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agar–gelatin–bioactive glass composites

Biomedical Materials

PAPER

Evaluation of the antibacterial effects of vancomycin hydrochloride released from agar–gelatin–bioactive glass composites

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Abstract

The aim of this work was to evaluate the perfomance of agar–gelatin (AG) composites and AG-containing 45S5 bioactive glass (BG) microparticles (AGBG) in relation to their water uptake capacity, sustained release of a drug over time, and antibacterial effects. The composites were fabricated by the gel-casting method. To impart the local drug release capacity, vancomycin hydrochloride (VC) was loaded in the composites in concentrations of 0.5 and 1 mg ml^{-1}. VC release was assessed in distilled water at 37 °C up to 72 h and quantified spectrophotometrically. The antibacterial activity of composites was evaluated by the inhibition zone test and the plate count method. The experiments were performed *in vitro* up to 48 h on three staphylococcus strains: *Staphylococcus aureus* ATCC29213, *S. aureus* ATCC6538 and *Staphylococcus epidermidis* ATCC12228. The results showed that the addition of BG to AG composites did not affect the degree of water uptake. The release of VC was significantly affected by the presence of BG. VC release was higher from AGBGVC films than from AGVC ones over prolonged incubation times. Bacterial inhibition zones were found around the composites. The halos were larger when the cells were put in contact with AGVC composites than when they were put in contact with AGBGVC ones. Nevertheless, the viable count method demonstrated that the composites inhibited Staphylococcus cell growth with no statistical differences. In conclusion, the addition of BG did not reflect an improvement in the parameters studied. On the other hand, composites loaded with VC would have a role in prophylaxis against bacterial infection.

1. Introduction

Natural polymers for the delivery of an antibiotic have been extensively studied for wound dressing application [1–4]. One of the most investigated protein-based delivery system is represented by collagen [5]. Advantages of natural polymers are that they have good cytocompatibility [6], are biodegradable in nature, and do not require any surgery for removal of polymers [7]. However, the release of antibiotics directly from natural polymers also presents some disadvantages. Most natural polymers are hydrophilic and cannot counteract the rapid release of the small antibiotic molecules upon water uptake. In addition, natural polymers undergo *in vivo* degradation by proteases. Consequently, the active agent is rapidly released from these materials [8].

In the last two decades, considerable research efforts have been made using bioactive glass (BG)-based platforms as carriers for the encapsulation, delivery and controlled release of bioactive molecules and therapeutic drugs [9–14]. The investigations were mainly focused on bone repair and regeneration applications. However, several studies have revealed that 45S5 BG can bind well with soft tissue and can promote the regeneration of soft tissue such as the skin [15, 16]. In fact, BGs can promote

angiogenesis *in vitro* [17–20] and *in vivo* [18, 21, 22]. Microarray analysis has also shown that ionic products from BGs can activate the expression of genes related to wound healing [19, 23]. Recently, ointments containing different BG systems, including 45S5 BG, have been shown to accelerate the recovery of skin wounds in both normal and diabetes-impaired healing models [24]. BGs promote the proliferation of fibroblasts and growth of granulation tissue. They also stimulate the production of the growth factors VEGF and FGF2 during the healing process and promote the formation of new capillary microvessels by the seventh day after treatment [24].

Previous researches have demonstrated that BG concentrations can modulate the release of a drug from a polymeric matrix [25, 26].

Gelatin, which is derived from collagen, has been widely used for biomedical applications such as wound dressing due to its low cost, wide commercial availability and low antigenicity [27, 28]. Besides, gelatin has film-forming properties and can absorb excess exudates because of its excellent ability to absorb water more than 5–10 times its own weight [29, 30]. Nevertheless, gelatin dissolves rapidly in aqueous environments and at human temperature [31, 32].

Agar is a polysaccharide with high mechanical strength, slow degradation, but no cell moieties for the adhesion and proliferation, so it is necessary to composite it with some quickly degradable polymer such as gelatin [33]. AG hybrids have been developed for different applications [32, 34–37]. In particular, AG matrices containing agar and gelatin in a 2:1 weight ratio exhibit the best growth kinetics of the fibroblast cell line NIH 3T3 [38]. Nevertheless, there are no studies considering these types of matrices with antibiotic release capacity by introducing BG particles.

The present work focuses on the preparation of AG and AG composites incorporating microparticles of 4S55 BG. Different concentrations of vancomycin hydrochloride (VC), a potent glycopeptide antibiotic used to treat infections caused by Gram-positive bacteria [39, 40], were incorporated into composites. Differences in water uptake capacity, the release behavior of VC, and antimicrobial properties between AGVC and AGVC plus 45S5 BG (AGBGVC) were investigated.

2. Materials and methods

2.1. Materials
Melt-derived 45S5 BG micrometer particles were 5–100 μm. The composition was (in wt%): 45% SiO_2, 24.5% Na_2O, 24.5% CaO, and 6% P_2O_5. Agar–agar was purchased from Britania S.A. (Buenos Aires, Argentina). Edible gelatin Royal was obtained from Kraft Foods Argentina. VC was purchased from Laboratorios Fabra S.A. (Buenos Aires, Argentina).

2.2. Preparation of composite films
Composite films were prepared according to Rivadeneira *et al* [40] with some modifications. Briefly,

AG in a 2:1 ratio was dissolved in distilled water making 1% homogenous solution. Then, 0.02 g of 45S5 BG was dispersed in 50 ml of distilled water for 15 min. Once BG microparticles precipitated, the excess of distilled water was removed with a pipette. The BG microparticles were then resuspended in 50 ml of distilled water and kept at 25 °C for another 15 min. Finally, the water was removed and added to the AG solution. Glycerol was added as plasticizer in a concentration of 1.33% (v/v). To this end, 30 ml was poured in a petri dish plate and kept in an incubator for 48 h at 30 °C. The VC-loaded composites were prepared in a similar manner such that the final concentrations of the drug in solution were 1 or 0.5 mg ml^{-1}. The composites obtained using the above preparation were named according to the components and the initial concentration of VC. In that way, AGVC-05 and AGVC-1 correspond to composites prepared with AG plus VC 0.5 mg ml^{-1} and 1 mg ml^{-1} respectively. Conversely, AGBGVC-05 and AGBGVC-1 correspond to composites prepared with AG plus 45S5 BG microparticles and plus 0.5 mg ml^{-1} and 1 mg ml^{-1} of VC respectively.

2.3. 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 M phosphate-buffered saline 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 SEM (JEOL JSM 6480 LV, Japan).

2.4. Water uptake capacity
The fluid absorbing capacity of a wound dressing is an important criterion in maintaining a moist environment over the wound bed. A swelling test was performed to determine the water uptake capacity of the various samples. Composite films were introduced in 15 ml of distilled water and then incubated at 37 °C. At regular time intervals, the weight of the composites was recorded after removing the water and gently blotting the composites with a filter paper. The weights of gels were recorded until equilibrium swelling was reached. Water uptake capacity of the film was calculated using the following formula:

$$\% \text{ Water uptake} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100.$$

All the tests were performed in triplicate and the average and standard deviation (SD) were calculated.

2.5. Release of VC
Initially, a calibration curve was obtained by dissolving 1, 2, 4, 6, 8, 10, 12, and 14 mg of VC in 20 ml deionized water. The solutions were analyzed using a UV-Vis Thermo Spectronic Helios Beta v.460 (Thermo
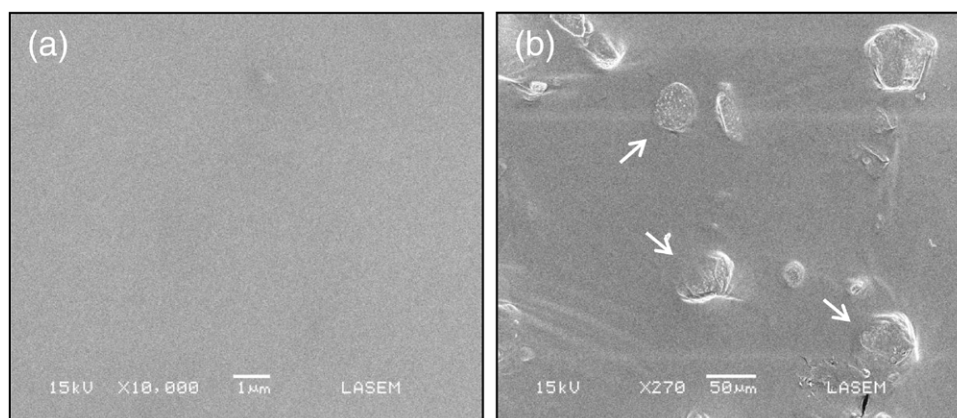
Figure 1. SEM images of composites. AGVC composites (*a*), AGBGVC composites (*b*), white arrows point to BG bulks.

Electron Corporation, Massachusetts, USA) at a wavelength of 280 nm.

The composites were cut in discs 5 mm in diameter with a paper punch circle (area = 0.2 cm^2) and then placed in 1 ml of pre-warmed water at 37 °C. At pre-determined time periods, the water was removed for sampling. The amount of VC released was determined spectrophotometrically using the above-mentioned spectrophotometer. Three samples in each condition were used for the *in vitro* drug-release tests, and the data were represented as means ± SD.

2.6. Bacterial culture and preparation of inoculum

The following strains were used: *S. epidermidis* ATCC12228, *S. aureus* ATCC29213 and *S. aureus* ATCC6538. All strains were grown for 24 h in Mueller Hinton broth (Britania S.A., Argentina) at 37 °C. For the experiments, bacterial cell suspensions were adjusted to 6–7 log cfu ml^{-1}, which correspond to infection [41–44]. For antibacterial activity tests, composite samples in disc form of 5 mm in diameter were obtained. The performance of samples was evaluated by the inhibition zone evaluation and the plate count method.

2.7. Inhibition zone evaluation

For the inhibition zone evaluation, 100 μl of the described bacterial suspension was seeded on Mueller Hinton agar plates. After that, the composites were placed upon lawns of each staphylococcus strain seeded on Mueller-Hinton agar. Non-releasing VC composites (controls) were also placed in the agar plates. After 24 h incubation, the inhibition zones were observed as a halo around samples where bacteria had not grown. The area of the inhibition zones was measured in mm. All tests were performed in duplicate and the average and SD were calculated.

2.8. Viable counts

For the plate count method, the experiments were carried out in Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg$^+$. The composite samples were incubated for 48 h at 37 °C in 1 ml of cellular
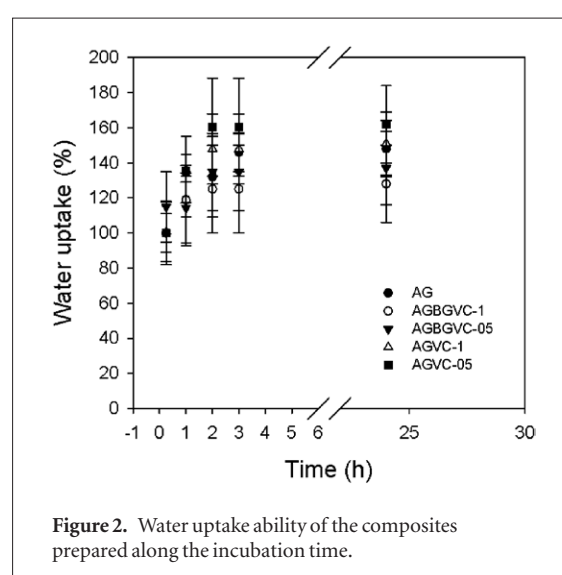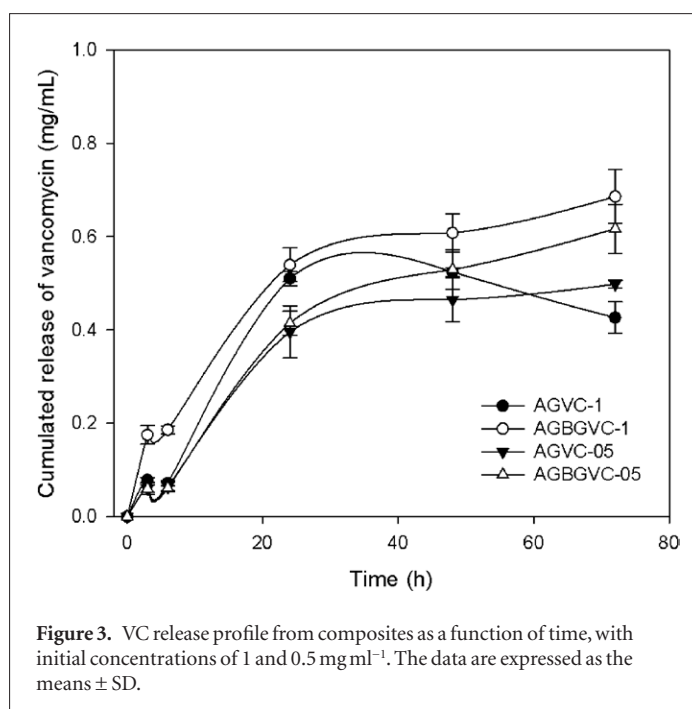

Figure 2. Water uptake ability of the composites prepared along the incubation time.

suspensions. Each staphylococcus suspension in the absence of biomaterial served as controls. Samples were collected after 24 and 48 h of incubation and the viability of cells at 37 °C was assessed by counting in Mueller-Hinton agar plates. After incubation, composites were prepared for SEM observation. The results are expressed as log$_{10}$ cfu ml^{-1} ± SD. All tests were performed in triplicate. The antibacterial activity was calculated as the difference between the log numbers of bacteria on the control and test composites. Finally, following the criteria of Gallant-Behn *et al* [45], low antimicrobial activity was considered to be less than a 1-log reduction, moderate activity between a 1- and 3-log reduction, and high antimicrobial activity as greater than a 3-log reduction.

2.9. Statistical analysis

Statistical analysis was performed using the SPSS 15.0 statistical package software with appropriate statistical tests such as one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-tests for inter-group analysis. The level of significance was set at a *P* value of <0.05.

Figure 3. VC release profile from composites as a function of time, with initial concentrations of 1 and 0.5 mg ml^{-1}. The data are expressed as the means ± SD.

3. Results

3.1. Preparation of composite films

The surface morphologies of composites examined by SEM are shown in figure 1. AGVC composites exhibited a smooth, dense and uniform surface (an example is shown in figure 1(*a*)). The addition of BG to composites introduced irregular bulks that made the surfaces rougher (figure 1(*b*)). As will be discussed later, this morphological change could have an impact on the interaction of the composite with Staphylococcus cells.

3.2. Water uptake capacity

The percentages of water uptake capacity for the composites are presented in figure 2. The water uptake values for both AGBGVC and AGVC were of the order of 125–160%. The addition of BG powder to composites did not reflect an increase or decrease in the water uptake capacity of the polymers along the incubation time. The swelling reached equilibrium after 15 min. Although some increase seemed to take place after that time, this was not statistically significant. The concentration of VC did not play a role in the swelling either. No decrease in water uptake was obtained after 24 h of incubation, presumably because no degradation occurred.

3.3. Release of VC

The cumulative release profiles from the four types of samples are presented in figure 3. The release profiles can be divided into two typically main stages: burst release during the first 24 h and approximately constant release rate from 24 to 72 h. BG presence affected the second stage of VC release. More VC was released from AGBGVC-1 and AGBGVC-05 composites over prolonged incubation periods (72 h). At this time,

the initial drug concentration had no effect on the cumulative release of VC on AGBGVC composites. On the other hand, increasing the drug content from 0.5 to 1 mg ml^{-1} had a significant effect only on the first stage of release; more VC was released until 24 h from AGVC-1 and AGBGVC-1 than from AGVC-05 or AGBGVC-05. After that period, a depletion of the drug was observed on AGVC-1 and a sustained released of VC took place on AGBGVC-05 composites.

3.4. Antibacterial effects

3.4.1. Inhibition zone evaluation

Representative images of the halos found around both AGBGVC and AGVC loaded with 0.5 and 1 mg ml^{-1} of VC are presented in figures 4(*a*)–(*c*). Also, table 1 shows the zone inhibition zone around the composites measured in mm. The three staphylococcus strains tested were inhibited in the presence of the composites. Nevertheless, *S. aureus* ATCC6538 produced inhibition zones of different size against the same composites compared with *S. aureus* ATCC29213 and *S. epidermidis* ATCC12228, which produced inhibition zones of similar size. For example, when AGVC-05 was incubated with the microorganisms, the inhibition zones around *S. aureus* ATCC6538 were 24 ± 0.00 mm, whereas those around *S. aureus* ATCC29213 or *S. epidermidis* ATCC12228 were 20 ± 1.41 mm and 20 ± 0.00 mm respectively.

The addition of BG powders to AGVC composites decreased the efficacy of bacterial inhibition nearly 20%. The halos found around AGVC-05 and AGVC-1 composites were always larger than those found around AGBGVC-05 or AGBGVC-1 (figure 4 and table 1).

The antibiotic concentration did not play a role in the size of the halos, which means that the diameters of the inhibition zones in the presence of AGBGVC-05 or AGBGVC-1 were similar. The same occurred for AGVC-05 and AGVC-1 (figure 4 and table 1).
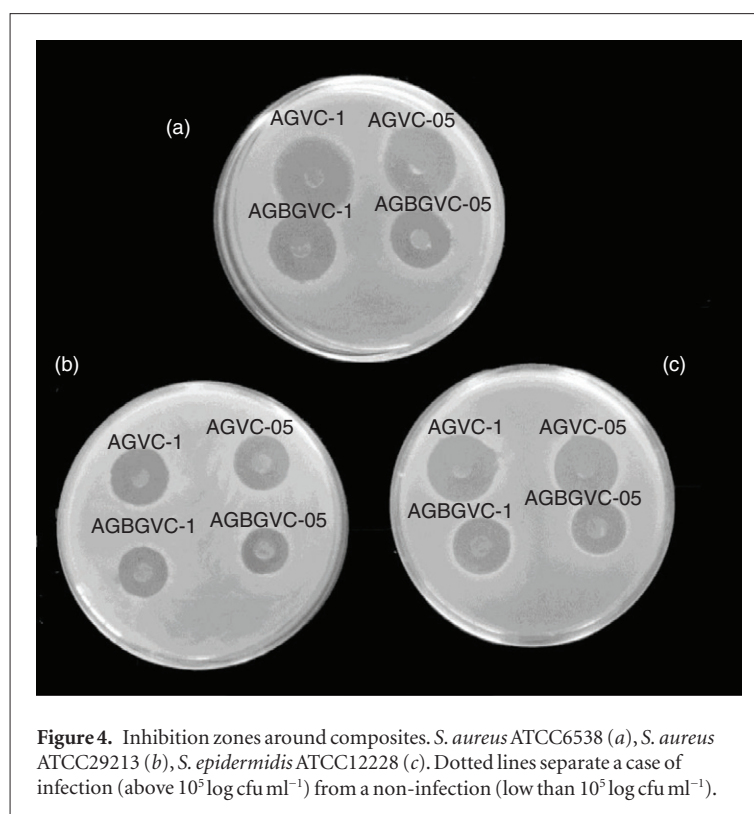
Figure 4. Inhibition zones around composites. *S. aureus* ATCC6538 (*a*), *S. aureus* ATCC29213 (*b*), *S. epidermidis* ATCC12228 (*c*). Dotted lines separate a case of infection (above $10^5 \log \mathrm{cfu\,ml^{-1}}$) from a non-infection (low than $10^5 \log \mathrm{cfu\,ml^{-1}}$).

Table 1. Inhibition zone around composites $N = 3$ (mm).

	Staphylococcus strain		
Composite	ATCC6538	ATCC29213	ATCC1228
AGVC-1	26 ± 0.00	20.5 ± 2.12	21.5 ± 0.71
AGBGVC-1	21.5 ± 0.71	15.67 ± 0.71	18 ± 0.00
AGVC-05	24 ± 0.00	20 ± 1.41	20 ± 0.00
AGBGVC-05	19.25 ± 0.5	15.5 ± 1.04	16.5 ± 0.71

3.5. Viable counts

The results of the viable counts are shown in figure 5. Cell viability was significantly inhibited ($p < 0.05$) on the three strains in comparison to the control after 24 and 48 h of incubation. A more prolonged incubation period did not significantly increase the reduction of cell numbers. Unexpectedly, there was no statistical significance between AGVC and AGBGVC composites. In addition, the efficacy of the samples was not dependent on VC concentration, as no statistically relevant differences between the samples exposed to the different concentrations of VC were found.

Both AGVC and AGBGVC composites failed to reduce the numbers of bacteria below 10^5 cfu ml^{-1}. Finally, following the criteria of Gallant-Behn *et al* [45], composites in general presented moderate antimicrobial activity, which means that the log reductions were between 1 and 3.

3.6. SEM analyses post-incubation

Highly porous surfaces were created on AGVC composites post-incubation in cell suspensions probably because of VC release (figure 6(*a*)). In AGBGVC composites (figure 6(*b*)), many bulk zones were found broken. The presence of bacteria was poor in both AGVC

and AGBGVC composites. Staphylococcus cells were found on plane AG surface (figure 6(*c*)) and around intact bulks of BG but not on exposed ones (figure 6(*d*)).
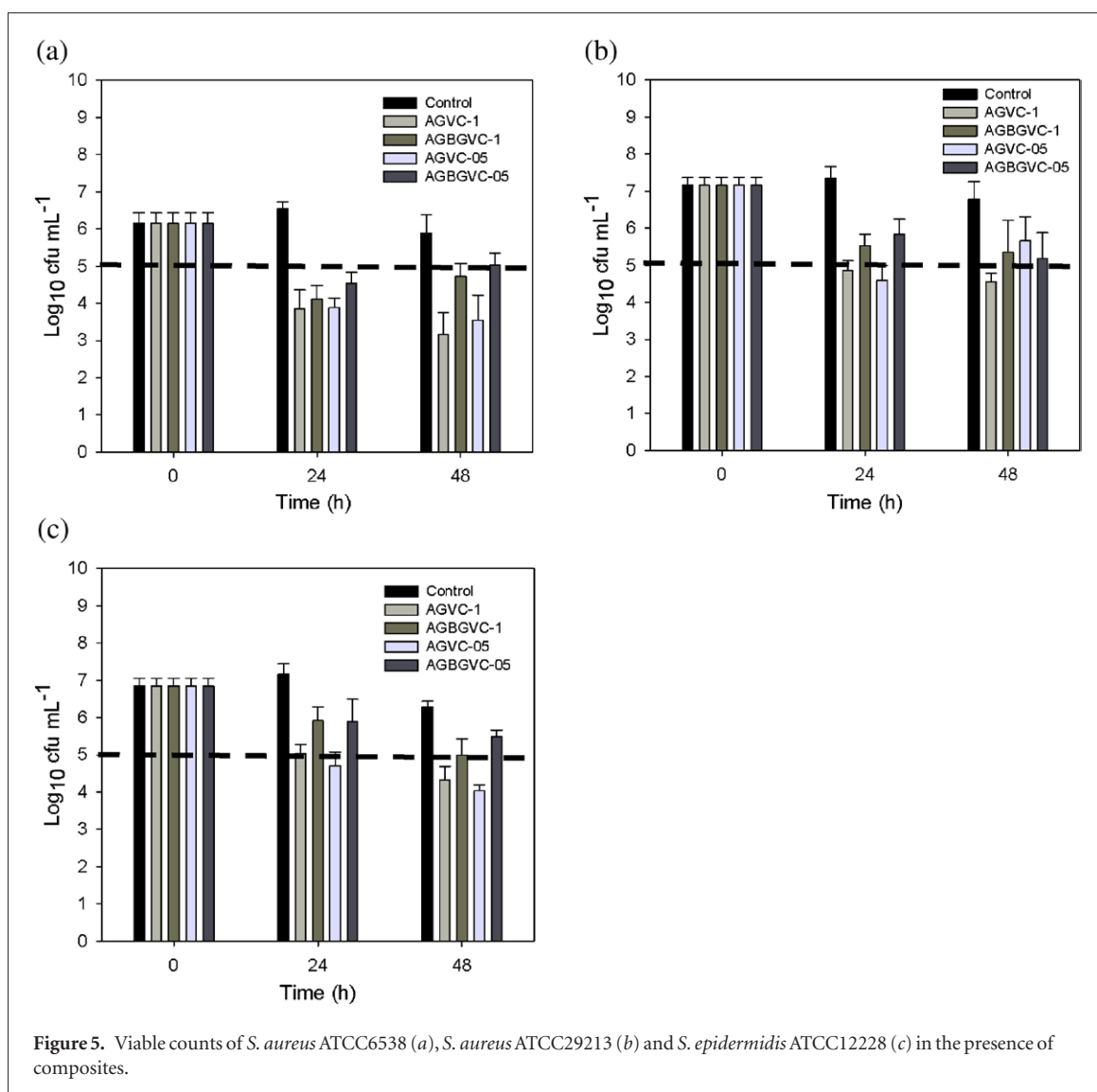
4. Discussion

In this work, AG composites and AGBG composites were developed for the delivery of VC. The effect of the addition of 45S5 BG on water uptake capacity, the release profile of the drug, and antibacterial performance of the composites were evaluated.

As mentioned above, it is important for wound dressings to absorb exudates quickly. Both AGVC and AGBGVC composites were able to reach the equilibrium of swelling of the order of 125–160% after 15 min. This was expected due to the hydrophilic nature of the components. According to the results, there were no differences in the swelling between the composites. Mota *et al* [46] found similar results with chitosan and chitosan with BG nanoparticle membranes. However, other authors reported opposite results. For example, Maquet *et al* [47, 48] showed that the addition of BG to a polymer increased water absorption, whereas Peter *et al* [49] found a significant reduction in water uptake with the addition of BG to scaffolds based on biopolymers.

The role of BGs as improvers of water uptake capacity is clearer when they are incorporated into hydrophobic polymers [25, 50]. In addition, the size particle of BG modifies the water uptake capacity. Nanoparticles lead to higher water uptake, attributable to the higher extent of exposure of the nano-BG particles on the composite surfaces as well as to their higher surface area [50].

Controlled drug release is a key factor for regenerative medicine. In general, the release curves show

Figure 5. Viable counts of *S. aureus* ATCC6538 (*a*), *S. aureus* ATCC29213 (*b*) and *S. epidermidis* ATCC12228 (*c*) in the presence of composites.

an initial burst effect followed by a slow release profile. The fast release is mainly caused by the dissolution of the antibiotic located on the surface of the composites and the slow release may be attributed to the chemically adsorbed drugs or to the drug molecules entrapped within the micropores present on the composite surfaces [12–14].

In the present study, we found that the release of VC from AG composites can be modulated by the addition of 45S5 BG microparticles. This was clearly significant at the second stage of the drug release when the total quantity of VC released increased on AGBGVC composites over prolonged incubation periods. One possible explanation is that an irregular surface constitutes a larger area for VC release.

Kouhi *et al* [26] reported that the presence of BG nanoparticles in nanofibers increases their diameter and that, consequently, the release path through the nanofibers increased. This phenomenon increased the release of a drug from nanofibers containing BG. On the other hand, the existence of a large number of Si–OH and P–OH groups in BG was the suggested mechanism of sustained drug release kinetics since those groups

interact with drugs and protein by hydrogen bonding [51]. Porosity has been related to a sustained release rate but not to the initial release rate [52]. The relationship between porosity of BG scaffolds and drug release kinetics is well documented elsewhere [51]. AGVC composites seem to be denser than AGBGVC ones, a fact that could also explain why the VC released from AGBGVC composites was greater.

The release profile of a dressing defines its role for example to combat an infection or for prophylaxis [8]. A burst release would be beneficial if the number of bacteria is large but a slow and sustained release would be more suitable for prophylaxis. In general, according to the results presented here, although the biomaterials clearly inhibited the cell growth of the three staphylococcus strains, both AGVC and AGBGVC composites would not be suitable to manage the large number of bacteria present in an infection since the numbers of bacteria were not lower than 10^5 cfu ml^{-1} after the incubation time. The exception would be *S. aureus* ATCC6538, which was strongly inhibited in the presence of both AGVC and AGBGVC composites. The selection of the antibiotic is relevant according to the
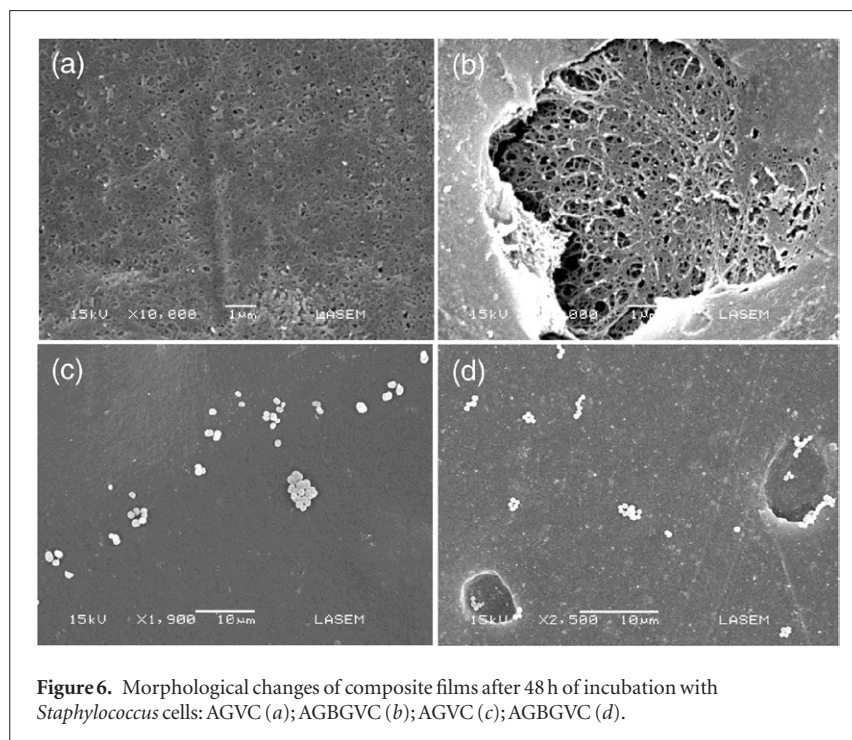
Figure 6. Morphological changes of composite films after 48 h of incubation with *Staphylococcus* cells: AGVC (*a*); AGBGVC (*b*); AGVC (*c*); AGBGVC (*d*).

strategy of wound management [53]. For treatment, the characteristics of the pathogen govern the choice of the drug. Conversely, for prophylaxis, the antibiotic substance should have a broad spectrum of activity, covering most pathogenic species usually implicated in implant infections in the given anatomic site [53]. In that context, the choice of VC could not be suitable for AG or AGBG matrices.

Interestingly, we found no correlation between the zone evaluation test and the viable count test. In the former, the halos found around AGVC composites were greater than those on AGBGVC ones, which allows assuming that AGVC composites would inhibit staphylococcus strains more strongly than AGBGVC ones. However, the viable count method demonstrated that, in fact, the composites inhibited staphylococcus cells with no statistical differences. The disc diffusion test is a good representation of the clinical situation, where the dressing material is applied to the wound surface, allowing the drug to diffuse to the wound bed [8]. However, the results of this method depend not only on the diffusion rate of the active agent from the dressing, set against the growth rate of the bacterial species growing on the lawn, but also on the physicochemical environment. Log reduction assays are used to determine the microbicidal activity of antimicrobial agents and provide valuable information for the evaluation of antimicrobial wound dressing efficacy [45].

Regarding the material components of wound dressings, surface morphology should present reduced presence of protective niches easily colonized by bacteria [53]. It is often acknowledged that rough surfaces increase the probability of bacterial adhesion, but, as reported previously [54], this is only valid for certain feature dimensions. Adhesion is enhanced when the dimensions or spacing of the surface are similar to those of the bacteria. Larger surface defects may not retain bacteria due to low cell–surface contact area. This could explain the reduced presence of Staphylococcus strains found on AGBGVC composites. Reduced presence of bacteria has also been observed on AG films coated with 45S5 BG [40] and on other polymers containing 45S5 BG [55, 56]. Another possible explanation may be the negative charge of the strain studied [57]. Since BG is negatively charged at physiological pH [58], some degree of electrostatic repulsion would occur between the bacterial cells and the BG surfaces. It has been shown that *S. aureus* exhibits a large zeta potential ($\zeta = 35.2$ mV) [59] and hence this could increase the electrostatic repulsion. In fact, the development of anti-adhesive materials against bacteria is another strong strategy besides materials eluting antimicrobial to prevent infections [60]. With less bacterial adhesion, the risk of biofilm formation could be reduced or delayed. In that context, AGBGVC composites would be a promising material.

5. Conclusions

This research work demonstrated that 45S5 BG can modulate the release profile of VC from AG matrices. Nevertheless, this modification did not reflect an improvement in the antibacterial effects of the composites nor the crescent VC concentration. On the other hand, the water uptake capacities of AGVC and AGBGVC were similar. The composites have potential use for wound dressing application. Future research would focus on the cell biological response of the developed composites in relation to wound healing.

Acknowledgments

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