The influence of pH and fluid dynamics on the antibacterial efficacy of 45S5 Bioglass

Short title: Antibacterial efficacy of 45S5 Bioglass

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In recent years, there has been considerable interest in the potential antibacterial properties that bioactive glasses may possess. However, there have been several conflicting reports on the antibacterial efficacy of 45S5 Bioglass®. Various mechanisms regarding its mode of action have been proposed, such as changes in the environmental pH, increased osmotic pressure, and 'needle like' sharp glass debris which could potentially damage prokaryotic cell walls and thus inactivate bacteria. In this current study, a systematic investigation was undertaken on the antibacterial efficacy of 45S5 Bioglass® on *Escherichia coli* NCTC 10538 and *Staphylococcus aureus* ATCO 6538 under a range of clinically relevant scenarios including varying Bioglass® concentration, direct and indirect contact between Bioglass® and microorganisms, static and shaking incubation conditions, elevated and neutralised pH environments. The results demonstrated that under elevated pH conditions, Bioglass® particles has no antibacterial effect on *S. aureus* whilst, a concentration dependent antibacterial effect against *E. coli* was observed. However, the antibacterial activity ceased when the pH of the media was neutralised. The results of this current study therefore suggest that the mechanism of antibacterial activity of Bioglass® is associated with changes in the environmental pH; an environment that is less likely to occur *in vivo* due to buffering of the system.

Keywords: Bioglass, 45S5, pH, antibacterial

Introduction

Since the discovery of the 45S5 Bioglass®, bioactive glasses (BAGs) have shown great success in many clinical applications particularly in the dental and orthopaedic fields [1-3]. 45S5 Bioglass has the ability to establish real chemical bonds with both soft (muscle) and hard (bone) tissue [4]. The glass is biodegradable and provides a controlled release of calcium and phosphorous ions under physiological conditions [1]. These ions then precipitate into amorphous calcium phosphate which later crystallise into hydroxyapatite to form new bone mineral [5-7]. In addition to providing the fundamental building blocks of bone (Ca and P) the dissolution products of bioglass were also found to stimulate osteoblast activity [8, 9].

In recent years, there has been considerable interest in the potential antibacterial properties that bioactive glasses may possess. However, there have been several conflicting reports on the antibacterial efficacy of 45S5 Bioglass. Bioactive glasses (BAGs) have been shown to exhibit an antibacterial effect against oral and skin pathogens [10-14]. BAGs have also been shown to reduce microbial infections when used as therapeutic materials to treat clinical conditions, such as infected frontal sinuses, periodontal defects, and atrophic rhinitis [10]. Based on these results Stoor *et al*. [15] explored the antibacterial properties of BAGs and in 1998 through experiments *in vitro*, they demonstrated that the bioactive glass S53P4 exhibited antibacterial efficacy on oral bacteria when cultured in the planktonic phase of growth [10]. Since then several *in vitro* studies have been conducted to determine whether 45S5 Bioglass exerted a similar antibacterial effect on skin pathogenic bacteria. Hu *et al*. [16] showed 45S5 Bioglass to possess broad-spectrum antibacterial activity, as it inhibited the growth of all three test microorganisms (*Staphylococcus aureus, Staphylococcus epidermidis* and *Escherichia coli*).

Unfortunately, the mechanisms of antibacterial activity are still not clearly understood. Various mechanisms regarding its mode of action have been proposed, such as changes in the environmental pH, increased osmotic pressure, and ‘needle like’ sharp glass debris which could potentially damage prokaryotic cell walls and thus inactivate bacteria [16]. Initial studies on the antibacterial efficacy of

BAGs revealed that the activity was attributed to the high aqueous pH values caused by the release of alkali ions such as, sodium and calcium from BAG particles, when immersed into an aqueous environment [11, 17]. It has also been reported that bacterial adhesion on 45S5 particles is a necessary step towards bacterial inactivation [16]. On the contrary, Allan *et* al. [17] has reported that direct contact between Bioglass and bacteria is not required to produce an antibacterial effect. Studies conducted by Stoor *et al.* [18] and Bellantone *et al*. [19] demonstrated that BAGs did not possess bacteriostatic or bactericidal activity. These contradictory findings cast doubt on whether BAGs are indeed antibacterial in nature. Furthermore it is important to note that different locations across an implant site will be subjected to different levels of contact /exposure to bioactive glass as illustrated in Figure 1. (a) illustrates a site that is in direct contact and has minimal flow of body fluids (represented by direct contact, static tests), (b) illustrates an area which is in direct contact with soft tissue and has body fluid buffering the system (represented by direct and indirect contact under shaking incubator conditions), and (c) illustrates an area where there is no direct contact and only dissolution products are acting on the tissue (represented by the indirect experiments). The location and nature of the site will determine the flow of body fluid and buffering of the site and therefore sites (b) and (c) have been modelled using pH neutralised and elevated pH experiments. Therefore the *in vitro* experimental conditions required to accurately test for antibacterial activity will need to reflect the range of environments that maybe present *in vivo*.

The objectives of this current study were to investigate the antibacterial efficacy of 45S5 Bioglass particles under a variety of clinically relevant conditions such as direct and indirect contact, the effect of the dissolution products, static and shaker incubation conditions and elevated or neutralised pH. *S. aureus* and *E. coli* were chosen to represent Gram-positive and Gram-negative bacteria, respectively. *S. aureus* has become a major concern regarding prosthetic devices and other surgery related-infections. Furthermore Gram-negative bacteria have been recovered from biomaterial-related infection sites [20, 21].

Materials and methods

Glass preparation

45S5 Bioglass was prepared using $CaCO_3$, SiO_2 (both Alfa Aesar, Lancashire, UK), Na_2O (Sigma-Aldrich, Dorset, UK), H_6NO_4P (Fluka, Dorset UK). The precursors were weighed in molar ratios of 46.1 SiO_2, 26.9 CaO, 24.4 Na_2O, and 2.6 % P_2O_5, thoroughly mixed and placed into a 59 ml volume (90% platinum and 10% rhodium) crucible. The crucible was placed into a furnace at room temperature and heated at 10ºC/min to 1450ºC and held at this temperature for 90 minutes. Glass was cast by pouring the melt into a preheated to (370ºC) graphite mould [6, 22]. The resultant glass was ground into particles and sieved (<63 microns).

Microorganisms

E. coli (NCTC 10538) and *S. aureus* (ATCO 6538) were used in this study. The microorganisms were stored at –70ºC on MicroBank beads (Pro-Lab Diagnostics, Neston, Cheshire, UK) until required. Starter cultures for each test microorganism were prepared following inoculation of nutrient broth (NB) with a single colony from nutrient agar (NA) plates. After 24 hour incubation at 37ºC in aerobic conditions, the concentration of microorganism in each starter culture was determined by an optical density (OD) (590 nm)/concentration standard curves. The cultures were then diluted in NB as required to generate a final working concentration of 10^6 colony forming unit (CFU)/ml.

Investigation of bacterial cell adhesion on 45S5 Bioglass particles by Scanning Electron Microscopy (SEM)

Ten mg of 45S5 Bioglass particles were added to one ml NB and inoculated with the test microorganisms at 10^6 CFU. Glass cover slips inoculated with the test microorganisms served as the control. Following a 24 and 96 hour incubation period the samples were fixed with 2.5% glutaraldehyde in 0.1% w/v sodium cacodylate buffer (pH 7.4) for 10 minutes, followed by

dehydration in graded ethanol series. Following the completion of the dehydration step the samples were treated with hexamethyldisilazane and left overnight at room temperature and gold-sputtered prior to observation with a Carl Zeiss EVO MA10 SEM.

Determination of the antibacterial activity of the 45S5 Bioglass

Direct contact

UV sterilised particles of 45S5 Bioglass at 2.5 mg, 5 mg and 10 mg were added to 1 ml NB. Bioglass free NB served as the control. Samples were inoculated with the test microorganisms to give a final microbial concentration of 10^6 CFU within the test carrier, incubated at 37ºC under aerobic conditions for 24, 48, 72 and 96 hours. Antibacterial properties of 45S5 Bioglass particles were evaluated under two different incubation conditions: - static and shaker (100 rpm). At each time point the samples were removed, and following a serial dilute 100 µl aliquots were dispensed onto NA plates, which were then incubated overnight at 37ºC under aerobic conditions. Following incubation, the total viable CFU count was determined, and growth reductions calculated. A logarithmic microbial growth reduction of less than 0.5 and 1 are regarded as a slight, values greater than 1 and less or equal to 3 as a significant, and values greater than 3 as a strong antibacterial activity [23].

Indirect contact

Stock solutions were prepared for (UV sterilised) using 45S5 Bioglass particles at 0 (control), 2.5, 5 and 10 mg/ml in NB. The stock solutions were incubated for 72 hours at 37ºC under aerobic conditions in both static and shaker (with a shaking speed of 100 rpm) incubators and then filtered using a 0.45 micron syringe filter. One ml was removed from the filtered stock solution and inoculated with the test microorganisms at a final concentration of 10^6 CFU. The inoculated samples were incubated at 37ºC under aerobic conditions in both static and shaker (100 rpm) incubators. The pH of the stock solution was measured using the Mettler Toledo pH probe. Following 24 and 96

hours of incubation serial dilutes were performed, and 100 µl aliquots were dispensed onto NA plates, which were incubated overnight at 37ºC. Following incubation, the total viable CFU count was determined, and growth reductions calculated.

Determination of the antibacterial activity of the 45S5 Bioglass following neutralisation

Direct contact

UV sterilised particles of 45S5 Bioglass at 2.5 mg, 5 mg and 10 mg were added to 1 ml NB. NB without the 45S5 Bioglass particles served as the control. The samples were incubated overnight at 37ºC under aerobic conditions in both static and shaker (100 rpm) incubators. After an overnight incubation period the samples were removed and the pH recorded. Once the pH was measured the samples were neutralised to pH 7.3 (2) to achieve this 0.1M of hydrochloric (HCl) acid was used. Neutralised samples were inoculated with the test microorganisms at 10^6 CFU, incubated overnight at 37ºC under aerobic conditions in both static and shaker (100 rpm) incubators. At each time point the samples were removed and the pH was measured, and if required the pH was further neutralised to 7.3 (2) to ensure the pH remained in the range. Subsequently, serial dilutions were performed, followed by 100 µl aliquots being dispensed onto NA plates that were incubated overnight at 37ºC. Following incubation total viable CFU count was determined, and growth reductions calculated.

Indirect contact

Stock solutions (mg/ml) were prepared for (UV sterilised) 45S5 Bioglass particles at 0 (control), 2.5, 5 and 10 mg in NB. The stock solutions were incubated for 72 hours at 37ºC under aerobic conditions in both static and shaker (with a shaking speed of 100 rpm) incubators. Following the 72 hour incubation period the stock solutions were filtered using a 0.45 micron syringe filter. One ml was removed and neutralised to achieve a pH of 7.3 (2) using 0.1M of HCl and inoculated with the test microorganisms at 10^6 CFU. Remainder of the stock solution was used to measure the pH. The samples were incubated at 37ºC under static and shaker (100 rpm) conditions. After 24 and 96 hours of incubation, the samples were removed, serial dilutions performed and 100 µl aliquots

dispensed onto NA plates, which were incubated overnight at 37ºC. Following incubation, the total viable CFU count was determined, and growth reductions calculated.

Statistical analysis

Two-way analysis of variance was carried out to determine statistical significances (GraphPad Prism). If a significant difference was detected a Tukey test and a Sidak's test (for the neutralised experiments) were carried out to determine which values were significantly different. Differences were considered statistically significantly at a level of $P < 0.05$.

Results

Bacterial adhesion on 45S5 Bioglass particles

The SEM images shown in figure 2 demonstrate bacterial adhesion on the surface of 45S5 Bioglass particles. *S. aureus* and *E. coli* were both able to adhere to 45S5 Bioglass, however a higher level of adherence was observed with *S. aureus*.

Determination of the antibacterial activity of the 45S5 Bioglas

Direct contact

Figure 3 shows the antibacterial effect of 45S5 Bioglass particles over a 96 hour period under static and shaker incubation conditions. 45S5 Bioglass particles exhibited a strong antibacterial effect against *E. coli* at the concentration of 10 mg/ml. Under shaker incubation conditions 45S5 Bioglass particles totally inhibited *E. coli* growth within 48 hours (< 0.001), with a log reduction of 9.45. However, under static conditions the 45S5 Bioglass at 10 mg/ml the particles gradually reduced the number of mean viable cells. A log reduction of 7.73 was observed at 96 hours. Also a smaller but significant difference was observed for lower concentrations; a 1.87 log reduction was seen for 5 mg/ml at 96 hours (< 0.0001) under shaking conditions. 45S5 Bioglass particles did not possess a strong antibacterial effect against *S. aureus*.

Indirect contact

Figure 4 shows the antibacterial effect of the dissolution products under static and shaker incubation conditions. The dissolution products of 45S5 Bioglass particles showed no antibacterial activity against the microorganisms tested under static conditions. However, a strong antibacterial effect was demonstrated for *E. coli* under shaking incubation conditions (10mg/ml), with a log reduction of 3.99 at 96 hours (< 0.001). The dissolution products also exhibited a slight antibacterial effect against *S. aureus* with a log reduction of 1 at 96 hours (< 0.05). Table 1 shows the pH values of the solutions as a function of 45S5 Bioglass particle concentration. As expected the pH increased with increasing concentrations of 45S5 Bioglass particles.

Determination of the antibacterial activity of the 45S5 Bioglass following neutralisation

Direct contact

As shown in figure 5 45S5 Bioglass particles showed no antibacterial activity against the test microorganisms under both static and shaker incubation conditions once the pH was neutralised.

Indirect contact

As shown in figure 6 the dissolution products of 45S5 Bioglass particles showed no antibacterial effect against *E. coli* and *S. aureus* under both static and shaker incubation conditions, following the neutralisation of the pH from alkaline to pH 7.3 (2).

Discussion

In the present study, the results show that particulate 45S5 Bioglass does not possess broad-spectrum antibacterial properties, as growth inhibition could only be demonstrated for *E. coli* but not for *S. aureus*. In agreement with the results presented here, Hu *et al*. [16] reported a significant difference between Gram-negative bacterium (*E. coli*) and Gram-positive bacteria (*S. aureus* and *S. epidermidis*), when tested against 45S5 Bioglass particles. The difference observed between *S.*

aureus and *E. coli* could be attributed to their cell structures. The cell wall of Gram-positive bacteria is primarily composed of a thick peptidoglycan layer however it also contains large amounts of teichoic and lipotechoic acids. Doyle *et al* [24] and Hughes *et al* [25] demonstrated that both the peptidoglycan layer and teichoic acids (which provide highly charged anionic clusters) contribute to the binding of metal (such as Na and Ca) ions to the cell wall. This could explain why no growth inhibition was seen for *S. aureus*, as the metal binding sites present in the cell wall promoted attachment onto the surface of 45S5 Bioglass particles, evident in the SEM images. Furthermore, Stoor *et al*. [18] discovered that BAGs transiently promote growth and are subject to bacterial adhesion due to the presence of Ca ions in the Si-rich layer.

However, this does not apply to *E.* coli, which is a Gram-negative bacterium. The cell wall of *E.* coli is composed of a thin peptidoglycan layer with no teichoic acids enclosed within an outer membrane. Hu *et al*. [16] reported that needle like BAG debris was adsorbed onto the surface of *E.* coli, which led to the destruction of the cell wall. In addition, the higher local pH value measured on the surface of the 45S5 Bioglass particles, was believed to potentiate the antibacterial effect of 45S5 Bioglass particles by destructing the bacterial structure.

Allan *et* al. [17] found the surface reactions of Bioglass to be capable of killing oral microorganisms. The indirect contact experiments were conducted to elucidate whether the dissolution products would be sufficient at inducing bacterial cell death, and also if particle presence is necessary for bacterial inactivation. BAGs release Na and Ca ions in aqueous conditions, and their release elevates the pH. The results for the indirect contact experiments show that 45S5 Bioglass particles were only effective at the highest concentration tested (10 mg/ml) at reducing *E. coli* growth under shaking conditions. *E. coli* has an outer membrane which is embedded with pore proteins. The dissolution products released from the 45S5 Bioglass particles are small enough to leach into these proteins, and once inside elevate the intracellular pH, resulting in cell death. Elevated pH levels of the medium can alter the pH gradient present in the cytoplasmic membrane of bacteria, which is important for the movement of nutrients into the cell. Therefore, by

influencing the transfer and permeability of the cytoplasmic membrane, the high pH can induce inhibition and toxic effects on the bacteria [10]. In contrast *S. aureus* lacks an outer membrane and its cell wall binds Ca and Na ions, therefore rendering the dissolution products ineffective. Furthermore, Cutinelli and Galdiero [26] demonstrated that an increased pH increased the amount of ions bound by *S. aureus* cell wall. As an increased pH deprotonates functional groups within the cell wall, resulting in an increasing number of sites available for metal adsorption [27]. These results indicate that the presence of bioactive glass particles play an important role in the antibacterial effect along with higher aqueous pH [16].

An *in vivo* study conducted on the antibacterial activity of BAGs revealed that particulate Bioglass does not exhibit antibacterial activity *in vivo*. Xie *et al*. [10] suggested that the inactivity of particulate Bioglass *in vivo* was due to the '*big buffer system'* in body fluid, which could swiftly exchange dissolution products with nearby blood and other body fluid, so the ionic concentration and pH in body fluid at the site of implantation cannot increase to sufficient high levels required to inhibit bacterial growth. In this current study the pH was neutralised to represent and recreate a physiological environment, from alkaline to 7.3 (2). The results for the neutralised experiments are in agreement with the *in vivo* study. BAGs have a negative surface potential when in aqueous solutions and at physiological pH the microorganisms also carry a net negative charge [15]. This may explain why no growth inhibition was observed for both *E. coli* and *S. aureus* as they were potentially unable to bind to the surface of the particles. The release of Ca and Na ions are not solely associated with pH elevation but also with osmotic pressure [11, 14, 18]. Allan *et al*. [17] postulated that pH reduction of Bioglass supernates may alter the solubility of particular ions, which at higher pH values could be responsible for the antibacterial action. Thus, pH neutralisation of 45S5 Bioglass particles, may have affected the solubility of Na and Ca ions, preventing an increase in the osmotic pressure.

Furthermore, the incubation conditions influenced the antibacterial effect of 45S5 Bioglass particles. 45S5 Bioglass particles significantly inhibited *E. coli* growth under shaking conditions.

This could be contributed to the fact that particles under shaking conditions are suspended in solution. Therefore, are far more likely to come into contact with the solution resulting in increased dissolution, which resulted in higher pH values. Under static conditions, particles on the top layer are more likely to be exposed to the solution. However, even under shaking conditions 45S5 Bioglass particles were unable to exhibit a strong antibacterial effect against *S. aureus*. This could be a result of *S. aureus* adsorbing onto the surface of the particles, and reducing the dissolution rate.

Conclusion

A systematic evaluation of the antibacterial effect of 45S5 Bioglass has been undertaken for a range of clinically relevant scenarios. 45S5 was found not to possess broad-spectrum antibacterial activity as no significant growth inhibition was found for Gram positive *S. aureus*. Under direct contact conditions 45S5 was found to significantly inhibit Gram negative *E. coli* within 24 hours at the highest concentration of 10 mg/ml and partially inhibit growth at 5 mg/ml. An increase in antibacterial activity was observed under shaking incubator conditions compared to static incubation and 10 mg/ml exhibited a complete kill within 48 hours. For indirect contact the dissolution products had negligible antibacterial effect with the exception of 10 mg/ml under shaking conditions which exhibited a 4 log reduction. Following neutralisation of the pH 45S5 no antibacterial activity was observed for either *S. aureus* or *E. coli* even at 10mg/ml under shaking incubator conditions.

The fact that the bacterial cells could withstand the antibacterial effect of the particles following pH neutralisation suggests 45S5 Bioglass would not be an effective antibacterial agent *in vivo*. These results highlight the importance of methodology when testing antibacterial properties of biodegradable implants. This study illustrates that the antibacterial effect of 45S5 Bioglass particles is primarily driven by pH and that contact between ‘needle like’ particles and bacterial cells or changes is osmotic pressure have minimal antibacterial efficacy.

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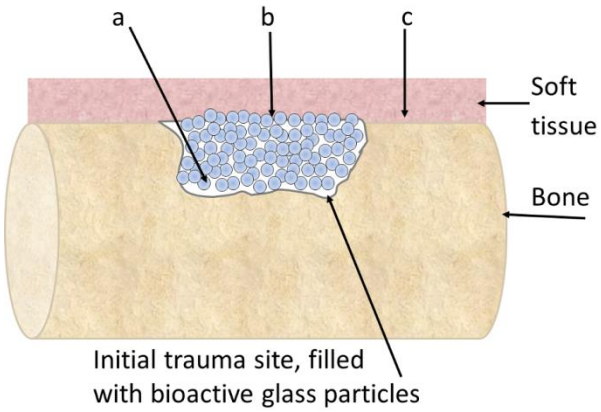

Figure 1 a schematic illustration of the different types of environments present within a bone defect filled with bioactive glass particles.

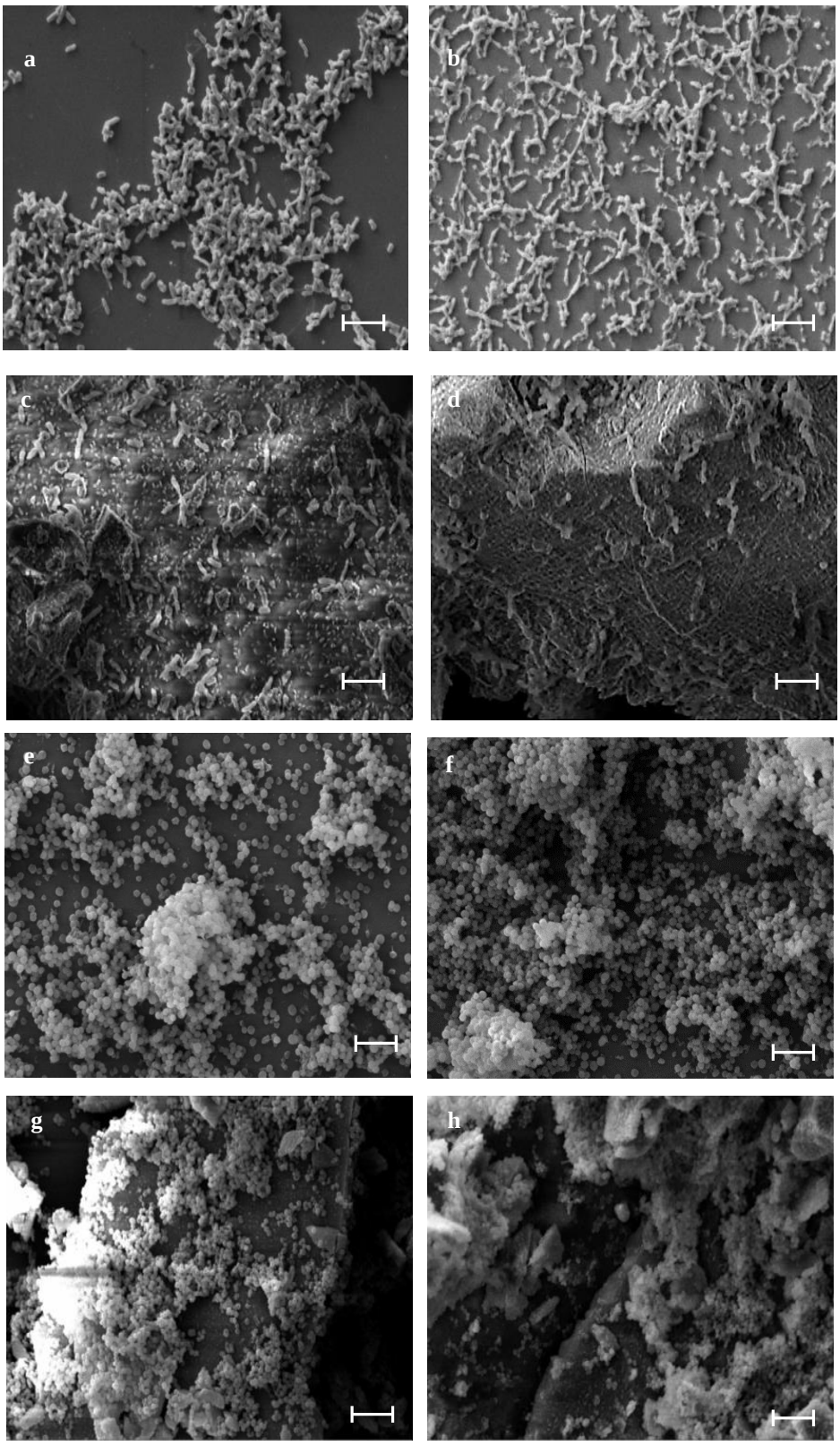

Fig.2 SEM images of *S. aureus* and *E. coli* cultured on 45S5 Bioglass particles and glass cover slips (control). *E. coli* cultured on glass cover slips at (a) 24 and (b) 96 hours and on 45S5 Bioglass particles at (c) 24 and (d) 96 hours. *S. aureus* cultured on glass cover slips at (e) 24 and (f) 96 hours, and on 45S5 Bioglass particles at (g) 24 and (h) 96 hours. Scale bar represents 2µm.

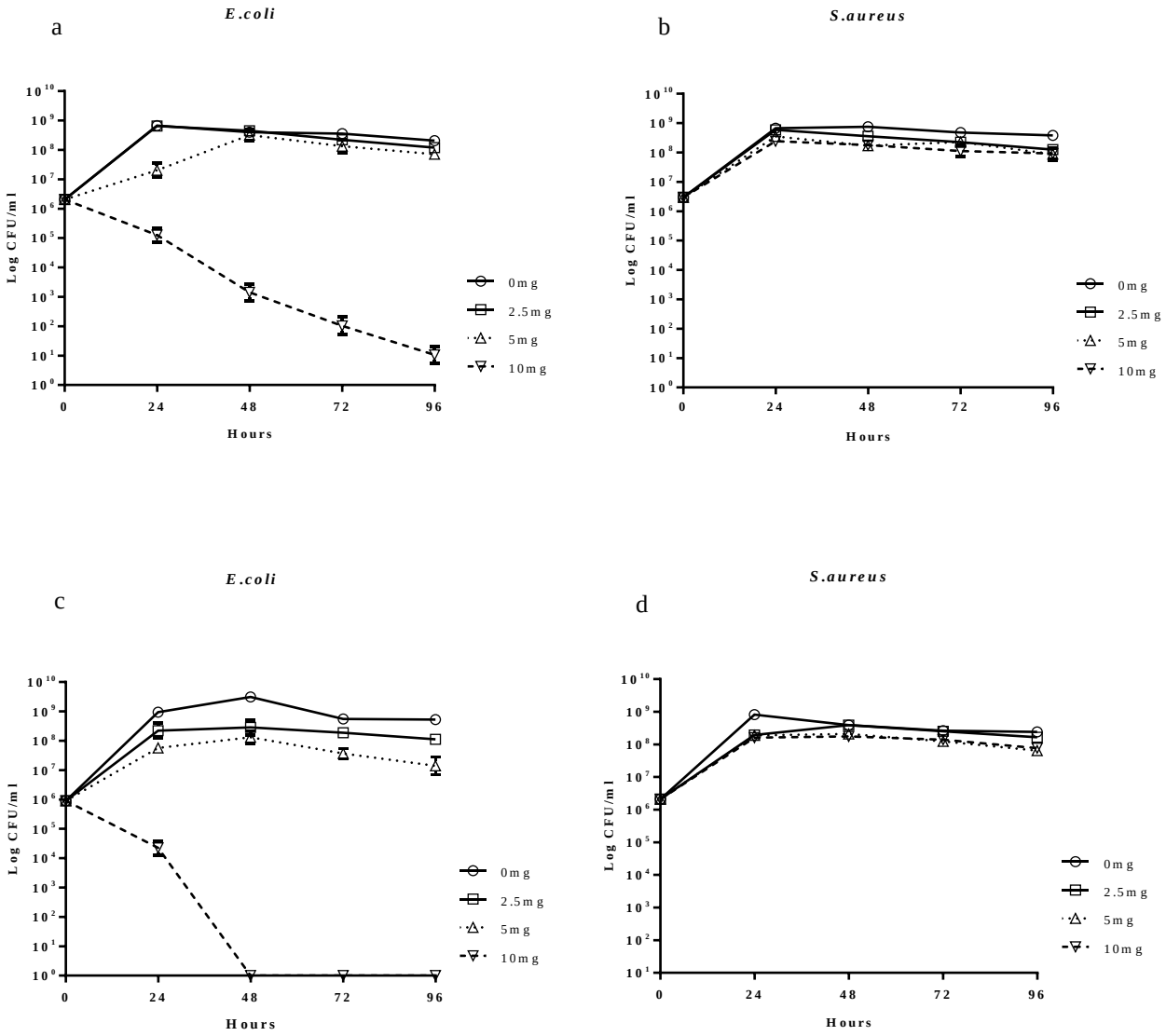

Fig.3 The antibacterial effect of 45S5 Bioglass particles on the viability of *E. coli* and *S. aureus* under static (a and b) and shaker (c and d) incubation conditions over a 96 hour period.

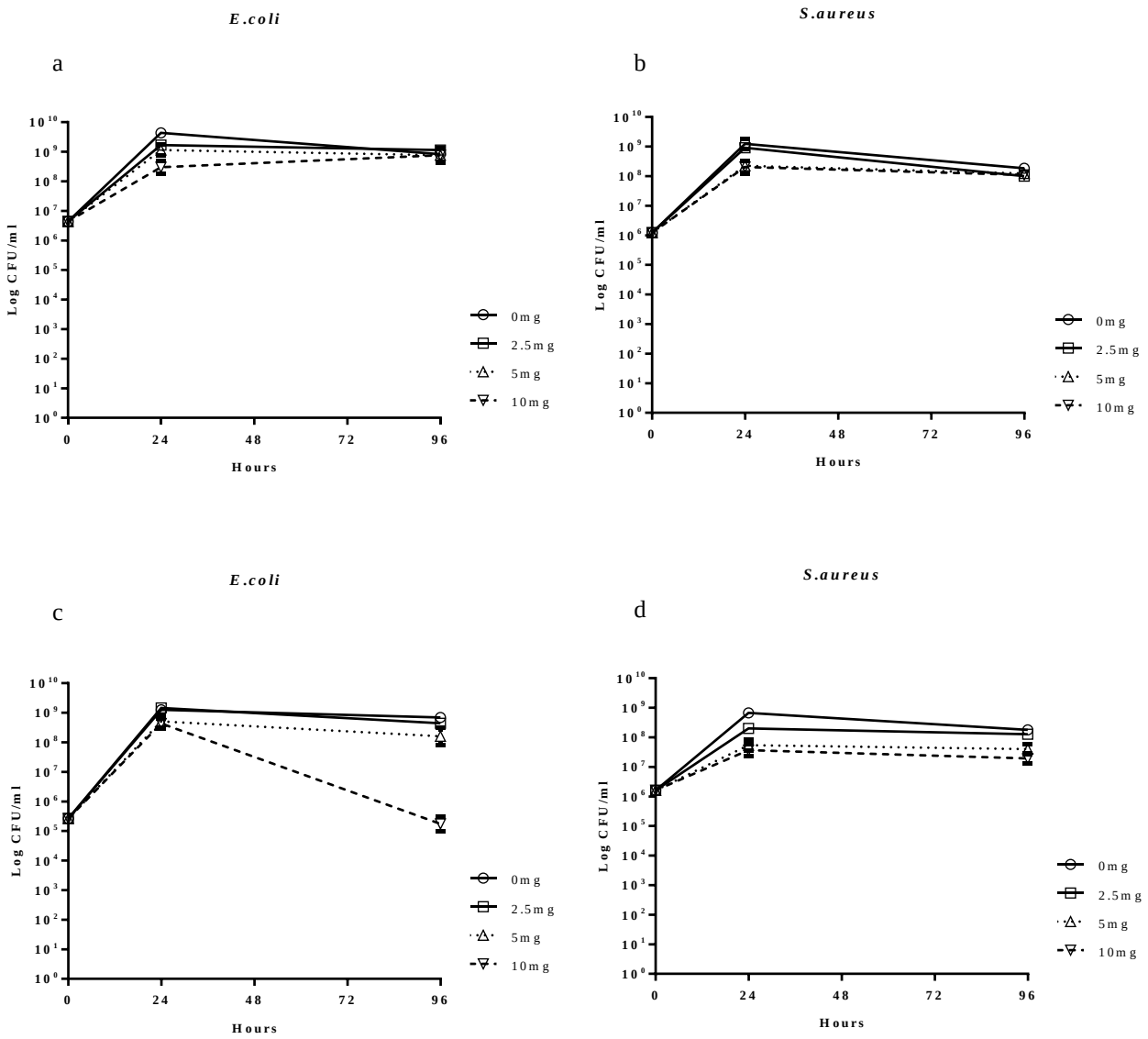

Fig. 4 The antibacterial effect of the dissolution products on the viability of *E. coli* and *S. aureus* under static (a and b) and shaker (c and d) incubation conditions.

Concentration of 45S5 Bioglass particles (mg/ml)	0	2.5	5	10
Static	7.4	8.70	9.09	9.29
Shaker	7.4	8.72	9.25	9.70

Table 1 The aqueous pH values of the 45S5 Bioglass particle suspension for static and shaker incubation conditions.

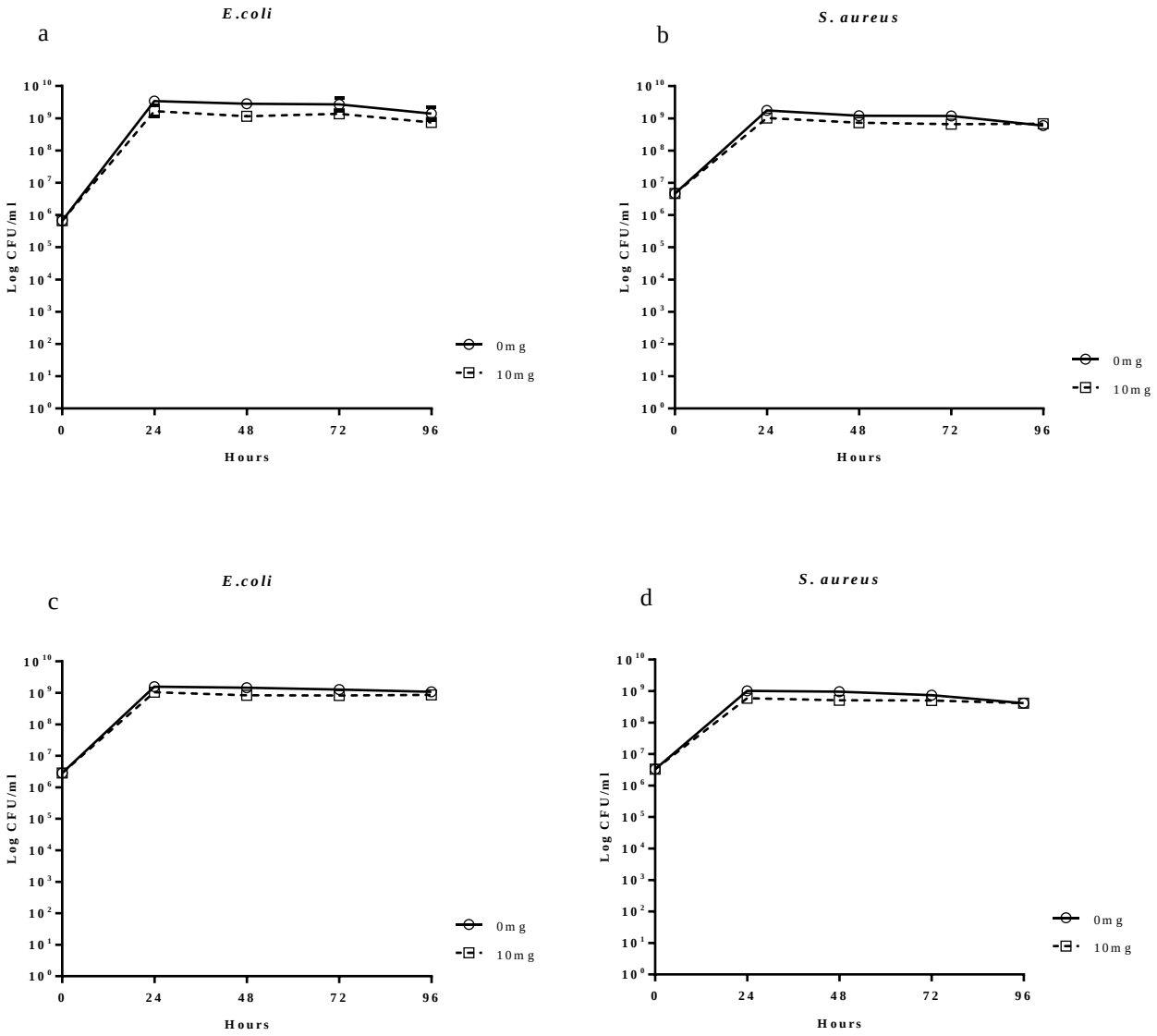

Fig.5 The antibacterial effect of 45S5 Bioglass particles on the viability of *E. coli* and *S. aureus* under static (a and b) and shaker (c and d) incubation conditions, once the pH was neutralised to pH 7.3 (2).

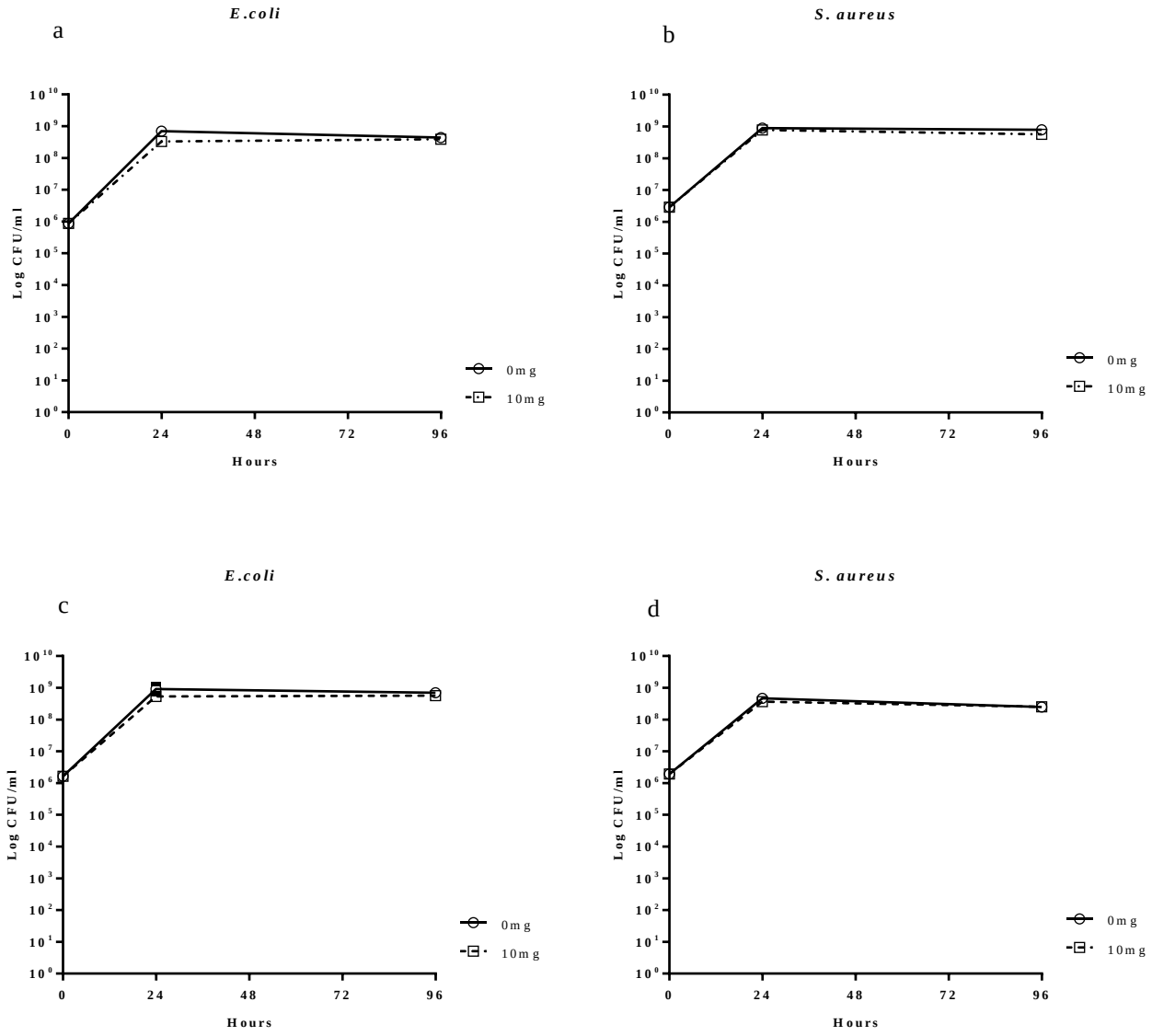

Fig.6 The antibacterial effect of the dissolution products of 45S5 Bioglass particles at a concentration of 10 mg/ml on the viability of *E. coli* and *S. aureus* under static (a and b) and shaker (c and d) incubation conditions, following the neutralisation of the pH from alkaline to pH 7.3 (2).