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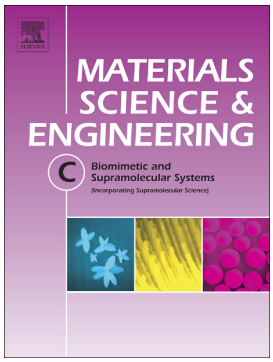

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The antimicrobial efficacy of zinc doped phosphate-based glass for treating catheter associated urinary tract infections.

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ABSTRACT

In this study, a series of phosphate-based glasses; $(P_2O_5)_{50}(Na_2O)_{20}(CaO)_{30-x}(ZnO)_x$ were prepared with increasing concentration of zinc oxide to determine the antimicrobial effect against clinically relevant microorganisms. The addition of 1 and 3 mol% zinc oxide decreased glass degradation however a higher dissolution rate was observed for 5 and 10 mol % ZnO. The antimicrobial results showed a concentration dependent effect on the viability of microorganisms. When in direct contact zinc doped glasses showed a complete kill, within 24 hours, against *Escherichia coli* and a significant ($p<0.01$) kill was observed against *Staphylococcus aureus* however the effect of dissolution products was not seen until 48 hours. Furthermore, the cytotoxic studies showed no toxic effects on the viability of uroepithelial cells. This study has shown that zinc doped phosphate-based glasses can potentially be used to prevent/treat catheter associated urinary tract infections.

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1. INTRODUCTION

Medical devices are an integral part of modern medicine. Whilst there has been a significant improvements in healthcare facilities, the occurrence of infections and undesirable complications arising from such devices have well been recognized and continue to escalate [1]. Since the presence of a foreign body can create a favourable environments for microbial colonisation, device related infections therefore account for at least half of the healthcare associated infection [2]. One of the most commonly reported device related infection is catheter associated urinary tract infection (CA-UTI) due to the inability of the material to prevent microbial colonisation [3, 4].

Urinary tract infections (UTIs) have been reported as the most common health-care associated infections where 80% of these infections are due to insertion of a urinary catheter. Due to frequent and sometimes unnecessary use of catheters, CA-UTIs, equate for 8-35% of healthcare-related infections [5, 6], and are the second most common cause of septicaemia which is associated with substantially increased mortality [7-9]. Studies have shown that between 15-25% of the patients admitted in hospital require urinary catheterisation [10] which increases their risk of developing urinary tract infection by 5% per day [11, 12]. In 30 - 40% of the patients suffering from CA-UTI microorganisms may enter blood and progress to septicaemia, which accounts for 8% of all nosocomial infections [10]. 1 in 3 patients who acquire bacteraemia in blood will eventually die of the infection [13].

Technological innovations such as antibiotic/antiseptic impregnated catheters have been introduced and evaluated over the past two decades, however clinically insignificant results and lack of any definitive evidence for silver coated catheters [14-16] call for development of new materials with a sustained release of antimicrobials. Phosphate based glasses containing sodium and calcium have been widely researched as controlled release glasses, as they dissolve completely in aqueous media with minimal or no cytotoxic effects [17-21]. Not only does their dissolution remain constant for as long as the material remains in the body but also the components of the glass can be easily excreted as these elements are found naturally in human body [22]. Previously phosphate-based glasses were

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developed as a vehicle for delivering silver as an antimicrobial agent for preventing urinary tract infections [23] and controlled released silver phosphate glasses have already been used to help control infections in in patients requiring long-term indwelling catheters [24]. Although several studies show silver possess broad antimicrobial properties against a range of microorganisms [25, 26], many studies have reported emerging silver resistance. McHugh *et al*. reported the first silver resistant strain, *Salmonella typhimurium* [27] and since then silver resistance has been described in members of the *Enterobacteriaceae* i.e. *Klebsiella pneumoniae, Enterobacter cloacae, Escherichia coli* [28, 29], *P. aeruginosa, Pseudomonas stutzeri* [30, 31] *Candida albicans* [32]. Therefore, due to the emerging resistance it is essential to expand the range of antimicrobial glasses to combat existing infections and reduce the risk of developing resistant strains. It is also possible that certain metals may have better antimicrobial efficacy against different pathogens, understanding which metals are most effective against which pathogens can optimize the effectiveness of bioactive glass use.

Zinc is known as an essential trace mineral for living organisms due to its role in many cellular processes. More than 200 enzymes need zinc as a cofactor to carry out metabolic processes. Both prokaryotic and eukaryotic cells have mechanisms such as transporters to regulate zinc concentration within the physiological range [33], however at higher concentrations, zinc ions can exert a cytotoxic effect. The antibacterial and antifungal properties of zinc ions have long been studied in the form of pure zinc and zinc oxide nanoparticles [34-36]. Similar antimicrobial effect could potentially be achieved by the dissolution products of bioactive glasses doped with zinc oxide. The aim of this study was to investigate the dissolution behaviour of zinc oxide doped phosphate-based glasses and to elucidate the antimicrobial effect using both direct contact and indirect contact methods (i.e. using the glass dissolution products) against clinically relevant Gram positive and Gram negative bacteria.

Escherichia coli (*E. coli*) is the most widespread pathogen associated with urinary tract infections and can be responsible for up to 80% of non-complicated UTIs. Recent studies have suggested

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Staphylococcus aureus (*S. aureus*) is associated with complicated UTIs and in particular catheter associated UTIs. The presence of a catheter has been shown to alter the ecology of the bladder making it much more susceptible to *S. aureus* [37]. It has also been reported that *E. coli* and *S. aureus* are the most common pathogens causing healthcare-associated infections and in particular for older age populations [38]. We therefore selected *E. coli* and *S. aureus* for this study.

2. MATERIALS AND METHODS

2.1. Glass synthesis: Phosphate based glasses were prepared by using P_2O_5 (Fisher Scientific, UK), NaH_2PO_4 (Sigma Aldrich, UK), and $CaCO_3$ (Alfa Aesar, UK) as starting materials. To prepare different mol % compositions of zinc containing phosphate based glasses ZnO (Fisher Scientific) was used (Table 1). The required chemicals were placed into a 59 ml Pt - 10% Rh crucible (GLC alloys Ltd, UK), mixed thoroughly and placed in a furnace at room temperature. The furnace was heated to 300°C (10°C per minute) and allowed to dwell for an hour. The temperature was then raised to 600°C (60°C per minute) and after dwelling for 30 minutes, once again temperature was rapidly increased up to 1050°C and held at this temperature for 30 minutes. The molten glass was then poured into a split graphite mould, with 10 mm aperture, which had been preheated at 350°C. The glass samples were allowed to slowly cool down to room temperature (inside furnace previously heated to 350°C) and the resulting glass rods were cut into ~2 mm thick discs with IsoMet™ 1000 Precision saw using Cool 2 cutting fluid as lubricant (Buehler). Discs were polished using MetaServ® (Buehler) polishing machine using a series of polishing clothes (30µm, 15µm using lapping oil) followed by a final polish using 0.02µ colloidal silica.

2.2. Degradation analysis: Glass discs with 10 mm diameter and ~2 mm thickness (the precise thickness of the discs was measured using Vernier callipers) were then placed in 60 ml plastic containers (Fisher Scientific) filled with 25 ml of standard distilled water. The containers were then placed in a 37°C incubator at 200 rpm. For 7 days weigh loss measurements were conducted at 24

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hour intervals by removing the glass discs from their respective containers. Prior to taking weight measurement, the excess moisture was removed. To determine the degradation rate, weight loss was calculated using

$$\text{Weight loss} = (M_0 - M_t)/A \qquad (1)$$

where M_0 is initial weight in mg, M_t is weight in mg at time *t* and A is the surface area, cm^2.

Weight loss was plotted against time and the slope of the graph gave a degradation value ($mgcm^{-2}\ h^{-1}$). Degradation analysis was also conducted in synthetic urine (to represent the environment for use in catheters), in cell culture media (to reflect the physiological environment for soft tissue) and nutrient broth which was used for the antimicrobial studies. Each measurement was carried out in triplicate.

2.3. pH measurements: pH measurements of the degradation media were simultaneously taken at each time point using pH meter (Mettler Toledo, Switzerland). Three-point calibration of the pH electrode was achieved using pH calibration standards (Fisher Scientific, UK).

2.4 Ion release study: The media obtained at each time point from degradation studies was analysed for the presence of cation (Na^+, Ca^{2+}, Zn^{2+}) and anion (PO_4^{3-}) using inductively coupled plasma optical emission spectrometry, ICP-OES (iCAP^{TM} 7000 Plus Series). The dissolution products were filtered prior to measurements using 0.2 µm Ministart filters (Fisher Scientific, UK) and the concentration of each ion was calculated from the linear portion of the generated standard curve as ppm.

2.5 Antimicrobial studies: The antimicrobial efficacy of the zinc doped phosphate glasses was studied using direct and indirect methods according to the international standards; film contact method (ISO-22196) [39] and shaking flask method [40] detailed below.

2.5.1 Bacterial strains: Two bacterial strains, *Escherichia coli* (NCTC 10538) and *Staphylococcus aureus* (ATCC 6538), which are representative Gram negative and Gram positive strains typically

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responsible for CA-UTIs, were used in this study. These strains were maintained at -80°C on MicroBank beads (Pro-Lab Diagnostics Neston, Cheshire, UK). When required the beads were cultured in nutrients broth by incubating overnight at 37°C in an aerobic atmosphere.

2.5.2 Evaluation of antimicrobial activity of glass surfaces using ISO 22196: To conduct experiments an overnight microbial culture was adjusted in fresh nutrient broth to produce ~10^6 CFU bacterial density on the top surface of the glass discs. A thin sterile glass cover slip (Fisher Scientific, UK) was placed on the top of the inoculum and pressed gently so that the test inoculum spreads to the edges. The cover slip not only helps to create a thin bacterial film on the glass discs but also prevents the bacterial death due to desiccation. Standard plastic coverslips with 12 mm diameter were used as the control. Inoculated glass discs and control samples were then placed in a Petri dish and incubated at 37°C. At each time point; 1, 2, 4, 6, 24 and 48 hours two glass discs were removed from each set of glass composition along with untreated glass and washed with 5 ml of D/E broth (Becton Dickinson UK Ltd). This was achieved by placing the test glass discs in 5 ml D/E broth and vortexing for two minutes. Viable count was determined using the spread plate method. In addition, immediately after inoculation, three untreated test specimens were processed and used as the recovery rate of the bacteria from the test specimens under investigation. The antibacterial effect was evaluated according to the standard outlined in ISO-standard 222196. The number of viable bacteria, N, for each specimen was determined using

$$N = (100 \times C \times D \times V)/A \qquad (2)$$

where C is the average plate count, D is the dilution factor for the plates counted, V is the volume in ml of D/E broth and A is the surface area of the cover film.

The antibacterial activity, R, was determined using

$$R = (U_t - U_0) - (A_t - U_0) = U_t - A_t \qquad (3)$$

where U_0 and U_t are the average of the common logarithm of the number of viable bacteria, in cells/cm^2 recovered from the untreated test specimens immediately after inoculation (t=0) and at

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time *t* respectively; and A_t is the average of the common logarithm of the number of viable bacteria, in cells/cm^2 recovered from the treated test specimens after time *t*.

2.5.3 Effect of dissolution products from the glasses on microbial growth: In this method, sterilised glass discs were placed in 25 ml of nutrient broth containing ~ 10^6cfu/ml of the bacterial culture. The test samples along with control (broth without glass discs) were then incubated in an aerobic incubator at 37°C with a shaking speed of 200 rpm. At various time intervals; 24, 48, 72, 96 and 120 hours, a 100 µl of broth was taken out from each test container and serially diluted to determine the viable count. All experiments were undertaken in triplicate.

2.6. Cytotoxic studies: Medical devices when *in vivo* are subject to degradation, therefore it is vital to evaluate the cytotoxic effect of the dissolution products of the test specimen on the growth and proliferation of mammalian cells. To test the cytotoxic effect of dissolution products, conditioned media was prepared as described in ISO-10993-5 (Biological evaluation of medical devices - Tests for *in vitro* cytotoxicity) [41].

2.6.1 Cell culture: transitional epithelium lines the human urinary tract including renal pelvis, bladder, ureters and urethra, therefore cytotoxic studies were conducted on epithelial cells. T24 (ATCC® HTB-4™), the human bladder transitional epithelial cells, were purchased from the American Tissue Culture Collection and cultured in McCoy's 5A medium (ATCC) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). Cells were maintained in a humidified atmosphere of 5% CO_2 and at 37°C. When 80 - 90% confluent cells were harvested using 0.25% trypsin, 0.03% EDTA solution.

2.6.2 Preparation of conditioned media: Sterile glass discs were incubated in complete cell media at 37ºC for 24 hours. Since phosphate glasses have uniform dissolution over an extended time period and the glass products are likely to get flushed out of the body within 24 hours, hence the time chosen for the extraction of glass products. After 24 hours incubation, conditioned media was filtered through 0.2µm Ministart syringe filter (Sartorius Biotech, Germany) and pH readings were taken using accumet® pH meter (Fisher Scientific, UK).

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2.6.3. Live/Dead cell viability assay: A cell suspension containing 10,000 cells in complete media was added in each well of a 24 well plate. Cells were incubated overnight to allow adherence of the cells onto the well surface. Following incubation, media was discarded, and cells were treated with conditioned media for 24, 48 and 72 hours. At each time point, a positive control was established by incubating cells with 70% ethanol for 45 minutes. Cells treated with complete media served as negative control. 100 µl staining solution containing 0.5 µM calcein-green and 2.5 µM Ethidium homodimer - 1 was added to T24 cells. The cells along with staining solution was incubated for 45 minutes. Fluorescence microscopy (Leica DM16000 B microscope) was used to photograph the cells at 100X.

2.6.4. Cell cytotoxicity and proliferation: Cells were seeded, in quadruplicate, into 96 wells plates at a density of 6 x 10^3 cells per well and treated with 100 µl conditioned media for 24, 48 and 72 hours. During incubation, cells were treated with fresh conditioned media every 24 hours. Ethanol was used as positive control whereas cells grown in complete cell media served as negative control. At each time point cell viability was determined using MTT test as described below and the treatments were rendered cytotoxic if the cell viability was less than 70% compared to the negative control. A 12mM stock solution of 3-(4.5-dimethylthiazol-2-yl)-2.5 diphenyltetrazolium bromide (MTT) was prepared by adding 1 ml PBS to 5 mg MTT reagent. At each time point, conditioned media was removed from each well and MTT reagent, diluted 1/10 in phenol free media, was added to the cells. The plates were then incubated in the dark for 3 hours at 37ºC / 5% CO_2. Following incubation, the solution was removed from the wells and 100 µl of DMSO (Sigma – Aldrich, UK) was added to each well and left to incubate at 37ºC for 10 minutes. The optical density was measured using an Ascent MultiScan GO spectrophotometer (Fisher Scientific, UK) at 570 nm (test) and 675 nm (background).

2.7. Statistical analysis: Each experiment was performed in triplicates or quadruplicates and repeated three times, data are represented as the mean ± standard deviation. Statistical analysis was undertaken using Graphpad Prism 7.0. A two-way ANOVA was used to test significance, if

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significance was detected a Tukey's multiple comparisons test was conducted. Levels of significance are given in the text and considered statistically significant at a level of $P < 0.05$.

3. RESULTS

3.1. Glass preparation and degradation analysis: a series of zinc oxide doped calcium-sodium phosphate glasses were successfully prepared with nominal compositions as given in Table 1. The glasses were visibly clear and no Bragg peaks were observed using X-ray diffraction (data not shown). The mass of glasses produced were consistent with the anticipated batch size (allowing for volatisation of carbonates and water) indicating that no unexpected mass loss or change in expected compositions had occurred.

Figure 1 shows weight loss measured per unit area (mg/cm^2) in four different aqueous media over 7 days. In comparison to un-doped glass composition, glass degradation decreased with an increasing zinc oxide content up till 3 mol % ZnO, however an increase in dissolution/weight loss was observed for 5 and 10 mol % ZnO compared to 3 mol % ZnO. The weight loss for 10 mol % ZnO was similar to zinc free composition, for example in distilled water at day 7 the weight loss for un-doped and 10 mol % glass was 33.97 ± 0.02 mg/cm^2 and 31.01 ± 0.04 mg/cm^2 respectively, whereas 3 mol % ZnO was least soluble composition (24.41 ± 0.55 mg/cm^2) of the glasses analysed. The dissolution trend described above was also observed in other media; nutrients broth, synthetic urine and cell culture media however, the cumulative mass loss observed in nutrient broth, cell culture media and synthetic urine was almost half compared to distilled water.

3.2. pH study: The pH values of zinc free composition, in distilled water, decreased sharply in first 24 hours. A gradual decrease was seen in the remaining period with the lowest value 3.8 on day 7 of the analysis. Similarly, the pH of zinc oxide doped glasses decreased rapidly within 24 hours falling to between 5.6 and 5.7. Nevertheless, the pH of zinc doped glasses did not decrease as much as un-doped glass by day 7. It is known that changes in pH can cause false positive antimicrobial results

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[42]. However a negligible change in the pH was observed for the glasses in nutrient broth, cell culture media and synthetic urine.

3.3. Ion release study: The ion release (Figure 2) reflected the degradation behaviour of the glass compositions. A linear increase in the release of anion (PO_4^{3-}) from all compositions was observed for the period investigated. By day 7, the release was highest from the un-doped glass (1064 ppm) followed by 10% mol zinc oxide doped glass (950 ppm). A linear increase in ion release was also observed for cations over 7-day period. Cation release increased with increasing dissolution rates, the lowest level of ion leaching was seen for the least soluble glass that is 3 mol % zinc oxide phosphate glass. The release trend of sodium was similar to anion (PO_4^{3-}). There was no significant difference in the release rate of Na^+ between the compositions, 220-280 ppm. On the other hand, calcium release was highest in glass without zinc oxide, followed by 1 mol % zinc oxide doped glass. By day 7, 85-300 ppm of calcium ions were leached from the glass compositions, where the lowest concentration of Ca^{2+} leaching was observed for 3 mol % zinc oxide glass composition i.e. 85 ppm. As with Zn^{2+} ion release, an increase in the quantity of zinc ions was observed with increasing zinc oxide content in glass compositions i.e. 20, 46, 83 and 173 ppm leached from 1, 3, 5 and 10 mol % zinc oxide doped glasses respectively.

3.4. Evaluation of antimicrobial activity of glass surfaces using ISO 22196: The antimicrobial effect of 1, 3, 5 and 10 mol % zinc oxide doped phosphate glasses compared to un-doped phosphate glass against *E. coli* is illustrated in Figure 3. An antimicrobial activity of ≥ 2 was seen within initial 2 hours, however at 4 hours a significant difference was observed for 1, 3, 5 and 10 mol % zinc doped glasses compared to un-doped glass ($p = 0.0233$). Furthermore, at this time point (4 hour) complete kill was exhibited by all the zinc oxide doped phosphate based glasses against *E. coli.*

In contrast, *S. aureus* (Figure 4) showed less susceptibility to zinc oxide doped phosphate glasses compared to *E. coli*. In the initial 2 hours, only 10 mol % zinc oxide doped glass showed an antimicrobial activity of ≥ 2, however the difference was non-significant when compared to un-

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doped phosphate glass. At the 4 hour time point, zinc doped glass composition showed an antimicrobial activity greater than 2, and 10 mol % zinc oxide doped glass exhibited a significant difference in comparison to un-doped phosphate glass ($p < 0.0001$). A similar trend in antimicrobial activity was observed at 6 and 24 hours, however significant difference was only detected at 24 hours for 5 and 10 mol % zinc oxide doped glass ($p = 0.0031$).

3.5. Effect of dissolution products of glasses on microbial growth: Figure 5 shows the antimicrobial effect of the dissolution products of un-doped phosphate glass and zinc oxide doped glasses against *E. coli* over 5 days period. It was found that dissolution products did not exhibit antimicrobial action for 96 hours. At time 120 hours the dissolution products of 5 and 10 mol % zinc oxide glasses demonstrated a significant antibacterial effect when compared to control culture ($p = 0.0011$ and $p < 0.0001$ respectively). Additionally, when compared to un-doped phosphate glass, the antibacterial effect of 5 and 10 mol % zinc oxide doped glass was significant ($p < 0.0001$). Significant differences in the antimicrobial activity was also seen for 5 and 10 mol % glasses compared to 1 and 3 mol % zinc oxide doped glasses.

During the initial 24 hours, the dissolution products of the glasses failed to show antibacterial effect against *S. aureus* (Figure 6). Similarly, all glass compositions exhibited a non-significant decrease in bacterial log reduction at 48 hours except for 10 mol % zinc oxide doped glass ($p < 0.0001$). Both 5 and 10 mol % zinc oxide doped glasses exhibited a significant decrease in bacterial viability at 72, 96 and 120 hours compared to control culture as well as un-doped phosphate glass dissolution products ($p < 0.0001$). Similarly, the dissolution products of 5 and 10 mol % zinc oxide doped glasses exhibited a significant decrease in bacterial density compared to 1 and 3 mol % dissolution products of zinc oxide doped glasses. Interestingly, dissolution products of un-doped phosphate glass seemed to improve bacterial viability compared to control *S. aureus* cultures. A significant difference ($p = 0.0067$) was detected at 24 and 48 hours.

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3.6. Live/Dead cell viability of T24 cells: The cytotoxic effects of dissolution products were assessed by Live/Dead staining of the cells with Calcein-AM green and EthD-1. As seen in Figure 7, when compared to negative control, for 3 and 5 mol % zinc oxide glass dissolution products a large number of cells were stained with Calcein-AM (green – live) and a very few or none with EthD-1 (red – dead) at 24, 48 and 72 hours. However, quite a few cells were stained with EthD-1 in 10 mol % zinc oxide doped glasses. Overall, by looking at the confluency of the culture in Figure 7 glass dissolution products appear to have a non-cytotoxic effect on the growth of T24 cells.

3.7. Cell cytotoxicity and proliferation using MTT assay: The metabolic activity of the cells after treating the cells with conditioned media, for 24, 48 and 72 hours, was also quantified using MTT assay. Figure 8 shows non-cytotoxic effects of dissolution products of 3, 5 and 10 mol % ZnO doped phosphate glasses on viability of T24 cells. A non-significant decrease in the cell viability was seen for 3, 5 and 10 mol % ZnO glasses compared to negative control as well as the un-doped phosphate-based glass. For 3 and 5 mol % glass dissolution products the viability was 103.0%, 90.1%, 90.3% and 94.1%, 86.1%, 83.3% at 24, 48 and 72 hours respectively. For 10 mol % ZnO glasses a slight cytotoxic effect was observed at 72 hours where the cell viability reduced to 67.6%, nonetheless the effect was non-significant.

4. DISCUSSION

Phosphate-based glasses are unique class of biomaterials that dissolve at a constant rate over a period and their dissolution can be tailored according to the end application. Therefore, these glasses can be used as a vehicle to deliver antimicrobial ions to treat or prevent infections. Phosphate-based glasses doped with silver have been researched for their potential use as coatings or controlled released cartridge for treating catheter associated urinary tract infections. However,

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not only do these studies lack microbiological and/or cytotoxic evaluation of the glasses, silver resistance has also been reported. Since the antimicrobial effect of silver and copper doped bioactive glasses has received much more attention in the previous years, therefore the current study aims to develop a bioactive glass with sustained release of zinc as antimicrobial ions to treat catheter related urinary tract infections with minimal toxic effects on uroepithelial cells. This was achieved by assessing the antimicrobial efficacy of increasing zinc oxide content (1, 3, 5 and 10 mol %) in a phosphate-based glass system (P_2O_5-Na_2O-CaO).

The antimicrobial study demonstrated a strong antimicrobial effect of zinc doped glasses against both Gram positive and Gram negative bacteria. Direct and indirect methods were undertaken to evaluate the antimicrobial effect of the glasses. Results from both assays revealed time and concentration dependent antimicrobial effect against *S. aureus* and *E. coli*. When in direct contact 10 mol % zinc oxide doped glass composition was able to exert significant antimicrobial effect in as little as 2 hours against *E. coli* and 4 hours against *S. aureus*. Whilst a strong antimicrobial effect was seen against both strains within 24 hours, *E. coli* showed higher susceptibility compared to *S. aureus*. The difference in the activity could be due the differences in the outer cell wall. Gram positive bacteria such as *S. aureus* contains a thick peptidoglycan layer in its cell wall along with teichoic and lipoteichoic acids. Teichoic acids are polyol phosphate polymers with a strong negative charge whereas in Gram negative bacteria these are not present [43]. These charged anionic clusters contribute to the binding of ions such as sodium, calcium, copper and cobalt etc. thereby promoting bacterial adhesion to the metallic surfaces. This cationic sequestering mechanism therefore confers resistance to bacterial killing in Gram positive bacteria. Furthermore, thick peptidoglycan layer also protects bacteria from penetration of ions and reactive oxygen species generated by metallic ions.

Part of catheter comes in contact with the cells lining the wall of urinary bladder, ureter and urethra and therefore should be biocompatible. Not only did the antimicrobial study show promising results but also the cytotoxic evaluation of the glass dissolution products revealed proliferative effect on T24 bladder cell line. The cell viability following exposure to zinc dissolution products was not

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only similar to the negative control but also higher compared to the dissolution products of un-doped phosphate-based glass. Whilst a growing body of research is available on phosphate and silicate glasses doped with zinc for orthopaedic tissue engineering, contradictory results on cytocompatibility have been reported. A dose dependent effect of zinc ions was observed on MG-63 cells, where zinc concentration in the range of 2-8 ppm was shown to damage cells via oxidative stress [44]. Similarly zinc concentration of 2.7 ppm decrease proliferation of endothelial cells whereas a proliferative effect was observed at zinc concentration of 1.1 ppm [45]. Recently Haimi *et al.*, [46] and Lusvardi *et al.*, [47] showed no significant effect of zinc ions on the activity and proliferation of human adipose stem cells and osteoblast-like cells (MC3T3-E1) respectively. Even though there have been reports of zinc ion cytotoxicity there have also been reports on the proliferative effect of zinc ions. This inconsistency is attributed to the type of cells; different cells have different sensitivity to the zinc concentration. Zinc concentration is strictly controlled within cells via several mechanisms such as zinc transporters, zinc importers and metallothioneins. Additionally, many mammalian cells contain membrane bound vesicles called zincosomes that sequesters high amount of zinc ions from cells. The toxicity of zinc ions is mainly dependent on the entry of the free ions into the cell via ion channels. Studies have shown that these channels are also shared by iron and calcium ions. As the given glass dissolve calcium is leached out which is taken up by the cells. A study conducted by Joost *et al*., [48] has shown the level of calcium as high as 100 ppm has no toxic effect on epithelial cells. Therefore, it is likely that the cytotoxic effect of zinc in the given glass system was suppressed by the leached calcium ions.

In the present study glasses were manufactured by substituting calcium with zinc that showed a varied dissolution pattern, with minimum solubility at 3 mol % ZnO. Whilst studies have shown reduced dissolution of the calcium-sodium-phosphate system with an increasing content of silver [49], cobalt [50] or copper[51], zinc showed a varied dissolution behaviour. It was observed that the glass composition with highest zinc content had the highest dissolution rate. Although both elements are divalent the smaller atomic radii of Zn compared to Ca results in a higher charge to

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size ratio that makes the crosslinked bonds stronger and therefore makes 1 and 3 % mol glasses durable compared to zinc free compositions. However, the glass composition with higher zinc oxide content had higher degradation rates. The local environment surrounding zinc is not believed to have changed as a function of ZnO composition and it has been reported in similar glasses that Zn enters the glass as a direct replacement for Ca causing no structural variation [52]. The change in the dissolution behaviour could be explained based on the network clustering of modifier cations. According to Christie *et al.,* [53] clustering of modifier cations is more likely to occur at low concentrations which strengthens the network and reduces the solubility. Similar trend in glass dissolution was observed by Salih *et al.* and Mustafa *et al.* [54, 55], where the highest zinc oxide containing glass showed significant weight loss compared to compositions containing lower zinc oxide content. The difference in the dissolution rates in different media can be explained by the diverse composition of the media used. Nutrients broth, cell culture media and synthetic urine contains various ions such as sodium, calcium, potassium, chloride and magnesium etc. along with amino acids, vitamins and glucose. As the ionic strength of the solution is already relatively higher than that of distilled water therefore the dissolution of glasses is supressed. Similar effect was observed by Uo *et al*., [17] where the authors observed reduced dissolution rate of the glasses studied in SBF compared to distilled water.

5. CONCLUSIONS

A series of zinc oxide glasses were successfully prepared and characterised. A decrease in glass dissolution was observed for glasses containing 1 and 3 mol% ZnO and this was attributed to clustering which has been reported for low concentrations. Glasses with 5 and 10 mol% ZnO showed an increase in glass dissolution relative to the 3 mol% with the 10 mol% glass having a similar weight loss to the zinc free control. The rate of dissolution was almost twice as high in distilled water compared to broth, media and synthetic urine. Zinc ion release correlated with zinc

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concentration whilst calcium released reduced as expected due to the replacement of CaO with ZnO. The release of P and Na ions were relatively independent of composition.

The antimicrobial results show a concentration dependent effect on the viability of microorganisms. Zinc doped glasses showed a complete kill, within 24 hours, when in direct contact against *Escherichia coli* and a significant ($p<0.01$) kill was observed against *Staphylococcus aureus*. Dissolution products showed a reduced kill 48 hours. Cytotoxic studies showed no toxic effects on the viability of uroepithelial cells. In conclusion this study has shown that zinc doped phosphate-based glasses can potentially be used to prevent/treat catheter associated urinary tract infections.

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Notes

The authors declare no competing financial interest.

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Table 1: Zinc doped phosphate glass nominal compositions investigated in the study.

Figure 1: The degradation profiles obtained for 0, 1 3, 5 and 10 mol % ZnO compositions, investigated in distilled water (A), nutrient broth (B), cell media (C) and synthetic urine (D). The data shows variable degradation rate in different media. (Error bars = ±SD for triplicate samples).

Figure 2: Accumulative ion release (A) phosphorous, (B) calcium, (C) sodium and (D) zinc as a function of time for 0, 1, 3, 5 and 10 mol% zinc doped glasses. (Error bars = ±SD for triplicate samples).

Figure 3: The antimicrobial efficacy of un-doped phosphate glass and 1, 3, 5 and 10 mol % zinc doped phosphate glass against *E. coli* over a period. Data shown are expressed as mean ± SD (N=3) antibacterial activity as determined by ISO-22196. The p value <0.05, marked by * shows statistically significant differences between ZnO doped and un-doped compositions.

Figure 4: The antimicrobial efficacy of un-doped phosphate glass and 1, 3, 5 and 10 mol % zinc doped phosphate glass against *S. aureus* over a 24 hours period. Data shown are expressed as mean ± SD (N=3) antibacterial activity as determined by ISO-22196. Statistically significant differences between un-doped and zinc oxide doped compositions are shown by ** ($p<0.01$) and **** ($p<0.0001$)

Figure 5: The effect of dissolution products of un-doped phosphate glass and 1, 3, 5 and 10 mol % zinc doped phosphate glass against *E. coli* over a 5 day period. Data shown are expressed as mean ± SD (N=3). At 120 hours, a statistically significant difference ** ($p<0.01$) and **** ($p<0.0001$) was observed for 5 and 10% zinc oxide doped compositions compared to un-doped glass.

Figure 6: The effect of dissolution products of un-doped phosphate glass and 1, 3, 5 and 10 mol % zinc doped phosphate glass against *S. aureus* over a 5 days period. Data shown are expressed as mean ± SD (N=3). The p value <0.0001 is indicated by **** which shows a significant difference in log reduction of various zinc oxide doped compositions in comparison to un-doped composition.

Figure 7: Live/dead staining of T24 cells cultured with media conditioned with zinc doped phosphate-based glasses at 24, 48 and 72 hours. Cells grown in **(A)** complete media, media conditioned with **(B)** un-doped phosphate glass, **(C)** 3% zinc glass, **(D)** 5% zinc glass **(E)** 10% zinc glass and **(F)** cells treated with 70% (v/v) ethanol. Images were taken using a fluorescent microscope at 100x magnification. Live cells are shown in green whereas red represents dead cells. Scale bar indicates 100µm.

Figure 8: The effect of the dissolution products of zinc doped phosphate-based glasses (3%, 5% and 10 mol %) and un-doped phosphate glass on T24 cells. MTT assay was used to determine T24

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cell viability following 24, 48 and 72 hours incubation with conditioned media. The data is represented as Mean ± SD (N=3). A statistically significant ($p<0.0001$ marked by ****) was seen in positive control.

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Table 1: Zinc doped phosphate glass nominal compositions investigated in the study.

	Glass composition (Mol %)			
	P_2O_5	CaO	Na_2O	ZnO
0% Zn	50	30	20	0
1% Zn	50	29	20	1
3% Zn	50	27	20	3
5% Zn	50	25	20	5
10% Zn	50	20	20	10

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Highlights

- Zinc doped glasses showed a complete kill, within 24 hours, against E. coli
- Zinc doped glasses showed a significant kill against S aureus
- Cytotoxic studies showed no toxic effects on the viability of uroepithelial cells
- zinc glasses can potentially be used to prevent/treat CA-UTIs

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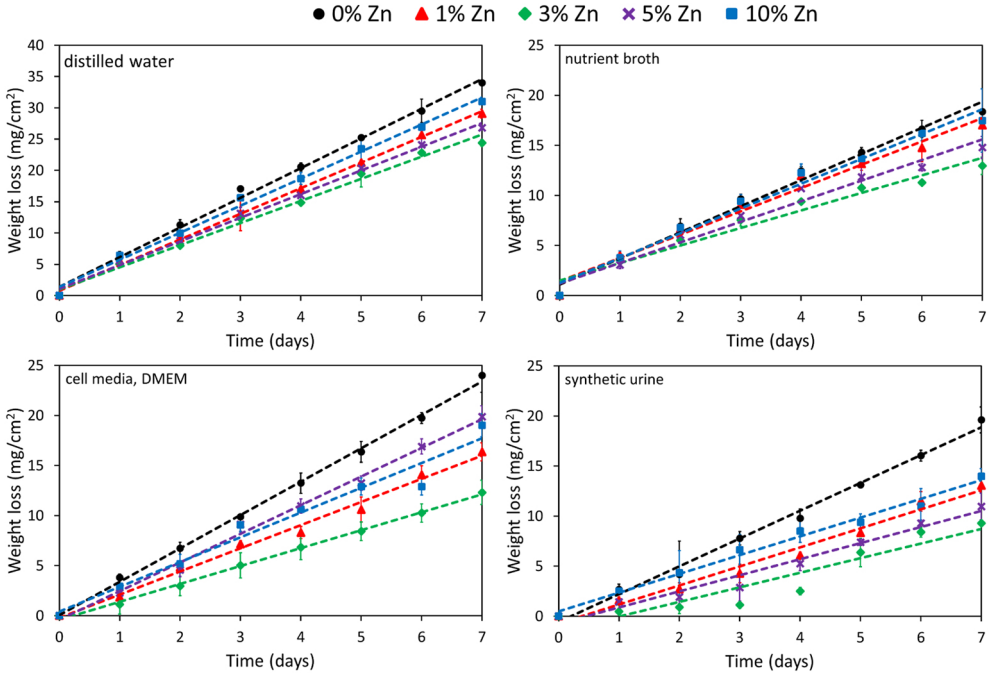
0% Zn
1% Zn
3% Zn
5% Zn
10% Zn
distilled water
nutrient broth
cell media, DMEM
synthetic urine
Weight loss (mg/cm²)
Time (days)

Figure 1

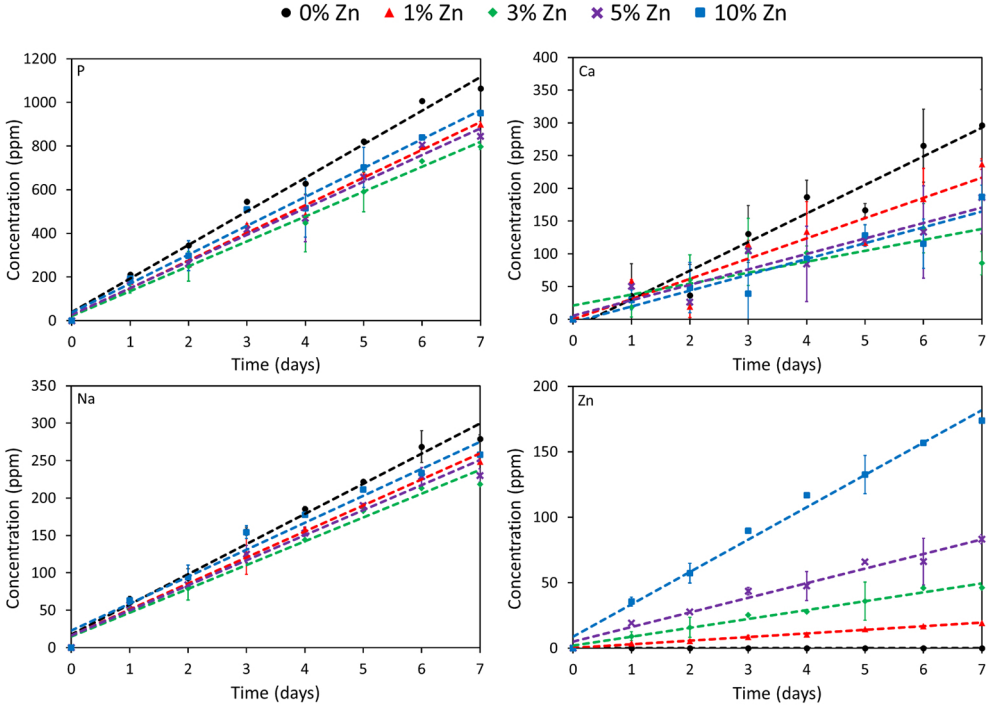

Figure 2

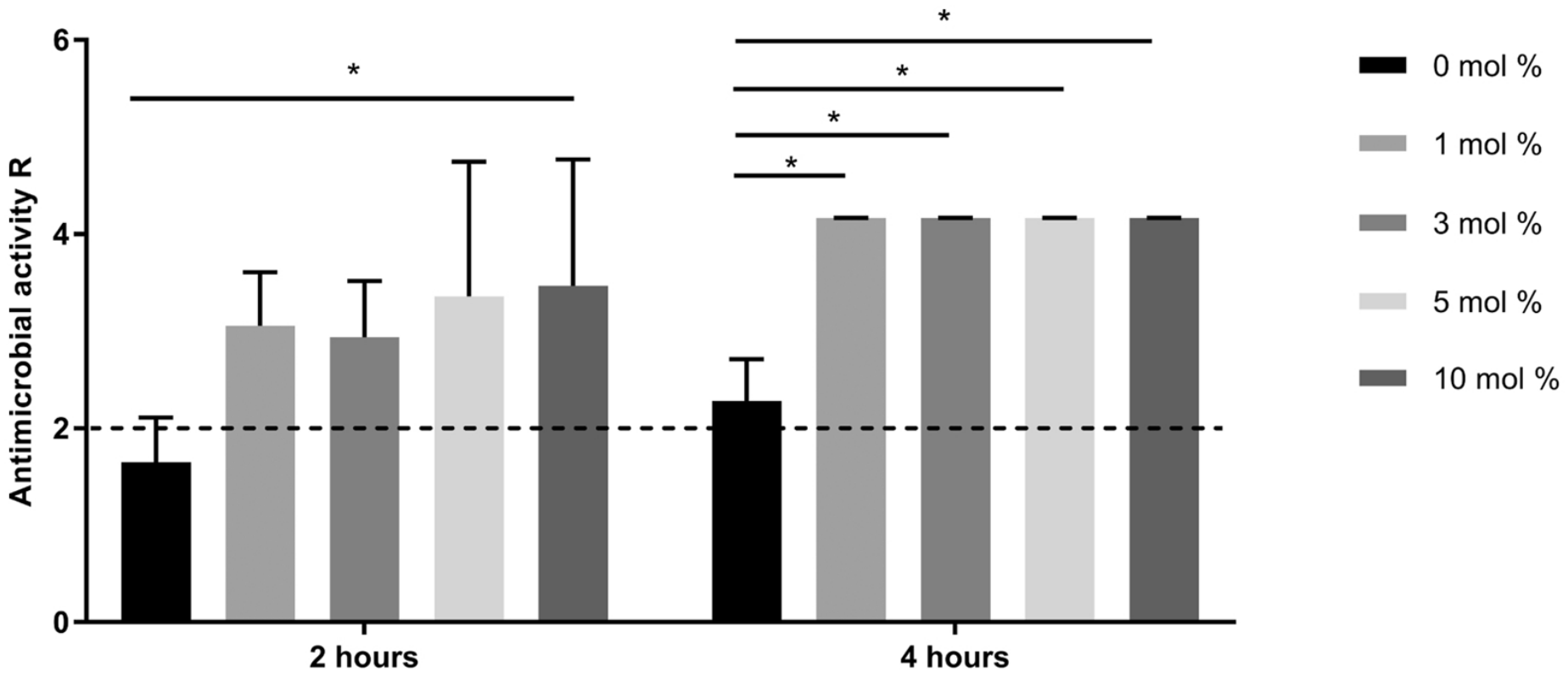
Antimicrobial activity R
6
4
2
0
*
*
*
*
*
2 hours
4 hours
0 mol %
1 mol %
3 mol %
5 mol %
10 mol %

Figure 3

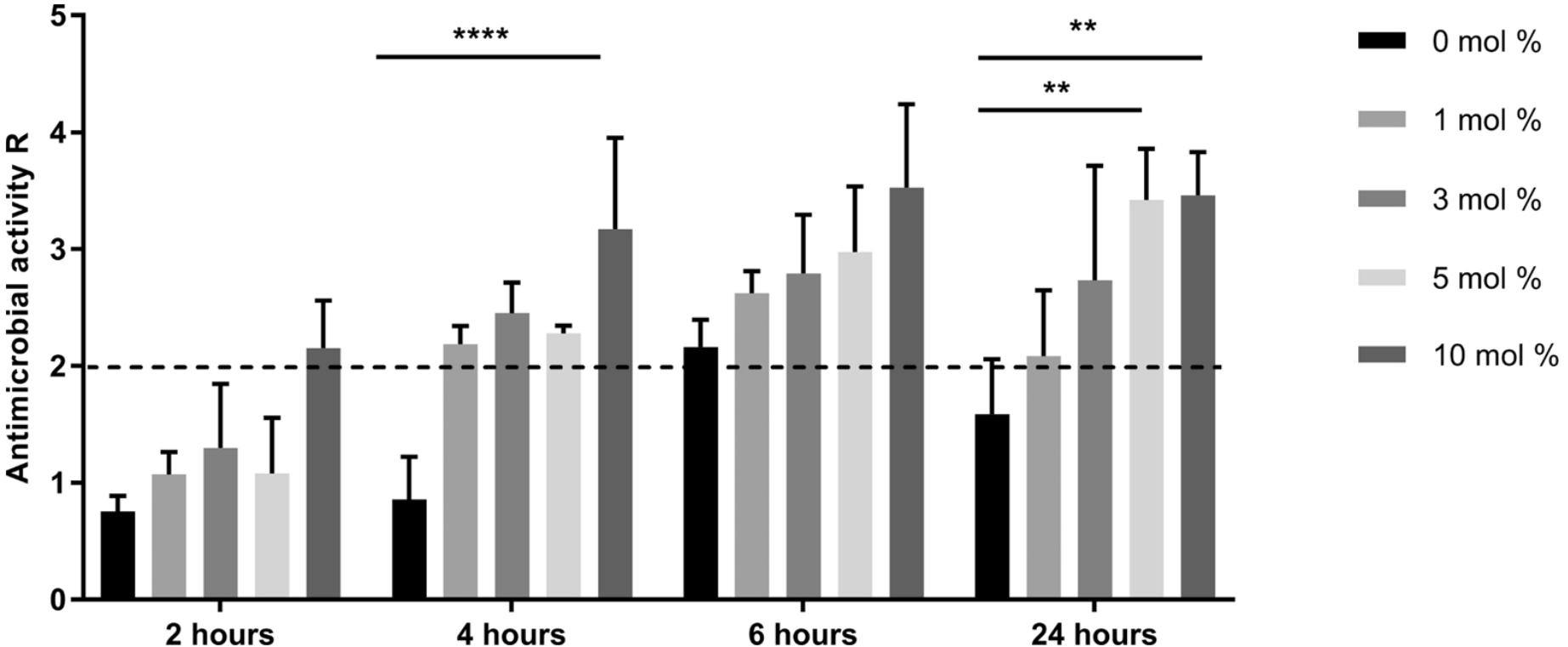
Antimicrobial activity R
5
4
3
2
1
0
****
**
**
2 hours
4 hours
6 hours
24 hours
0 mol %
1 mol %
3 mol %
5 mol %
10 mol %

Figure 4

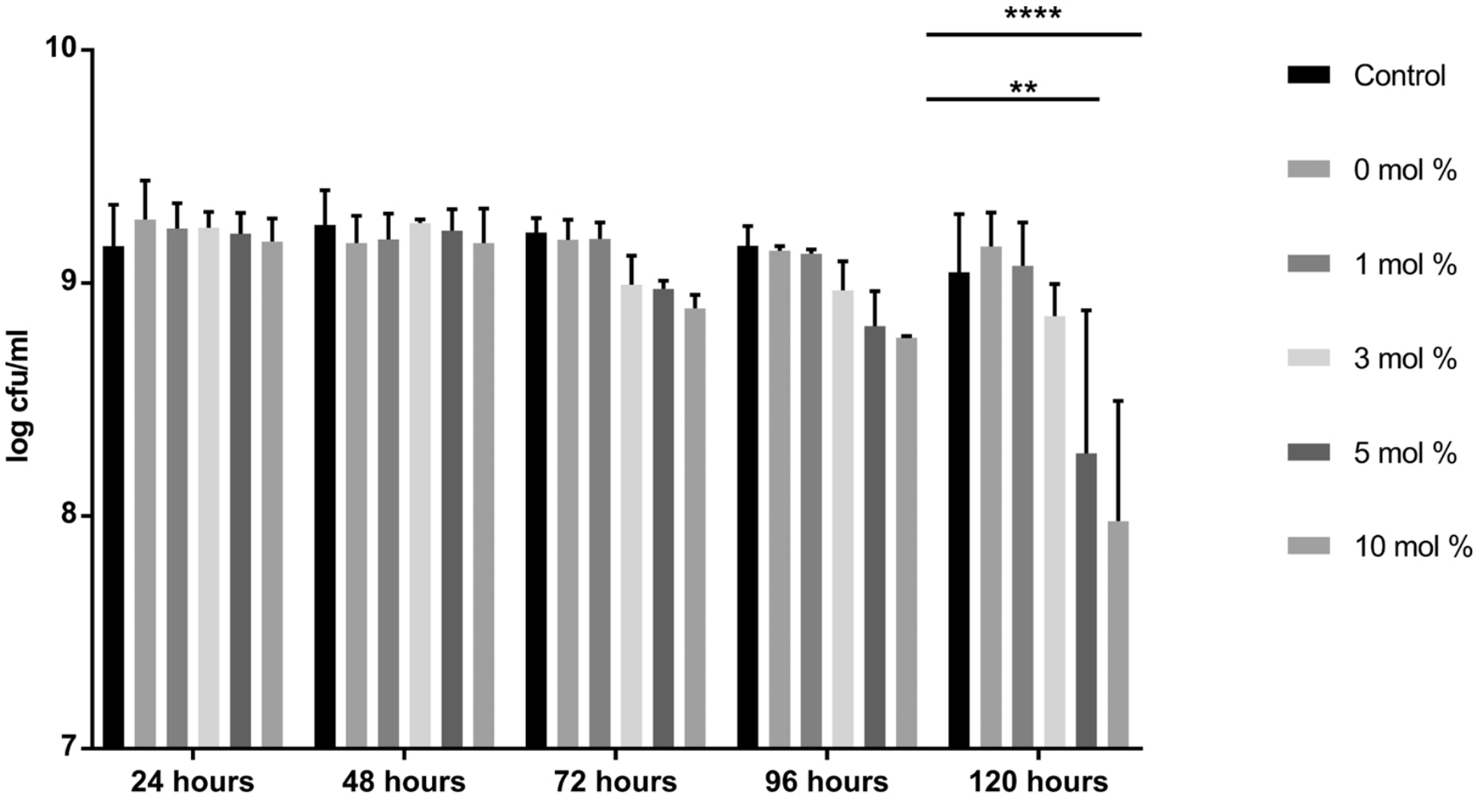
****
**
10
9
8
7
log cfu/ml
24 hours
48 hours
72 hours
96 hours
120 hours
Control
0 mol %
1 mol %
3 mol %
5 mol %
10 mol %

Figure 5

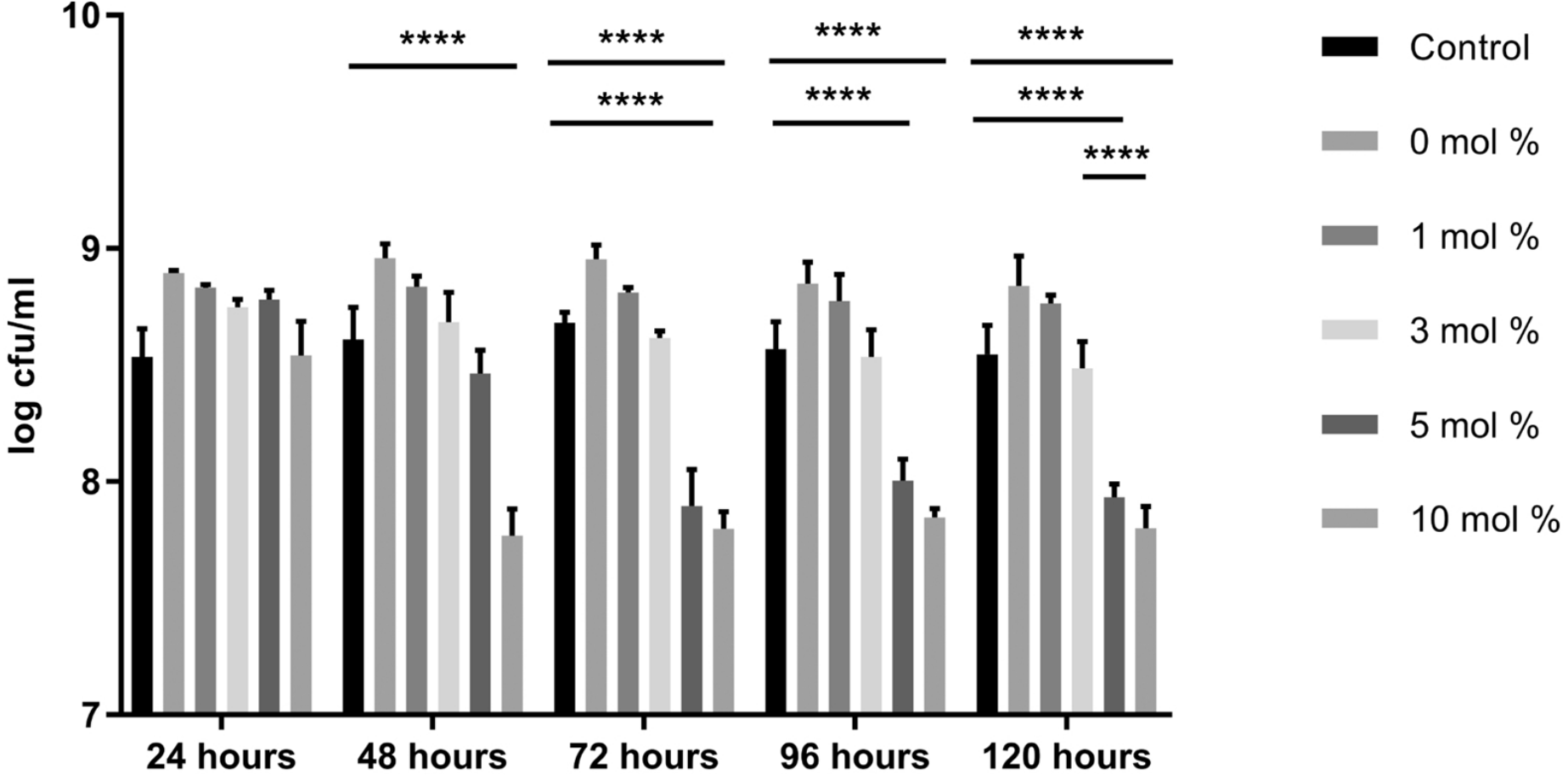
10
9
8
7
log cfu/ml
****
****
****
****
****
****
****
24 hours
48 hours
72 hours
96 hours
120 hours
Control
0 mol %
1 mol %
3 mol %
5 mol %
10 mol %

Figure 6

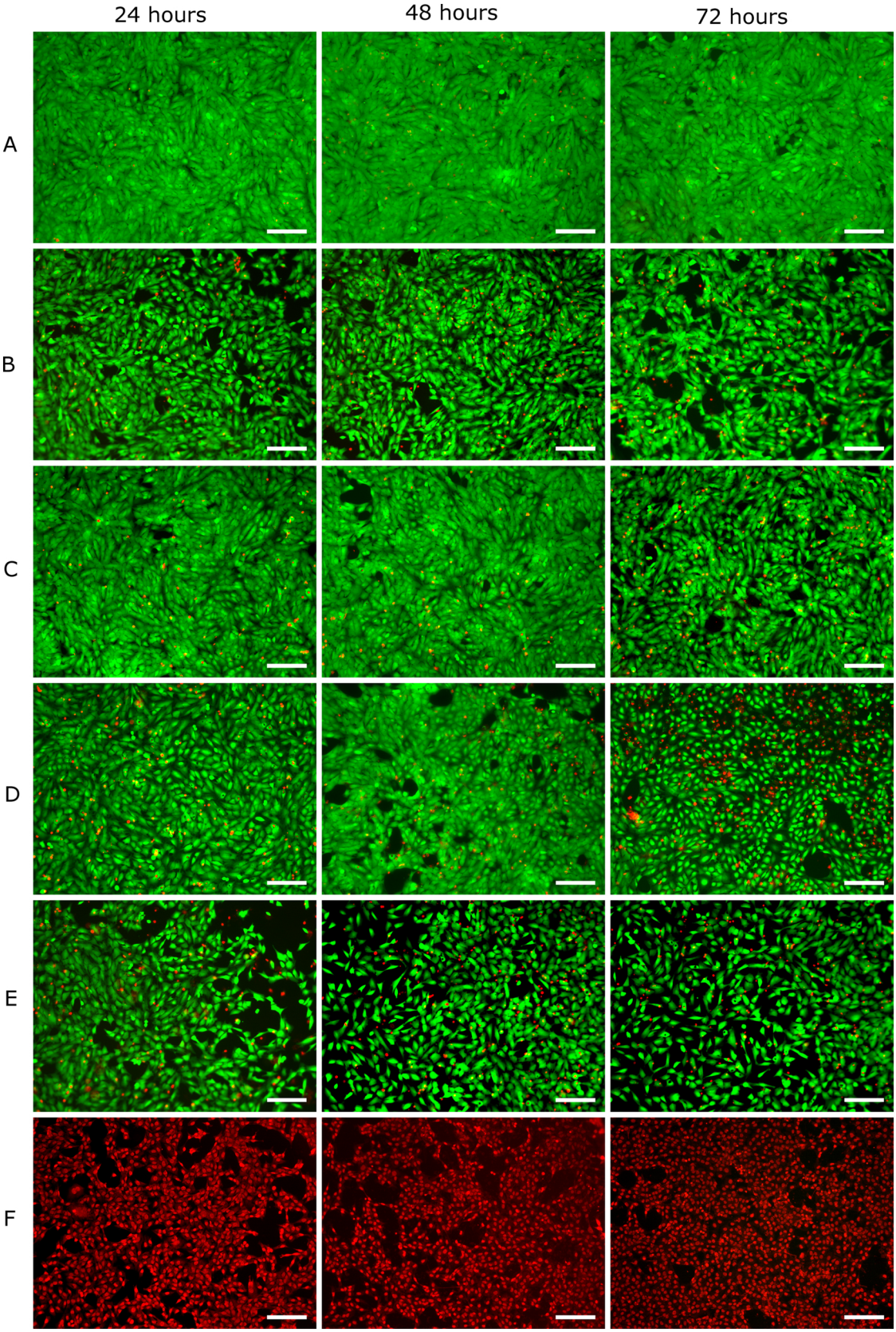

Figure 7

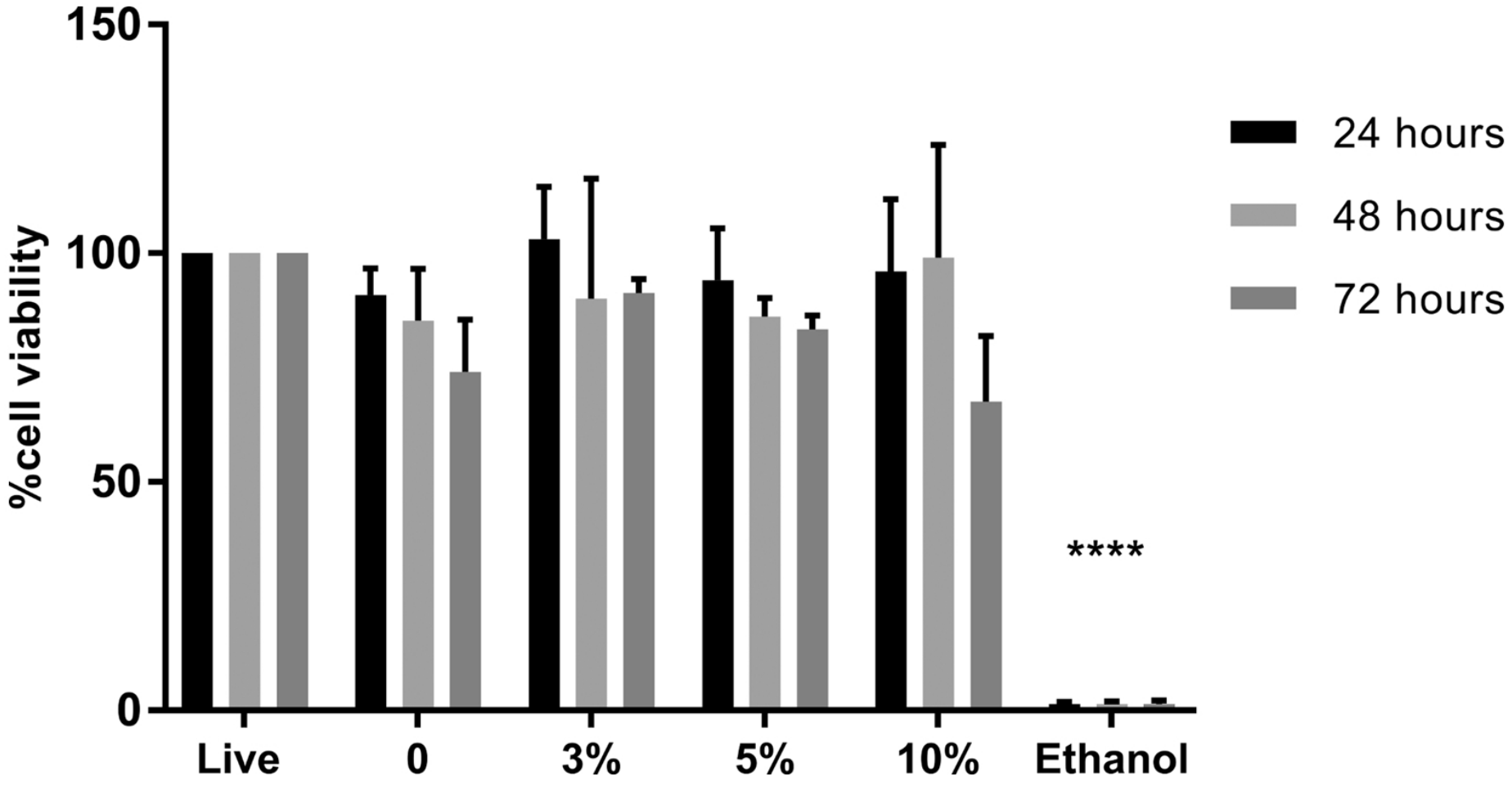
150
100
50
0
%cell viability
24 hours
48 hours
72 hours
****
Live
0
3%
5%
10%
Ethanol

Figure 8