silver nanoparticles.

$$Ag^{+} + NaBH_4 \rightarrow Ag + H_2\uparrow + B_2H_6 + Na^{+}$$

1. Stir a mixed solution of 200 μL 5% $AgNO_3$ solution and 20 mL 1% sodium citrate solution with a speed of 1000 r/min.
2. Add 1 mL 0.1% $NaBH_4$ solution into the mixture of step 1 dropwise.
3. Continue to stir the solution for 5 min. Citrate capped silver nanoparticles (negative surface potential) can be obtained by this method [28,29]. All the percentages are mass-to-volume ratio (1% = 1 g/100 mL), and solution means aqueous solution (The following is the same).

PVA-COOH was synthesized as Gaio Paradossi et al. described [20]. In a bath of 60° centigrade water, pH value of 100 mL 5% PVA solution was regulated to 4 by adding 2 mol/L HCl solution, then add 0.2 g $NaIO_4$. After the reaction has completed, 0.8 g $NaClO_2$ was added and let the solution rest for 5 h. Then PVA-COOH was obtained by evaporating the solution to dryness in a water bath.

2.3. Microcapsule preparation and surface modification

Preparation of microcapsules was achieved by using water-oil-water emulsion (w/o/w) double emulsion method as previous study [21,30]. Briefly, first emulsification – an ultrasound emulsification (Cell disruptor, Branson, USA) of "water phase 1" (1.8% CA solution) and "oil phase" (PLLA-COOH/dichloromethane = 3 g/100 mL), the volume ratio of "water phase 1"/"oil phase" is 1/10; second emulsification – stir the emulsion and "water phase 2" (5% PVA-COOH and 0.8% CA solution) with a speed of 2000 r/min (T-25 digital ULTRA-TURRAX, IKA, Germany), the volume ratio of emulsion/"water phase 2" is 1/6. Finally, the prepared microcapsules by this two-step emulsion method were modified with 0.5% PEI solution to change their surface potential to be positive. Because opposite charges attract each other (electrostatic adherence), we can finally create composite antibacterial microcapsules by mixing microcapsule and prepared silver nanoparticles. Microcapsules only carry CA or silver nanoparticles were also prepared for releasing test.

Microcapsule size was analyzed by Particle Size Analysis software (Nano Measurer 1.2.0, Fudan University, Department of Chemistry).

The polyurethane (PU) films were prepared by evaporating THF from polyurethane solution. Then in order to immobilize microcapsules, we planted a layer of sulfhydryl group on the surface of polyurethane films (sulfhydryl group can "grab" the silver nanoparticles on the microcapsule). The pretreating of polyurethane films with $C_6H_{16}O_3SSi$ solution ($C_6H_{16}O_3SSi$/95% ethanol = 1/50, mass ratio) was utilized by soaking films in the solution for 1–2 s. After that, to finally immobilize microcapsules, we can soak the ready-processed PU film into concentrated microcapsule suspension (0.1 g/mL) for 1–2 s and then dry the film by airing. The modified film still needed to be rinsed for 5 times, cleaned ultrasonically for a short-time (≤1 min) and finally dried under nitrogen.

2.4. Characterization and modifying efficiency

The morphologies of the composite antibacterial microcapsules were investigated by scanning electron microscope (SEM) and transmission electron microscope (TEM). Flame atomic absorption spectrometry (FAAS) was used both to determine the mass fraction of silver and to analyses the modifying efficiency on the PU films.

2.5. Determination of release of antibacterial ingredients

The release of CA and silver ions from the microcapsules was measured from leach liquor of microcapsules (1 mg/mL) in a 37 °C environment (water bath) by using ultraviolet spectrophotometer (UV-3600, Shimadzu, Japan). Silver-Spectrophotometric method with 3,5-Br2-PADAP was applied to investigate the silver ion release (absorbance

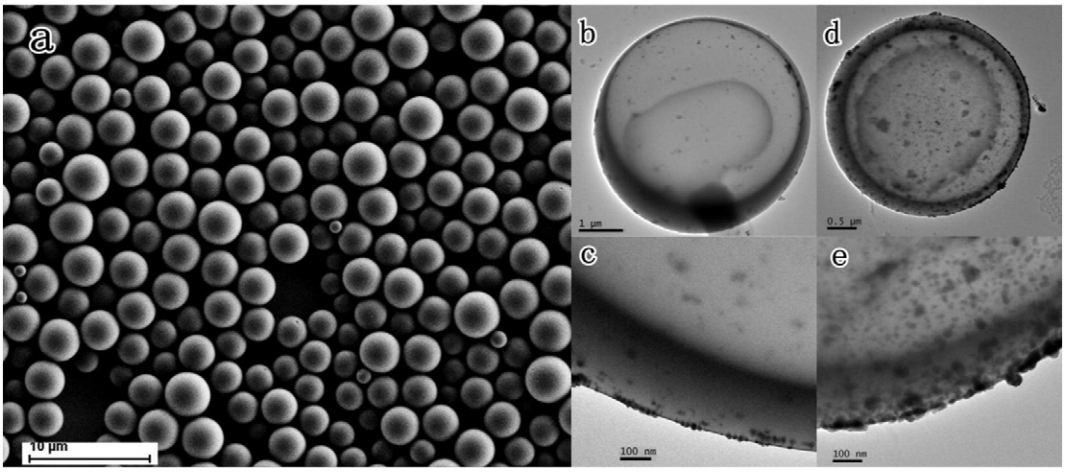

Fig. 2. SEM image of (a) composite microcapsules, TEM images of (b) composite microcapsules, (d) composite microcapsules with excessive silver, (c) and (e) are detail views of (b) and (d) respectively.

was measured at 570 nm) [31]. Maximum absorbance for CA is at 254 nm according to Pharmacopoeia [32].

65 mg dry microcapsules powder were redispersed in 65 mL distilled water for sample preparation. Then 1.5 mL microcapsules suspension was added into a 2 mL centrifugal tube to make a sample. Total 42 samples were prepared.

In CA release experiment, 21 samples were used totally. Sampling timetable is 1 h, 4 h, 12 h, 20 h, 28 h, 48 h and 72 h, 3 samples would be tested each time. In silver ions release experiment, Sampling timetable is once per day for 6 days. 18 samples were used totally, 3 samples each time.

Samples are independent, and the initial state of each sample was the same, only the soaking times were different. The average value of 3 samples would be used to plot release curve.

2.6. Detection of antibacterial property

To evaluate the antibacterial property of composite antibacterial microcapsules and modified PU films, we made references to the method described by Joon Myong Song et al. and ISO 22196:2011 [22]. We selected the concentration of microcapsules as 1 mg/mL, and initial microbial concentration as 10^5 cfu/mL; then we cultivated the mixture on a constant temperature (37 °C) shaker for 24 h; finally after centrifugation (500 r/min, 5 min), we measured the concentration of bacteria in the supernatant. For the test of films, 500 μL of 10^5 cfu/mL bacteria solution was added to a 2 cm diameter modified PU film, then we cover a 1.8 cm diameter PU film on it, after a 24 h cultivation, we rinsed the modified PU film with 2 mL water and counted the number of bacteria in the 2 mL rinsing water. PU films contained Ag nanoparticles were used as control [23]. During a long term test, living bacterium would be added to each sample every day, keeping the initial concentration of bacterium as 10^5 cfu/mL. Two kinds of tested bacteria: *Staphylococcus aureus* and *Escherichia coli* were used. Bacterial count was estimated by plate count method, and each of the data was determined by three parallels. Antibacterial ratio = 1 - (bacterial count of control sample/bacterial count of test sample).

3. Result and discussion

3.1. Characterization and optimization of preparation

By using the method described latter, we could promise our microcapsules with regular shape, uniform size and controllable silver content. Fig. 2 showed the SEM and TEM characterization of microcapsules,

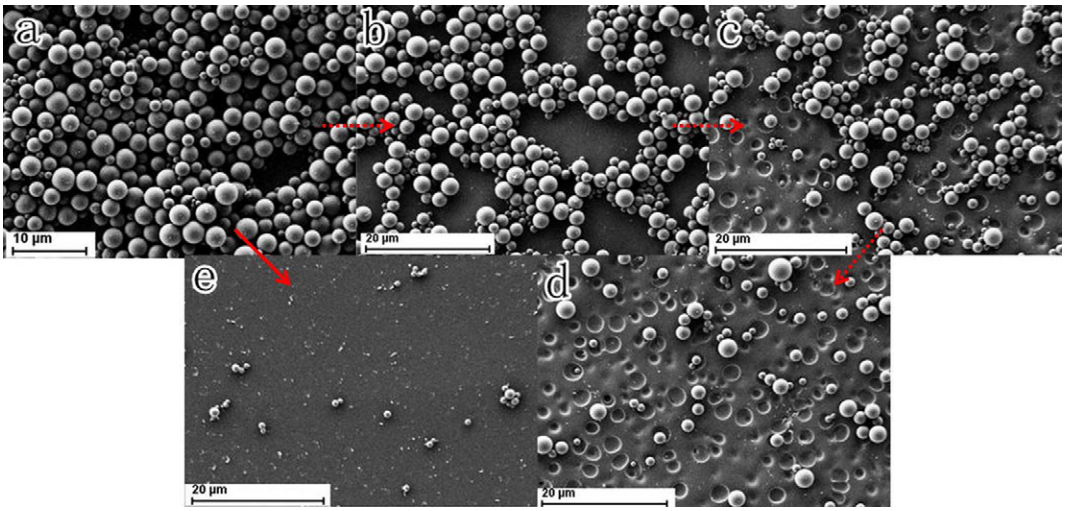

Fig. 3. SEM image of (a) modified polyurethane film without ultrasonic cleaning, (b) modified films after a 30s ultrasonic cleaning, (c) after a 60 s ultrasonic cleaning, (d) after a 5 min ultrasonic cleaning, (e) modified films after repeated swabbing by a wet cotton swab. The porous polyurethane films in (c) and (d) resulted from breath figure effect during THF evaporation.

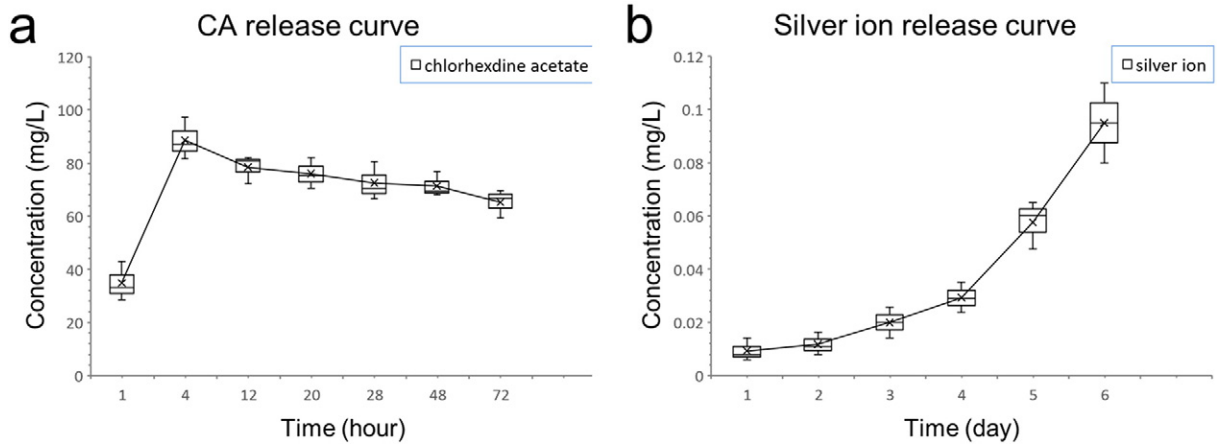

Fig. 4. Release curves of (a) chlorhexidine acetate, (b) and silver ions (37 °C water bath temperature). Dosage of microcapsules was all 1 mg/mL, and every milligram of microcapsules carries 0.113 mg CA and 3.2 μg silver initially.

sphere with a smooth surface. Microcapsules had good monodispersity, 98% of them were controlled between 2.4 and 4 μm, and 80% were between 2.8 and 3.6 μm (data were analyzed by Particle Size Analysis software). However, if we introduced other emulsifiers (like Tween 80), it would complicate the experimental operation and make us harder to control the morphology of microcapsules (Fig. S2). Fig. 2 also showed that silver nanoparticles (Fig. S1) were randomly distributed on the surface, and mass fraction of silver could be controlled between 0.0‰ and 6.1‰ (Fig. 2b and c, PEI, $M_w = 600$). Maximum mass fraction of silver would increase with higher concentration of PEI solution and greater molecular weight of PEI, for instance, treating blank microcapsules with 0.5% PEI ($M_w = 10{,}000$) solution overnight made the mass fraction of silver to 30.8‰ (Fig. 2d and e). The microcapsule used in releasing and antibacterial test carried 0.113 mg CA and 3.2 μg silver per milligram.

3.2. Modifying efficiency

After a simple pretreatment of polymer, PLLA microcapsules can be modified on the surface of biomedical material. In this article, we use polyurethane (PU) film as example. Without ultrasonic cleaning, modifying efficiency of microcapsules on PU films was 3.5 mg/cm^2 (Fig. 3a), microcapsules wouldn't lose after repeated rinse and soak (at least 5 times). Yet, a 30 s ultrasonic cleaning will reduce modifying efficiency to 1.32 mg/cm^2 (Fig. 3b), and variation tendency has been showed in Fig. 3 by dashed arrows. Long-time ultrasonic cleaning and scratch by hard objects would extremely reduce the number of immobilized microcapsules, a wet cotton swab could erase most of the microcapsules by repeated swabbing (Fig. 3e with solid arrow).

3.3. Release of antibacterial constituents

We measured the release curve of antibacterial constituents to value the antibacterial property of microcapsule theoretically. Within a 37 °C environment, CA released rapidly, reaching minimal bactericidal concentration (MBC) of *Escherichia coli* (2 mg/L) in 1 h and MBC of *Staphylococcus aureus* (62.5 mg/L) in 4 h, and microcapsules could keep the concentration for at least 72 h (Fig. 4a). Because, it took at least 1–2 days for concentration of silver ions to barely reach mean MBC (0.01 mg/L) (Fig. 4b), released CA could compensate for the lack of silver ions in first one or two days, realizing an immediate bactericidal effect. Then silver ions from nanoparticles promised a sustained bactericidal effect. This combination could both guarantee the function and reducing the production cost and change in material's color by decreasing the usage of silver nanoparticles.

3.4. Antibacterial property

According to the research of Miguel Jose Yacaman et al., the bactericidal property of the Ag nanoparticles depends on their size [24]. Ag nanoparticles with small diameter (~10 nm) can directly interact with the bacteria (affect bacterial signal transduction and inhibit the growth of the organisms [25]). In addition, smaller Ag nanoparticles present a higher releasing speed of Ag ions base on a larger specific surface area. However, during centrifuge-redispersion process, smaller Ag nanoparticle (<10 nm) shows instability in our study. Thus, in order to balance the strong antibacterial property and stability of the Ag nanoparticles, we controlled the diameter of our silver nanoparticles within 10–15 nm (Fig. S1).

Before determination of the antibacterial ratio of microcapsules, we took inhibition zone test as a preliminary experiment, which proved microcapsules possessed antibacterial property (Fig. 5). Below is a quantitative analysis of antibacterial property of microcapsules by comparing antibacterial ratios of different samples.

We chose the same microcapsules used in determination of release (1 mg microcapsules load 0.113 mg CA and 3.2 μg silver). Dispersed microcapsules (in water) could be effective for 18 days (antibacterial ratio ≥ 99.9%) against both *Escherichia coli* and *Staphylococcus aureus* (initial concentration of bacterium is 10^5 cfu/mL). Period of validity could be extended to 30 days by doubling the mass fraction of silver nanoparticle (1 mg microcapsules load 0.113 mg CA and 6.2 μg silver). Yet, higher

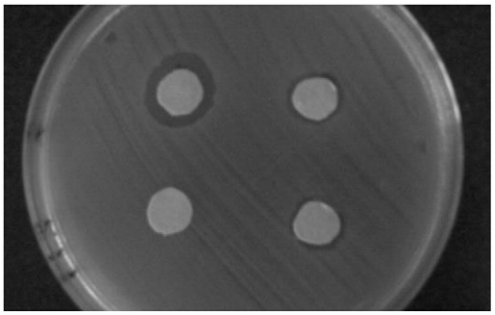

Fig. 5. As a qualitative experiment, inhibition zone test proves the antibacterial property of microcapsules. Diameter of filter paper is 1 cm, each piece of filter paper contains 100 μg microcapsules, bacteria concentration (*Escherichia coli*) used to cover the agar culture-medium is 107 cfu/mL. Microcapsules (load both silver nanoparticles and chlorhexidine acetate, upper left) shows a 1.6 cm inhibition zone; Microcapsules (only load chlorhexidine acetate, upper right) shows a 1.2 cm inhibition zone; Microcapsules (only load c silver nanoparticles, lower left) shows a 1.2 cm inhibition zone; blank microcapsules (lower right) as a control shows no inhibition zone.

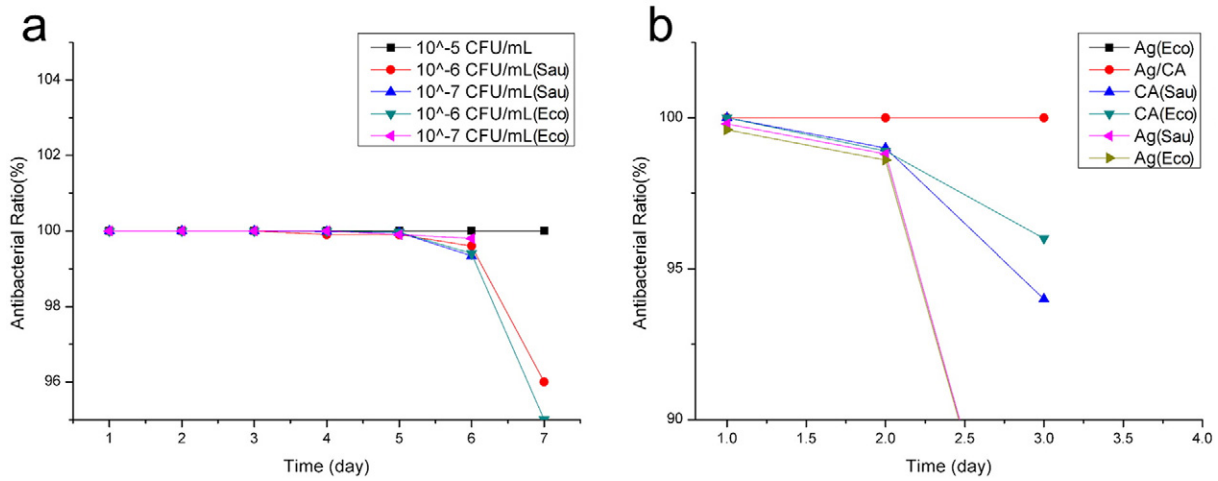

Fig. 6. (a) The impact of concentration of bacterium on antibacterial effectiveness of microcapsule; (b) antibacterial effectiveness of CA microcapsules and Ag microcapsules.

concentration of bacterium (10^6 cfu/mL and 10^7 cfu/mL) would restrict effective time substantially. A higher concentration of bacterium means more quickly the antibacterial ratio decline (Fig. 6a). But microcapsules (1 mg microcapsules load 0.113 mg CA and 3.2 μg silver) could still be active for at least 3 days, even initial concentration of bacterium reached 10^8 cfu/mL. CA microcapsules (1 mg microcapsules only load 0.113 mg CA) could only stand for one day. And Ag microcapsules (1 mg microcapsules only load 3.2 μg silver) could not promise an antibacterial ratio >98% the first day (Fig. 6b), even increasing the carrying capacity of silver to 12.33 μg/mg, it wouldn't acquire the same effectiveness of composite microcapsules. So combining these two antibacterial agents to achieve rapid and sustained antibacterial property was shown to be necessary.

Films modified with microcapsules (1.32 mg/cm^2, 4.22 μg silver per piece of film) could be powerful for at least one week. However, when Ag-nanoparticle-contained PU films can possess a similar firepower, the mass of silver should be >26.98 μg per piece of film. Immobilized microcapsule performed less activity than dispersed microcapsules, cause of less chance for Ag nanoparticle to contact with bacterium. Even though, immobilized microcapsules on the surface of films still performed better than embedding Ag nanoparticles inside. And because microcapsules could be easily modified on the surface of other material, this composite antibacterial microcapsule could promise a good application prospect.

4. Conclusions

In this research, novel composite antibacterial microcapsules with silver nanoparticles on the surface were prepared, realizing an immediate and sustained antibacterial effectiveness. Compared with the microcapsules which only carry silver nanoparticles and the PU film with silver nanoparticles on the surface, composite microcapsules performed a better antibacterial property with less usage of silver and lowered the cost. And surface modification of microcapsules could provide most possibility for microcapsules to contact with bacterium, magnifying the antibacterial property. This kind of composite microcapsules might have perspectives in treating medical materials, like medical proof fabric.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.msec.2017.04.100.

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