J Mater Sci: Mater Med (2009) 20:281–286
DOI 10.1007/s10856-008-3564-5

Study on antibacterial effect of 45S5 Bioglass®

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Received: 1 July 2008 / Accepted: 14 August 2008 / Published online: 3 September 2008
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Abstract Previous studies have shown that bioactive glasses possessed antibacterial effect on common bacteria due to the high aqueous pH value caused by the bioactive glass dissolution. In the present study, the efficiency of the antibacterial effect of 45S5 Bioglass® (45S5 BAG) against *S. aureus*, *S. epidermidis* and *E. coli* and its mechanism were investigated. The results showed that 45S5 BAG exhibited a strong antibacterial effect against the bacteria, and the sensitivity of gram-negative and gram-positive bacteria to Bioglass was different. Furthermore, a dose-dependent bacterial adhesion on 45S5 BAG particles and the formation of needle-like Bioglass debris were observed, which resulted in the damage of cell walls and inactivation of bacteria. The results suggested that both the high pH and bioglass debris on the surface of bacteria may be the possible mechanisms of the antibacterial effect of 45S5 BAG particulates.

1 Introduction

45S5 Bioglass® (45S5 BAG), a bioactive implant material invented in 1971, had excellent biocompatibility and could bond to bone and soft tissues. 45S5 BAG had been widely used as bone filling material in clinic for its ability of bone regeneration [1]. Moreover, previous studies had shown that bioactive glasses could enhance the wound healing of soft tissues [2, 3]. Infection prevention is always an important issue during skin wound healing. In general, antibiotics are used against infection in clinic, and it would be idea if the wound dressing materials not only enhance the healing, but also possess antibacterial activity.

Recently, some studies on the antibacterial activity of bioactive glasses were published. Stoor et al. [4] and Yli-Urpo et al. [5] found that S53P4 BAG possessed an antibacterial effect on four oral classic microorganisms. Recently, Munukka et al. [6, 7] reported sol–gel derived bioactive glasses had a broad spectrum antibacterial effect on classic clinical pathogenic bacteria. Allan et al. [8] showed that particulate 45S5 BAG exerted a considerable antibacterial effect against certain oral bacteria including supra- and subgingival bacteria. Meanwhile, a study about *Streptococcus sanguis* in vitro suggested that 45S5 BAG particulates had the potential to reduce the bacterial colonization on its surface [9]. In 2004, Pratten et al. [10] found that bacterial colonization decreased significantly on surgical sutures with 45S5 BAG coating as compared to that without coating. However, the efficiency and mechanisms of 45S5 BAG against some pathogenic bacteria in wound healing was still unclear.

In previous studies, it was found that the antibacterial activity of bioactive glasses was attributed to the high aqueous pH value caused by release of alkali ions from BAG particles [4, 8]. In aqueous environment, a series of reactions occurred on the surface of BAG particles, including release of soluble silica, sodium and calcium, which resulted in an increase of the aqueous pH value [11]. But the mechanisms of the antibacterial effect of the 45S5 BAG were still in discussion.

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In the present work, the efficiency of 45S5 BAG against some pathogenic bacteria was investigated. The antibacterial activities were compared among a series of dose of bioactive glasses and various bacteria. The possible mechanisms of the antibacterial effect of the bioactive glass were also discussed.

2 Materials and methods

2.1 Materials

Melt-derived 45S5 Bioglass® were obtained from the NovaBone Products LLC (Alachua, FL, USA). The mean grain size was below 50 μm. The composition of the Bioglass powder was (in wt%): 45% SiO_2, 24.5% Na_2O, 24.5% CaO,6% P_2O_5 [11]. To evaluate its antibacterial activity, silicon dioxide particles and benzylpenicillin G (PG) were the control groups [4]. Sterilized silicon dioxide particles (Analytical grade) were obtained from Shanghai Lingfeng Company, and PG was 800,000 U per 0.5 g (Shanghai Pioneer Antibiotic Company).

2.2 Bacterial cultivation

Three classic pathogenic bacteria (*Staphylococcus aureus* ATCC25923, *Staphylococcus epidermidis*, and *Escherichia coli* ATCC25922), which could be found in wound healing, were used in the present study [12]. *S. aureus* were grown at 37°C for 8 h in Lysogeny Broth (LB), a medium for bacterial culture. *S. epidermidis* and *E. coli* were grown under same condition for 3 h. Before experiments, they were diluted to 0.5 to 2 × 10^8/ml approximately [13]. LB was made from 10 g tryptone (Oxoid), 5 g yeast extract (Oxoid), 10 g NaCl and 1 liter water (The electric resistance was 18.2 MΩ), and adjusted to the final pH 7.0 by adding NaOH [14].

2.3 The antibacterial activity of 45S5 BAG

To determine the variance of antibacterial activity with concentration of 45S5 BAG, 10, 50 and 100 mg 45S5 BAG particles (BAG10, BAG50, BAG100) were added into 1 ml bacterial suspension in 1.5 ml eppendorf tubes, respectively [8]. Under the same condition, SiO_2 particles and benzylpenicillin were also added into 1 ml bacterial suspension at final concentration of 500 mg/ml and 200 U/ml. After stirring with a vortex mixer for 1 min, eppendorf tubes were placed in an aerobic environment at 37°C for 1 h [5]. Then, after a serial dilute, 100 μl suspensions were moved onto LB-agar plates followed by a further overnight incubation at 37°C [13]. The resultant colonies were counted as CFU/ml (colony forming units) and the percentages of antibacterial activity were calculated.

2.4 Aqueous pH value measurement

45S5 BAG particles were added into flask containing 10 ml LB medium at final concentration of 10, 50 and 100 mg/ml. After stirring with vortex for 1 min, the solutions were placed at 37°C for 1 h. The pH values of the media were measured with a PHSJ-3F pH meter (Shanghai Precision Scientific Instrument Co., LTD).

2.5 The morphology of 45S5 BAG particle and bacteria

To observe morphology of 45S5 BAG particle and bacteria, (*S. aureus* and *S. epidermidis* were selected because of their blue color in Gram-stain. Hundred milligram 45S5 BAG particles were added into 1 ml bacteria followed a stirring at a vortex mixer, followed by incubation at 37°C aerobically for 1 h. Then a drop of solution was moved onto a slide for a convention Gram-stain. The morphologies were observed under a Hirox KH-7700 3D video microscope (Hirox Co. LTD, Japan).

Escherichia coli was selected because of its relatively large size under transmission electronic microscope (TEM). One milliliter *E. coli* suspension and 100 mg 45S5 BAG particle were mixed before incubated at 37°C for 1 h. The supernate was moved out and the depositions containing 45S5 particle and bacteria were immersed in phosphate buffered saline (PBS) containing 2.5% glutaraldehyde solution and washed with PBS for 12 h, followed by treatment in 1% osmium tetroxide solution for 8 h at room temperature. After dehydration in a graded series of alcohol and propylene oxide, they were embedded in epoxy resin. Ultrathin (60 nm) sections were prepared on an ultramicrotome with a diamond knife, and stained with lead citrate and Uranyl acetate. The morphology of the bacterium (*E. coli*) was observed under a HITACHI H-7000FA TEM.

2.6 Measurement of bacterial adhesion on 45S5 BAG particles

To detect the bacterial adhesion on 45S5 particle surface, 0, 10 and 50 mg 45S5 BAG particles were mixed with 1 ml bacteria suspension in 1.5 ml eppendorf tubes with a vortex mixer for 1 min, followed by maintained unstirred for 5 min. Then, the suspensions were centrifugated at a low speed of 1,000 rpm for 1 min, which leaded to deposition of 45S5 particles and bacteria adhered on 45S5 surface when free bacteria remained in solution [15]. After 0.8 ml

supernate were moved out under microscope, bacterial numbers of supernate were counted using a cytometer [16]. The bacterial adhesion rates were calculated as the percent of the number of lost cells to the number of addition cells [17].

2.7 Comparison of antibacterial activity of 45S5 BAG particle and extract

To evaluate the antibacterial activities of 45S5 BAG particles and extracts, a comparative study between 45S5 BAG solutions with and without 45S5 BAG particles was conducted. For *extract group*, 1 ml LB medium was added into eppendorf tubes containing 10 mg 45S5 BAG particle. They were stirred on a vortex mixer for 1 min, followed by incubation at 37°C for 1 h. Then 0.9 ml of the supernate was transferred to other eppendorf tubes. For *particle group*, 0.9 ml LB medium and 10 mg 45S5 BAG particle were stirred on a vortex mixer for 1 min in eppendorf tubes. After those previous work, 0.1 ml bacterial suspension added into the eppendorf tubes of two groups and the solutions cultured at 37°C for 1 h. After incubation stopped, the resultant bacterial colonies were counted and the bacteriostasis percentages were calculated as above. Meanwhile, the aqueous pH values of both groups were measured after incubation.

3 Results

3.1 The antibacterial activity of 45S5 BAG

45S5 BAG exhibited a visible antibacterial effect against three pathogenic bacteria as shown in Fig. 1. The bactericidal percentages for *S. aureus, S. epidermidis* and *E. coli* were 55%, 50% and 80%, respectively, at the concentration of 10 mg/ml. A remarkable difference ($P < 0.01$) was seen between gram-negative bacterium (*E. coli*) and gram-positive bacteria (*S. aureus* and *S. epidermidis*), while no difference was found between two gram-positive bacteria. At concentration over 50 mg/ml, the bactericidal percentages increased up to 98%. Meanwhile, PG showed an effective bactericidal ability to all those bacteria [18, 19]. On the contrary, antibacterial effect of inert SiO_2 was below 10% [4].

3.2 The pH value

The aqueous pH values of 45S5 BAG suspension increased with the increase of BAG concentration. The value of 0 mg/ml represented the pH of the media prior to the addition of 45S5 BAG. The aqueous pH values were 8.7 at 10 mg/ml, 9.8 at 50 mg/ml and 10.3 at 100 mg/ml, respectively. (Table 1)

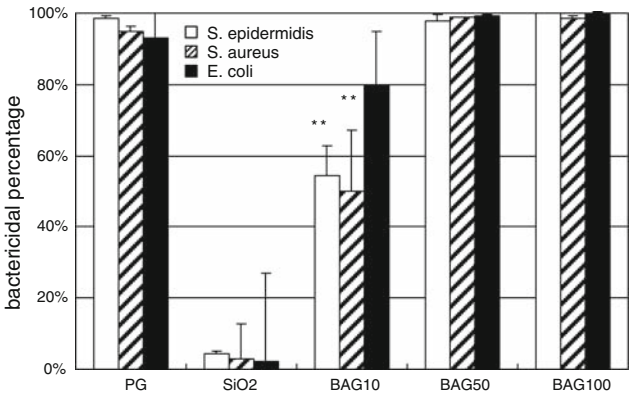

Fig. 1 The bactericidal percentages of 45S5 BAG, SiO_2 and PG. (BAG10: 10 mg/ml; BAG50: 50 mg/ml; BAG100: 100 mg/ml) **indicated significant difference for *S. aureus* and *S. epidermidis* groups ($P < 0.01$) compared to *E. coli* group

Table 1 The aqueous pH values of the 45S5 BAG suspension

Concentration of BAG (mg/ml)	0	10	50	100
pH value	7.0	8.7	9.8	10.3

3.3 The morphology of 45S5 BAG and bacteria

On gram stain slides, the 45S5 BAG particles appeared as large red masses of 1–50 μm in size (Fig. 2a), while cells of *S. epidermidis* and *S. aureus* appeared as smaller blue hollow circles of 0.5 μm in size (Fig. 2b, c). It was visible that most of the cells adhered on 45S5 BAG particle surface, and only a few cells were distributed in the blank area far from 45S5 BAG particles (see Fig. 2b, c).

Under TEM, 45S5 BAG particles were revealed in deep dark color (Fig. 3a), and living *E. coli* cells showed regular cellular structure clearly (Fig. 3b). In Fig. 3c, *E. coli* cells adhesion on 45S5 BAG particle surface showed complete cell wall but unclear and irregular cellular structure. Some needle-like BAG debris with a size of 100×300 nm^2 were observed on the surface of 45S5 BAG particles and near cellular area (Fig. 3a, d). In Fig. 3d, it was clear to see that the cell wall and cellular structure of one *E. coli* was damaged by 45S5 debris, which resulted in the death of the bacteria.

3.4 Bacterial adhesion on 45S5 BAG particles

The results of bacterial adhesion rates on 45S5 BAG particles were shown in Fig. 4. It was clear to see that the bacterial adhesion on 45S5 BAG was dose-dependent. High bacterial adhesion was found in the 50 mg/ml group. In addition, differences of bacterial adhesion were

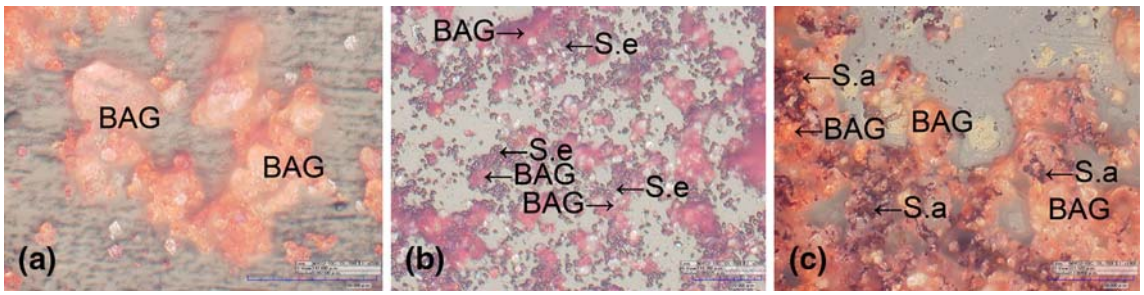

Fig. 2 The morphologies of bacteria surrounding the 45S5 BAG particles. (**a**, scale bar = 50 μm) 45S5 BAG particles (BAG) without bacteria. (**b**, scale bar = 20 μm) *S. epidermidis* (S.e) cells with 45S5 BAG particles. (**c**, scale bar = 50 μm) *S. aureus* (S.a) cells with 45S5 BAG particles

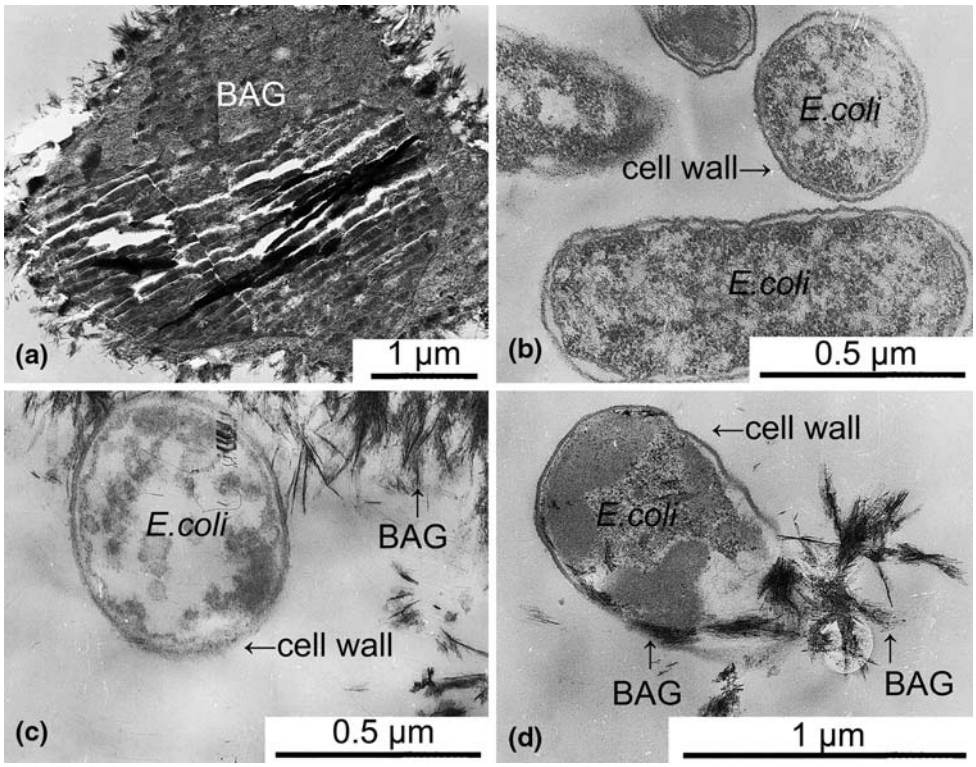

Fig. 3 The morphologies of bacteria adhered on the 45S5 BAG particle surface. (**a**) 45S5 BAG (BAG) particles without bacteria. (**b**) Living *E. coli* cell without 45S5 BAG particles. (**c**) A *E. coli* cell on BAG particle. (**d**) A dead *E. coli* cell with damaged cell wall and 45S5 BAG debris

observed between the three bacteria. *S. epidermidis* showed a higher adhesion compared with *S. aureus* and *E. coli*.

3.5 Antibacterial activity of 45S5 BAG particle and extract

Table 2 and Fig. 5 show the pH value of the solutions with and without 45S5 BAG particles and the corresponding bactericidal percentages respectively. There was no significant difference in the pH values of the solutions with or without 45S5 BAG particles (Table 2). In contrast, antibacterial percentages of extract group were significant lower than that of particle groups (Fig. 5), which suggests other factors contributing to the antibacterial activity besides high aqueous pH value.

4 Discussion

The results described above show that 45S5 BAG exhibited a broad-spectrum antibacterial effect against the classic skin pathogenic bacteria, just as oral microorganisms [4, 8]. The antibacterial effects against all three bacteria were dose-dependent at concentration of 50 mg/ml and below: the bactericidal percentage increased with the increase of particle concentrations. The efficient antibacterial concentration of 45S5 BAG was 50 mg/ml and above, since the

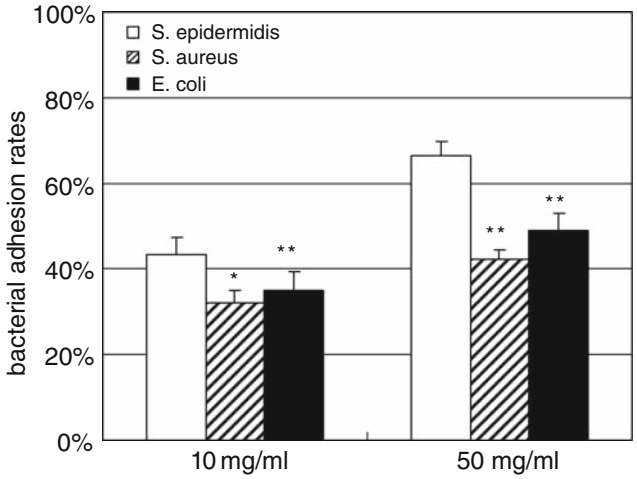

Fig. 4 The bacterial adhesion rates on 45S5 BAG particles at concentration of 10 and 50 mg/ml. *indicates significant difference for *S. aureus* and *E. coli* group ($P < 0.05$) compared to *S. epidermidis* groups, and **means $P < 0.01$

Table 2 The pH values of solutions with (particle) and without 45S5 BAG (extract) particles at 10 mg/ml

	S. epidermidis	*S. aureus*	*E. coli*
Particle	8.79	8.71	8.77
Extract	8.73	8.69	8.72

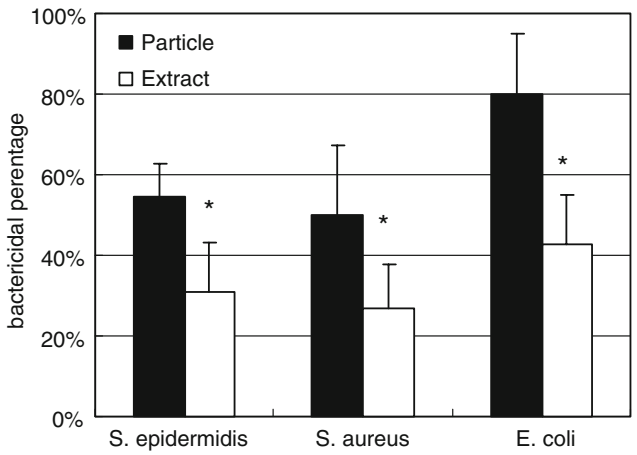

Fig. 5 The bactericidal percentages of the bacteria solutions with and without 45S5 BAG particles at 10 mg/ml. *indicates statistically significant difference between extract groups and particle groups ($P < 0.05$)

bactericidal percentages were more than 98%. In addition, a clear difference of antibacterial effects between the two gram-positive bacteria (*S. aureus* and *S. epidermidis*) and one gram-negative bacteria (*E. coli*) was observed at concentration of 45S5 BAG 10 mg/ml. Gram-negative cell wall was composed of high proportion of phospholipids, lipopolysaccharides and proteins, whereas peptidoglycan was the major component of gram-positive cell wall [20]. This fact would possibly contribute to the difference of antibacterial effects between gram-negative and positive bacteria, but further investigation was necessary to be conducted to confirm this assumption.

After immersion in aqueous environment, 45S5 BAG particles underwent a series of surface reactions what included release of soluble Na and Ca ions resulting in an increase of aqueous pH value [11]. The high pH value of 45S5 BAG extracts was certainly a critical factor for 45S5 BAG antibacterial effects [4, 8]. This conclusion was also supported by our results that the antibacterial effect of 45S5 BAG suspension increased with the increase of the aqueous pH of the suspension (Fig. 1; Table 1). Furthermore, antibacterial effects of solution with 45S5 BAG were visible higher than that of 45S5 BAG extracts without particles (Fig. 5), but at same aqueous pH value (Table 2). This fact implied that the 45S5 BAG particles also played important role in the antibacterial effect besides high aqueous pH.

45S5 BAG had a dose-dependent and bacteria-dependent bacterial adhesion (Fig. 4). The remarkable difference among bacteria was attributed to the different properties of bacterial surface [16]. Therefore, bacterial adhesion in solution with 45S5 particle was undoubted higher than that of extract without particle, which indicated bacterial adhesion enhanced its antibacterial activity. Jacobs et al. [16] showed that the bacterial adhesion on sand could not inhibit bacterial growth. In the present work, we also found that inert SiO_2 had a poor antibacterial effect (Fig. 1). The cause of enhancement of bacterial adhesion was a noticeable problem, since bacterial adhesion had poor effect on bacterial proliferation.

Furthermore, the TEM observation revealed that needle-like BAG debris on bacterial surface destructed bacterial structure (see Fig. 3d). This result indicated that 45S5 debris from BAG particle was one of the reasons for destruction of cellular structure and bacterial death. A similar phenomenon had been reported that HAp particles absorption leads to the death of cancer cells [21, 22]. Therefore, the possible mechanisms of the antibacterial effect of 45S5 BAG may include two aspects, one was the higher pH proximal to the 45S5 BAG particles which were closely surrounded by bacteria, and the other was the destruction of the cell walls by 45S5 BAG debris. A schematic illustration of the bactericidal process of 45S5 BAG was given here as shown in Fig. 6. First, when bioglass particles immerse in solution, alkali ions began to be released. The local pH value on the surface of 45S5 BAG particles was higher than that in the area far from the particles [23]. Secondly, bacteria were adhered onto the surface of 45S5 BAG particle. The higher local pH value would be benefit to kill the bacteria around the 45S5 BAG

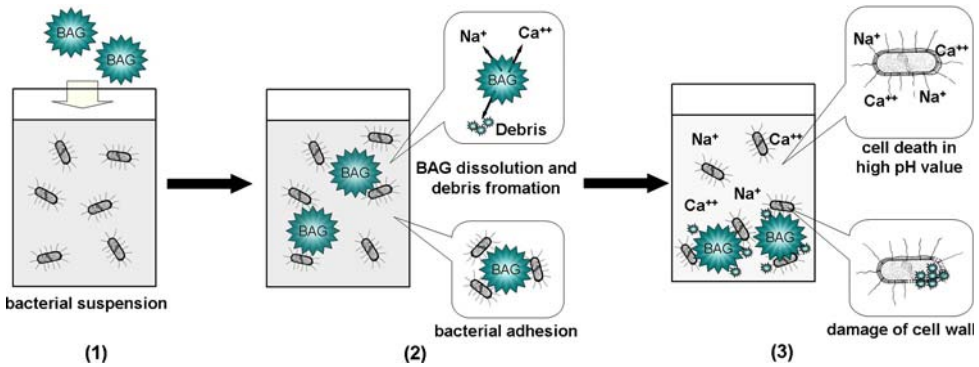

Fig. 6 A schematic illustration of the bactericidal process of 45S5 BAG. (1) BAG particles are added into bacterial suspension. (2) After immersion into bacterial suspension, BAG particles dissolution leads to increase of aqueous pH value and debris formation. Bacteria are adhered on the surface of BAG particles simultaneously. (3) Bacteria are killed by high aqueous pH value and the damage of cell walls caused by BAG debris

particles. In addition, the 45S5 BAG debris could damage cell walls and lead to bacterial death.

5 Conclusions

45S5 BAG particle exhibited strong antibacterial activity against skin pathogenic bacteria. For all the three bacteria studied (*S. aureus* ATCC25923, *S. epidermidis*, and *E. coli* ATCC25922), the bactericidal percent over 98% could be achieved at the 45S5 BAG concentration of 50 mg/ml. The antibacterial rates increased with the increase in the 45S5 BAG concentration. In addition, bacterial adhesion on 45S5 BAG particles was observed, which may play an important role in the antibacterial effect of 45S5 BAG particles, since this adhesion resulted in a high local aqueous pH value to bacteria and contact of bacteria with 45S5 BAG debris, which caused damage of cell walls of the bacteria. Therefore, it may be concluded that the high pH and 45S5 BAG debris on the surface of bacteria may be the possible mechanisms of the antibacterial effect of 45S5 BAG particles.

Acknowledgements This study was supported by China Postdoctoral Science Foundation (20060400679), Science and Technology Commission of Shanghai Municipality (Grant No.: 07DZ05012) and Project supported by Funds of the Chinese Academy of Sciences for Key Topics in Innovation Engineering (Grant No. KGCX2-YW-207). The authors would like to show appreciations to Mr. Wanyin Zhai, Mr. Weiming Gu and Mr. Kaili Lin for their kind discussions.

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