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ORIGINAL ARTICLE

In vitro antistaphylococcal effects of a novel 45S5 bioglass/agar–gelatin biocomposite films

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Abstract

Aims: To assess the antibacterial efficacy of new composite materials developed from microparticles of 45S5 bioactive glass (BG) and agar–gelatin films.
Methods and Results: *In vitro* antibacterial activity was evaluated against *Staphylococcus* spp. because of the importance of this pathogen in damaged tissues and in failures associated with biomaterial implants. To our knowledge, this is the first paper reporting on the suitable combination of BG and agar–gelatin for bioactive and antibacterial films. Bacterial suspensions up or below 10^5 CFU ml^{-1} reflecting situations of wound infection and of noninfection, respectively, were prepared and then put in contact with the biomaterials at 37°C. After 24 and 48 h of incubation, the pH value was measured and the staphylococci strains viability was determined by counting in Mueller–Hinton agar plates. Moreover, the biomaterials were prepared for observation under scanning electron microscopy (SEM). Biocomposites (BCs) showed a strong antibacterial effect against all staphylococci strains tested. Some differences were found depending on the strain, the inoculum size and the contact time. This effect was correlated with an alkalinization of the media. By SEM analyses, no bacterial presence was observed on the surface of BCs in any of the cell concentrations tested at any time.
Conclusions: Overall, the coating of 45S5 BG on agar–gelatin films promoted BCs with strong antistaphylococcal activity. The effect was efficient under bacterial concentration up or below 10^5 CFU ml^{-1}. Additionally, none of the strains were found on BCs surfaces.
Significance and Impact of Study: 45S5 bioglass/agar–gelatin biocomposite films are reported for the first time. The results suggest a potential application as wound dressing.

Introduction

Staphylococcus aureus and *Staphylococcus epidermidis* are Gram-positive bacteria that are normal colonizers of human skin and mucous membranes, but can become pathogenic in the presence of wounds causing in some cases serious diseases (Lowy 1998; Mack *et al.* 2007). They are also associated with infection problems during orthopaedic failure of implants (Krimmer *et al.* 1999; Campoccia *et al.* 2008; Campoccia *et al.* 2009). Treatment of these infections is associated with high complication rates and implies in many cases prolonged hospital stay, increased morbidity and mortality, and serious economic costs (Gollwitzer *et al.* 2003). The infections may be difficult to treat with traditional antibiotics due to the emergence of some antibiotic resistance like methicillin-resistant *S. aureus* (MRSA) or because of biofilms formation (Raja *et al.* 2011).

Diverse strategies have been developed to avoid the staphylococci survival and adhesion on wound and implants including the development of antibacterial drug-delivery systems using different substrates (Itokazu *et al.* 1997; Schierholz and Beuth 2001; Gollwitzer *et al.* 2003; Von Eiff *et al.* 2005) or the alteration of the surface topography to prevent bacterial adhesion (Balazs *et al.* 2003; Chua *et al.* 2008). The disadvantages of these methods are the cytotoxicity of some antibacterial agents (Albers *et al.* 2013) or the persistent risk of an antibiotic resistance. Indeed many of them have a negative effect on the attachment of the host cells (Misra *et al.* 2010).

Bioactive glasses (BGs) are inorganic material with the ability to stimulate specific cellular responses at the molecular level as a result of the controlled release of ions from them (Hench and Polak 2002; Hoppe *et al.* 2011). The first system, 45S5 Bioglass®, constituted by (in wt%) 45% SiO_2, 24·5% Na_2O, 24·5% CaO and 6% P_2O_5 was developed by Hench and coworkers in 1971 (Hench *et al.* 1971). Subsequently, other compositions were obtained by replacing or incorporating different ions (Brink *et al.* 1997; Gorustovich *et al.* 2006, 2010; Vallet-Regí *et al.* 2006). 45S5 BG has been successfully employed to fill bone defects in clinic for its ability to promote osteogenesis. In addition, it was also demonstrated that 45S5 BG enhances the wound healing of soft tissues (Moosvi and Day 2009; Lin *et al.* 2012). On the other hand, numerous works reported on the antibacterial potential of 45S5 BG particles (Allan *et al.* 2001; Waltimo *et al.* 2007, 2009; Hu *et al.* 2009) including those of staphylococci (Hu *et al.* 2009; Misra *et al.* 2010). Biofilm viability was also prevented by particulate 45S5 BG (Allan *et al.* 2002). In recent years, 45S5 BGs have been incorporated into many natural or synthetic polymers in order to improve some characteristic of both materials like delivery or degradation (Blaker *et al.* 2005; Day *et al.* 2005; Hong *et al.* 2009; Gentile *et al.* 2010; Marelli *et al.* 2010). In that sense, some biocomposites (BCs) with antistaphylococcal activity were reported (Pratten *et al.* 2004; Misra *et al.* 2010). Nevertheless, to date, there have been no scientific reports of BCs based on 45S5 BG and agar–gelatin hybrids. Gelatin is obtained by thermal denaturation, physical or chemical degradation of collagen and has been used for medical applications such as wound dressings and adsorbent pads during surgery (Sakai *et al.* 2007). It has gained interest in biomedical engineering due to its low cost, wide commercial availability and characteristic of biocompatibility and biodegradability (Gentile *et al.* 2010). The disadvantage of gelatin is that it dissolves at the temperature of the human body as well as temperatures used for culturing cells (Van den Bosch and Gielens 2003; Sakai *et al.* 2007). Agar is a polysaccharide with higher thermostability than gelatin. Besides, agar gels behave like a sponge. Even though, agar has no significant moieties for the adhesion and proliferation of cells (Gruber *et al.* 1997).

Agar–gelatin scaffolds have been developed with promising application in tissue engineering (Sakai *et al.* 2007), evaluation of the toxicity of drugs and chemicals (Verma *et al.* 2009) and drug delivery (Saxena *et al.* 2011; Shome *et al.* 2011). The cytocompatibility of different weight ratio of these hybrids was investigated, and films and scaffolds containing agar and gelatin in 2 : 1 weight ratio exhibited the best growth kinetics of mouse fibroblast cell line NIH3T3 (Verma *et al.* 2007).

Wound infections are defined as a bacterial count over 10^5 micro-organisms g^{-1} tissue (Robson *et al.* 1999; Edwards and Harding 2004; O'Meara *et al.* 2006; Jacobsen *et al.* 2011). In the present work, micrometre 45S5 BG was employed to coat agar–gelatin films, and a series of experiments were carried out to evaluate the antibacterial properties of the BCs against four staphylococci strains. Two different inoculum sizes of *Staphylococcus* spp. strains were assessed to reflect a case of infection and one of noninfection.

Materials and methods

Materials for biocomposites

Melt-derived 45S5 BG micrometre particles were in range size of 5–100 μm. The composition was (in%w/w): 45% SiO_2, 24·5% Na_2O, 24·5% CaO, 6% P_2O_5. Agar-agar was purchased from Britannia S.A. (Buenos Aires, Argentina). Edible gelatin Royal was obtained from Mondelez International (Buenos Aires, Argentina).

Films preparation and coating with 45S5 Bioglass

The agar–gelatin films were prepared in 2 : 1 weight ratio. Briefly, agar–gelatin 2 : 1 ratio was dissolved in distilled water making 1% homogenous solution. Twenty millilitres was poured in a Petri dish plate and kept in an incubator for 24 h at 30°C. Then, 0·54 g of 45S5 BG was suspended in 25 ml of isopropanol alcohol and finally incorporated to agar–gelatin gel. Once BG microparticles precipitated, the excess of isopropanol was removed with a pipette (this process accelerates the drying time). The new BCs were then incubated for 24 h to complete the drying. Subsequently, they were cut in discs shape of 5 mm of diameter. For biological assays, BCs and control films discs were UV sterilized for 20 min on each side. To determine the weight of each disc, coated and uncoated samples discs were weighed. The subtraction of both averages allowed determining the average weight in mg of 45S5 BG in each disc.

Morphological characterization

The materials obtained were morphologically characterized by scanning electron microscopy (SEM). For this, biomaterials were fixed with a 2·5% glutaraldehyde 0·1 mol l^{-1} PBS solution overnight at 4°C. The samples were then washed with distilled water and sequentially dehydrated through a graded series of ethanol solutions. After mounting on stubs and gold sputtering, the samples were examined with a scanning electron microscope (JSM 6480 LV, JEOL Ltd, Tokyo, Japan).

Bacterial cultivation

The following strains were used in this study: *S. aureus* ATCC29213, *S. aureus* ATCC25923, *S. aureus* ATCC 6538P and *S. epidemidis* ATCC12228. All strains were grown for 24 h in Muller–Hinton broth (Britannia S.A.) at 37°C. For the experiments, the bacterial cell suspensions were diluted to 4 and 6 log CFU ml^{-1}, approximately.

Antibacterial properties of biocomposites

The experiments were carried out in Hank's balanced saline solution (HBSS) without Ca^{2+} and Mg^{+} (Life Technologies, Carlsbad, CA, USA). The biomaterials (3 discs) were incubated for 48 h at 37°C in 1 ml of cellular suspensions. Each staphylococci suspension in absence of biomaterial served as controls. Samples were collected after 24 and 48 h of incubation, and the viability of cells at 37°C were assessed by counting in Muller–Hinton agar plates. Also, at the end of each period, the pH value of the culture was determined. The results were expressed as $\log_{10}$ CFU ml^{-1} ± SD. On the other hand, the BCs and uncoated films were prepared for SEM observation. Previously, samples were rinsed in distilled water and vortexed for 1 min to wash away free bacteria.

Statistical analysis

Statistical analysis was performed using SPSS 15·0 statistical package software (IBM, Armonk, NY, USA) with appropriate statistical tests such as one-way analysis of (ANOVA) with Dunnett's and Tukey's multiple comparison post-tests for intergroup analysis. Specifically, the data from cell suspension were used as a controland compared to the coated and uncoated films. The level of significance was set at a *P*-value of <0·05.

Results

Films preparation and coating with 45S5 Bioglass

The mean weight of BCs discs was 2·93 ± 0·60 mg, of the uncoated films was 0·10 ± 0·01 mg. This means that the 45S5 BGs incorporation on each disc was 2·83 ± 0·59 mg.

Morphological characterization

The microstructure of agar–gelatin films and BCs are shown in Fig. 1. Figure 1(a) corresponds to agar–gelatin pure films. It can be seen that the surface of pure film is homogenous. Also, it is a continuous matrix without cracks with good structural integrity. Figure 1(b) shows the morphology of BCs. A homogeneous and continuous coating can be observed; pores were created as a consequence of the irregular size presented by 45S5 BG microparticles.

Antibacterial properties of biocomposites

Against a concentration of Staphylococcus spp. below 10^5 CFU ml^{-1}

The antibacterial effects of BCs at different incubation periods are shown in Fig. 2. The initial cell concentration of the strains expressed as log CFU ml^{-1} was: *S. epidermidis* ATCC1228, 4·00 ± 0·43; *S. aureus* ATCC25923,

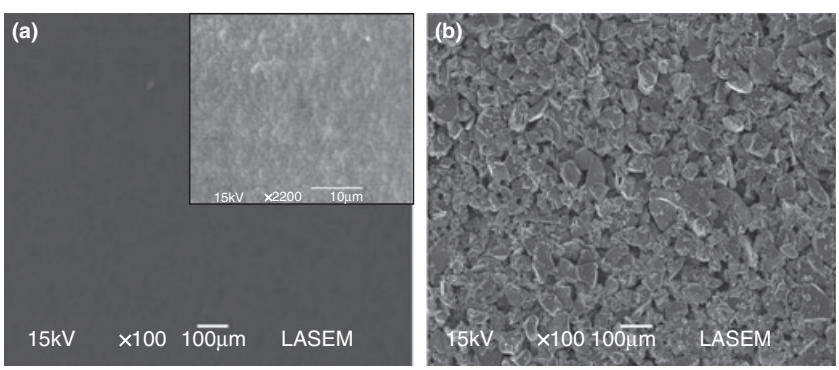

Figure 1 Scanning electron microscopy micrographs of (a) Agar–gelatin 2 : 1 pure film and (b) 45S5 bioglass coating on agar–gelatin film.

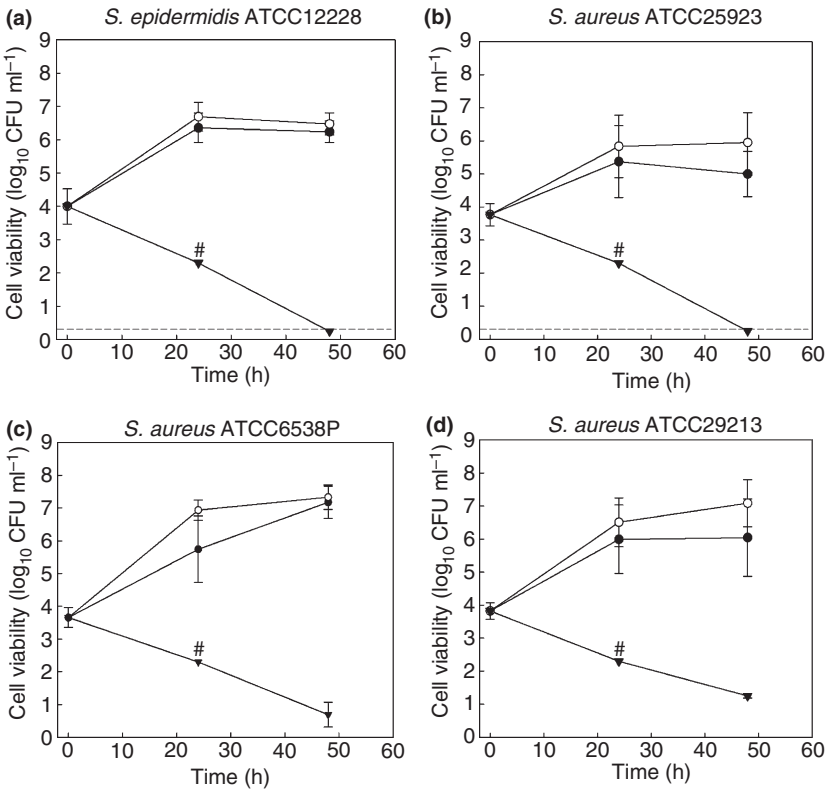

Figure 2 Effects of biocomposites (BCs) and pure films on viability of (a) *Staphylococcus epidermidis* ATCC12228 and *S. aureus* (b) ATCC25923, (c) ATCC6538P and (d) ATCC29213 at a inoculum size below 10^5 CFU ml^{-1}: (○) control cells suspensions, (●) cells suspensions plus agar/pure films, (▲) cells suspensions plus BCs. Error bars represent ± standard deviation. # significative when compared with control time 0.

3·77 ± 0·34; *S. aureus* ATCC6538P, 3·65 ± 0·30; and *S. aureus*ATCC29213, 3·82 ± 0·25. No statistical differences were found in the inoculum size between the strains, and pure films had no effects on the viability of the strains. On the other hand, the BCs strongly inhibited the cell growth of all strains. The inhibition increased with the time of exposition. After 24 h of incubation, the cell viability of the strains was reduced to 2·30 log CFU ml^{-1}. While, after 48 h, *S. epidermidis* and *S. aureus* ATCC25923 were the most sensitive to BCs because their viability was below the detection limit of the technique (<2 CFU ml^{-1}). In the case of *S. aureus* ATCC6538P, the bacterial count was reduced to 0·70 ± 0·38 log CFU ml^{-1}. Even though *S. aureus* ATCC29213 was the least sensitive of the strains, at the end of the assays, the cell count for this strain was 1·25 ± 0·07 log CFU $ml^{-1.}$

In all cases, it was observed a positive correlation between the growth inhibition of the cells and the alkalinization of the media culture containing BCs, indicating that the aqueous pH values increased with the increase of the incubation periods. Table 1 shows the values of these modifications.

Against a concentration of Staphylococcus spp. up 10^5 CFU ml^{-1} The results are shown in Fig. 3. The initial cell concentration expressed as log CFU ml^{-1} was: *S. epidermidis* ATCC1228, 6·40 ± 0·15; *S. aureus* ATCC25923,

Table 1 pH value at different conditions

	Time of incubation (h)		
Conditions	0	24	48
Cells suspensions	7	7	7
Agar–gelatin films	7	7	7
Biocomposites	7	10	11

6·16 ± 0·30; *S. aureus* ATCC6538P, 6·24 ± 0·09; and *S. aureus*ATCC29213, 6·39 ± 0·13. No statistical differences were found in the inoculum size between the strains. Similar to the results shown above, the BCs strongly inhibited *Staphylococcus* spp. cell viability. The growth of *S. epidermidis* ATCC12228 and *S. aureus* ATCC25923 was the most strongly inhibited in presence of BCs after 24 h. After this period time, the cell viability was 3·80 ± 0·11 log CFU ml^{-1} for *S. epidermidis* and 2·90 ± 0·16 log CFU ml^{-1} for *S. aureus* ATCC25923. That means a reduction of 2·61 and 3·26 log CFU ml^{-1}. After 48 h, no significant differences were found in cell viability for these strains.

After 24 h, BCs inhibited *S. aureus* ATCC6538P and ATCC29213 cells almost 1 order of magnitude. However, after 48 h, the cell viability for *S. aureus* ATCC6538P was 2·90 ± 0·45 and 4·25 ± 0·14 log CFU ml^{-1} for *S. aureus* ATCC29213. Once again, this last strain results the less

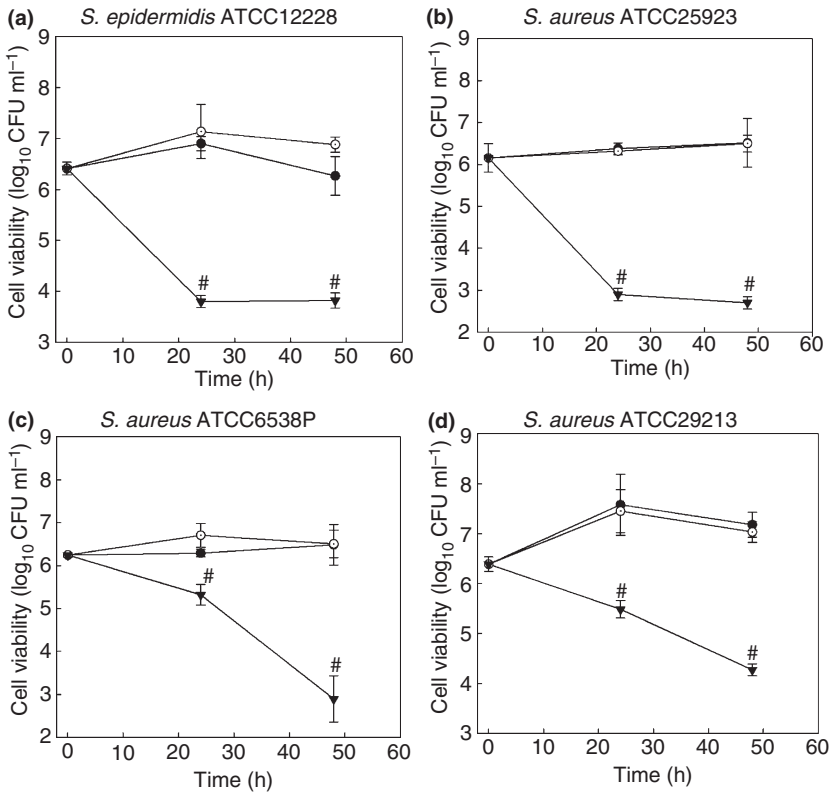

Figure 3 Effects of biocomposites (BCs) and pure films on viability of (a) *Staphylococcus epidermidis* ATCC12228 and *S. aureus* (b) ATCC25923, (c) ATCC6538P and (d) ATCC29213 at a inoculum size up 10^5 CFU ml^{-1}: (○) control cells suspensions, (●) cells suspensions plus agar/pure films, (▲) cells suspensions plus BCs. Error bars represent ± standard deviation. # significative when compared with control time 0.

sensitive to BCs at the end of the experiments. The pH of the culture also constantly increased during the incubations periods (see Table 1).

SEM analyses post-incubation

Representative morphological changes in samples with conditioning time are illustrated in Fig. 4. No cells of *Staphylococcus* spp. were found on BC-coated surfaces after 24 or 48 h post-incubation (a,b). No cells were found either on the uncoated faces of BCs (c). When bacterial cells were incubated with control samples, *S. aureus* ATCC 29213 cells were found on gelatin–agar surface, but only after 48 h of incubation period and when the inoculum size were the highest. It was the only case of bacterial presence found on agar–gelatin films. None of the rest of the strains was found on pure films.

Discussion

In this work, the antibacterial effects of novel 45S5 BG/agar–gelatin films against four staphylococci strains at different incubation periods were investigated. The experiments were performed at bacterial concentrations up and below 10^5 CFU reflecting an infective and a subinfective level of bacteria, respectively (Robson *et al.* 1999; Edwards and Harding 2004; Jacobsen *et al.* 2011). It has been proposed that subinfective levels would accelerate wound healing and formation of granulation tissue and an increase of collagen formation (Edwards and Harding 2004). The results obtained in this work showed that BCs were highly effective in keeping or reducing the bacterial number to this level, even though some differences were obtained depending on the strain and the incubation periods.

The antistaphylococcal effects observed in this study are in good agreement with a previous investigation demonstrating that a biocomposite made of Poly(3-hydroxybutyrate) and nanoparticles of 45S5 BG (P(3HB)/n-BG 10% wt) inhibited the cell growth of *S. aureus* NCTC6571. The inhibitory effects in that work increased with the incubation periods (up to 48 h) and also were in correlation with a pH increment of the media culture (Misra *et al.* 2010).

Also, the results of the current study for ATCC29123 concur with those of Hu *et al.* who reported a strong antibacterial effects *in vitro* of 45S5 microparticles (<50 μm) against this strain and also against a strain of *S. epidermidis* ($0{\cdot}5–2 \times 10^8$ CFU ml^{-1}) in a high alkaline environment (Hu *et al.* 2009).

Nevertheless, Bellantone *et al.* reported that 45S5 particles (90–710 μm) had no effect *in vitro* on *S. aureus* NCIMB11852 even at a 10 mg ml^{-1} of concentration (Bellantone *et al.* 2002). Also, Gorriti *et al.* (2009) reported that scaffolds made from 45S5 BGs did not exhibit antibacterial effect against *S. aureus* ATCC25923, ATCC29213 and ATCC6538P after 1 and 24 h of incubation period.

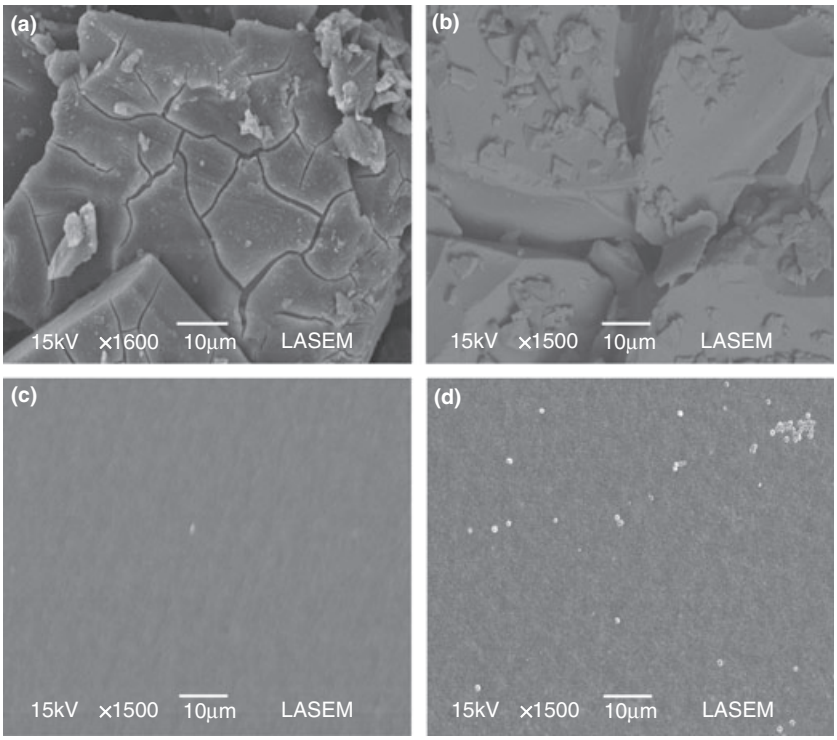

Figure 4 Interaction among *Staphylococcus* cells and the different films by Scanning electron microscopy micrographs analyses: (a) with biocomposites (BCs) and incubated for 24 h; (b) with BCs after 48 h; (c) uncoated surface of BCs; (d) *S. aureus* ATCC29213 cells on pure film after 48 h of incubation.

Finally, Xie *et al.* (2008) showed that 300 mg of 45S5 BG (355–500 μm) failed to prevent *in vivo S. aureus* ATCC25923 infection of open tibial fractures in rabbits; these results were obtained for a single *S. aureus* strain and a single body site, a limitation that was recognized by the authors. Moreover, the common feature of all these works was the lack of pH increments in milieu in presence of 45S5 BG that could explain the discrepancies with the results of this work. In fact, the available evidence strongly suggests that the increase in aqueous pH value plays a critical role in 45S5 BG antibacterial effects (Allan *et al.* 2001; Hu *et al.* 2009) because it is well known that in general, a high alkaline environment is not well tolerated by the micro-organisms (Waltimo *et al.* 2007). The optimum pH value for the growth of staphylococci cells is between 7·0 and 7·5 (Misra *et al.* 2010). Thus, the increase in the pH value during the time of incubation found in this work explains, in part, the cell growth inhibition. Furthermore, there was a positive correlation between the antibacterial effects with the increase in pH during the incubation periods. Other reasons of the antibacterial properties may be related to an increment of the ionic strength, as the leaching of ions occurs from the BGs (Stoor *et al.* 1998), the high concentration of calcium and silica that could inhibit bacterial viability (Stoor *et al.* 1999; Zehnder *et al.* 2006), and also a physical damage to cell wall coming from BG debris could be related to its antibacterial effect (Hu *et al.* 2009).

It has been proved that the adhesion of bacteria on to mammalian tissue surfaces or biomaterials constitutes an important initial step in the pathogenesis of an infectious process (Von Eiff *et al.* 2005). The SEM observations showed that up to 48 h, none of the cells of the strains tested were present on BCs surfaces supporting the strongly antibacterial effects determined by plate count. These results are consistent with those found by Gorriti *et al.* on *S. aureus* ATCC 29213 (Gorriti *et al.* 2009). In other works, 45S5 BG just limited the bacterial attachment when present on Poly(3-hydroxybutyrate) (Misra *et al.* 2010) or sutures (Pratten *et al.* 2004). Conversely, it has been reported that 45S5 BG presented a dose-dependent and bacteria-dependent bacterial adhesion (Hu *et al.* 2009). On the other hand, when cells were incubated with uncoated films, a differential behaviour among *S. aureus* ATCC29213 and the rest of the staphylococci tested was detected. *S. aureus* ATCC29213 were found on pure films only in the case of infection model and after 48 h. It is well known that bacterial adhesion is a complex process affected by many factors such as bacterial properties (hydrophobicity, cell concentration), environmental conditions (ionic strength, pH, etc.) and the material surface characteristics (regularities and porosity) (Katsikogianni and Missirlis 2004; McWhirter *et al.* 2002; Dass *et al.* 2009; Ketonis *et al.* 2010; Crawford *et al.* 2012).

Taken an overview of the results, the BCs may have a potential application as wound dressing as they have

some advantageous features. Gelatin possesses the RGD (arginine-glycine-aspartic acid) sequences of collagen responsible of the cell adhesion and proliferation (Rosellini *et al.* 2009). Furthermore, gelatin results more convenient than collagen because it is known to have no antigenicity and also results more economical than collagen (Ulubayram *et al.* 2001). Because agar gels behave like a sponge, it will be ideal for adsorbing wound exudates. Another plus for these BCs is that agar–gelatin 2 : 1 weight ratio was proved to have good cytocompatibility on eukaryotic cells (Verma *et al.* 2007, 2009). Finally, there have been no reports of bacterial resistance to BG, and the preparation costs are relatively inexpensive compared to other antibacterial agents such as silver (Ong *et al.* 2008; Madhumathi *et al.* 2010) antibiotics (Choi *et al.* 1999; Denkbas *et al.* 2004) or iodine (Ignatova *et al.* 2008; Misra *et al.* 2010). Moreover, angiogenic effects have been reported *in vitro* and *in vivo* for 45S5 system (Day *et al.* 2004, 2005; Gorustovich *et al.* 2010) that could enhance the wound healing process.

Finally, it may be discussed if the the harmful effect in bacteria cells could be also negative for eukaryotic cells because of the alkaline pH. In reference to this, Misra *et al.* 2010; reported that P(3HB)/Bioglass BCs exhibited bactericidal properties in alkaline environment. *In vitro* study demonstrated that the biomaterials were suitable for MG-63 osteoblast cell attachment and proliferation. Also, when the foams were implanted in rats as subcutaneous implants resulted in a nontoxic and foreign body response after 1 week of implantation. Other investigations have shown that in fact, some healing processes such as the take rate of skin-grafts requires an alkaline milieu (Schneider *et al.* 2007).

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