2017-05-10

Antifungal properties and biocompatibility of silver nanoparticle coatings on silicone maxillofacial prostheses in vitro

Meran, Z

10.1002/jbm.b.33917
Journal of Biomedical Materials Research Part B: Applied Biomaterials
Wiley

Title:

Antifungal Properties and Biocompatibility of Silver Nanoparticle Coatings on Silicone Maxillofacial Prostheses *In Vitro*.

Author names and affiliations:

Zhala Meran [a,c], Alexandros Besinis [a,b,d,*], Tracy De Peralta [b,e], Richard D. Handy [a]

[a] School of Biomedical and Biological Sciences, University of Plymouth, Drake Circus, Plymouth, PL4 8AA, UK

[b] Plymouth University Peninsula Schools of Medicine and Dentistry, University of Plymouth, John Bull Building, Tamar Science Park, Plymouth, PL6 8BU, UK

[c] Present address: Department of Prosthodontic, Hawler Medical University, Erbil, Kurdistan, Iraq.

[d] Present address: School of Engineering, University of Plymouth, Drake Circus, Plymouth, PL4 8AA, UK

[e] Present address: School of Dentistry, University of Michigan, Ann Arbor, Michigan, USA

*** Corresponding author:**

Alexandros Besinis, email: alexander.besinis@plymouth.ac.uk; abessinis@hotmail.com

Telephone: Tel. +44 (0)1752 586193; Fax: +44 (0) 1752 586103

Abstract

Patients with facial prostheses suffer from yeast, *Candida albicans*, infections. This study aimed to determine the biocompatibility and antifungal properties of silicone facial prostheses coated with silver nanoparticles (Ag NPs) *in vitro*. Medical grade silicone discs were coated with 5 and 50 mg l^{-1} dispersions of either Ag NPs or $AgNO_3$. Coatings were fully characterised using scanning electron microscopy and energy dispersive X-ray spectroscopy. The biocompatibility was examined using human dermal fibroblasts (Hs68), whereas antifungal efficacy was tested against *C. albicans* (NCPF-3179). The fibroblast viability was assessed by measuring lactate dehydrogenase (LDH) activity, protein content and tissue electrolytes. There were no effects on the LDH activity of fibroblast cell homogenates, and leak of LDH activity into external media remained low (0.1-0.2 IU ml^{-1}). Sublethal effects of Ag NP coatings on membrane permeability/ion balance was not observed, as measured by stable homogenate Na^+ and K^+ concentrations. Some Ag (13 mg l^{-1}) was detected from the $AgNO_3$ coatings in the media, but total Ag remained below detection limit (<1.2 µg l^{-1}) for the Ag NP coatings; indicating the latter were stable. When fibroblasts grown on silver coatings were challenged with *C. albicans*, the Ag NP coating was effective at preventing fungal growth as measured by ethanol production by the yeast, and without damaging the fibroblasts. Ethanol production decreased from 43.2±25.02 in controls to 3.6 µmol ml^{-1} in all the silver treatments. Data shows that silicone prosthetic materials coated with Ag NPs are biocompatible with fibroblast cells *in vitro* and show antifungal properties.

Running Head: Silicone prosthetics and silver nanoparticles

Keywords: Maxillofacial prosthetics; nanotechnology; yeast infection; oral bacteria; fibroblast cells

1. Introduction

Maxillofacial prostheses made of medical grade silicone elastomers are routinely used to replace facial parts lost through disease or trauma. The prosthesis needs to be biocompatible to enable wound healing and the restoration of healthy tissue; but the material also needs to be aesthetically acceptable to the patient.[1,2] Maxillofacial prostheses are exposed to saliva and nasal secretions[3] and thus they are inevitably susceptible to bacterial colonisation, which usually leads to the subsequent degradation of the material and infection of the surrounding tissues.[4] There is a wide range of microbial species that are known to colonise the biomaterials used for prostheses.[5]

Yeast infections are of particular concern,[6] with *Candida albicans* being responsible for the most prevalent fungal infections in the human oral cavity and skin.[7] The growth of *C. albicans* is observed on facial prosthetic materials and dentures; causing denture stomatitis in the latter.[8] Moisture, body warmth and the nutrient-rich residue from skin secretions promote fungal growth on the silicone elastomer surface.[9] Facial skin has a pH that ranges between 4.0 and 4.9,[10] and the acidic conditions also favour the growth of *C. albicans*.[11] However, the acidic pH may also cause degradation of the prosthetic material, leading to increased surface roughness and thus render it more susceptible to the microbial adherence;[12] exacerbating the infection risk for the patient.

Traditionally, patients are instructed to maintain their prostheses by regularly washing them using soap and water. However, Kurtulmus et al. have demonstrated that even after washing high numbers of microorganisms remain on the surface.[10] Facial prostheses also have the tendency to retain water after washing, which alters their physical properties and the perception of colour matching of the prosthesis to the surrounding facial tissues.[1] Disinfectants have been suggested for infection control; such as benzene, xylene and chlorhexidine gluconate. However, all of these chemicals generally deteriorate the colour stability of maxillofacial prostheses.[13,14] An additional difficulty for cleaning is that yeasts are able to infiltrate prosthetic materials such as silicone elastomers; either by enzymatic degradation of the silicone or by directly utilising silicone as a nutrient source.[4,15] The most popular medical grade silicone material is the A-2186, which despite its long clinical use, also suffers from durability and infection control issues.[16] Estimates suggest over a thousand maxillofacial silicone prostheses are made in the UK alone each year with the implant remaining serviceable from 7-24 months before repair or replacement.[17] Failure rates for prostheses are on average about 5%, but can be much higher in certain groups of patients, such as those following radiotherapy.[18] Thus

extending the serviceable life of the prosthesis before needing a replacement and reducing failure rates is clinically desirable, as well as alleviating the financial burden on the patients. The use of nanomaterials with antimicrobial properties in conjunction with silicone elastomer prostheses may offer an improvement.

Nanomaterials have found applications in many areas of medicine including drug delivery, vaccine development, medical imaging, diagnostics and medical implants.[19-22] Nanotechnology can be defined as the branch of technology using materials and structures with nano scale dimensions, usually in the range of 1-100 nm.[23] The small size, colloidal behaviour and propensity to adhere to surfaces[24] suggests that some nanomaterials can be used in coating applications. For instance, nanoforms of silver have been used as antimicrobial coatings in catheters[25] and wound dressings.[26] However, research effort has mainly focused on the antibacterial properties of silver nanoparticles (Ag NPs)[27,28] and limited information is available about their antifungal properties. Nevertheless, recent studies suggest that Ag NPs may be a good antifungal agent. Ag NPs have fungicidal activity against *C. albicans* at low milligram concentrations (e.g., 0.4-3.3 mg l^{-1});[29] perhaps better than their antimicrobial properties for some bacteria. For example, the minimum inhibitory concentration to prevent bacterial growth of *Streptococcus mutans*, one of the common oral bacteria, is considerably higher (50 mg l^{-1}).[30]

Our hypothesis is that applying an Ag NP coating on the surface of silicone elastomers may inhibit fungal infections caused by *C. albicans* and also prevent the ingrowth of the yeast into the prosthetic material without compromising its biocompatibility. So far, Ag NPs have not been used as an antifungal coating on silicone elastomer facial prostheses in patients. Nonetheless, medical devices, similar to medicines containing nanomaterials, need to be tested for their safety and biocompatibility.[21,23] The aim of the present study was to determine the biocompatibility of the Ag NP coating on the silicone elastomer prosthetic materials. The study used human dermal fibroblast cells as a model system, since these cells are functionally important to skin and essential to wound healing. Then the cells grown on the silicone elastomer were challenged to a fungal infection of *C. albicans* in the presence and absence of an Ag NP coating on the silicone prosthetic material. Finally, the biocompatibility and antifungal properties of Ag NPs were compared to silver nitrate because of the historic use of dissolved silver as an antiseptic, and to bench mark against the potentially toxic effects of Ag ions.[31]

2. Methods and materials

In this study, three experiment series were performed. The first series determined the biocompatible concentration of Ag NPs compared to $AgNO_3$ by direct additions to the cell culture media over confluent monolayers of fibroblast cells. The second series demonstrated the effect of Ag NPs on fibroblast cells, when applied as a coating to the silicone elastomer surface. The third series investigated the antifungal properties of silver-coated silicone elastomer against *C. albicans* in the presence of fibroblast cells.

2.1. Experimental design

The first experiment investigated the effect of Ag NPs and $AgNO_3$ in culture media on human fibroblast cells. The unit of replication in the experimental design was the cell culture plate. The cells were seeded in 6-well plates (*n = 6)* and cultured in Dulbecco's modified Eagle's medium (DMEM) for 48 h until confluent. The culture media was then aspirated from the wells and replaced with fresh DMEM containing 5 and 50 mg l^{-1} of either Ag NPs or $AgNO_3$. The initial 1 g l^{-1} stock solutions and dispersions were prepared in deionised ultrapure sterile Milli-Q water (see below). The controls on each plate included a seeded well containing DMEM only (i.e., untreated cells with no other additions) and a second well with DMEM that contained a volume of sterile ultrapure water (Milli-Q water, no added silver) equivalent to the water introduced to DMEM due to the dilution of the stock solutions, to check for osmotic stress (hereafter called "control-MQ").

After 24 h of exposure to the Ag NPs and $AgNO_3$ solutions, the overlying media were carefully collected to measure lactate dehydrogenase (LDH) activity, total Ag, Na^+ and K^+ concentrations, and pH (see below). The cells remaining adhered to the bottom of the wells were washed twice with 2 ml of a sucrose washing buffer (300 mmol l^{-1} sucrose, 0.1 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA), 20 mmol l^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered to pH 7.4 with a few drops of trizma base). Then the cell morphology was examined *in situ* on the plates by light microscopy (Olympus Microscope SZ-1145 CHI equipped with a Scope Tek MDC 560 camera). After acquiring images, the bottom of the wells was scraped to collect the cells (Fisher Scientific cell scraper, 250 mm handle, 18 mm blade) and then 1 ml of a sucrose lysis buffer was added to each well (similar to the washing buffer above, but hypotonic, made with 30 mmol l^{-1} sucrose). The lysed cells were transferred to Eppendorf tubes. The harvested cells were sonicated for 30 sec (100 Watt, speed setting 8, 22.5 kHz, Misonix incorporated, XL2000-010, New York) to ensure that lysed samples were homogenised. The total Ag, K^+, and Na^+ concentrations were determined in the cell homogenates to access any ionoregulatory effects associated with silver toxicity to

the cells, as well as LDH activity for cell viability/membrane leak and total protein concentration (see below).

The second experiment aimed to examine the effect of Ag NPs and $AgNO_3$ on the fibroblast cells when applied as coatings to the silicone discs. Silver-coated silicone discs were prepared as described below. The experimental design was similar to that of the first experiment; except that fibroblasts were grown on silver-coated, or uncoated silicone discs for 72 h (until confluence was reached on the controls). The controls included cells grown on normal untreated silicone (control), and cells grown on silicone discs that had been spiked with Milli-Q water instead of silver solution/dispersion to simulate the water additions during the preparation of the coatings (control-MQ). The culture media were carefully removed from the wells and replaced with fresh media every 24 h. The removed media was subjected to metal analysis and measurements of the LDH activity. Additional 6-well plates were prepared to investigate the morphology of the fibroblasts adhered to the silicone discs at the end of the 72 h exposure using light microscopy. For the latter, cells were fixed *in situ* with 100% methanol and stained with 1% Giemsa solution for 3 min.

The third experiment investigated the antifungal activity of the silver-coated silicone discs against *C. albicans* (NCPF-3179) in the presence of fibroblasts. Fibroblast cells were cultured on the surface of silver-coated and control discs for 72 h (as above). Then the culture media was removed from the wells containing the discs and replaced with 2 ml of fresh media inoculated with *C. albicans* (see below). The plates were incubated for another 24h at 37^{o}C. The cell and bacterial morphology was examined by light microscopy (0.1% methylene blue stain for 1 min was used for the *C. albicans*) and scanning electron microscopy (SEM). Measurements of the metal concentrations, LDH activity and protein concentration in the media were also taken as before. Ethanol production was also measured to determine the metabolic activity specific to *C. albicans* (n = 4 discs/treatment, see below).

2.2. Preparation of silicone discs and application of antibacterial coatings

Silicone discs (37 mm in diameter, n = 6/treatment) were prepared using a platinum-catalysed, vinyl-terminated poly(dimethyl siloxane) elastomer (A-2186, Factor II, Lakeside, AZ). A-2186 is a medical grade maxillofacial silicone elastomer. The preparation method followed the manufacturer's instructions. Briefly, the elastomer was combined with a poly(methyl hydrogen siloxane) cross-linking agent at a 10:1 ratio by weight. The elastomer and the cross-linker were thoroughly mixed using a spatula; then a functional intrinsic cosmetic pigment (Naturelle Fl-SK01) was added. The homogenised mixture was poured into 6-well plates, which were placed

in a vacuum chamber for 15 min to remove any air pockets trapped within the material. The silicone discs were then left to cure for 24 h at room temperature. Once the elastomer was set in the plates, each silicone disc was treated with 5 ml of 0.5% (v/v) chlorhexidine digluconate (R4, Septodont Ltd, UK) for 5 min to ensure sterility. Chlorhexidine digluconate was then aspirated from the surface of the discs and the discs were washed twice with 5 ml of phosphate buffered saline (PBS). The disc surfaces were then coated with Ag NPs and $AgNO_3$ aqueous solutions (5 and 50 mg l^{-1}) prepared in ultrapure Milli-Q water (see below). The coating process involved adding 2 ml of the appropriate silver solution to each disc for 24 h to allow particle precipitation on the surface of the specimens. Control discs were treated with sterile ultrapure water without added silver. At the end of the coating process, the excess solution was gently aspirated leaving a thin silver coating (as appropriate) on the surface of the silicone discs (Figure 1).

Additional silicone discs (n = 3/treatment) were prepared for examination by SEM to verify whether the application of the coatings was successful. Routine SEM preparation techniques involving serial dehydration through alcohols, or similar solutions, risk potentially dislodging or dissolving the nanoparticles from the nanocoatings on the disc surface.[32] Thus, specimens were left to air-dry thoroughly at room temperature for 72 h instead. The resulting discs were then chromium sputtered prior to SEM examination. One half of each specimen was assessed by SEM (JEOL7001F SEM, with an Oxford Instruments Aztec X-Ray analysis system) and the other half with energy dispersive X-ray spectroscopy (EDS) to verify the metal composition of the Ag NPs and $AgNO_3$ coatings. Identical operating conditions and scanning parameter settings were used for all EDS scans (spot size: 10; accelerating voltage: 15 kV; working distance 10 mm). Data and spectra analysis was achieved using Aztec 2.0 software.

2.3. Preparation of the stock dispersions and nanomaterial characterisation

The materials used for the experiments were exactly the same batches as previously described and characterised in detail by Besinis et al.[30] Briefly, the materials were silver nanopowder (Sigma-Aldrich, Wisconsin, USA, 99.5 % purity, lot number 7721KH) and with a measured primary particle diameter of 56 nm, and BET surface area of 4.8 m^2 g^{-1}; $AgNO_3$ (99.9 % purity, Fisher, Loughborough, UK). The Ag NPs and $AgNO_3$ stock dispersions and solutions were prepared according Besinis et al.[30] In brief, 1 g l^{-1} stock dispersions of Ag NPs and $AgNO_3$ were initially prepared in ultrapure Milli-Q water. Stocks were sonicated for 4 h to disperse the nanomaterials (35 kHz frequency, Fisherbrand FB 11010, Germany), before preparing secondary stocks of 5 and 50 mg l^{-1}. Both primary and secondary Ag NPs and $AgNO_3$ stock

dispersions and solutions were autoclaved (121 ^{o}C for 15 min at 15 psi pressure) to ensure sterility. Additionally, 5 and 50 mg l^{-1} secondary stocks were prepared in DMEM culture media (the 1 g l^{-1} stock dispersions diluted 1:20 and 1:200 with culture media) to estimate the behaviour of the Ag NPs in this more complex media compared to ultrapure water. The Ag NPs and $AgNO_3$ dispersions were analysed by nanoparticle tracking analysis (NTA) to measure the particle size distribution and mean aggregate size (hydrodynamic diameters) using a Nanosight LM 10 (Nanosight, Salisbury, UK, laser output set at 30 mW at 640 nm). Example particle size distributions in the different media are shown (Figure 2). The resulting dispersion of Ag NPs in Milli-Q water gave average aggregate sizes of 114 ± 43 and 177 ± 52 nm (mean hydrodynamic diameter ± SEM, n = 3) at concentrations of 5 and 50 mg l^{-1} total silver respectively. When Ag NPs were dispersed in DMEM culture media the hydrodynamic diameters were 189 ± 19 nm in control culture media (no added silver); and 159 ± 17, 82 ± 2 nm at concentrations of 5 and 50 mg l^{-1} Ag NPs respectively. The average aggregate size of $AgNO_3$ were 176 ± 19 and 149 ± 12 nm at concentrations of 5 and 50 mg l^{-1} respectively. Total metal concentrations were also measured (see below).

2.4. Cell and yeast cultures

Human dermal fibroblasts (HS-68 cell line; Health Protection Agency, Salisbury, UK) were cultured at a density of 1.5 x 10^6 in 75 cm^2 flasks containing 15 ml of DMEM supplemented with L-glutamine, 10% foetal bovine serum and 1% penicillin-streptomycin (all obtained from Lonza, Nottingham, UK). The cells were sub-cultured every six days (when 80% confluent) and the medium was changed every three days as routine maintenance. For experiments, antibiotics were withdrawn two passages before seeding the cells into the 6-well plates. The cells were washed twice with phosphate-buffered saline (PBS; containing 9.5 mmol l^{-1} of phosphates without added calcium or magnesium), detached from the stock culture flasks using trypsin (0.25% with EDTA) and re-suspended in fresh DMEM. The cells were then introduced to the 6-well plates at a density of 10^6 cells ml^{-1}. Cell viability was examined by tryphan blue staining and 96% of the cells were found to be viable prior to seeding the cells in the 6-well plates. The plates were left in the incubator for 48 h at 37^{o}C until the cells were confluent.

C. albicans were cultured for 24 h at 37^{o}C in Sabouraud's dextrose agar (SDA). Then, 30 ml of Sabouraud's dextrose broth (SDB) were inoculated with a loop-full of the organisms and incubated for 72 h. The turbidity of yeast suspension was adjusted to a McFarland standard of 1.5. Then, the yeast suspension was diluted in culture media (DMEM with L-glutamine, 10% foetal bovine serum, without antibiotics) to a density of 10^6 cells ml^{-1}.

263

264 *2.5. Metal analysis*

265 Metal analysis of the cell culture media and cell homogenates was performed to confirm any
266 potential Ag exposure of the cells and metal toxicity on ionic regulation of the cells.
267 Measurements were conducted according to Besinis et al.[30] The total Ag, Na^+ and K^+
268 concentrations were determined by inductively coupled plasma optical emission spectrometry
269 (ICP-OES, Varian 725-ES, Melbourne, Australia fitted with v-groove nebuliser and Sturman-
270 Masters spray chamber) using acidified matrix matched standards. All samples were sonicated
271 for 1 h immediately prior to ICP-OES analysis to ensure homogenous distribution of the
272 particles in the solutions. The culture media and cell homogenates were acid digested. For Ag
273 analysis, 3 ml of 70 % concentrated nitric acid was added to 400 µl of the cell homogenate,
274 and then 3 ml of 10 mmol l^{-1} sodium citrate (as a stabiliser) was added to the mixture. For total
275 Na^+ and K^+ concentrations, a separate 400 µl of the cell homogenate was similarly treated with
276 concentrated nitric acid; but without the stabiliser. In the absence of certified reference
277 materials for total silver from Ag NPs in tissues or media, procedural spike recovery test was
278 performed. Samples of culture media were spiked with 50 mg l^{-1} of Ag NPs or $AgNO_3$ in culture
279 media without the cells; then subjected to the preparation protocol above. The samples showed
280 a good recovery for $AgNO_3$ recovery (measured value: 49 ± 1.6 mg l^{-1} or 97% recovery).
281 However, the detection of silver as Ag NPs was problematic in the culture media at the high
282 mg concentrations of the stocks with only 12 % recovery (measured mean ± SEM: 6.12 ± 0.7
283 mg l^{-1}, n = 5). However, complete acid digestion gave good recovery, regardless of the form of
284 silver, and consequently measurements of total silver concentrations in/on the cells (i.e., cell
285 homogenates) rather than the external media were used to verify the exposure. Instrument
286 detection limits for total Ag were calculated for the metal analysis in each experiment and were
287 1.26, 1.03, 1.15 µg l^{-1} respectively. Values for silver in the media are reported as mg l^{-1} of total
288 metal, and metals in the cell homogenates as µmol mg^{-1} protein to allow comparison with the
289 literature.

290

291 *2.6. Lactate dehydrogenase activity*

292 Lactate dehydrogenase activity was determined in the media, and in cell homogenates at the
293 end of each experiment. The assay was performed according to Plummer.[33] The cell culture
294 media from each well was gently centrifuged for 1 min to remove any cell debris. The assay
295 was performed by adding 2.8 ml of a reaction mixture (0.6 mmol l^{-1} pyruvate in 50 mmol l^{-1}
296 phosphate buffer at pH 7.5) to 0.1 ml of 0.6 mmol l^{-1} NADH solution and 0.1 ml of the sample

297 (cell culture media or cell homogenate), in 3 ml cuvettes. Absorbances were read at 340 nm
298 (Helios Beta spectrophotometer, Thermo Scientific, UK) for 2 min. The LDH activity was
299 calculated using an extinction coefficient of 6.3 mM for a path length of 1 cm. LDH activity
300 was expressed as µmol min^{-1} ml^{-1} for media and µmol min^{-1} mg^{-1} cell protein for homogenates.
301 Protein in the homogenates was determined using the bicinchoninic acid method with a
302 commercial kit (MC155208, Pierce, Rockford, USA), using 25 µl of homogenate in triplicate
303 and measured against bovine serum albumin standards (0-2 mg ml^{-1}). Samples were read at
304 562 nm on a plate reader (VERSA max, Molecular Devices, Berkshire, UK).
305
306 *2.7. Ethanol assay*
307 Ethanol production was determined only for the third experiment to measure the metabolic
308 activity associated with *C. albicans*. The rationale was that fibroblasts do not normally produce
309 ethanol and thus any ethanol present in the media would have been produced by yeast
310 fermentation. The enzymatic method for ethanol determination (K-ETOH 12/12, Megazyme
311 International Ireland Ltd) was used to measure the ethanol production according to the
312 manufacturer's protocol. Following incubation of the fibroblast cell cultures for 24 h in the
313 presence of *C. albicans*, the culture media was collected for immediate ethanol determination.
314 Ten µl from each sample (in triplicate) were mixed with 20 µl of β-nicotinamide-
315 adeninedinucleotide (NAD^+), 5 µl alcohol dehydrogenase enzyme (ADH) and 20 µl ml of the
316 buffer supplied with the kit (pH 9.0, plus sodium azide as a preservative). Background
317 absorbance was measured for each sample at 340 nm for 2 min (VERSA max, Molecular
318 Devices, Berkshire, UK); then the reaction was initiated by adding 2 µl of NADH and the
319 absorbance at 340 nm was monitored for 10 min. Ethanol concentration was calculated using
320 a 0-5 µg ml^{-1} standard curve. The ethanol assay was checked for interference caused by the
321 presence of Ag NPs or $AgNO_3$ in the media, but no interference was found for silver
322 concentration up to 50 mg l^{-1} (data not shown).
323
324 *2.8. Statistical analysis*
325 All data are presented as mean ± SEM and were analysed using Stat Graphics Plus Version 5.1.
326 Following descriptive statistics and a variance check (Bartlett's test), differences between
327 treatments within each experiment were evaluated using one-way ANOVA for parametric data.
328 Where appropriate, time effects within treatment were also analysed by one-way ANOVA.
329 Differences were located using Fisher's Least Significant Difference (LSD) multiple range test.
330 Bonferroni correction was not needed in the one-way ANOVA as the differences were large in

individual comparisons with Fisher's LSD, far exceeding the theoretical 5% risk of a false difference within individual comparisons in the post hoc test. However, for treatment x time effects two-way ANOVA was used with the alternative post hoc tests. The Kruskal-Wallis test was used for non-parametric data that could not be transformed and differences were located using notched box and whisker plots. The student's *t*-test was also used to investigate the differences between the reference controls and the controls with added Milli-Q water, and sometimes as additional confirmation of the Fisher's LSD. All statistical analysis used a 95% confidence limit, so that p values ≥ 0.05 were not considered statistically significant.

3. Results

3.1. Effects of direct additions of Ag NPs or $AgNO_3$ to the culture media on fibroblasts.

The first experiment explored the effects of adding Ag NPs or $AgNO_3$ directly to the culture media on confluent layers of fibroblasts, using a 24 h exposure period. The exposure was confirmed by measuring the total silver concentrations in the media and in the cell homogenates at the end of the experiment. For $AgNO_3$, the measured total Ag concentrations in the liquid phase of the culture media were lower than the nominal concentrations with measured values of 23.2 ± 3.7 and 2.9 ± 0.0 mg l^{-1} for the 50 and 5 mg l^{-1} exposures respectively, and was partly due to observed spontaneous precipitation of insoluble silver chloride from the media onto the cells in the culture dishes. For the total Ag in the media from the Ag NPs exposures, the values were hampered by the inability to measure Ag from Ag NPs directly in the media (poor spike recovery) and were therefore not reflective of the nominal concentrations added to the culture dishes; with measured values of 13.6 ± 2.1 and 1.0 ± 0.2 mg l^{-1} for the 50 and 5 mg l^{-1} exposures respectively. Nonetheless, all the values were above the controls (not detectable), indicating that exposure had occurred. However, the exposure was mainly confirmed by measuring the total Ag concentrations in the cell homogenates at the end of the experiment (Table 1); which had been subject to a complete acid digestion prior to Ag determination. The cell homogenates showed the expected concentration-dependent increase in total Ag, and the measured total Ag was statistically different between all groups (Kruskal-Wallis $p < 0.05$; Table 1), with the exposure to $AgNO_3$ causing greater Ag accumulation than the equivalent Ag NPs exposure. There were also some changes in the Na^+ and K^+ concentrations in the cell homogenates, which were not due to changes in salt concentrations in the external media (culture media Na^+ and K^+ did not alter, data not shown). Exposure to Ag NPs caused statistically significant decreases in both Na^+ and K^+ concentrations in the cell homogenates compared to the controls, and for the

364 Na^+ at least, there was a material-type effect with lower concentrations in the cells exposed to
365 Ag NPs compared to $AgNO_3$ (Table 1).

366 The morphology of the fibroblast cultures exposed by direct addition of either $AgNO_3$ or
367 Ag NPs to the culture media for 24 h compared to controls is shown in Figures 3A-D. The
368 control cells showed normal elongated morphology, with defined cell membranes and nuclei.
369 The morphology of Ag NP-treated cells, regardless of the exposure concentration, were not
370 discernibly different form the controls. In contrast, cells exposed to $AgNO_3$ showed a loss of
371 morphology, and the cells were detached from the dishes at both exposure concentrations. The
372 loss of LDH to the external media was also used as a viability measure (Figure 3E) and reflected
373 the morphology; with a background LDH leak of 1 μmol ml^{-1} or much less in the controls and
374 Ag NPs-treated cells, while the LDH concentrations in the media were higher in both $AgNO_3$
375 treatments compared to the controls or the equivalent Ag NP treatment. The LDH activity in
376 the cell homogenates was also measured (Table 1). There were no statistically significant
377 differences between the control, nor either Ag NP treatments; only the $AgNO_3$ showed a
378 statistically significant decrease in LDH activity compared to all other treatments. The total
379 protein concentrations in cell homogenates (Table 1) from controls and cells treated with Ag
380 NPs were not statistically different and remained around 0.1-0.2 mg ml^{-1}. However, a
381 statistically significant loss of protein was observed in the $AgNO_3$ treatments (one-way
382 ANOVA $p > 0.05$).
383
384 *3.2. The effect of silver as a coating over the silicone elastomer surface on fibroblast cells.*
385 In this experiment, the intention was to attach the silver to the silicone elastomer rather than
386 exposing the fibroblasts to Ag additions via the cell culture media. The newly prepared coatings
387 were confirmed, prior to introducing the cells, using SEM and EDS (Figure 1). Notably, the
388 $AgNO_3$ coating formed nanoscale crystals on the surface of the elastomer, with a composition
389 of Ag and some Cl, suggesting the presence of insoluble AgCl. At the low Ag concentrations,
390 for both Ag NPs and $AgNO_3$, there was a sparse but consistent coverage of the surface with the
391 relevant test material. However, when using a 50 mg l^{-1} coating solution/dispersion, the
392 coverage was much denser, over the entire surface of the elastomer (Figure 1). In the case of
393 Ag NPs, an increase in the concentration resulted in a higher degree of particle agglomeration
394 on the elastomer surface (Figure 1B). The Ag NP coating also remained intact during the
395 experiment, as determined by apparent release of silver into the culture media. Measurement
396 of total silver concentrations in the media did not detect any Ag from either the control or Ag

NP treatments (below detection limit). However, some Ag was detected in the media from the $AgNO_3$ coating. For example, in the latter, at the 50 mg l^{-1} concentration of $AgNO_3$ used for coating, the measured total Ag concentrations in the media were: 13.3 ± 3.0, 13.3 ± 2.3, and 12.8 ± 4.9 mg l^{-1} on days 1, 2 and 3 respectively (no time effect, ANOVA, $p > 0.05$); suggesting some steady leaching of either dissolved Ag or AgCl particles from the surface of the elastomer.

Table 1 shows the Ag concentrations in the cell homogenates after 72 h growing on the Ag-coated silicone elastomers. Overall, the fibroblasts showed small, but statistically significant elevations in the total Ag concentration in the cells when grown on both the Ag NP-coated surface, but the highest values were for cells grown on the 50 mg l^{-1} $AgNO_3$-coated surface. Compared to the first experiment with exposure via the media, all the values were much lower when Ag was added as a coating, even though the exposure was several days longer; suggesting the coating is less bioavailable to the cells. The application of Ag as a coating also had fewer effects on the cell electrolyte composition. There were no statistically significant effects on cell K^+ concentrations in any treatment, but the cell Na^+ concentration showed an apparent rise in both $AgNO_3$ treatments compared to controls when expressed per mg of cell protein (Table 1).

Figure 4 shows the morphology of fibroblast cells grown over Ag-coated silicone for 72 h. Fibroblasts exposed to either concentration of the Ag NP coatings showed normal morphology, with the cells remaining confluent and firmly attached to the well plates. In contrast, both coating concentrations of $AgNO_3$ caused mortality and the cells to detach from the well plates. Figure 5A shows the daily cumulative LDH activity in the external media, which remained low in the control treatments, and throughout in the 5 mg l^{-1} Ag NP coating treatment. There was a transient rise in the LDH activity on day 1 in the 50 mg l^{-1} Ag NP coating treatment, but this did not persist (Figure 5A). In the $AgNO_3$ coating treatments, the LDH activity was lost, simply because the cells detached at day 1 and were lost from the culture media during the necessary media changes. LDH activity in the cell homogenates derived from the adherent cells at the end of the experiment (72 h) are shown in Table 1. Values were below detection limit for both $AgNO_3$ treatments as insufficient cells survived. There were no statistical differences between the controls or the Ag NP treatments (ANOVA, $p > 0.05$) for cell homogenate protein concentration (Table 1), but protein concentration of the homogenates from both $AgNO_3$ coating treatments were significantly lower (ANOVA, $p < 0.05$) due to poor survival.

3.3. The antifungal properties of silver-coated silicone elastomer against C. albicans.

This experiment was identical to the experiment above with fibroblasts grown on Ag-coated silicone elastomer for 72 h, except that the fibroblasts were then challenged with an inoculum of *C. albicans* for a further 24 h (96 h of fibroblast growth on the Ag-coated surfaces in total). The measured total Ag concentrations in the culture media were similar to the previous experiment with no detectable Ag in the controls or from the plates with the Ag NP coatings. Similar to the previous experiment, some Ag was detectable in the culture media for the $AgNO_3$ coating treatments. For example, with the 50 mg l^{-1} $AgNO_3$ coating treatment the total Ag concentrations in the culture media were: 13.5 ± 1.1, 11.9 ± 1.4, 10.8 ± 0.8, 11.6 ± 0.9 mg l^{-1} on days 1, 2, 3, and 4 respectively. The Ag accumulation, as measured by cell homogenate total Ag concentrations (Table 1) was also broadly similar to the previous experiment with the biggest increases in the cells from the $AgNO_3$ coating treatment. However, although the Ag NP coating caused a trend of increasing Ag compared to the controls, this was not statistically significant (Table 1); suggesting the addition of the yeast challenge may have limited Ag availability to the fibroblasts. The electrolyte concentrations in the homogenates also showed changes following the infection challenge; with statistically significant increases in the homogenate K^+ concentration in only the Ag NP coating treatment compared to the control, and conversely, an elevation of cell homogenate Na^+ concentration; but only in the $AgNO_3$ treatment (Table 1).

The morphology of fibroblast cells grown on the silicone elastomer for 96 h and inoculated with *C. albicans* in the last 24 h of the experiment are shown in Figure 4. In the controls (no added Ag) *C. albicans* was attached to the uncoated silicone elastomer and the fibroblast cells were absent (i.e., dead). Similarly, when the silicone elastomer was coated with either concentration of $AgNO_3$ no fibroblast cells were observed growing on the surface by the end of the experiment, as the cells had been already detached or had died. The $AgNO_3$ also prevented the growth of the yeast, as only a few yeast cells were observed when the silicone elastomer was coated using treatments of 5 or 50 mg l^{-1} $AgNO_3$. In contrast, the fibroblast cells were viable and protected when the silicone elastomer was coated with Ag NPs compared to controls or $AgNO_3$. An apparent dose effect was also observed as more cells were attached when the silicone elastomer was coated with 50 mg l^{-1} than 5 mg l^{-1} Ag NPs (Figure 4).

Measurements of LDH activity in culture media each day (Figure 5B) reflected the morphological observations. The controls showed low LDH leak until they were infected with yeast cells on day 4. Similar, to the previous trials, few fibroblasts from the $AgNO_3$ coating treatments survived, reflecting the largest LDH activity in the media in the first 24 h of exposure to the coatings. In contrast, the Ag NP coating treatments showed reasonably steady

LDH activity in the media, even during the yeast challenge (Figure 5B). LDH activity in the cell homogenates at the end of the experiment (after attempts to wash off the yeast) are shown (Table 1). All the silver treatments were lower than the control, but with no material-type effects for the form of silver (ANOVA, $p < 0.05$). Similar to the previous experiment, the protein concentrations in the homogenates of cells from the controls and Ag NP coating treatments remained normal around 0.08-0.15 mg ml^{-1}, but the $AgNO_3$ coatings caused some statistically significant protein depletion compared to the other treatments (Table 1).

Extracellular ethanol produced by *C. albicans* was also measured to investigate whether the yeast cells were capable of aerobic metabolism (i.e., no ethanol production) or if they used fermentation to make ATP and therefore produced ethanol (Figure 6). The main findings were that ethanol production before incubating the plates (mean ± SEM, $n = 6$) was about 0.07 ± 0.02 µmol ml^{-1}, and after 24 h incubations with the yeast ($n = 4$ plates) the ethanol production was 43.2 ± 25.02 µmol ml^{-1} in controls (uncoated silicone elastomer). Both forms of Ag coatings resulted in a statistically significant decrease in apparent ethanol production to around 3.6 µmol ml^{-1} or much less (ANOVA test, $p < 0.05$), although there was no material-type effect between Ag NPs and $AgNO_3$ as a coating (Figure 6).

4. Discussion

This study demonstrates that medical grade silicone elastomer coated with Ag NPs allows the growth to confluence of normal, healthy, fibroblasts. The Ag NP coating is also antifungal, delaying or preventing the proliferation of *C. albicans*; and inhibiting ethanol production by the yeast. In contrast, coatings made from $AgNO_3$ were toxic to both fibroblasts and yeast. Fibroblasts grown on uncoated silicone elastomer as controls were not protected from fungal infection. Overall, the results suggest that coating the silicone elastomer material used for facial prosthesis with Ag NPs derived from a 50 mg l^{-1} dispersion is biocompatible and able to prevent clinically relevant fungal infection.

4.1. Composition and stability of Ag NP and $AgNO_3$ coatings on silicone elastomer.

This study successfully coated silicone elastomer with either Ag NPs or $AgNO_3$ (Figure 1). The method of allowing gravimetrically settling of the silver, followed by oven drying, gave a coating of each material on the silicone elastomer, although the coverage was much more complete using 50 mg l^{-1} dispersions and solutions. The coatings were not washed off by the various preparation steps in the SEM, suggesting they were reasonably attached to the surface. The absence of detectable total Ag in the cell culture media from the Ag NP coatings at least,

499 also supports good adherence of the particles to the silicone. Interestingly, when the surface
500 was coated with $AgNO_3$, the sterilisation procedure using PBS may have played a vital role in
501 attaching the Ag to the surface of silicone elastomer. The PBS contained around 140 mmol l^{-1}
502 of chloride ions. In high ionic strength solutions that contain mmolar amounts of chloride, Ag
503 ions will rapidly form insoluble AgCl.[34] In the presence of PBS, particles of insoluble AgCl
504 appear to have formed on the surface of silicone elastomer (Figure 1). The EDS analysis
505 showed that the material was rich in both Ag and Cl (Figure 1). It may also be possible that
506 during the formation of AgCl, the crystals became annealed to the surface of the silicone
507 elastomer. However, some total Ag was detected in the culture media during the experiments
508 to grow fibroblasts. This observation suggests that either intact particles of the amorphous
509 AgCl detached from the coating surface during the cell culture, or that silver (form unknown)
510 was accumulated (see below) and then excreted by the cells into the culture media. It is also
511 possible that the culture media contained some debris from dead fibroblast cells that had some
512 associated Ag from the coating in/on their membrane fragments, or that the necessary daily
513 changes of the culture media itself caused damage to the coating. The latter seems unlikely.
514 Further research is needed to explore the possibility that fibroblasts might mobilise Ag from
515 AgCl precipitates on the silicone elastomer, and the forms (soluble or particulate) of the Ag
516 involved.
517
518 *4.2. Accumulation and toxicity of silver to fibroblasts by direct addition to the culture media.*
519 The first experiment provided a bench mark and demonstrated, as expected, that additions of
520 mg l^{-1} concentrations of $AgNO_3$ to the culture media are toxic to fibroblasts as measured by
521 loss of morphology and leak of LDH activity to the external media (Figure 3). In the high ionic
522 strength culture media, the silver speciation will be mainly insoluble AgCl (discussed above).
523 However, AgCl is also toxic to fibroblasts. Contreras et al. demonstrated 100% mortality of
524 human gingival fibroblasts cells exposed to nominal concentrations of 0.5 mmol l^{-1} AgCl over
525 24 h[35] (similar to the 50 mg l^{-1} here, equivalent 0.34 mmol l^{-1} over 24 h in the present study).
526 In contrast, additions of Ag in the form of Ag NPs were less toxic than the equivalent
527 nominal concentration of $AgNO_3$; with the cells exposed to Ag NPs showing normal
528 morphology and limited leak of LDH activity. Arora et al. report a concentration for 50%
529 viability (IC_{50}) for primary fibroblasts grown in DMEM for 24 h of 61 mg l^{-1} for Ag NPs.[36]
530 The cultured cell lines used in the present study are slightly hardier. Panáček et al. also found
531 limited effects of using 30 mg l^{-1} Ag NPs against human fibroblast cells.[37] Nonetheless, the

difference in toxicity between $AgNO_3$ and Ag NP additions to culture media has been reported previously for mammalian cells;[31] with the nanoform being generally less hazardous.

4.3. Accumulation and toxicity of silver-coated silicone elastomer to fibroblasts.

In comparison with the first experiment where Ag was added to the culture media for 24 h, exposure of fibroblasts to Ag as a coating for 72 h produced generally less apparent Ag accumulation by the cells, as measured by the total Ag in the cell homogenates at the end of the experiments. This suggests that the Ag, regardless of whether it was originally as $AgNO_3$ or Ag NPs, is less bioavailable as a coating. Nonetheless, similar to direct additions to the media, the $AgNO_3$ coating resulted in mortality of the fibroblasts; with up to around 2 mg l^{-1} of total silver in/on the remaining washed cells (Table 1). In contrast, cells exposed to the Ag NP coatings survived, and at the highest Ag concentration had less Ag in the cell homogenates compared to the equivalent $AgNO_3$ coating treatment (Table 1).

It is unclear how Ag as a coating may become bioavailable to fibroblast cells, and was not the purpose of the current experiments. The possibilities include the uptake of dissolved Ag species from the coating at the elastomer-cell interface, or the uptake of intact particles from the coating directly. Understanding the former would require some detailed investigations of the chemistry in the microenvironment between the elastomer and cell membrane. However, Besinis et al. found that $AgNO_3$ rapidly forms AgCl crystals in saline, and dialysis experiments estimated a maximum release rate of dissolved Ag of 0.17 µg min^{-1}.[30] Over 72 h there may therefore be sufficient dissolution of dissolved Ag species directly at the cell membrane. In contrast, the dissolution of the same Ag NPs as used in the present study was much lower (0.058 µg min^{-1}).[30] It is also theoretically possible that fibroblasts may erode or detach Ag particles directly from the coating, although how this might occur from apparently robust coatings is unclear. Arora et al. suggest that cultures of primary fibroblasts can internalise particles from Ag NPs exposures when the particles are added directly to the culture media,[36] although the composition of the apparently internalised particles were not confirmed by EDS.

4.4. Effect of silver exposures on electrolyte concentrations in cell homogenates.

Ionic silver is well known for its ability to inhibit Na^+,K^+-ATPase activity[38] and compete with Na^+ ions for uptake through sodium channels.[39] Ag additions directly to the culture media caused the loss of either Na^+ and/or K^+ from the cells (Table 1). This is most easily explained by electrolyte leak through increased permeability of the cell membrane and may involve the free Ag ion toxicity to the cells.[31] However, when the cells were exposed to Ag NP- or $AgNO_3$-

coated silicone elastomer they showed no K^+ depletion (Table 5); implying insufficient bioavailable Ag was released from the coating to block the Na^+ pump. However, the $AgNO_3$ coating, unlike the Ag NP coating also showed an increase of homogenate Na^+ concentration, implying some additional diffusional influx of Na^+ down the electrochemical gradient (inward membrane leak).

Following inoculation with *C. albicans*, the electrolyte composition of the fibroblast showed a slightly different response; with increased K^+ but not Na^+ for the Ag NP coating treatment compared to controls, and vice versa for the $AgNO_3$ coating treatment (elevated Na^+, but not K^+, Table 1). This data following the yeast inoculations is difficult to interpret as electrolytes from fragments of the yeast cells cannot be excluded (although the plates were carefully washed); and because *C. albicans* has some unusual features to its salt regulation. For example, the Na^+/H^+ exchanger in *C. albicans* is not as specific as in higher organisms, and may also transport K^+.[40,41] Nonetheless, Ag NPs are suggested to increase the cell permeability *C. albicans*[42,43] and therefore could alter the apparent electrolyte content of cellular material remaining in the culture dishes.

4.5. Antifungal properties of silver nanoparticles and ethanol production by C. albicans.

Fibroblasts grown on the Ag-NP-coated silicone elastomer survived the yeast challenge, because the proliferation of *C. albicans* was prevented (Figure 4). In contrast, the $AgNO_3$ coating was toxic to both fibroblasts and yeast cells. The toxicity of $AgNO_3$ to the yeast cells could be explanation by some free ion toxicity from Ag leached in the media from the coating. The minimum inhibitory concentration (MIC value) to prevent biofilm growth of *C. albicans* within 5 h is around 1.2 mmol l^{-1} (129 mg l^{-1}) for dissolved silver.[44] The measured total silver concentrations in the media for the $AgNO_3$ coatings was around 10 mg l^{-1}; and over several days would give a dose exceeding the MIC estimate for *C. albicans* above. Therefore, the silver concentration in the media derived from the $AgNO_3$ coatings would be sufficient to kill the yeast in the current experiment. However, the antifungal effect of Ag NPs has received much less attention. Monteiro et al. showed that Ag NPs added to the media exhibit fungicidal activity against *C. albicans* growth at 0.4-3.3 mg l^{-1} Ag NPs after 48 h.[29] This indicates that *C. albicans* is at least sensitive to Ag NP additions to the external media. In the present study, Ag NP coatings were used instead, but also showed fungicidal activity. The hyphae of *C. albicans* may have penetrating through the fibroblast layer to have direct contact toxicity with the Ag NP coating, or the coating may simply prevent the attachment of the yeast to the fibroblast culture.

Furthermore, both types of silver coatings decreased ethanol production by *C. albicans* (Figure 6). Yeast use aerobic metabolism and can switch to anaerobic fermentation in less favourable conditions; but would normally produce measurable quantities of ethanol during a growth phase.[45] The loss of ethanol production due to exposure to either Ag NP or $AgNO_3$ coatings could have several explanations: (i) the yeast are not growing and therefore there are less cells to produce ethanol, (ii) the yeast cells are growing normally but favour aerobic metabolism and therefore decrease ethanol production, or (iii) the yeasts are quiescent and are not able to ferment to alcohol. Ag NP interference with the ethanol assay (false negatives) is excluded at the concentrations used in this experiment (data not shown). The former seems a likely explanation as fewer yeast cells were present in both Ag treatments, but some inhibition of fermentation by Ag is also possible. Low ethanol production is associated with mitochondrial dysfunction during Ag exposure.[46] However, even with normal mitochondria, ethanol production can be inhibited when *C. albicans* are growing at low pH.[45] After 24 h incubation with silicone elastomer coated with Ag NPs, the media had a pH of about 6; suggesting some metabolic acidic production by the yeast (i.e., lactic acid), but not fermentation all the way to alcohol. Moreover, in yeasts, acetaldehyde is fermented to ethanol using alcohol dehydrogenase,[47] and interference of Ag NPs or Ag ions with this enzyme cannot be excluded. Clearly, further work is needed to investigate the inhibition of ethanol production by Ag NPs.

5. Conclusion

This study demonstrates that Ag NP-coated silicone elastomer has antifungal activity without appreciable adverse effects on human dermal fibroblast cells *in vitro*. The current experiments used high doses of the yeast to challenge the fibroblast cultures (10^6 yeast cells ml^{-1}), and yet the nanocoatings were very effective in preventing growth of the infection. In clinical situations *Candida* infection occur at lower doses, suggesting that the current coating would be very effective indeed. In the present study, the coatings were prepared gravimetrically, and the Ag NP coatings did not leach appreciable silver over a maximum of 96 h. From the perspective of the chemical safety aspects of regulatory approvals for medical devices, achieving a new prosthetic material coated with Ag NPs without the need for additional chemicals (adhesive, solvents, etc.,) is highly desirable. The Ag NP coatings were not eroded with the short duration and experimental conditions here, but longer *in vitro* studies are needed to confirm durability of the nanocoatings; and whether or not the antifungal properties remain. There were no colour changes that would cause an aesthetic concern to the patient with the nanocoatings here.

However, one limitation is that surface nanocoatings can be scratched, and a next step would be properly incorporate the Ag NPs in the matrix of the elastomer during the preparation of the silicone material. This would also require studies of the mechanical properties of the prosthesis to ensure the silicone elastomer is not hardened or altered in a way that would be uncomfortable to the patient.

Acknowledgements

Zhala Meran was supported by a scholarship from the Ministry of Higher Education and Scientific Research, Kurdistan Regional Government of Iraq. This work was supported by strategic funding from the University of Plymouth awarded to Handy and De Peralta. Technical support from M. Emery on microbiology, and L. Cooper on cell cultures is gratefully acknowledged.

References

[1] Aziz T, Waters M, Jagger R. Analysis of the properties of silicone rubber maxillofacial prosthetic materials. J Dent 2003;31:67-74.

[2] Hooper SM, Westcott T, Evans PLL, Bocca AP, Jagger DC. Implant-Supported Facial Prostheses Provided by a Maxillofacial Unit in a U.K. Regional Hospital: Longevity and Patient Opinions. J Prosthodont 2005;14:32-38.

[3] Kurtulmus H, Kumbuloglu O, Özcan M, Ozdemir G, Vural C. *Candida albicans* adherence on silicone elastomers: Effect of polymerisation duration and exposure to simulated saliva and nasal secretion. Dent Mater 2010;26:76-82.

[4] Busscher HJ, de Boer CE, Verkerke GJ, Kalicharan R, Schutte HK, van der Mei HC. *In vitro* ingrowth of yeasts into medical grade silicone rubber. Int Biodeter Biodegr 1994;33:383-390.

[5] Campoccia D, Montanaro L, Arciolaa CR. A review of the clinical implications of anti-infective biomaterials and infection-resistant surfaces. Biomaterials 2013;34:8018-8029.

[6] Sobolewska E, Fraczak B, Czarnomysy-Furowicz D, Ey-Chmielewska H, Karakulska J. Bacteria adhesion to the surface of various prosthetics materials. Ann Acad Med Stetinen 2007;53:68-71.

[7] Coenye T, de Prijck K, Nailis H, Nelis H. Prevention of *Candida albicans* biofilm formation. Open Mycol J 2011;5:9-20.

[8] Thein ZM, Samaranayake YH, Samaranayake LP. Characteristics of dual species *Candida* biofilms on denture acrylic surfaces. Arch Oral Biol 2007;52:1200-1208.

[9] Leow M, Kour A, Inglis T, Kumarasinghe G, Pho R. Fungal colonisation in digital silicone rubber prostheses. Prosthet Orthot Int 1997;21:195-198.

[10] Korting HC, Braun-Falco O. The effect of detergents on skin pH and its consequences. Clin Dermatol 1996;14:23-28.

[11] Spielmann N, Wong DT. Saliva: diagnostics and therapeutic perspectives. Oral Dis 2011;17:345-354.

[12] Mandracci P, Mussano F, Ricciardi C, Ceruti P, Pirri F, Carossa S. Low temperature growth of thin film coatings for the surface modification of dental prostheses. Surf Coat Tech 2008;202:2477-2481.

[13] Deng HY, Zwetchkenbaum S, Noone AM. Bond strength of silicone to polyurethane following immersion of silicone in cleaning solutions. J Prosthet Dent 2004;91:582-585.

[14] Kiat-amnuay S, Johnston DA, Powers JM, Jacob RF. Color stability of dry earth pigmented maxillofacial silicone A-2186 subjected to microwave energy exposure. J Prosthodont 2005;14:91-96.

[15] Lemon JC, Chambers MS, Jacobsen ML, Powers JM. Color stability of facial prostheses. J Prosthet Dent 1995;74:613-618.

[16] Montgomery PC, Kiat-Amnuay S. Survey of currently used materials for fabrication of extraoral maxillofacial prostheses in North America, Europe, Asia, and Australia. J Prosthodont 2010;19:482-490.

[17] Hatamleh MM, Haylock C, Watson J, Watts DC. Maxillofacial prosthetic rehabilitation in the UK: a survey of maxillofacial prosthetists' and technologists' attitudes and opinions. Int J Oral Maxillofac Surg 2010;39:1186-1192.

[18] Chrcanovic BR, Nilsson J, Thor A. Survival and complications of implants to support craniofacial prosthesis: A systematic review. J Craniomaxillofac Surg 2016;44:1536-1552.

[19] Fadeel B, Garcia-Bennett AE. Better safe than sorry: Understanding the toxicological properties of inorganic nanoparticles manufactured for biomedical applications. Adv Drug Deliv Rev 2010;62:362-374.

[20] Sozer N, Kokini JL. The applications of nanotechnology. In: Picó Y,editor. Chemical Analysis of Food: Techniques and Applications. Amsterdam: Academic Press, 2012. p.145-176.

[21] Juillerat-Jeanneret L, Dusinska M, Fjellsbo LM, Collins AR, Handy RD, Riediker M. Biological impact assessment of nanomaterial used in nanomedicine. Introduction to the NanoTEST project. Nanotoxicology 2013 doi: 10.3109/17435390.2013.826743.

[22] Schütz CA, Juillerat-Jeanneret L, Mueller H, Lynch I, Riediker M. Therapeutic Nanoparticles in Clinics and under Clinical Evaluation: a Review. Nanomedicine 2013;8:449-467.

[23] Handy RD, Shaw BJ. Toxic effects of nanoparticles and nanomaterials: Implications for public health, risk assessment and the public perception of nanotechnology. Health Risk Soc 2007;9:125-144.

[24] Christian P, von der Kammer F, Baalousha M, Hofmann T. Nanoparticles: structure, properties, preparation and behaviour in environmental media. Ecotoxicology 2008;17:326-343.

[25] Roe D, Karandikar B, Bonn-Savage N, Gibbins B, Roullet JB. Antimicrobial surface functionalization of plastic catheters by silver nanoparticles. J Antimicrob Chemoth 2008;61:869-876.

[26] Tian J, Wong KK, Ho CM, Lok CN, Yu WY, Che CM, et al. Topical delivery of silver nanoparticles promotes wound healing. Chem Med Chem 2007;2:129-136.

[27] Rai M, Yadav A, Gade A. Silver nanoparticles as a new generation of antimicrobials. Biotechnol Adv 2009;27:76-83.

[28] Xiu ZM, Zhang QB, Puppala HL, Colvin VL, Alvarez PJ. Negligible particle-specific antibacterial activity of silver nanoparticles. Nano letters 2012;12:4271-4275.

[29] Monteiro DR, Gorup LF, Silva S, Negri M, de Camargo ER, Oliveira R, et al. Silver colloidal nanoparticles: antifungal effect against adhered cells and biofilms of *Candida albicans* and *Candida glabrata.* Biofouling 2011;27:711-719.

[30] Besinis A, de Peralta T, Handy RD. The antibacterial effects of silver, titanium dioxide and silica dioxide nanoparticles compared to the dental disinfectant chlorhexidine on *Streptococcus mutans* using a suite of bioassays. Nanotoxicology 2014;8:1-16.

[31] Reidy B, Haase A, Luch A, Dawson KA, Lynch I. Mechanisms of silver nanoparticle release, transformation and toxicity: A critical review of current knowledge and recommendations for future studies and applications. Materials 2013;6:2295-2350.

[32] Besinis A, van Noort R, Martin N. Infiltration of demineralized dentin with silica and hydroxyapatite nanoparticles. Dent Mater 2012;28:1012-1023.

[33] Plummer DT. An Introduction to Practical Biochemistry. London: McGraw Hill,1971. p. 288-289.

[34] Bradford A, Handy RD, Readman JW, Atfield A, Mühling, M. Impact of silver nanoparticle contamination on the genetic diversity of natural bacterial assemblages in estuarine sediments. Environ Sci Technol 2009;43:4530-4536.

[35] Contreras RG, Sakagami H, Nakajlama H, Shimada J. Type of cell death induced by various metal cations in cultured human gingival fibroblasts. In Vivo 2010;24:513-517.

[36] Arora S, Jain J, Rajwade J, Paknikar K. Interactions of silver nanoparticles with primary mouse fibroblasts and liver cells. Toxicol Appl Pharm 2009;236:310-318.

[37] Panáček A, Kolář M, Večeřová R, Prucek R, Soukupová J, Kryštof V, et al. Antifungal activity of silver nanoparticles against *Candida* spp. Biomaterials 2009;30:6333-6340.

[38] Hussain S, Meneghini E, Moosmayer M, Lacotte D, Anner BM. Potent and reversible interaction of silver with pure Na,K-ATPase and Na,K-ATPase-liposomes. BBA-Biomembranes 1994;1190:402-408.

[39] Bury NR, Wood CM. Mechanism of branchial apical silver uptake by rainbow trout is via the proton-coupled Na^+ channel. Am J Physiol-Reg I 1999;277:1385-1391.

[40] Kinclová O, Potier S, Sychrová H. The Candida albicans Na^+/H^+ antiporter exports potassium and rubidium. FEBS Lett 2001;504:11-15.

[41] Kinclová O, Potier S. Sychrová H. Difference in substrate specificity divides the yeast alkali-metal-cation/H^+ antiporters into two subfamilies. Microbiology 2002;148:1225-1232.

[42] Kim KJ, Sung WS, Suh BK, Moon SK, Choi JS, Kim JG, et al. Antifungal activity and mode of action of silver nano-particles on *Candida albicans*. Biometals 2009;22:235-242.

[43] Das D, Ahmed G. Silver nanoparticles damage yeast cell wall. J. Biotechnol 2012;3:37-39.

[44] Harrison JJ, Rabiei M, Turner RJ, Badry EA, Sproule KM, Ceri H. Metal resistance in *Candida* biofilms. FEMS Microbiol Ecol 2006;55:479-491.

[45] Ogasawara A, Iino Y, Sato K, Nakajima Y, Bessho S, Watanabe T, et al. Anti-*Candida* activity of sodium sulfite. Biol Pharm Bull 2008;31:1101-1103

[46] Hwang IS, Lee J, Hwang JH, Kim KJ, Lee DG. Silver nanoparticles induce apoptotic cell death in *Candida albicans* through the increase of hydroxyl radicals. FEBS J 2012;279:1327-1338.

[47] Strijbis K, Distel B. Intracellular acetyl unit transport in fungal carbon metabolism. Eukaryot Cell 2010;9:1809-1815.

Figure legends

Figure 1. Scanning electron micrographs showing surface morphology of newly prepared silicone discs coated with A) 5 mg l^{-1} Ag NPs, B) 50 mg l^{-1} Ag NPs, C) detail of a Ag NPs agglomerate adhered to the silicone disc surface, D) discs coated with 5 mg l^{-1} $AgNO_3$, E) discs coated with 50 mg l^{-1} $AgNO_3$ and F) detail of $AgNO_3$ crystals adhered to the silicone disc surface. All images were acquired at x1000 magnification, except panels C and F taken at x15000 magnification. The EDS spectra show the elemental composition of the nano-silver coated silicone disc surfaces.

Figure 2: Particle size distributions determined by nanoparticle tracking analysis (Nanosight, LM10) in culture media (DMEM, supplemented with glutamine and 10% FBS). The plots are individual examples from triplicate measurements. A) 5 mg l^{-1} Ag NPs, B) 50 mg l^{-1} Ag NPs, C) 5 mg l^{-1} $AgNO_3$, D) 50 mg l^{-1} $AgNO_3$ and E) culture media control (no added silver).

Figure 3. Fibroblast cell morphology *in situ* on cell culture dishes (no silicone elastomer) following 24 h exposure to direct additions of Ag NPs or $AgNO_3$ to the culture media. A) control (no added silver), B) 5 mg l^{-1} Ag NPs, C) 5 mg l^{-1} $AgNO_3$, D) 50 mg l^{-1} Ag NPs, and E) measurement of the LDH activity in the external media. Optical light microscope images were obtained using an Olympus SZ-1145 microscope (magnification 40x), which was equipped with a ScopeTek MDC 560 CCD camera (magnification 0.6x). Data are means ± SEM (n = 6). Different letters indicate statistical difference (one-way ANOVA, $p < 0.05$).

Figure 4. Optical light microscopy of fibroblasts grown for 72 h on silicone elastomer coated with Ag NPs or $AgNO_3$ (n = 6 plates/treatment). Panels on the left are fibroblasts alone (stained with Giemsa), panel on the right are the cells following an infection challenge with an inoculum of *C. albicans* (10^6 cells ml^{-1}) for a further 24 h (96 h on the elastomer in total, additional staining with methyl blue). Images were obtained using an Olympus SZ-1145 microscope (magnification 40x), which was equipped with a ScopeTek MDC 560 CCD camera (magnification 0.6x).

Figure 5. LDH activity in the culture media from fibroblasts grown on silver-coated silicone elastomer surfaces. A) Daily measurements over 72 h for fibroblasts grown on the elastomer without a yeast infection challenge, and B) with a *C. albicans* challenge for a further 24 h. The media was inoculated with *C. albicans* immediately after the 72 h endpoint. Data are means ± SEM (n = 6 plates per treatment). Values on the x- axis (1,2,3,4) are the number of days on the silicone elastomer. * shows a statistical difference between the control-MQ and treated groups for the same endpoints (one-way ANOVA, $p < 0.05$). # indicates statistical difference (one-way ANOVA, $p < 0.05$) between the two concentrations of the same coating material (concentration-effect within time point). Within the same treatment group, different letters indicate significant differences (one-way ANOVA, $p < 0.05$) between days (time-effect within treatment).

Figure 6: Ethanol production by *C. albicans* measured in the external media of fibroblast cultures inoculated with the yeast in the final 24 h following fibroblast growth on silicone elastomer (control), sham coated with Milli-Q water (control-MQ), and coated with Ag NPs or $AgNO_3$. Data are means ± SEM (n = 4) measurements of ethanol from separate dishes. On the x-axis, 5 and 50 refer to the mg l^{-1} concentrations of silver metal used to coat the elastomer surface. Different letters indicate statistical difference between treatments (one-way ANOVA, $p < 0.05$).

Figure 1

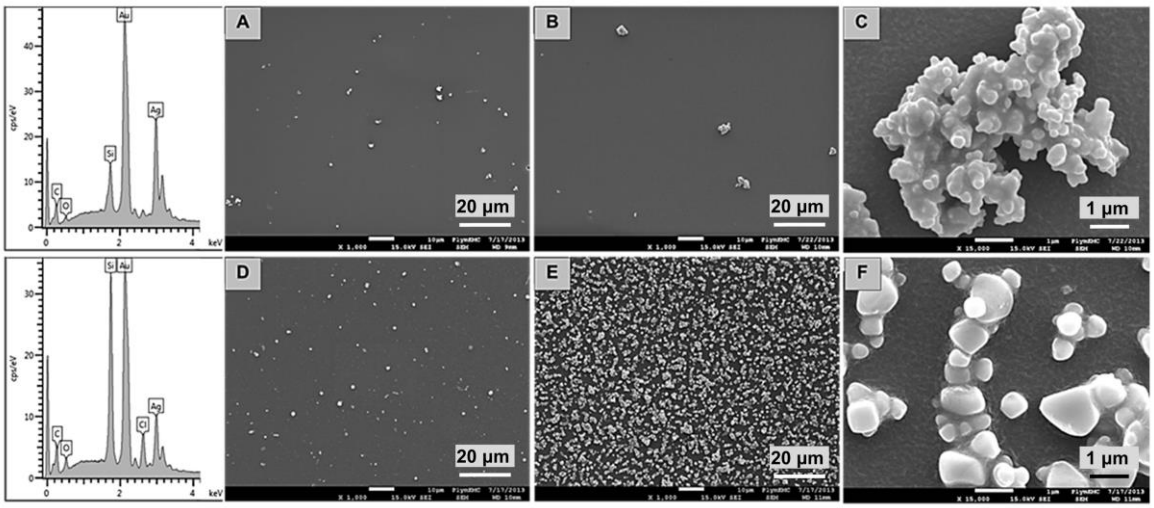

Figure 2

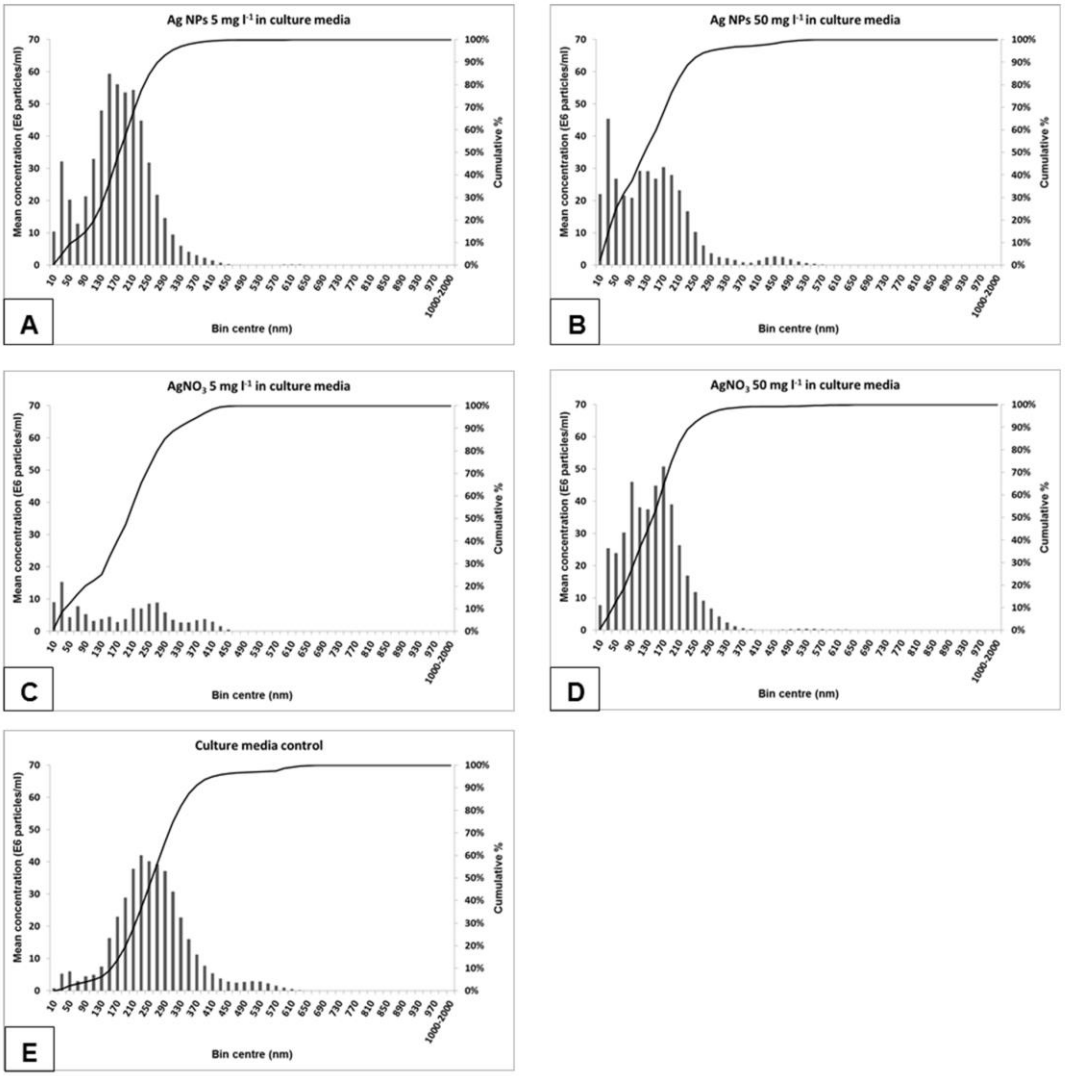
Ag NPs 5 mg l-1 in culture media
Ag NPs 50 mg l-1 in culture media
AgNO3 5 mg l-1 in culture media
AgNO3 50 mg l-1 in culture media
Culture media control
Mean concentration (E6 particles/ml)
Cumulative %
Bin centre (nm)
A
B
C
D
E

Figure 3

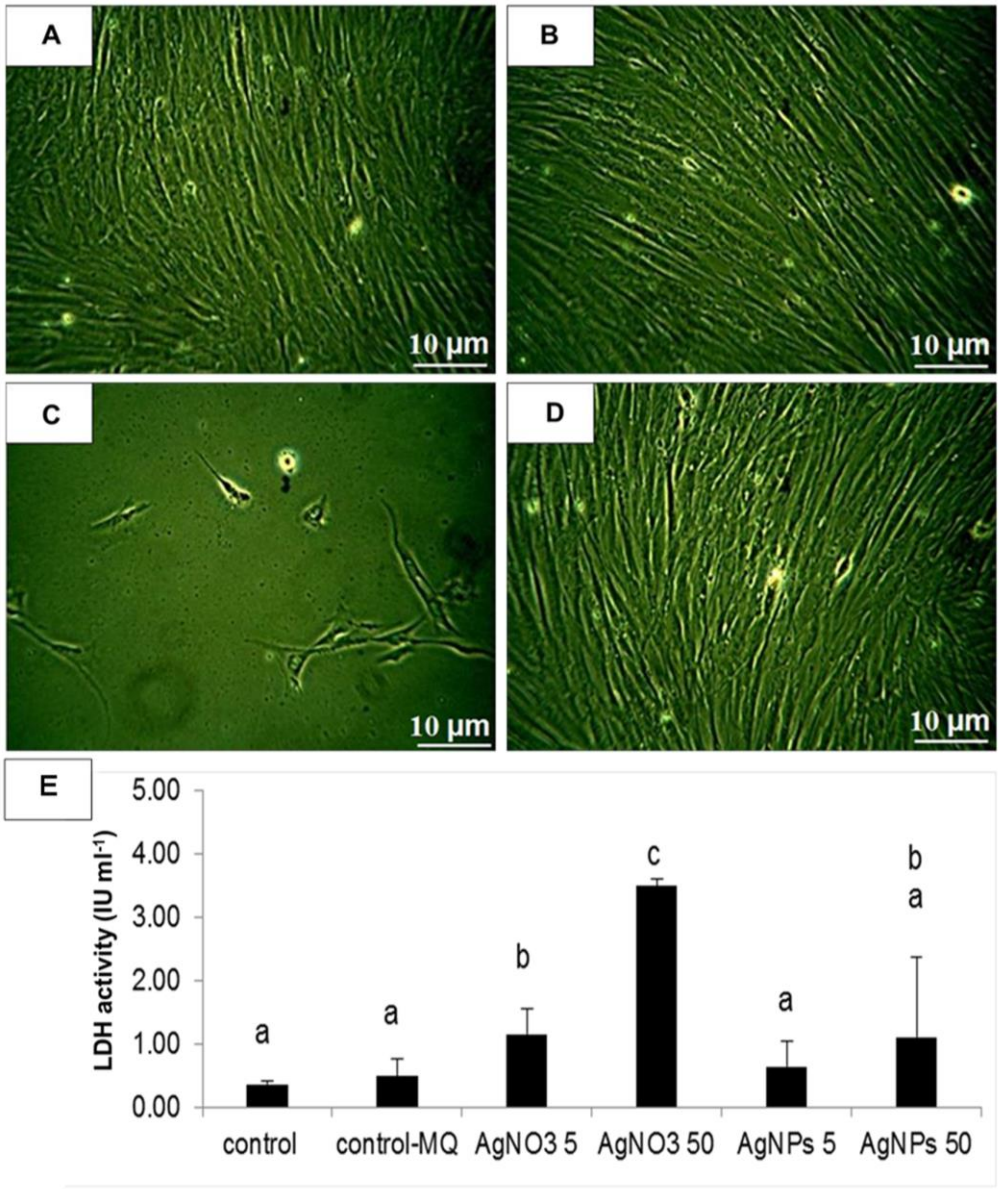
A
10 µm
B
10 µm
C
10 µm
D
10 µm
E
LDH activity (IU ml-1)
5.00
4.00
3.00
2.00
1.00
0.00
a
a
b
c
a
b
a
control
control-MQ
AgNO3 5
AgNO3 50
AgNPs 5
AgNPs 50

Figure 4

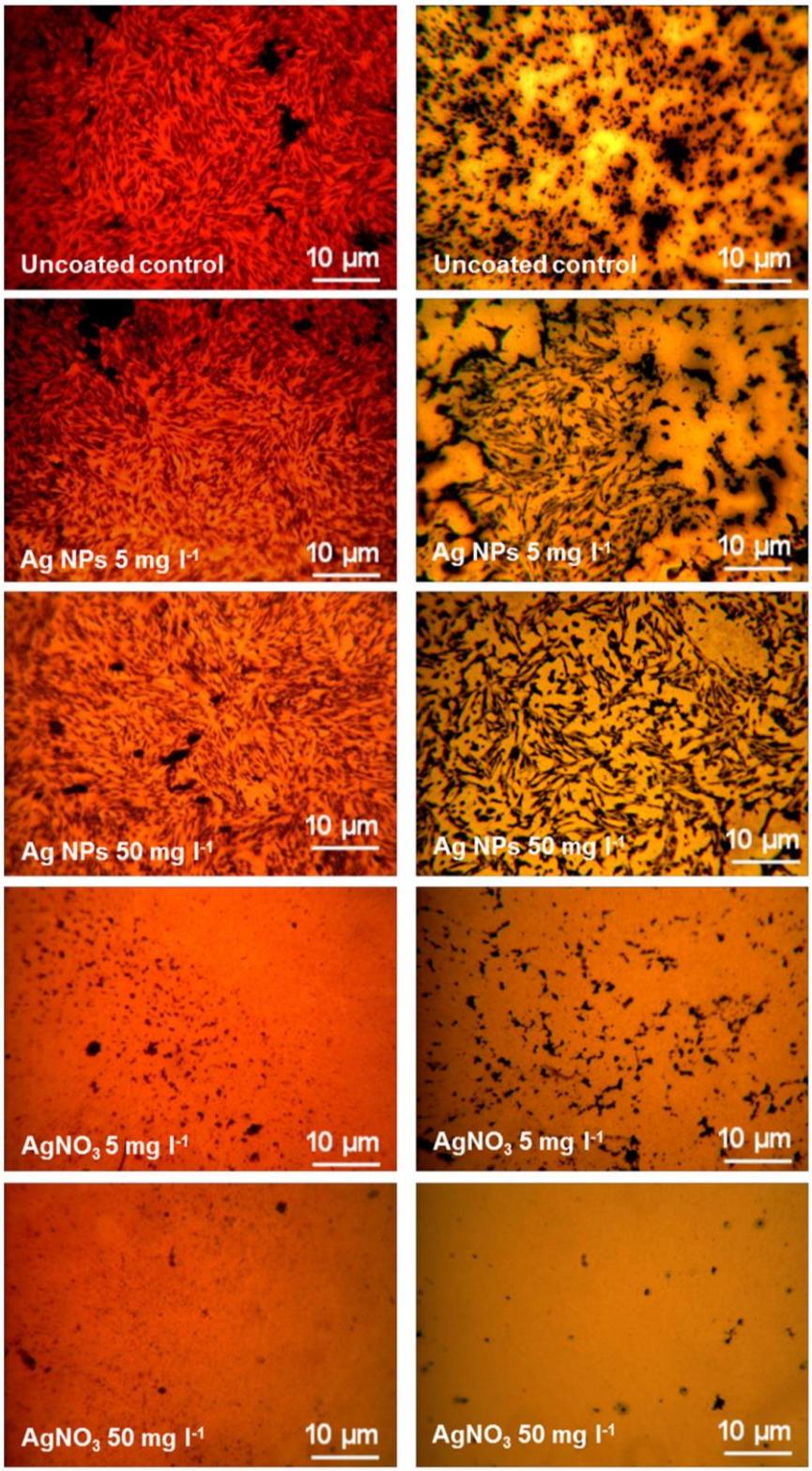
Uncoated control
10 µm
Uncoated control
10 µm
Ag NPs 5 mg l-1
10 µm
Ag NPs 5 mg l-1
10 µm
Ag NPs 50 mg l-1
10 µm
Ag NPs 50 mg l-1
10 µm
AgNO3 5 mg l-1
10 µm
AgNO3 5 mg l-1
10 µm
AgNO3 50 mg l-1
10 µm
AgNO3 50 mg l-1
10 µm

Figure 5

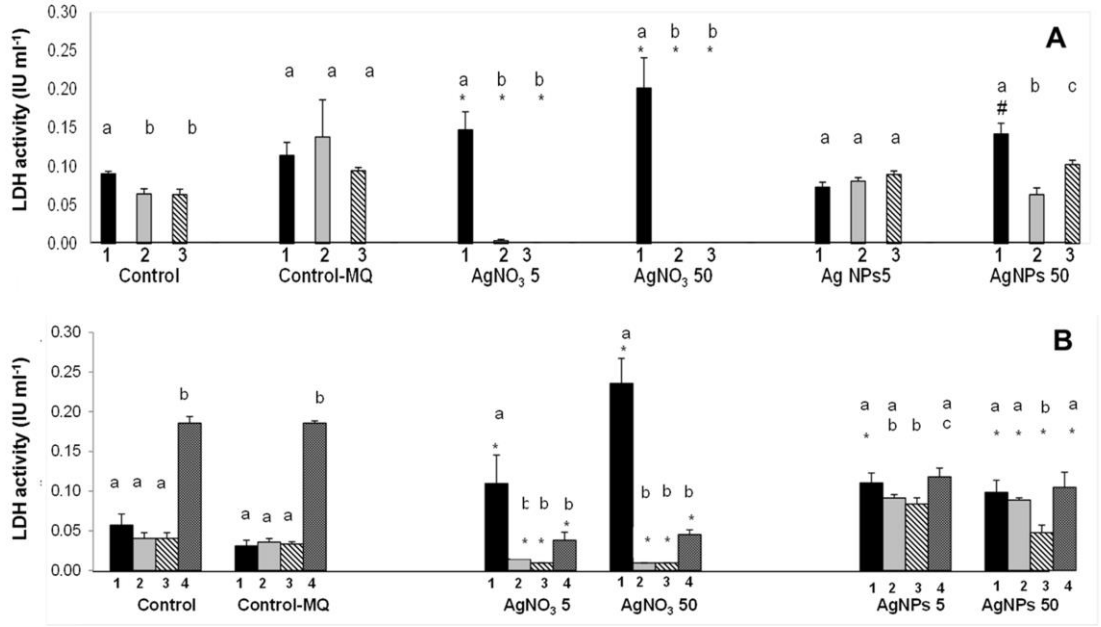

Figure 6