The Use of Silver Nanoparticles as an Antifungal Coating on Silicone Facial Prosthesis

By

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Copyright Statement

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Abstract

Introduction

Maxillofacial prostheses are used to substitute lost facial parts, but give variable clinical results due to complications such as contamination and infection. *Candida albicans* infection remains a significant problem for facial prostheses made of silicone, as the organism causes degradation of the material and infection of the surrounding tissue. This study investigated the antifungal properties of silver nanoparticles (Ag NPs) as a coating on silicone facial prostheses to eliminate infection.

Methods

The experimental approach used fibroblast cell cultures to test the biocompatibility of Ag NPs and to demonstrate the safety of Ag NPs compared to $AgNO_3$ at concentrations of 5 and 50 mg l^{-1}. Cells cultures with and without the prosthetic material coated with Ag NPs were tested for cell viability and adherence to the prosthetic. An addition of yeast (*C. albicans*) was used as a pathogenic challenge to test the “antifungal” properties of the new facial prosthetics. End points included the determination of the cytotoxic response of the cells to nanoparticles including; protein and lactate dehydrogenase enzyme (LDH) leak, as well as electrolytes relating to the osmotic health of the cells (measuring pH, Na^+, K^+). Total Ag concentrations were also measured. In addition, an ethanol assay was used to follow metabolic activity associated with *C. albicans*, as fibroblasts do not produce ethanol.

Results

This study revealed that facial prostheses coated with Ag NPs preserved fibroblast cells from fungal infection compared to uncoated silicone prosthesis at a concentration of 50 mg l^{-1}. Cell viability measures showed Ag NPs were not toxic to fibroblast cells compared to $AgNO_3$ after 24h exposure. LDH leak was 9.7 % at a concentration of 50 mg l^{-l} Ag NPs, while LDH leak for cells exposed to $AgNO_3$ was 97%. Additionally, fibroblast cells exposed to $AgNO_3$ showed significant change in cell Na^+ content, as a coating, and when silver was added directly to the culture media (Kruskal Wallis, $p< 0.05$). However, when cells were exposed for 24 h to Ag NPs prepared in the media, this caused a significant change in cell K^+ content (One way ANOVA, $p< 0.05$) and inhibition of Na^+ K^+-ATPase activity. In contrast, when the cells were exposed to the same concentrations of Ag NPs as a coating on silicone prostheses for 72 h, there was no effect on the Na^+ pump or on cell K^+. This study also showed that silver released from Ag NPs as a coating was less compared to that from $AgNO_3$. Moreover, the production of ethanol by *C. albicans* was 30-fold lower when the silicone coated with Ag NPs compered to uncoated silicone, and 8-fold lower when coated with $AgNO_3$.

Conclusion

The coating of silicone materials with Ag NPs could be of great use clinically to prevent fungal infection for patients with maxillofacial prostheses. A practical coating on the surface of the silicone prosthesis of 50 mg l^{-1} Ag NPs should be antifungal without any toxic effects to human dermal fibroblast cells. In clinical situations *Candida* infection occur at lower innoculations than used here and a lower concentration of Ag NPs could therefore also be effective in real patients.

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Dedication

This thesis is dedicated to my parents

Dr. Zhian & **Dr. Dara Meran**

Thank you for all your love and support

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Author’s Declaration

At no time during the registration for the degree of Research Masters has the author been registered for any other University award without prior agreement of the Graduate Committee. A programme of advanced study was undertaken, which included a course in Microbial Diseases and Biotechnology and postgraduate courses on Research skills and Methods.

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Zhala Meran

Introduction

Maxillofacial prostheses are made of artificial substitutes like silicone and used to replace facial parts lost through disease or trauma. They are also used to restore and maintain the health of the tissues, and to improve aesthetics for better social acceptance of facial injuries (Aziz et al., 2003; Hooper et al., 2005). Some of the materials used for facial prostheses give variable clinical results in terms of quality and stability, due to problems such as contamination and infection (Lewis et al., 1980; Montgomery et al., 2010). Some examples of different prosthetic materials used for facial replacement are presented in Table 1. The surface of maxillofacial prostheses appliances, which are used for nasal, mid-facial, or combinations of facial prostheses with a defect in maxilla, are exposed to body fluids such as saliva and nasal secretions (Kurtulmus et al., 2010). Inevitably, these prostheses are susceptible to surface colonisation by microorganisms, with subsequent degradation of the material and infection of the surrounding tissue (Busscher et al., 1994).

Table 1. The advantages and disadvantages of available materials used for maxillofacial prostheses modified from Maller et al. (2010).

Name of materials used for prostheses.	Advantages	Disadvantages
Acrylic Resin	The benefits of acrylic are that they are long lasting and easily coloured for cosmetic use. Additionally, acrylic materials can be relined or repaired.	This material is characterised by rigidity, thus the mould needs to be destroyed after the processing, therefore, duplication is not possible. Additionally this material absorbs water after one week, leading to increases in weight about 0.5%.

Acrylic Co-polymers	Acrylic Co-polymers are soft and elastic in texture compared to acrylic resin.	This material has not received wide acceptance due to poor edge strength and lack of toughness. Furthermore, when this material is exposed to sunlight, this lead to degradation. Dust collection and staining of the material which may damage the prostheses.
Polyvinyl chloride co-polymers	Polyvinyl chloride co-polymers are flexible and suitable for both intrinsic and extrinsic colouration; thus better cosmetic results can be obtained.	This material can discolour over time, the edges may show tearing. The material may also absorbs sebaceous secretions, which will compromise the physical properties of the material. Additionally, it is sensitive to UV Light.
Polyurethane Elastomers	This material is elastic and it can be coloured extrinsically and intrinsically, which gives better cosmetic results.	This material is moisture sensitive and water contamination is difficult to control, leading to discoloration of the prostheses and poor compatibility.
High temperature vulcanization silicones HTV	This material has excellent thermal stability and colour stability and it is biologically inert.	Not adequately elastic, low edge strength, opaque and life less appearance.
Room temperature vulcanizing silicone RTV	This material retains physical and chemical properties at a wide ranges of temperatures. Additionally, it is biocompatible, and easily manipulated.	Cosmetic appearance of the material is inferior to that of polyurethanes, acrylic resins and polyvinyl chloride. Additionally, discolouration with the time occurs and it has poor edge strength, besides it is expensive.

There are many microorganisms that thrive and proliferate in the space between the prostheses and the surrounding biological environment. These include bacteria such as *Porphyromonas gingivalis* (Zortuk et al., 2010) *Staphylococcus epidermidis, Pseudomonas. aeruginosa* (Benson et al., 1996) and *Candida albicans*

(Sobolewska et al., 2007), resulting in a complex biofilm formation on the prosthesis. Yeast infections are of particular concern. One of the most prevalent fungal infections in the human oral cavity as well as on the skin is *C. albicans* (Coenye et al., 2011). Growth of *C. albicans* has been observed on facial prosthetic materials, as well as on the dentures causing denture stomatitis, and is also responsible for a great number of infections related to implanted devices such as prosthetic heart valves and joint replacements (Dominic et al., 2007; Thein et al., 2007; Estivill et al., 2011; Borghi et al., 2011).

Furthermore, facial prostheses come into direct contact with the skin, where various microbes are present including *C. albicans*. In addition, the moisture arising from sweating and body warmth assists fungal growth on the silicone surface. Residual salts from the sweat and sebum from sebaceous glands can adhere to the surfaces of the prostheses; providing nutrients for fungal growth (Leow et al., 1997). The facial skin also has a pH range between 4.0 to 4.9 (Korting et al., 1996), which is favourable for the growth of *C. albicans*. Although *C. albicans* is able to grow over a broad range of pH conditions (Ghosh et al., 2008), most yeast cells tend to colonise more in acidic conditions (Spielmann et al., 2011). Together, the presence of optimal pH, nutrients, moisture and warmth lead to the colonisation on the surface of the prostheses, and acidic metabolic waste from organisms such as oral strains of *C. albicans* can support this environment (Kurtulmus et al., 2010). The acid conditions may lead to degradation of the prosthetic materials and eventually will produce a rough surface which is more susceptible to microorganisms attachment (Mandracci et al., 2008), exacerbating the problem of infection for the patient.

Traditionally, patients are instructed to wash their prostheses regularly with water, which is the most common cleaning medium used to prevent colonisation by

bacteria (Denge et al., 2004). This is a simple measure that can help prevent microorganism-related damage to maxillofacial prostheses. However, recently Kurtulmus et al. (2010) demonstrated that even after washing, high number of microorganisms remained on the surface. Another particular problem is that yeasts are able to infiltrate prosthetic materials such as silicone rubber; either by enzymatic degradation of silicone or by using the silicone directly as a nutrient source (Busscher et al., 1994; Lemon et al., 1995). Additionally, facial prostheses absorb water after washing; affecting the physical properties and the perception of colour matching of the prosthesis to the surrounding facial tissue (Aziz et al., 2003). To overcome these problems microwave sterilisation, strong solvents such as benzene and xylene, and chlorhexidine gluconate have all been used to disinfect maxillofacial prostheses. However, all these disinfectant substances affect colour stability of maxillofacial prostheses (Deng et al., 2004; Kiat-amnuay et al., 2005). A study by Jani et al. (1978) revealed that more than 69% of the prostheses were remade (e.g., repaired or replaced) within a year because of changing colour of prosthetic material. Currently, silicone elastomers also exhibit colour change over time and require replacement (Goiato et al., 2009). The most popular material is A-2186, but despite its long clinical success, A-2186 has a number of disadvantages in terms of durability and infection control and it is not an “ideal” material for maxillofacial prostheses (Montgomery et al., 2010). Nevertheless, many experiments has been conducted to improve A-2186 facial prostheses, for instance, Han and co-workers added nano scale metal oxides (Ti, Zn, or Ce) into A-2186 to improve its mechanical properties, and found that 2.0 and 2.5% of these oxides strengthened the mechanical properties (Han et al., 2008). But, still further research should be done to develop A-2186

material to fulfil the needs of patients; to overcome these problems nanotechnology offers some advantages.

Recently nanotechnology has opened up new avenues of research and offers many applications in human health (Sozer et al., 2012). Nanotechnology can be defined as using materials and structures with nano scale dimensions usually in the range 1-100 nm (Handy et al., 2007). Keck et al. (2013) has also defined nanoparticles as a range of particle size from 1 nm to just below 1000 nm. Nanomaterials can potentially be applied to refine and improve maxillofacial prostheses. For example, due to their small size and their colloidal behaviour to adhere on to surfaces, they could be used as a coating or incorporated into the products (Christian et al., 2008). Furthermore, nanostructure materials can produce better responses (e.g., improved biocompatibility) between the material surface and biological entities (Engel et al., 2008). Due to these properties, nanoparticles have been used in many biomedical applications (Fadeel et al., 2010). For instance, nano silver (Ag NPs) has been used as an antimicrobial coating for catheters. Roe et al. (2008) applied a thin coating of 100 nm Ag NPs on the surface of the catheters and revealed that the coating was able to prevent biofilm formation of a wide range of species including *Escherichia coli, Enterococcus, Staphylococcus aureus*, coagulase-negative *Staphylococci*, *Pseudomonas aeruginosa* and C.*albicans.* Furthermore, Ag NPs have been used as an antimicrobial agent in wound dressings. Tian et al. (2007) used Ag NPs in an animal model and found that rapid healing with a better cosmetic appearance occurred in a dose-dependent manner.

Nanomaterials are also being used in dentistry, in an attempt to make dental procedures faster, more reliable, safer, and much less painful. The use of nanomaterials has had a great effect on dental treatment methodologies and

potentially can reduce recovery times (Subramani et al., 2012). For example, simple measures include using Ag NPsin toothpastes as an antibacterial agents to retard bacterial growth and reduce tooth decay. Similarly, quaternary ammonium polyethylenimine (PEI) nanoparticles were incorporated into composite resin restorations to prevent biofilm formation and secondary caries (Beyth et al., 2006). Nanotechnology has been applied to dental implants by coating the surface of the implant to improve biocompatibility and biointegration. For example, implant surfaces coated with nano-textured titanium or nano hydroxyapatite may provide a better biological response as it improves the osseointegration properties (Kanaparthy et al., 2011).

Silver metal has been used in dentistry in the amalgam filling materials to inhibit microbial attachment and colonisation (Davies et al., 1997; Tweden et al., 1997). This is because silver ions can retard the growth of bacteria, fungi, protozoa, and spirochetes by inactivating enzymes vital to microbial metabolism, and by disabling the mechanisms for DNA replications causing cell death (Oei et al., 2012). After the application of silver ions, some proteins (e.g., metal chelators) will be produced by the yeast cell to protect the nuclear region and especially DNA molecules. However, if the silver ions are toxic, the cell membrane may collapse or leak, leading to a loss of intracellular integrity and the cells will lose their ability to replicate (Feng et al., 2000). These toxic effects of Ag ions are in part due to their high affinity for thiol groups (-SH residues) in biological macromolecules such as the peptide chains used to make proteins (Graf et al., 2009; Liau et al., 1997). Ag NPs have a high surface area to volume ratio, thus in theory, it would allow for the more effective release of silver ions. However, the dissolution rates of Ag from Ag NPs are surprisingly low in saline conditions (Besinis et al., 2012), and this is in part due to the formation of

insoluble silver chloride which is not bioavailable. Consequently, silver metal salts are regarded as having low toxicity to human cells in culture conditions since the silver ions tend to form strong complexes with anions in the media that lower toxicity. For example, reports suggest that Ag NPs have only modest toxicity to mammalian cells in culture with lethal concentrations around at 1.0 mg l^{-1} (Kawata et al., 2009). Furthermore, 14×10^{-5} % of silver nitrate ($AgNO_3$) caused 76% inhibition of fibroblast growth after 2h of contact (Hidalgo et al., 1998). The apparent low toxicity of dispersions of Ag NPs in biological media may be due to the presence of biomolecules like proteins and solutes in the media that can make a complex coating on the surface of the particles called the “protein corona” (Hellstrand et al., 2009). This may have the effect of masking the particle surface so that the bioavailability of any silver ions released is lower than expected, and would also prevent the reactive surface of the particle coming into direct contact with the cell membranes. Additionally, the high ionic strength of the media used to culture cells may aggregate Ag NPs to increase settling in the media. Also, like the metal salts above, any dissolution of silver may precipitate or form poorly soluble salts like silver chloride due to the presence of chloride in the culture media (Greulich et al., 2012).

Some substantial experiments have been conducted on the antibacterial properties of Ag NPs (Rai et al., 2009; Xiu et al., 2012), but less work has been done on the antifungal properties of Ag NPs, although recent studies suggest that the fungicidal activity of the Ag NPs may be significantly stronger than their bactericidal effects. Ag NPs exhibited fungicidal activity against *C. albicans* at very low concentrations 0.4–3.3 mg l^{-1} prepared in RPMI 1640 medium (Monteiro et al., 2011), while the survival rate of one of the common oral bacteria *Streptococcus mutans* was 2% at 100 mg l^{-1} Ag NPs in saline–BHI media (Besinis et al., 2012).

To eliminate biofilm formation on facial prostheses, coating the surface of the silicone with antimicrobial nanomaterials might be the solution. So far, no material has been developed that meets all the ideal clinical requirements without any undesirable characteristics. Additionally Ag NPs have not been used as antifungal coating on silicone facial prosthetic before. One hypothesis is that the surface characteristics of maxillofacial prostheses can be changed by applying Ag NPs as a thin film or coating which might help to prevent infections such as *C. albicans* and the ingrowth of yeast into the prosthetic appliance. Nonetheless, medical devices that involve nanomaterials need to be tested for safety and biocompatibility, since these medical devices may provide a route for direct dermal exposure to a potentially toxic metal and may lead to penetration of nanoparticles into the (damaged) skin; which then may spread to other organs through the blood and lymphatic networks (Rothen-Rutishauser et al., 2006; Handy et al., 2008). Such exposures might lead to clinically relevant hazards to the patients such as harmful inflammatory responses (Atiyeh et al., 2007). It is mandatory at the early stages of product development to consider the human health risks and all adverse effects of nanoparticles that will be incorporated into biomedical materials (Chang et al., 2009). Additionally, many factors should be considered when nanoparticles are to be used, since the antimicrobial activity of Ag NPs changes according to size and shape; for example, 30 nm and 15 nm Ag NPs are more toxic than the 55 nm Ag NPs (Auffan et al., 2009). Furthermore, triangular Ag NPs displayed greater biocidal action than rod or spherical nanoparticles (Monteiro et al., 2011).

Biofilm formation

It has been estimated that 65% of human microbial infections are due to biofilm formation (Dominic et al., 2007). Biofilms are highly structured, hydrated microbial communities containing sessile cells embedded in a self-produced extracellular polymeric matrix (Coenye et al., 2011). Several medical devices have been introduced in the human body which are susceptible to biofilm formation, including those made with polydimethylsiloxane (PDMS) also called “silicone”. Silicone prostheses, as any other medical device when introduced into the body, is first exposed to water and solutes, then to proteins in the body fluids. Because of the high surface hydrophobicity of silicone, it interacts strongly with macromolecules, thus adsorbs significant quantities of protein from the surrounding biological environment (Chen et al., 2004). Consequently, the protein-coated biomaterial will accelerate the initial attachment of microorganisms, forming a biofilm layer on the surface (Oustre et al., 2004; Dominic et al., 2007). As microorganisms reach the prostheses surface, they will be attracted or repelled depending on the nature of the bacterial cell wall (Pompilio et al., 2008), biomaterial surface structure such as surface charges or chemical composition (Engel et al., 2008), and environmental factors such as the presence of serum proteins and the associated flow conditions (Katsikogianni et al., 2004).

The adhesive nature of bacteria depends on cell surface structures such as, pili, flagella proteins, and lipopolysaccharides (LPS), which enable microorganisms to adhere initially to the surface and to form biofilms. In addition to bacterial cell structure, surface characteristics of the biomaterial are considered one of the most important factors. It is well known that the microbial cell’s response is affected by the

physico-chemical parameters of the biomaterial surface (Engel et al., 2008). For example, oral stomatitis may occur as a consequence of cell colonisation on polymeric materials, which is facilitated by van der Waals and electrostatic forces. In addition, surface energy also influences the adherence of microorganisms, as when the free surface energy is increased, it activates microbial adhesion and the number of adhered microbes also increases (Kurtulmus et al., 2010; Spielmann et al., 2011). Similarly, ions present on the material surfaces have a role in biofilm formation, as these ions may interact with cell receptors and cause cell adhesion and growth (Khorasani, et al., 2006). Furthermore, many studies have shown that the hydrophobicity of the prosthetic surface and irregularity on the surface can promote bacterial adhesion (Katsikogianni et al., 2004).

Apparently, large proportions of *C. albicans* infections are also associated with biofilm formation on the surface of medical devices (Borghi et al., 2011). Several studies have revealed that the strains of *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata* have been associated with failure of many medical devices (Von Eiff et al., 2005; Ramag et al, 2006; Estivill et al., 2011). These micro-organisms become pathogens and cause infections ranging from superficial mucous membrane infection (candidiasis) to life-threatening systemic diseases. To eliminate the opportunities for microbial adhesion and biofilm formation, traditional coatings aim to alter the physical chemical properties of the surface, such as surface roughness, to prevent biofilm formation. Different antimicrobial compound have also been incorporated into polymers to prevent biofilm formation such as antibiotics (Schierholz, 1997), antifungals (De Prijck et al., 2010), as well as silver metals and Ag NPs (Bengt 2002; Coenye et al., 2011; Padial-Molina et al., 2011).

Hypothesis

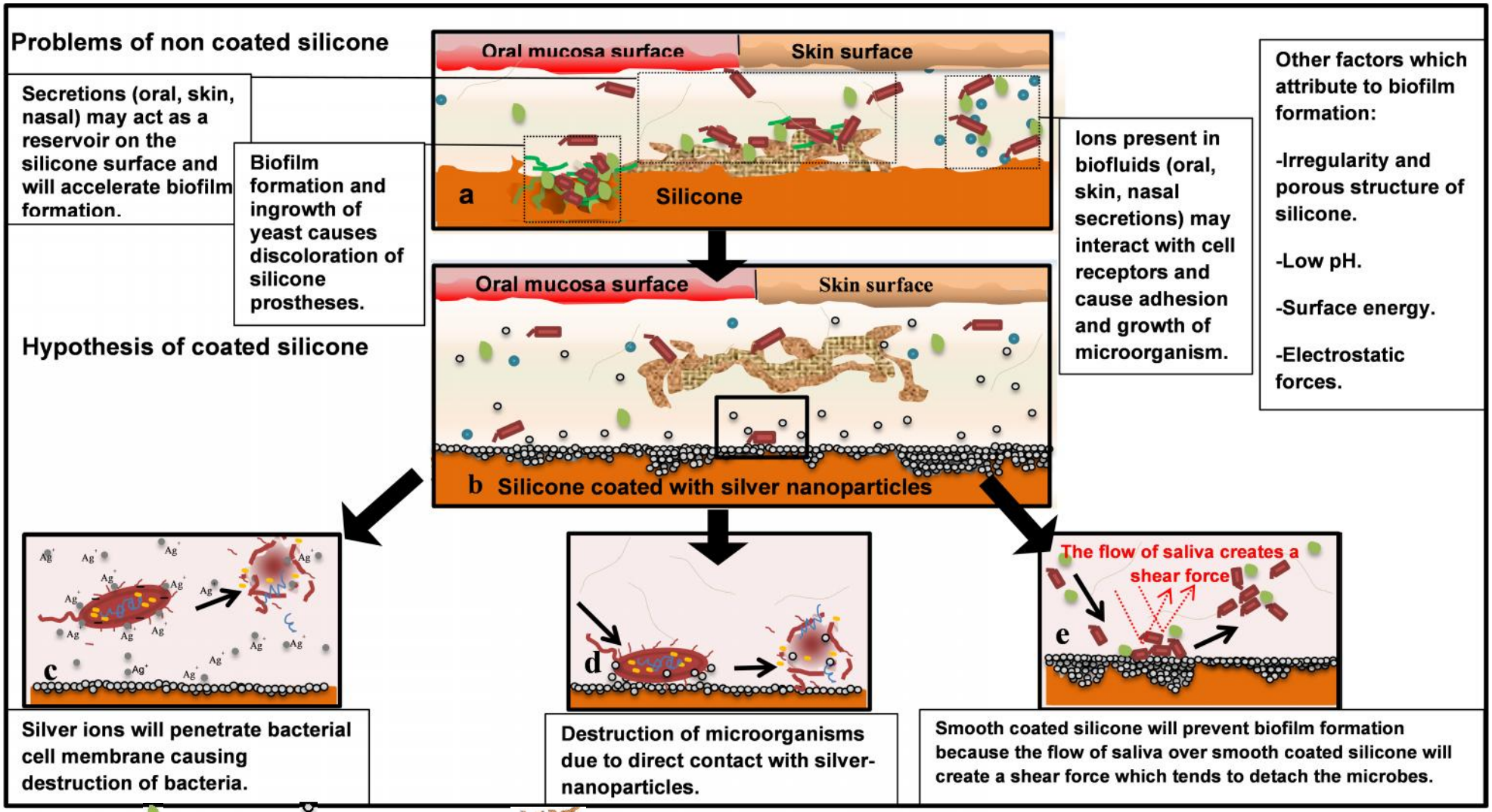
The hypothesis to be tested in the present study is that selected Ag NPs coatings can prevent fungal growth on silicone prostheses without any toxic effect on the cells involved in wound repair such as fibroblasts (i.e., biocompatibility). Ag NPs will inhibit microorganism adherence, as it causes damage to microbes via direct contact toxicity or via localised free metal ion toxicity due to silver ions being released from nanoparticles as shown in Figure 1. Thus, this project aimed to develop a method to overcome the deficiencies of silicone-based prostheses.

The main aim of the study was to apply Ag NPs as an antifungal coating against one of the most prevalent fungal infection in the human oral cavity and skin, *C. albicans*. The purpose was to prevent fungal infection and the in growth of yeast into the prosthetic materials without interfering with biocompatibility of fibroblast cells.

The specific objectives of this study were as follows:

- Demonstrate the biocompatibility of the Ag NPs compared to $AgNO_3$ using fibroblast cell cultures without the presence of silicone prostheses, by testing cell viability and adherences of the cells to culture plates. End points included the cytotoxic response of the cells.
- Demonstrate whether Ag NPs and $AgNO_3$ can be applied as coatings on the surface of the silicone prostheses.
- Demonstrate the effect of Ag NPs as a coating over the silicone surface on the fibroblast cells compared to $AgNO_3$, as well as to investigate whether or not fibroblast cells would adhere and grow on the coated prostheses.

- Investigate the antifungal properties of silver coated silicone against *C. albicans,* with the presence of fibroblast cells on the surface, in order to determine the antifungal properties of the coatings against *C. albicans* without damaging fibroblast cells.
- Demonstrate the effect of silver on the fibroblast and *C. albicans* on cell electrolytes relating to osmotic health.
- Demonstrate the effect of silver on the ethanol production by *C. albicans*.



(Bacteria, Yeast, Ions, silver nanoparticles, secretions from glands).

Figure 1 Problems of currently used prosthetic silicone material and the hypothesis that nano silver-coated silicone is antifungal. (a) Biofilm formation on surface of non-coated silicone which is exposed to oral mucosa and/or skin surface. Panels b,c,d, and e Illustrates the steps in the hypothesis of coating silicone surface with silver nanoparticles and the expected outcomes of the newly coated silicone.

Materials and methods

In this study, three series of experiments were performed (Fig 2). The first series demonstrated the safety/biocompatibility of the Ag NPs by using fibroblast cell cultures. The second series demonstrated the effect of Ag NPs as a coating over the silicone surface on fibroblast cells. The third series investigated the antifungal properties of new silver coated silicone against *C. albicans*.

Preparation of the stock dispersions and nanomaterial characterisation

Ag NPs and silver nitrate ($AgNO_3$) were used. The same batches of both Ag NPs and $AgNO_3$ have been used and characterised by Besinis et al (2012). Ag NPs was obtained from (Sigma-Aldrich, Wisconsin, USA, lot number 7721KH) and the manufacturer's information is: purity, 99.5%, <100 nm; trace elements in this batch of dry powder; Al 7.3, B 5.6, Ba 5.7, Ca 15, Cr 26, Cu 5.5, Fe 229, Mg 1.5, Mn 7.9, Na 8.0, Ni 7.0, Sn 15, Ti 4.9 ppm; measured BET surface area, 4.8 m^2 g^{-1}. While $AgNO_3$ was obtained from Fisher, Loughborough, UK (product code s/1280/46,) and manufacturer's information of $AgNO_3$ was: purity, 99.9%, main impurities <10 ppm Ca and <20 ppm Na. BET surface area measurements for $AgNO_3$ in our laboratory were below detection limit (<1 m^2 g^{-1}) and the analysis indicated the main elemental component was nitrogen (as expected, see Besinis et al, 2012).

In brief, the Ag NPs and $AgNO_3$ were dispersed in Milli-Q at a concentration of 100 mg l^{-1} and sonicated for 4 h to disperse the materials following the protocol of Besinis et al. (2012). The aggregate size of Ag NPs and $AgNO_3$ in Milli-Q water were (means ± SD, *n* = 3) 164 ± 70 and 130 ± 50 nm respectively, which was determined by nanoparticle tracking analysis (NTA, using a Nanosight LM 10, Nanosight, Salisbury, UK, laser output set at 30 MW at 640 NM). Transmission electron microscopy (TEM, JEOL-1200EX II) was used to measure particle diameters. The primary particle diameter of Ag NPs and $AgNO_3$ were 56.8 ± 18.6 and 52.8 ± 18.6 nm respectively. For further information see Besinis et al. (2012).

In this study Ag NPs and $AgNO_3$ were characterised at concentrations of 5, 50 and 100 mg l^{-1}. Stock dispersions of 1 g l^{-1} were initially prepared in sterile Milli-Q water for Ag NPs and $AgNO_3$, and then sonicated (35 kHz frequency, Fisherbrand FB 11010, Germany) for 4 h to disperse the materials. To analyse Ag NPs and $AgNO_3$ by NP tracking analysis (NTA, using a Nanosight LM 10, Nanosight, Salisbury, UK, laser output set at 30 mW at 640 nm), in Mili-Q water, secondary stocks were prepared at 5, 50 and 100 mg l^{-1} (samples were continuously sonicated for 3 h until they were analysed, *n* = 3). In addition, attempts were made to characterise the test materials at concentrations 5, 50 and 100 mg l^{-1} in culture media (Dulbecco's Modified Eagle's medium with L-Glutamine, 10% fetal bovine serum), (*n* = 3). Each secondary stock was prepared separately just as before and analysed by NP tracking analysis.

In order to prevent microbial contamination, all the stocks of Ag NPs and $AgNO_3$ were prepared in sterile Milli-Q water and autoclaved at (121 ^{o}C for 15 min at 15 psi pressure). Before preparing the secondary stocks for dosing the culture dishes, the dispersions were sonicated for 4 h (35 kHz frequency, Fisherbrand FB

11010, Germany) then diluted to the desired concentrations (5 mg l^{-l}, 50 mg l^{-1}) in culture media (Dulbecco's Modified Eagle's medium with L-glutamine, foetal bovine serum). In experiments where the coating was applied directly to the silicone surface, the stocks were diluted to the desired concentrations (5 mg l^{-l}, 50 mg l^{-l}) in Milli-Q water (see below).

Cell culture

Human dermal fibroblast HS-68 cells were obtained from the Health Protection Agency and cultured at a density of 1.5 x 10^6 in 75 cm^2 culture flasks containing 15 ml growth medium (Dulbecco's Modified Eagle's medium with L-glutamine, 10% foetal bovine serum and penicillin-streptomycin obtained from Lonza laboratories). The cells were sub-cultured every six days (when confluence was at 80%); for routine maintenance the medium was changed every three days. For the experiments, antibiotics were withdrawn two passages before seeding the cells into 6-well plates. The cells were washed twice with Phosphate-Buffered Saline, PBS, (Lonza, containing 9.5 mmol l^{-1} phosphates without added calcium and magnesium) and removed from 75 cm^2 tissue culture flasks using trypsin and re-suspended in fresh medium then counted using a haemocytometer. The cells were plated in 6-well plates (n = 6) at a density of 10^6 cells ml^{-1}. Cell viability was checked by tryphan blue staining, prior to seeding the cells into the 6-well plates. After overnight growth the cells showed confluence (visually inspected), but the cells were left for another 24 h to ensure that the confluence had been established.

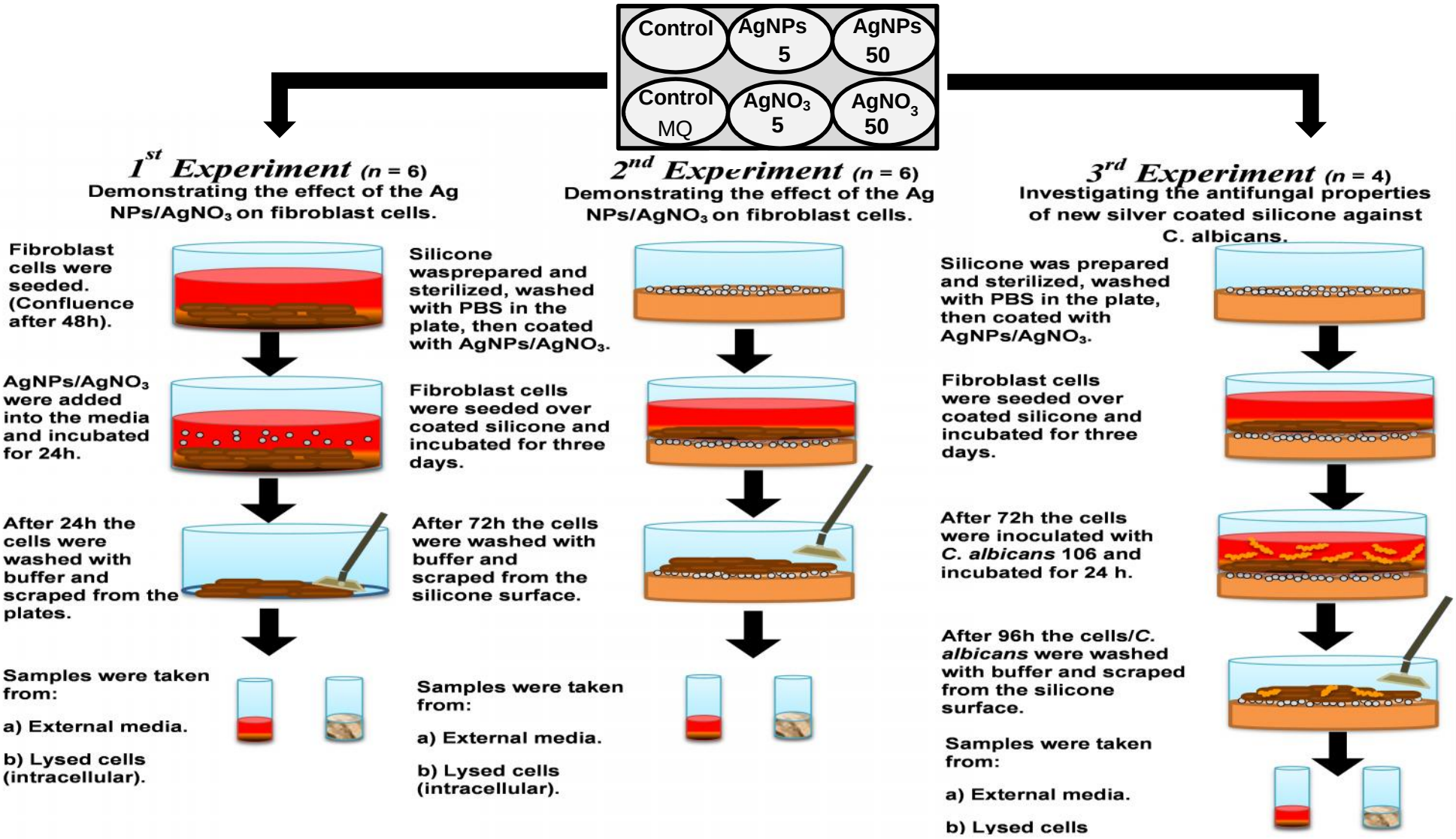


Figure 2. Experimental designs. The approach to the 1st experiment involved seeding fibroblast cells in culture flasks then exposing the cells to Ag NPs or $AgNO_3$ added to the media. In the 2nd experiment fibroblast cells were grown over Ag NPs or $AgNO_3$ coated silicone prostheses. The 3rd experiment was the same as 2nd experiment, but after 72h, the fibroblasts were challenged with a *C. albicans* infection for 24h.

Silicone Elastomer A-2186 and coatings preparation

A-2186 Silicone Elastomer used in the clinic for facial prostheses was supplied from (Factor 2, Inc., Lakeside USA) as a two part material system, silicone elastomer base (part A) and catalyst (part B), mixing of the two parts of A-2186 were processed according to manufacturer's instructions. Parts A and B were mixed in a 10:1 ratio by weight. Functional Intrinsic Skin Colour (Naturall Fl-SK01) was added into the part A silicone elastomer base and mixed thoroughly, then the mixture was mixed with the catalyst and placed under a vacuum (using a de-pressurised Tupperware box) for 15 min until all bubbles were visually removed. The mixture was then placed with special care to minimise air entrapment into 6 well plates and allowed to be cured at room temperature for 24 h. To ensure sterility, the 6-well plates with the silicone elastomer surface were soaked with 5 ml of 0.5% (v/v) of chlorhexidine digluconate (clinical antiseptic product R4, Septodont Ltd, UK). After 5 min the chlorhexidine digluconate was aspirated from the surface of silicone and then washed twice with 5 ml PBS. To coat the surface of silicone elastomer, Ag NPs and $AgNO_3$ at concentrations (5 mg l^{-l}, 50 mg l^{-l}) were prepared in Mili-Q water (as above) and 2 ml were added to each well of 6-well plates and left for 24 h to allow the particles to precipitate on the surface of silicone elastomer and the remaining were aspirated.

Experimental design

In the first experiment, to demonstrate the effect of the Ag NPs/$AgNO_3$ on fibroblast cells, the cells were seeded into 6-well plates (*n* = 6 plates/treatment), then supernatant from the well plates were aspirated out after 48 h growth and replaced

with fresh medium containing Ag NPs compared to $AgNO_3$ at concentrations 5 mg l^{-l} or 50 mg l^{-l}. There were two controls, one as a reference control (only media and cells), the second control-Milli-Q water (volume of Milli-Q water without nanoparticles were added to the well in order to investigate the effect of osmolality on the cells).

After 24 h of exposure, the overlying media were carefully collected to measure lactate dehydrogenase (LDH) leakage, and total Ag, Na^+, K^+ concentrations and pH of media (see below). The remaining cells which were adhered to plates 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 dishes by light microscopy (Olympus Microscope SZ-1145 CHI with transmitted light magnification 40x and camera Scope Tek MDC 560 with magnification 0.6 x). Normal fibroblast cells appears elongated with a spindle-shape (Bell et al., 1979). After photographing, the plates were scraped to collect the cells (Fisher scientific cell scarper 250 mm handle, 18 mm blade) and then 1 ml of sucrose lysis buffer was added (as the washing buffer above but hypotonic made with 30 mmol l^{-1} sucrose). The scraped cells were then sonicated for 30 s to ensure the lysed samples were well mixed. The homogenised cells were used to measure total Ag, K^+, and Na^+ by inductively coupled plasma optical emission spectrometer (ICP-OES, Varian 725ES), as well as the protein concentration (BCA assay), and LDH activity (see below).

In the second experiment, to demonstrate the effect of silver as a coating over the silicone surface on fibroblast cells, instead of applying the silver directly into the culture media, the materials were applied to the silicone surface in order to investigate the effect of silicone coated with Ag NPs and $AgNO_3$ on the viability and

growth of the fibroblast cells. The same measurements were performed as above, but for the cells to be confluent, they were incubated for 3 days over the Ag NP or $AgNO_3$ coated-silicone with appropriate controls as above. In order to ensure cell growth, the supernatant from the well plates was replaced with fresh growth media every 24 h (*n* = 6 plates/treatment). Photographs were taken following 72 h exposure (*n* = 4), the supernatant from the well plates was aspirated and cells were fixed and stained with Giemsa 1% for 3 min (Giemsa's stain solution, IVD, England). The same microscope was used as above.

The third experiment investigated the antifungal properties of silicone prostheses against *C. albicans* with and without a silver coating in the presence of fibroblasts. *C.albicans* NCPF-3179 was first cultured at 37 ^{o}C for 24 h in Sabouraud's dextrose agar (SDA). Then, a loop full of yeast was inoculated in 30 ml of Sabouraud's dextrose broth (SDB) and incubated for three days. The SDB was adjusted to a turbidity equivalent to a 1.5 McFarland standard in SDB (used as a reference to adjust the turbidity) then, the yeast suspension was diluted in culture media (Dulbecco's Modified Eagle's medium with L-glutamine, 10% foetal bovine serum) so that the density equals to 10^6 cells ml^{-1}. To test antifungal properties of silver coated silicone, fibroblast cells were grown over the appropriate silver-coated silicone for 72 h (the same as the second experiment above), but after 72 h the media was replaced with 2 ml culture media inoculated with *C. albicans* at a density of 10^6 cells ml^{-1} and incubated at 37 ^{o}C for another 24 h. Measurements on the fibroblast cells and culture media for this experiment were the same as the first experiment, except for cell morphology. The cells of *C. albicans* were stained with methylene blue stain for 1 min. In addition, an ethanol assay was also used to measure ethanol production by *C. albicans* (*n* = 4, see below).

Additional experiments were conducted to determine whether or not the sterilisation step caused any effect on silver coatings. Thus two separate sets of experiments were conducted. First, SEM images were taken of the silicone surface which were prepared following the same protocol as described above with fibroblast cells grown for 72h (*n* = 3). In the second method, SEM images were taken from silicone surface coated with Ag NPs/$AgNO_3$ without sterilising and washing with PBS (*n* = 3). Energy dispersive spectroscopy (EDS) was also used to confirm the presence of silver on the silicone surface.

For scanning electron microscopy (SEM), the samples were fixed and silicone was cut out of the well plate for mounting on SEM grids. Briefly, cells were fixed in 3 ml of (2.5 % glutaldehyde in 0.1 M phosphate buffer) for 1 h. The cells were dehydrated in ascending ethanol steps (30, 50, 70, 90 and 100 % ethanol. Each concentration of ethanol was left on the surface of silicone for 15 min and then the cells were air dried for 24 h. The samples were coated with gold using (Emitech K550 gold sputter coater). Samples were imaged and elementally analysed using (SEM, JEOL / JSM-7001F, Oxford Instruments INCA X-ray analysis system) with a voltage 15 KV, at a working distance of 10 mm.

Metal analysis

Metal analysis of the overlying cell culture media, and cell homogenates removed from the dishes were conducted according to Besinis et al. (2012). Metal analysis was determined by inductively coupled plasma optical emission spectrometer (ICP-OES, Varian 725ES) to measure the total Ag, Na^+ and K^+ concentrations in the cell homogenates, and in the media.

For Ag analysis, to 400 µl of the cell homogenate, three ml of 70% concentrated nitric acid were added, and then three ml of 10 mmol l^{-1} sodium citrate (as a stabiliser) were added to the mixture. For total Na^+ and K^+ concentrations, to 400 µl of the cell homogenate, three ml of 70% concentrated nitric acid were added without the stabiliser. All samples were sonicated for 1h prior to being analysed to disperse the particles and to ensure uniform introduction of the samples into the instrument. Samples were analysed by closely matching the standards to the concentrations of samples. The standards used for ICP-OES calibrations were between 0-2, 0-80 and 0-800 mg l^{-1} for Ag, K^+ and Na^+, respectively. In the absence of certified reference materials for Ag NPs in cells or tissue, spike recovery tests was performed. For the spike recovery test, samples were spiked with 50 mg l^{-1} of Ag NPs or $AgNO_3$ in culture media without the cells, then subjected to the digestion protocol above. The samples showed a procedural recovery of 12 % for Ag NPs and 97 % for $AgNO_3$ (n = 5). Most of the blank samples produce signals which represent the background noise of the instrument, and in order to determine the instrument detection limit, 3 x standard deviation of the blank were used which required producing a signal greater than three times the standard deviation of the noised level, and the instrument detection limits were 1.26, 1.03, 1.15 µg l^{-1} for each experiments.

Lactate dehydrogenase activity and protein determination

The lactate dehydrogenase assay was used as a method for measuring the cytotoxicity or cell injury which was produced by exposure to Ag NPs or $AgNO_3$ compared to unexposed controls. The assay measures the appearance of LDH in the medium, which leaks from injured cells. In addition, the LDH assay was used to

measure the leakage of LDH from inside the cells by following the LDH content of the cell homogenates. The assay was performed according to Plummer (1971), using 100 µl of test sample (medium from the experiment, or cell homogenate). The cell culture media from each well was gently centrifuged for 1 min to remove any cell debris/turbidity. The assay was performed by adding 2.8 ml of a reaction mixture (0.6 mmol l^{-1} pyruvate in 50 mmol l^{-1} phosphate buffers at pH 7.5) to a 3 ml curvette, and then adding 0.1 ml of 0.6 mmol l^{-1} NADH solution, then mixed with 0.1 ml of sample in last. In the assay, the oxidation of NADH was followed at 340 nm (Helios ß Spectrophotometer, manufacturer, England) for 2 min. The LDH concentration was calculated using an extinction coefficient of 6.3 mM for a path length of 1 cm. LDH activity was expressed as IU mgl^{-1} (µmol min^{-1} ml^{-1}) for media and (µmol min^{-1} mg^{-1}) cell protein, and total LDH leak was measured by calculating (total LDH leak into external media/ total LDH in well plate).

The (BCA) assay bicinchoninic acid (MC155208, Pierce, Rockford, USA) was used to determine the protein content of the cell homogenates. Briefly, 200 µl of the BCA reagents was added to 10 µl of sample. A series of standard were prepared from bovine serum albumin (BSA) 2 mg ml^{-1} stock (2, 1, 0.5, 0.25, 0.125, 0.0625, 0 mg ml^{-1}). Afterward the plates were incubated at 37 ^{o}C for 30 min (a colour changes were observed when the reaction was carried out at 37°C). Then absorbance was read at 562 nm on a plate reader (VERSA max, Molecular Devices, Berkshire, UK). Standard curves were used to calculate the concentration of protein in the samples.

Ethanol assay

Ethanol production was determined only in the third experiment to follow metabolic activity associated with *C. albicans*. The rationale being that fibroblasts do not usually produce ethanol and so any ethanol present in the media will have come from fermentation by the yeast. The enzymatic method for ethanol determination (K-ETOH 12/12, Megazyme International Ireland Ltd) was used to measure the ethanol production according to the manufacturer's protocol. Following incubation of the fibroblast cell cultures for 24 h in the presence of *C. albicans*, the culture media was collected for immediate ethanol determination. 0.010 ml of the samples were mixed with 0.020ml β-nicotinamide-adeninedinucleotide (NAD^+), and 0.005 ml alcohol dehydrogenase enzyme (ADH) and buffer (pH 9.0 plus sodium azide as a preservative) and the absorbance was measured after 2 min; then the reactions were started by adding 0.002 ml NADH and the absorbance was measured for 10 min again. The reaction was observed by measuring the absorption of samples caused by the consumption of NAD^+, or the formation of NADH during the conversion of ethanol to acetaldehyde within a spectrophotometer at 340 nm (VERSA max, Molecular Devices, Berkshire, UK). A standard curve was used to calculate the concentration of ethanol ranging from 1 to 1000 (µg/ml) in the samples.

Statistical analysis

All data are presented as mean ± S.E and analysed using Stat Graphics Plus Version 5.1, while the Figures were drawn using Excel. The differences between the treated samples and controls were evaluated using one way ANOVA. Parametric data were tested by one-way ANOVA and differences found using the default least squares difference multiple range test in the software. Non-parametric data that

could not be transformed were analysed using the Kruskal–Wallis test and differences were located using notched box and whisker plots. The student's *t*-test was used as well to investigate the differences between the reference control and the control with added Milli-Q water. All statistical analysis used the default 95 % confidence limit, so that all P values equal to or less than 0.05 were considered statistically significant.

Results

Ag NPs and $AgNO_3$ Characterisation

Hydrodynamic diameter of aggregates was measured by nanoparticle tracking analysis (NTA) and shown in Fig 3. The resulting dispersion of Ag NPs in Milli-Q water gave a mean value of average aggregate size (Mean diameter ± S.E, in nm); 14 ± 8 in control and 114 ± 43, 177 ± 52, 201 ± 16 nm at concentrations of 5, 50, 100 mg l^{-1} respectively. While average aggregate size of $AgNO_3$ in Mili Q water were 169 ± 11, 171 ± 16, 263 ± 54 at concentrations of 5, 50, 100 mg l^{-1} respectively.

Figure 4 shows the particles dispersed in culture media, Ag NPs gave a mean value of average aggregate size (Mean diameter ± S.E, nm) of 189 ± 19 in control culture media and 159 ± 17, 82 ± 2, 122 ± 13 nm at concentrations of 5, 50, 100 mg l^{-1} Ag NPs respectively. The average aggregate size of $AgNO_3$ were 176 ± 19, 149 ± 12, 183 ± 14 at concentrations of 5, 50, 100 mg l^{-1} respectively.

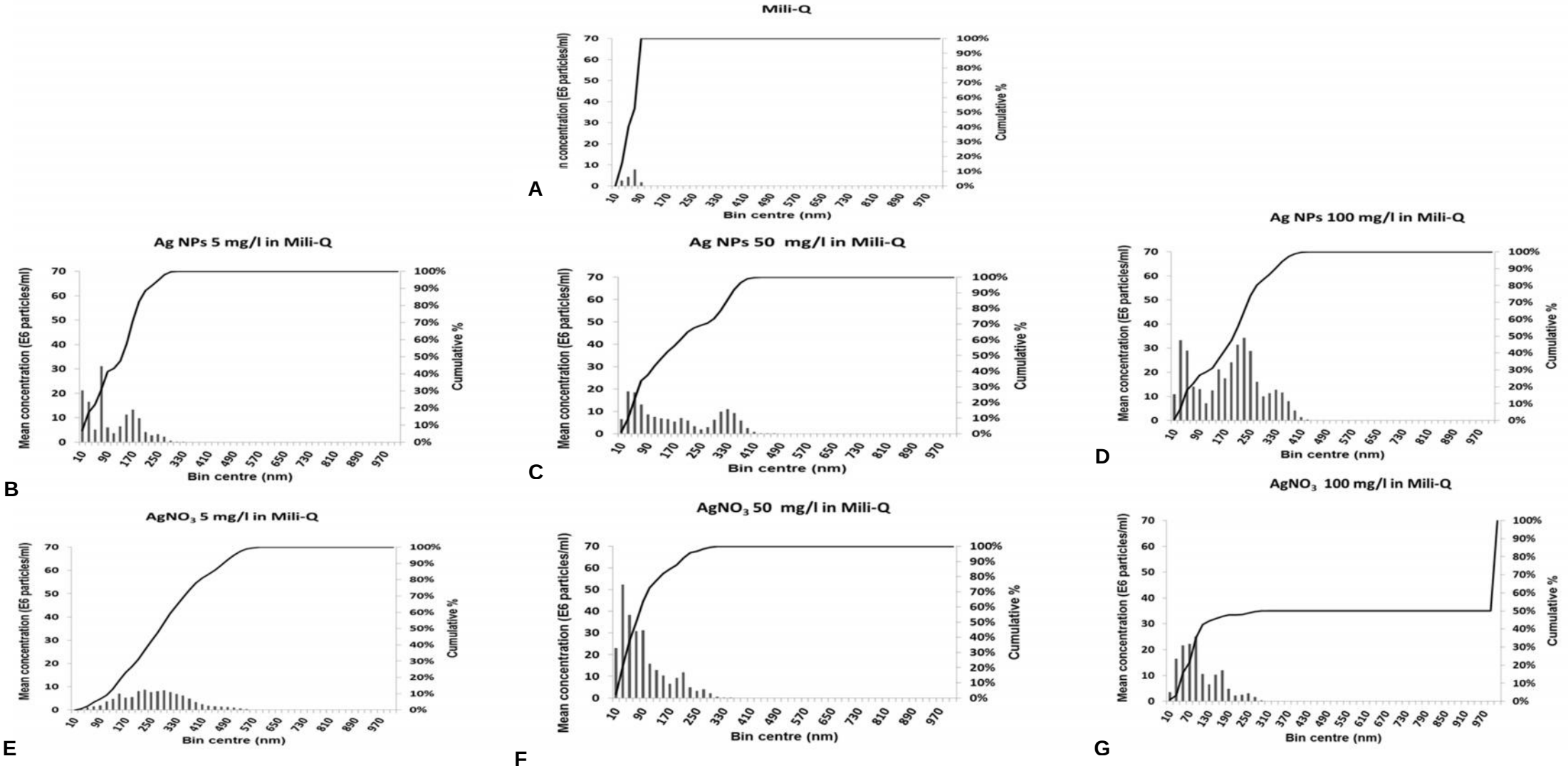


Figure 3 Nanosight graphs showing the particle size distributions in Mili-Q water, examples from triplicate; (A) Control Milli-Q water, (B) 5 mg l^{-1} Ag NPs, (C) 50 mg l^{-1} Ag NPs, (D) 100 mg l^{-1} Ag NPs, (E) 5 mg l^{-1} $AgNO_3$, (F) 50 mg l^{-1} $AgNO_3$, (G) 100 mg l^{-1} $AgNO_3$.

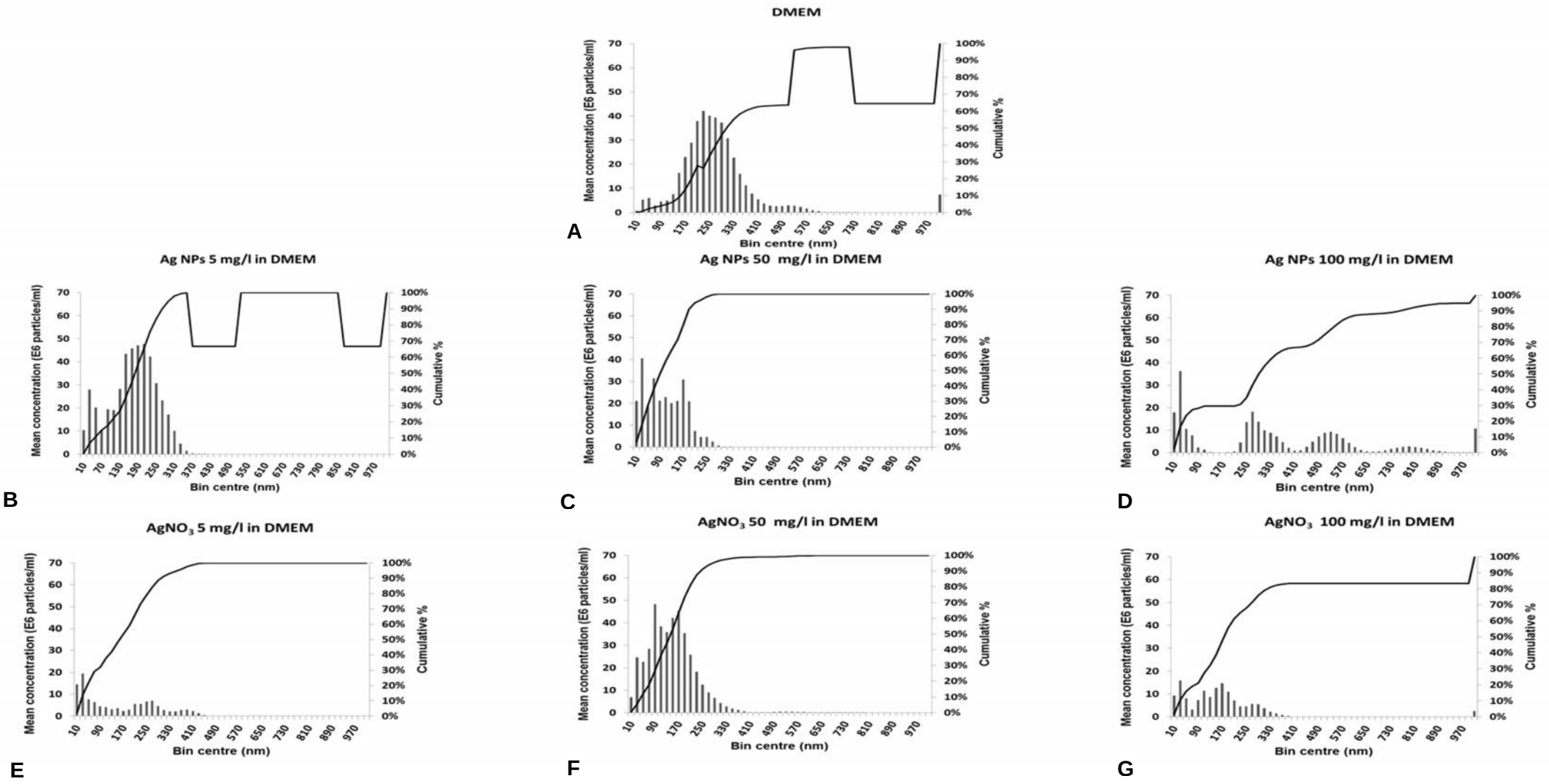


Figure 4 Nanosight graphs showing the particle size distributions in culture media, examples from triplicate; (A) Control Mili-Q water, (B) 5 mg l^{-1} Ag NPs, (C) 50 mg l^{-1} Ag NPs, (D) 100 mg l^{-1} Ag NPs, (E) 5 mg l^{-1} $AgNO_3$, (F) 50 mg l^{-1} $AgNO_3$, (G) 100 mg l^{-1} $AgNO_3$.

Controls of all experiments

The main findings were that the unexposed control cells showed normal elongated spindle-shape morphology during all the experiments with the cells remaining confluent and attached to the well plates (Fig 5a, 9a). Furthermore, silver was also measured in control cells and showed that the silver was below the detection limit when the cells were grown in all experiments.

Demonstrating the effect of the Ag NPs/$AgNO_3$ on fibroblast cells.

Silver was measured in the media when the cells were exposed to treatments for 24h, and showed that silver was statistically different between all groups (One way ANOVA, $p< 0.05$). The highest concentration in the media was when the cells were exposed to $AgNO_3$ at 50 mg l^{-1} followed by Ag NPs 50, $AgNO_3$ 5 and Ag NPs 5 mg l^{-1} (Table 2). Silver accumulation in the cells was also investigated when the cells were exposed for 24h, and silver was statistically different between all groups (Kruskal-Wallis, $p< 0.05$). The highest concentration in the homogenates cells was when exposed to $AgNO_3$ 50 mg l^{-1} followed by Ag NPs 50, $AgNO_3$ 5 and Ag NPs 5 mg l^{-1} (Table 5). Exposure of fibroblast cells to Ag NPs and $AgNO_3$ caused changes in the electrolyte levels (K^+ and Na^+) in the media, as well as, in the cell homogenates of exposed compared to unexposed cells. K^+ was measured in the external media after 24h exposure and showed that there was a rise when the cells were exposed to $AgNO_3$ compared to Ag NPs, while there was no change in K^+ concentration in the media when the cells were exposed to Ag NPs compared to controls (Table 3). Whereas, a decrease of the K^+ content in cell was observed (normalised to protein) when the cells were exposed to Ag NPs. The effect of the silver treatments on Na^+ in

the media was the same as controls (Table 4, no effect), whereas the Na^+ content in the cells showed a clear increase when cells were exposed to $AgNO_3$ compared to unexposed cells in all three experiment (Table 5).

Table 2 Total Ag concentrations in culture media after exposure to different concentrations of Ag NPs and $AgNO_3$

Experiments	Nominal concentration (mg l^{-1})	Treatment	Total Ag (mg l^{-1}) Day 1	Day 2	Day 3	Day 4
The effect of the Ag NPs/$AgNO_3$ on fibroblast cells After 24h		Control-MiliQ			0.02 ± 0.01 a	
	5	$AgNO_3$			2.75 ± 0.95 b	
	50	$AgNO_3$			23.20 ± 3.66 c	
	5	AgNPs			1.04 ± 0.22 a	
	50	AgNPs			13.60 ± 2.13 d	
The effect of silver as a coating over the silicone surface on fibroblast cells after 72 h		Control-MiliQ	0.19 ± 0.03 a	0.20 ± 0.04 a	0.23 ± 0.06 a	
	5	$AgNO_3$	3.49 ± 1.24 b	0.95 ± 0.15 a *	0.43 ± 0.17 a *	
	50	$AgNO_3$	13.27 ± 2.95 c	13.27 ± 2.30 b	12.79 ± 4.85 b	
	5	AgNPs	0.59 ± 0.25 a	0.38 ± 0.09 a	0.38 ± 0.12 a	
	50	AgNPs	0.36 ± 0.10 a	0.36 ± 0.09 a	0.43 ± 0.11 a	
Fibroblast cells grown over coated silicon and inoculated with *C. albican* 96 h		Control-MiliQ	0.03 ± 0.01 a	0.06 ± 0.01 a	0.06 ± 0.02 a	0.04± 0.03a
	5	$AgNO_3$	3.04 ± 0.712 b	2.09 ± 0.44 b	1.47 ± 0.54 b	4.91 ± 2.22 b
	50	$AgNO_3$	13.52 ± 1.10 c	11.85 ± 1.36 c	10.82 ± 0.83 c	11.60 ± 0.87 c
	5	AgNPs	0.19 ± 0.03 a	0.12 ± 0.02 a	0.19± 0.08 a	0.05 ± 0.02 a
	50	AgNPs	0.30 ± 0.03 a	0.31 ± 0.06 a	0.30± 0.07 a	0.44 ± 0.20 d

Total Ag in culture media after exposure to Ag NPs and $AgNO_3$. The first experiment cells were confluent then treatments were added into media for 24h (n = 6). Second experiment fibroblast cells were grown over coated silicone for 72h, silver was measured every day for three days in the external media (n = 6). Third experiment fibroblast cells were grown over coated silicone for 96h in last 24h the media was inoculated with *C. albicans* (n = 4), all data (Mean ± S.E).Numbers with different letters are statistically different from each other within the colum, * difference from day one within row, # difference from the previous time point within row (One way ANOVA or Kruskal-Wallis test, $p < 0.05$).

Table 3 Total K^+ concentrations in culture media after exposure to different concentrations of Ag NPs and $AgNO_3$.

			Total K (mmol l^{-1})			
Experiments	Nominal concentration ($mg\ l^{-1}$)	Treatment	Day 1	Day 2	Day 3	Day 4
The effect of the Ag NPs/AgNO3 on fibroblast cells After 24h		Control-MiliQ			3.53 ± 0.02 a	
	5	AgNO3			3.63 ± 0.06 b	
	50	AgNO3			3.66 ± 0.03 b	
	5	AgNPs			3.46 ± 0.07 a	
	50	AgNPs			3.47 ± 0.03 a	
The effect of silver as a coating over the silicone surface on fibroblast cells after 72 h		Control-MiliQ	5.45 ± 0.11 a	4.52 ± 1.42 a	5.39 ± 0.30 a	
	5	AgNO3	5.79 ± 0.30 b	7.05 ± 0.29 b	6.87 ± 0.62 b	
	50	AgNO3	6.59 ± 0.14 c	7.44 ± 0.48 b	7.84 ± 0.41 b	
	5	AgNPs	5.96 ± 0.14 a	5.29 ± 0.16 a	4.75 ± 0.49 a	
	50	AgNPs	5.97 ± 0.14 a	4.98 ± 0.69 a	7.03 ± 0.27 b *#	
Fibroblast cells grown over coated silicon and inoculated with C. albicans 96 h		Control-MiliQ	6.77 ± 0.63 a	6.04 ± 1.21 a	7.54 ± 0.89 a	4.93 ± 0.44 a *
	5	AgNO3	5.61 ± 0.20 b	6.87 ± 0.19 a	6.69 ± 0.42 a	7.53 ± 0.62 b
	50	AgNO3	6.36 ± 0.12 a	7.30 ± 0.32 b	7.70 ± 0.27 a	6.67 ± 0.07 b
	5	AgNPs	6.18 ± 0.26 a	5.92 ± 0.32 c	5.20 ± 0.33 b	4.96 ± 1.06 a *
	50	AgNPs	5.81 ± 0.09 b	4.82 ± 0.46 c	7.29 ± 0.24 a #	6.15 ± 0.48 a

Total K^+ in culture media after exposure to Ag NPs and $AgNO_3$. The first experiment cells were confluent then treatments were added into media for 24h (*n* = 6). Second experiment fibroblast cells were grown over coated silicone for 72h, silver was measured every day for three days in the external media (*n* = 6). Third experiment fibroblast cells were grown over coated silicone for 96h in last 24h the media was inoculated with *C. albicans* (*n* = 4), all data (Mean ± S.E) Numbers with different letters are statistically different from each other within the colum, * difference from day one within row ,# difference from the previous time point within row (One way ANOVA or Kruskal-Wallis test, $p < 0.05$).

Table 4 Total Na^+ concentrations in culture media after exposure to different concentrations of Ag NPs and $AgNO_3$.

			Total Na (mmol l^{-1})			
Experiments	Nominal concentration (mg l^{-1})	Treatment	Day 1	Day 2	Day 3	Day 4
					113.93 ± 3.81 a	
The effect of the		Control-MiliQ			108.89 ± 1.56 a	
Ag NPs/AgNO3	5	AgNO3			111.57 ± 1.21 a	
on fibroblast	50	AgNO3			115.77 ± 6.40 a	
cells	5	AgNPs			111.80 ± 3.48 a	
After 24h	50	AgNPs				
		Control-MiliQ	123.53 ± 0.64 a	99.69 ± 0.71 a *	98.10 ± 0.17 a #	
The effect of	5	AgNO3	110.28 ± 2.06 b	96.95 ± 0.08 b *	93.79 ± 0.12 b #	
silver as a	50	AgNO3	109.45 ± 0.31 b	96.22 ± 1.13 b *	92.92 ± 0.11 b #	
coating over the	5	AgNPs	113.98 ± 0.85 c	99.36 ± 0.77 a *	97.73 ± 0.78 a #	
silicone surface	50	AgNPs	112.60 ± 0.97 c	94.82 ± 0.51 b *	95.25 ± 0.15 c #	
on fibroblast cells after 72 h						
Fibroblast cells		Control-MiliQ	115.93 ± 7.25 a	99.71± 8.90 a	101.38 ± 3.63 a	97.27 ± 1.04 a*
grown over	5	AgNO3	109.94 ± 1.39 a	96.60 ± 0.06 a*	75.31 ± 6.44 b *	92.97 ± 0.25 b *#
coated silicon	50	AgNO3	91.00 ± 6.41 a	95.90 ± 0.76 a	81.71 ± 5.66 b *#	92.32 ± 0.16 b #
and inoculated	5	AgNPs	128.14 ± 8.04 a	117.14 ± 9.07 b	97.39 ± 0.52 a *#	95.62 ± 1.42 a *#
with C. albican	50	AgNPs	115.92 ± 3.31 a	94.52 ± 0.34 a*	94.94 ± 0.10 a *	95.77 ± 0.84 a *
96 h						

Total Na^+ in culture media after exposure to Ag NPs and $AgNO_3$. The first experiment cells were confluent then treatments were added into media for 24h (n = 6). Second experiment fibroblast cells were grown over coated silicone for 72h, silver was measured every day for three days in the external media (n = 6). Third experiment fibroblast cells were grown over coated silicone for 96h in last 24h the media was inoculated with *C. albicans* (n = 4), all data (Mean ± S.E) Numbers with different letters are statistically different from each other within the colum, * difference from day one within row,# difference from the previous time point within row (One way ANOVA or Kruskal-Wallis test, $p < 0.05$).

Table 5 Total Ag, K^+, Na^+ concentrations in fibroblast cells after exposure to different concentrations of Ag NPs and $AgNO_3$.

Experiments	Nominal concentration Treatment (mg l^{-1})		Metal concentrations (µmols mg^{-1} protein) Ag	K^+	Na^+
The effect of the Ag NPs/AgNO3 on fibroblast cells After 24h		Control-MiliQ	0.05 ± 0.03 a	2.44 ± 0.22 a	14.27 ± 1.33 a
	5	AgNO3	1.11 ± 0.22 a	2.89 ± 0.40 a	30.24 ± 4.82 b
	50	AgNO3	34.52 ± 6.15 b	2.98 ± 0.18 a	49.87 ± 4.34 c
	5	AgNPs	3.01 ± 0.47 c	2.11 ± 0.23 b	8.93 ± 1.42 d
	50	AgNPs	18.42 ± 1.11 d	2.17 ± 0.22 b	10.90 ± 1.51 d
The effect of silver as a coating over the silicone surface on fibroblast cells after 72 h		Control-MiliQ	0.04 ± 0.01 a	13.76 ± 1.34 a	34.13 ± 1.22 a
	5	AgNO3	0.02 ± 0.06 a	16.00 ± 2.02 a	73.70 ± 8.60 b
	50	AgNO3	2.08 ± 0.31 b	18.19 ± 1.212 a	95.77 ± 4.85 c
	5	AgNPs	0.12 ± 0.04 c	20.74 ± 1.33a	42.41 ± 6.20 a
	50	AgNPs	0.11 ± 0.02 c	18.42 ± 2.63 a	29.90 ± 2.69 a
Fibroblast cells grown over coated silicon and inoculated with C. albican 96 h		Control-MiliQ	0.00 ± 0.00 a	6.43 ± 1.72 a	8.84 ± 2.73 a
	5	AgNO3	2.25 ± 1.21 b	5.46 ± 2.53 a	47.17 ± 23.28 b
	50	AgNO3	5.52 ± 0.91 c	5.31 ± 1.89 a	53.15 ± 17.92 b
	5	AgNPs	0.04 ± 0.00 a	9.41 ± 1.91 b	11.47 ± 2.32 a
	50	AgNPs	0.06 ± 0.01 a	11.68 ± 1.82 b	12.86 ± 1.02 a

Total Ag, K^+, Na^+ in fibroblast cells after 24h exposure to Ag NPs and $AgNO_3$ (n = 6). Total Ag K^+, Na^+ in fibroblast cells grown over Ag NPs and $AgNO_3$ coated silicone for 72 h (n = 6). Total Ag K^+, Na^+ in fibroblast cells grown over silver coated silicone for 96 h in the last 24 h the cells were inoculated with *C. albicans* (10^6) cells/ml (n = 4). Data are expressed as (Mean ± S.E) numbers with different letters are statistically different from each other within row (One way ANOVA or Kruskal-Wallis test, $p < 0.05$).

The effect of nanoparticles was investigated on fibroblast cells grown in the dishes for 24h (Fig. 5), and the main findings were that the cells exposed to Ag NPs were morphologically in good health with no effect on the cell structure. In contrast, when cells were exposed to $AgNO_3$, the treatment caused the cells to detach from the dishes at both concentrations (5 and 50 mg l^{-l}).

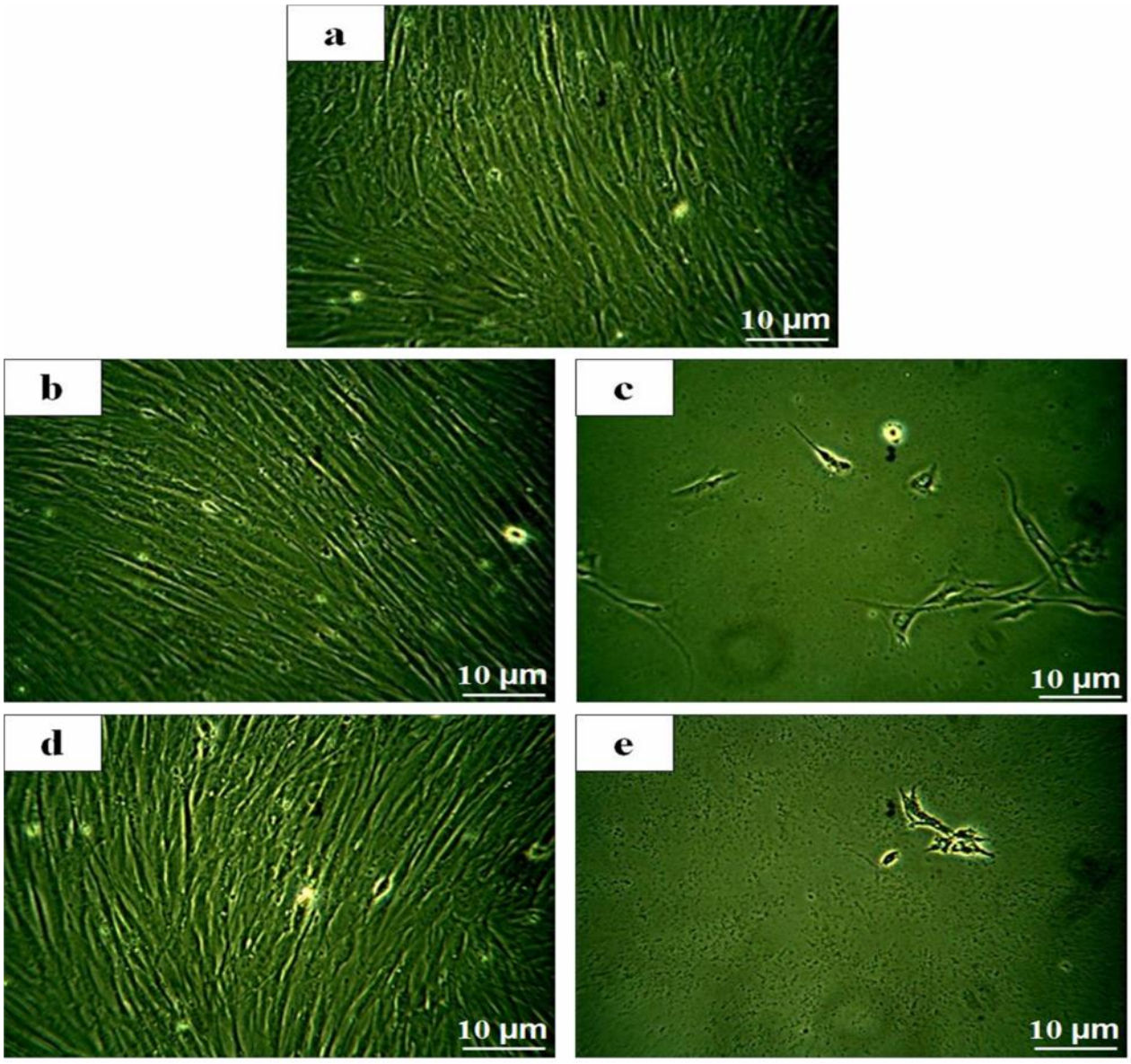


Figure 5 Morphology of fibroblast cells after 24h exposure to Ag NP and $AgNO_3$. Olympus microscope SZ-1145 CHI with transmitted light magnification 40x and camera Scope Tek MDC 560 with magnification 0.6 x, was used (*n* = 6) scale bar-10 µm (a) control (b) 5mg l^{-l} Ag NPs (c) 5mg l^{-1} $AgNO_3$ (d) 50 mg l^{-l} Ag NPs (e) 50 mg l^{-1} $AgNO_3$.

Lactate dehydrogenase leaked from the cells was also measured after 24 h as a method to determine injury to the cell membrane (cell permeability) and was statistically different between all groups (Kruskal Wallis, $p< 0.05$) (Fig 6). Total LDH leaked of control cells leaked 4.2 % of cell LDH and cells exposed to Ag NPs leaked 4.8% and 9.7 % of cell LDH at concentration 5 and 50 mg l^{-l} respectively. While LDH leakage for cells exposed to $AgNO_3$ were 44% and 97% at concentrations 5 and 50 mg l^{-l} respectively. Table 6 shows total LDH activity leaked in µmol min^{-1} ml^{-1}. After 24 h exposure to 5 and 50 mg l^{-1} Ag NPs or $AgNO_3$, the adherent cells were washed and lysed, and the cell homogenate quantified for LDH activity (Fig 7). The LDH in the cell homogenate was not statistically different between the control and when cells were exposed to Ag NPs, whereas, when the cells were exposed to $AgNO_3$ statistically they showed less LDH activity compared with those exposed to Ag NPs.

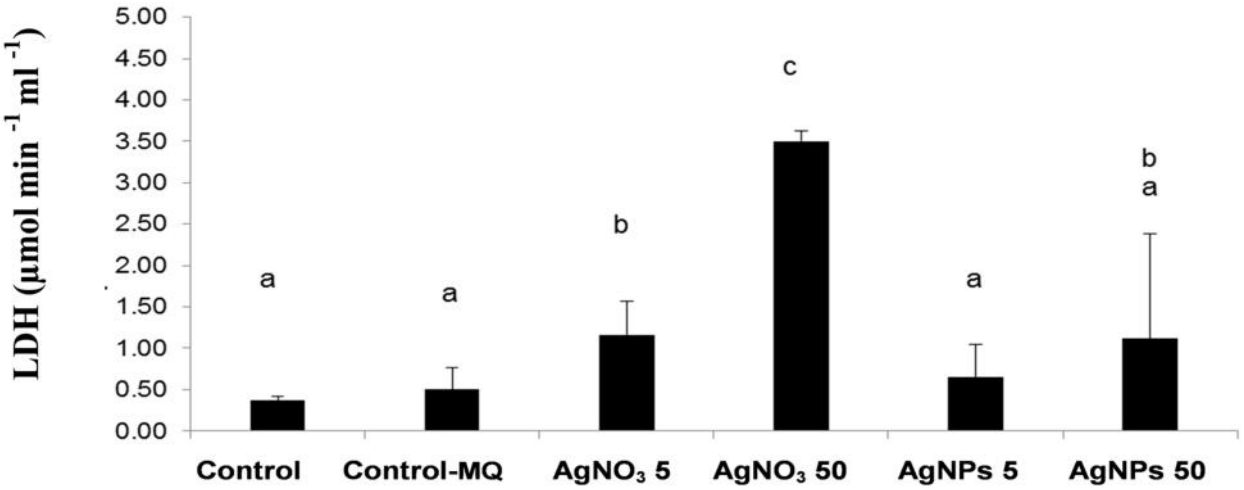


Figure 6 LDH activity leak into the external media after 24h. Data are means ± S.E (*n* = 6) Bars with different letters are statistically different from each other (Kruskal-Wallis test, $p<0.05$).

Table 6 LDH activity after 24 h incubation with different concentrations of Ag NPs and $AgNO_3$.

Treatment	Media LDH activity (µmol min-1 ml-1)	Cell homogenate LDH activity (µmol min-1 mg-1 protein)
control	0.37 ± 0.04 a	0.88 ± 0.11 a
control-MQ	0.50 ± 0.11 a	0.70 ± 0.12 a
AgNO3 5	1.16 ± 0.39 b	0.31 ± 0.09 b
AgNO3 50	3.50 ± 0.16 c	0.04 ± 0.01 c
AgNPs 5	0.65 ± 0.25 a	0.59 ± 0.08 a
AgNPs 50	1.11 ± 0.81 ab	0.60 ± 0.07 ab

*The effect of Ag NPs and AgNO3 exposure on total LDH activity release after 24h into media and in fibroblast cells. Values are means ± S.E (n = 6). Different letters are statistically different from each other within colum (One way ANOVA, Kruskal-Wallis test, p<0.05).

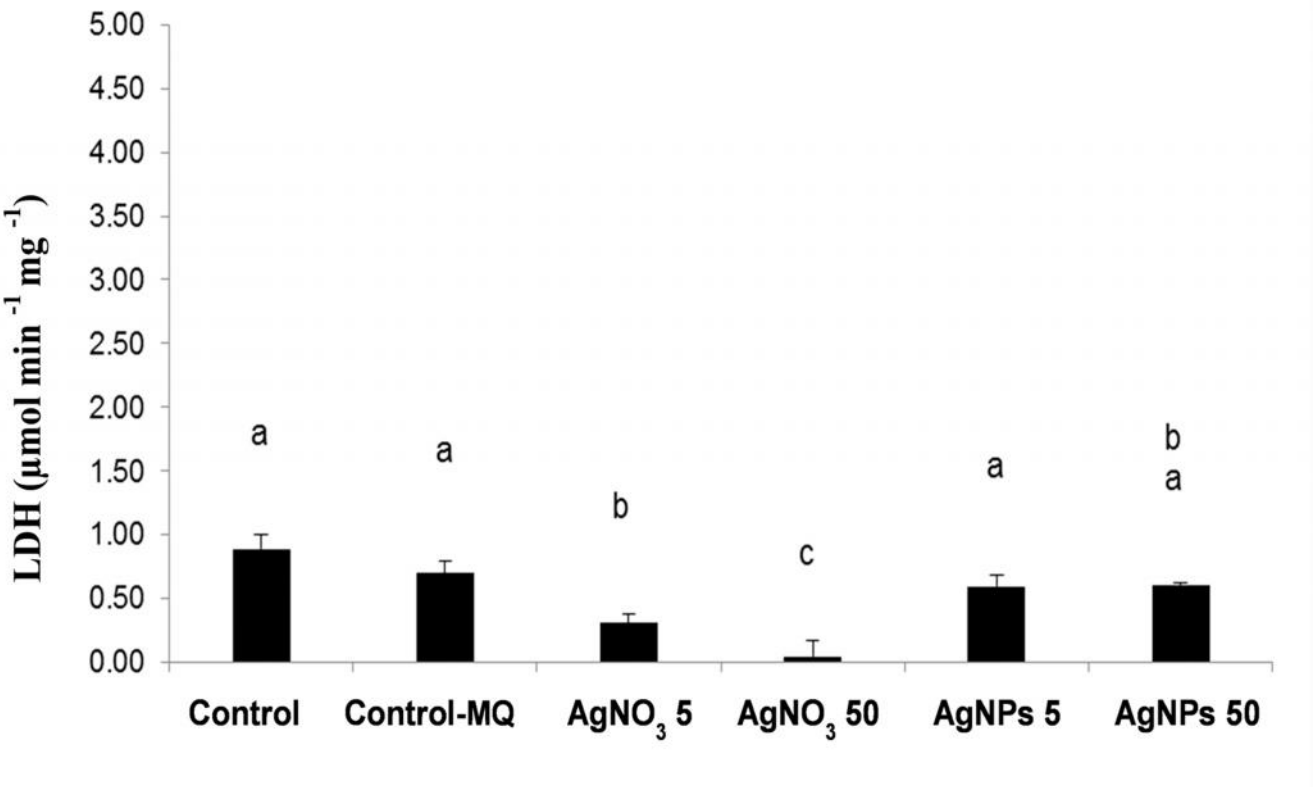


Figure 7 LDH activity from cell homogenate after 24h exposure. Data are means ± S.E. (*n* = 6). Bars with different letters are statistically different from each other (One way ANOVA, *p*<0.05).

The protein assay was used as an indication for measuring the cell viability since it measures the protein content of viable cells that were left after 24 h exposure. From the data in Figure 8, it is apparent that the protein content was not statistically different from control when cells were exposed to 5, 50 mg l^{-1} Ag NPs compared to $AgNO_3$. Protein content was (in mg ml^{-1}); 0.168 ± 0.013 in controls, and 0.226 ± 0.054, 0.188 ± 0.009 when the fibroblast cells were exposed to 5, 50 mg l^{-1} Ag NPs respectively. Whereas when the fibroblast cells were exposed to 5, 50 mg l^{-1} $AgNO_3$, protein content was lower than controls 0.083 ± 0.007, 0.081 ± 0.010 for 5, 50 mg l^{-1} $AgNO_3$ respectively.

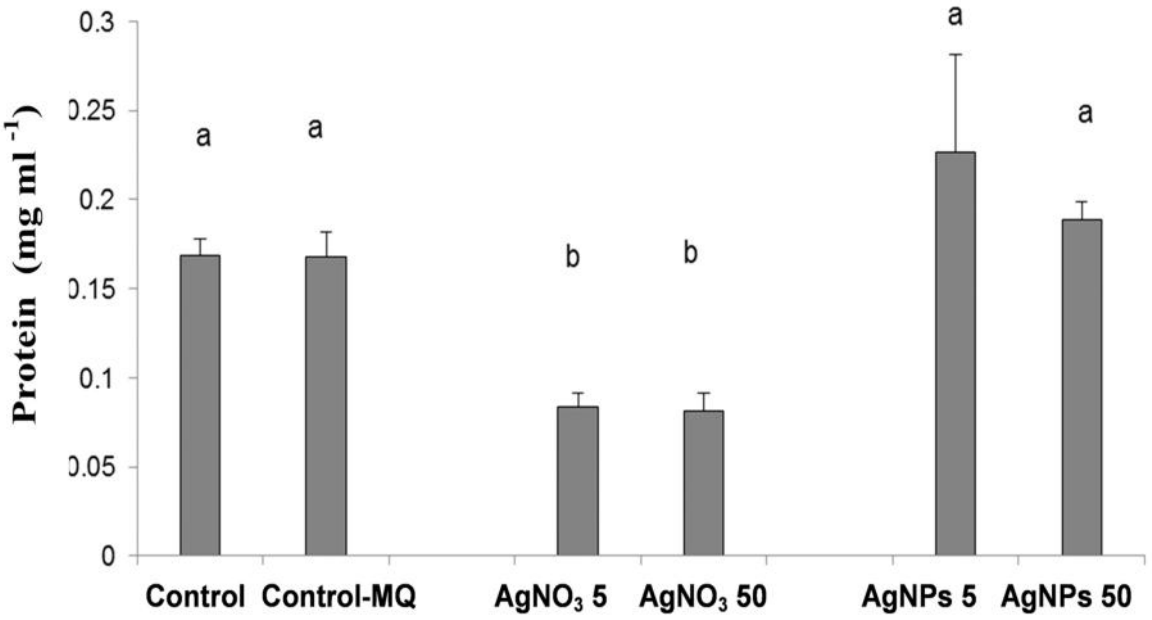


Figure 8 Protein concentration in cell homogenates from fibroblasts exposed to 5 or 50 mg l^{-1} Ag NPs or $AgNO_3$ for 24 h. Data are means ± SE (n = 6) culture plates/treatment). Bars with different letters are statistically different from each other (Kruskal-Wallis test, p<0.05).

Demonstrating the effect of silver as a coating over the silicone surface on fibroblast cells.

When the cells were grown over silicone coated prostheses for 72h, silver was measured in the external media every day. It is apparent from Table 2 that silver concentration in external media showed a constant increase when the cells were grown over silicone coated with $AgNO_3$ at concentration 50 mg l^{-1} compared to Ag NPs. Silver accumulation in the cells was also measured after 72h exposure (Table 5). Comparing the results of that with the first experiment (24h exposure) shows that silver accumulation, after washing the cells was higher when cells were exposed for 24 h to $AgNO_3$ and Ag NPs prepared in the media compared to silver accumulation in cells grown on silicone coated silver ($AgNO_3$ and Ag NPs). Although the cells were exposed for three days with direct contact with silver, the accumulation was less in fibroblast cells than without the silicone. Furthermore Ag accumulation in fibroblast cells were more when the cells were grown over silicone coated with $AgNO_3$ compared to Ag NPs. Cell electrolytes were also measured in cell homogenate after 72h exposure to silver coated silicone prostheses, and showed no effect on cell K^+ content, while Na^+ content showed a clear increase when cells were grown on the silicone coated with $AgNO_3$ (Table 5).

When the cells were grown on silver coated silicone for 72h, cell viability showed the same effect on cell morphology (Fig 9), indicating that the effect of $AgNO_3$ and Ag NPs on fibroblast cell viability was the same with and without the presence of silicone prostheses. The cells which were exposed to 5 and 50 mg l^{-1} Ag NPs showed normal morphology with the cells remaining confluent and well attached to the well plates (Fig 9 b, d). Whereas $AgNO_3$ at both concentrations were toxic to fibroblast cells as the cells were dead and detached from well plate (Fig 9 c, e).

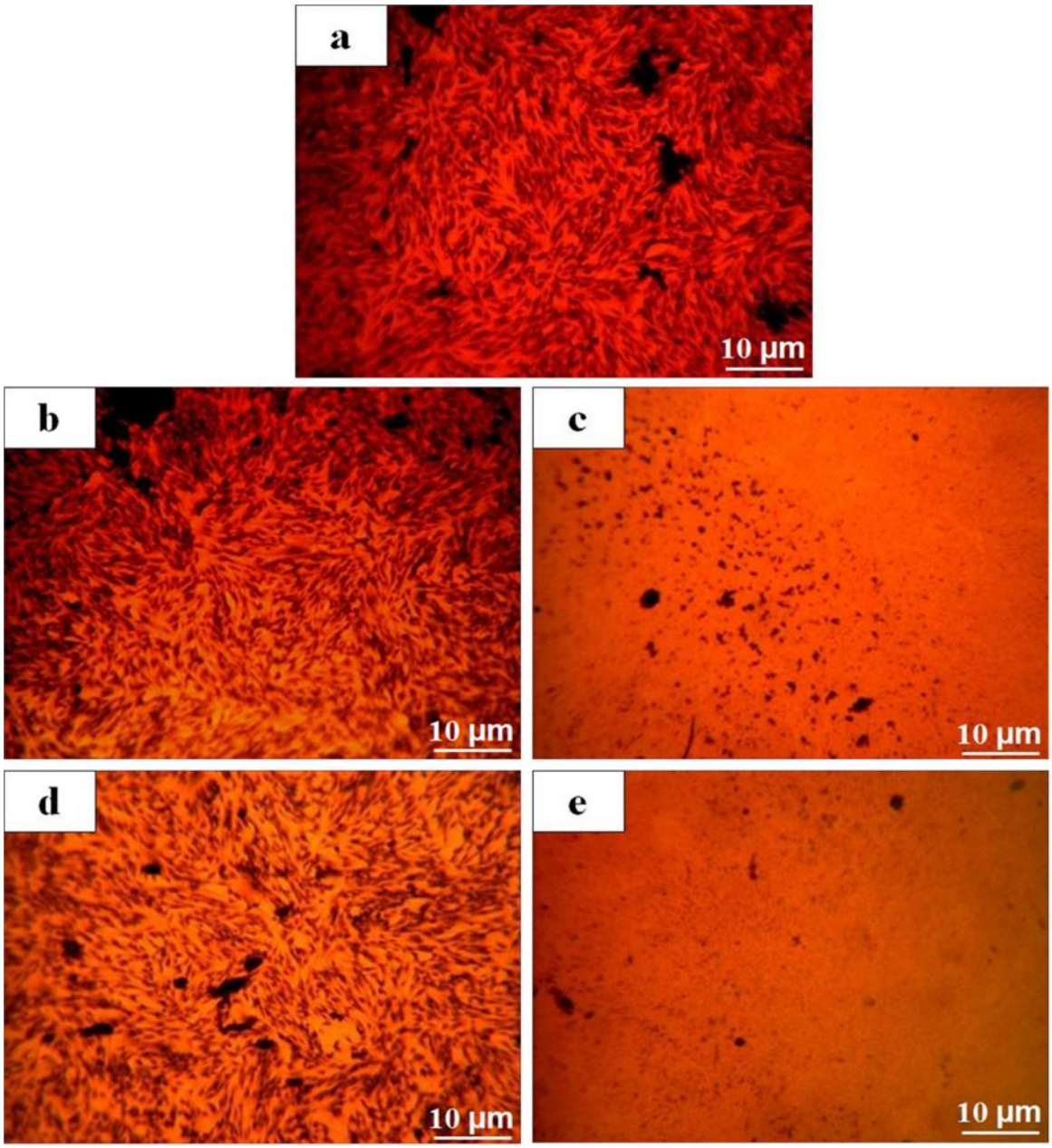


Figure 9 Morphology of fibroblast cells after 72h exposure to Ag NPs and $AgNO_3$. Olympus microscope SZ-1145 CHI with transmitted light magnification 40x and camera Scope Tek MDC 560 with magnification 0.6 x, was used (*n* = 6) scale bar = 10 µm. Fibroblast cells grown over silicone coated prostheses stained with Giemsa (a) control uncoated silicone (b) silicone coated with 5mg l^{-l} Ag NPs (c) silicone coated with 5mg l^{-1} $AgNO_3$ (d) silicone coated with 50 mg l^{-l} Ag NPs. (e) silicone coated with 50mg l^{-1} Ag NPs.

In relation to cumulative LDH leak to external media over three days exposure (Fig 10 A), LDH leak of unexposed cells every day was about 4.3 %, and at the end

of 72 h cumulative LDH leak was about 13 % of the cell LDH. Similarly cumulative LDH of cells grown over silicone coated with Ag NPs was 6.6 and 12.5 % of cell LDH at concentration 5 and 50 mg l^{-l} respectively after 72 h exposure. While cumulative LDH leak of cells grown over silicone coated with $AgNO_3$ was around 30.8 % and 36 % at concentration 5 and 50 mg l^{-l} after 72 h exposure (maximum leak was in the first day as the cells were all dead and detach after 24h exposure). After 72 h exposure to 5 and 50 mg l^{-1} Ag NPs and $AgNO_3$, the adherent cells were washed and lysed to measure LDH from the cell homogenate (Fig 10 B) and the LDH in the cell homogenate was not statistically different between the control and when cells were exposed to Ag NPs, whereas, when the cells were exposed to $AgNO_3$ they showed less LDH activity compared with those exposed to Ag NPs. Table 7 shows total LDH activity leaked into media every day (µmol min^{-1} ml^{-1}) and LDH from cell homogenate.

Table 7 LDH activity in the media from fibroblast cells every day for 72 h grown on silicone coated with different concentrations of Ag NPs and $AgNO_3$.

		Total LDH activity		
	Treatment	Total LDH activity after 24 h	Total LDH activity after 48 h	Total LDH activity after 72 h
Media LDH activity (µmol min^{-1} ml^{-1})	Control	0.09± 0.00 a	0.06± 0.00 b	0.06 ± 0.00 b
	Control-MQ	0.11± 0.01 a	0.13 ± 0.04 a	0.09 ± 0.00 a
	$AgNO_3$ 5	0.14 ± 0.02 a	0.00 ± 0.00 b	0.00 ± 0.03 b
	$AgNO_3$ 50	0.20 ± 0.03 a	0.00 ± 0.00 b	0.00 ± 0.00 b
	AgNPs 5	0.07 ± 0.00 a	0.08 ± 0.00 a	0.08 ± 0.00 a
	AgNPs 50	0.14 ± 0.01 a	0.06 ± 0.00 b	0.10 ± 0.00 c
Cell homogenate LDH activity after 72 h. (µmol min^{-1} mg^{-1} protein)	control	0.46 ± 0.05 a		
	control-MQ	0.31 ± 0.04 a		
	$AgNO_3$ 5	0.00 ± 0.00 b		
	$AgNO_3$ 50	0.00 ± 0.00 b		
	AgNPs 5	0.49 ± 0.08 a		
	AgNPs 50	0.32 ± 0.03 a		

*Media LDH activity: The effect of Ag NPs and $AgNO_3$ exposure on total LDH activity release after 72h from fibroblast cells. Values are means ± S.E (n = 6). Different letters are statistically different from each other within row (One way ANOVA or Kruskal-Wallis test, p<0.05). Cell homogenate LDH activity Total LDH in fibroblast cells after 72h. Values are means ± S.E (n = 6). Different letters are statistically different from each other (One way ANOVA, p<0.05).

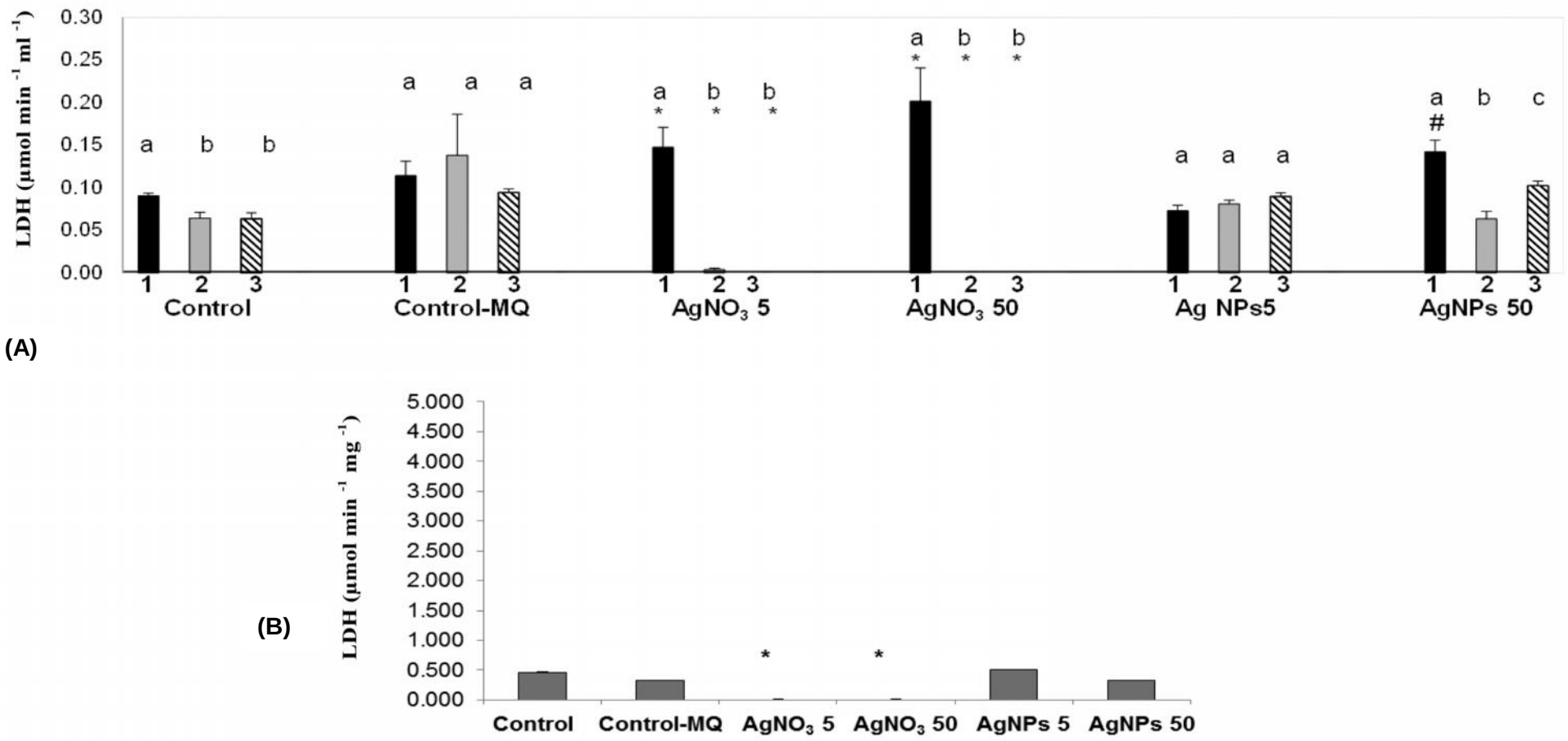


Figure 10 LDH activity after 72h exposure to Ag NPs and $AgNO_3$. (A) The effect of Ag NPs and $AgNO_3$ exposure on LDH activity leaked to external media per 2 ml over 24 h (B) LDH activity from cell homogenate after 72 h exposure. Data are means ± S.E (*n* = 6).* difference between control and treatment with in day, different letters with in treatment indicates a time effect, # concentration effect within day. (One way ANOVA or Kruskal-Wallis test, $p<0.05$).

The protein assay was again used to measure the protein content of viable cells that were left after 72 h exposure. From the data in Figure 11, it is apparent that the protein content remained the same when cells were exposed to 5, or 50 mg l^{-1} Ag NPs compared to $AgNO_3$. Protein content were (in mg ml^{-1}); 0.166 ± 0.007, in controls, and 0.145 ± 0.02, 0.151 ± 0.012 when the fibroblast cells were exposed to 5, 50 mg l^{-1} Ag NPs respectively. Whereas when the fibroblast cells were exposed to 5, 50 mg l^{-1} $AgNO_3$, protein content was lower than controls 0.111 ± 0.03, 0.110 ± 0.005 for 5, 50 mg l^{-1} $AgNO_3$ respectively.

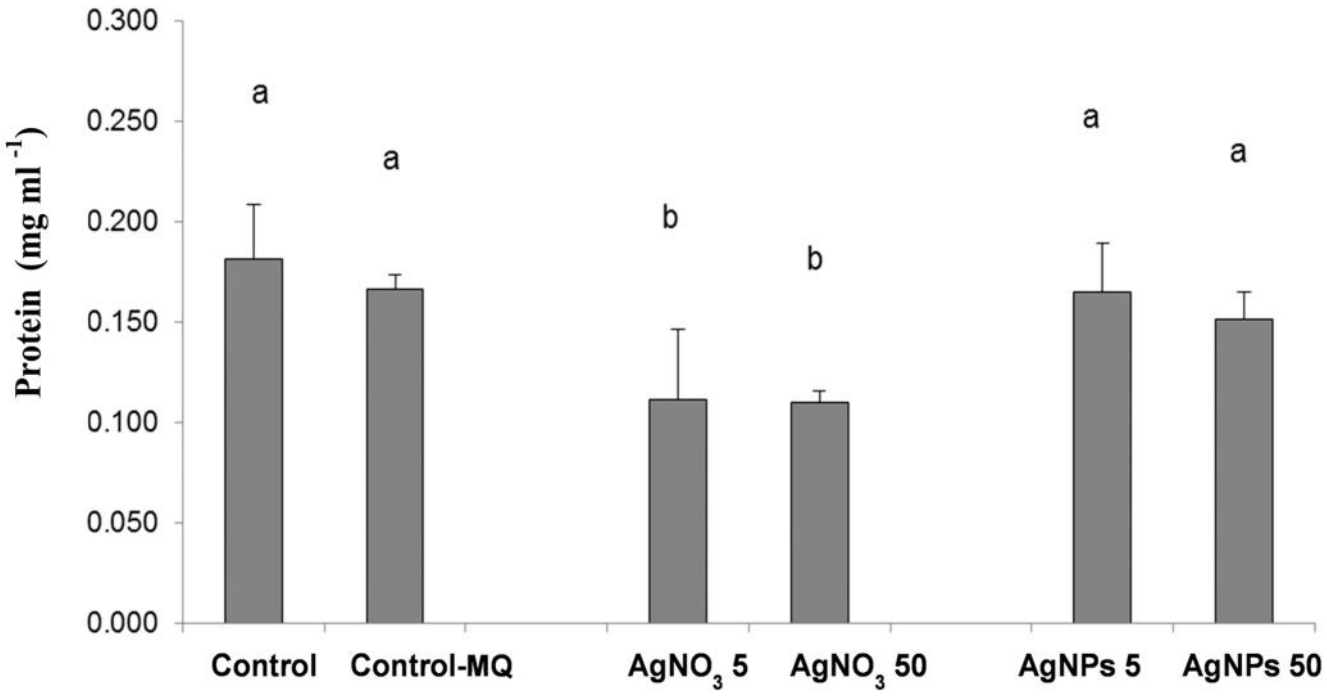


Figure 11 Protein content after 72h exposure to Ag NPs and $AgNO_3$. (n = 6) Bars with different letters are statistically different from each other (One way ANOVA, p<0.05).

Quality of silver coating on the silicone surface and contact with the cells.

Some preliminary experiments were conducted to determine whether or not sterilisation of the silicone surfaces with (chlorhexidine and PBS) had caused any changes to the silver coatings. SEM investigations showed that when the silicone surface was sterilised, it had no effect on Ag NPs distribution as a coating. Similarly, when the surface of silicone was coated with Ag NPs but without sterilising and the washing step, it showed no effect as well (Fig 12 a, b). Another three sets of plates were prepared separately (silicone coated prostheses with fibroblast cells grown for 72h), (Fig 12 c, d). They showed that fibroblast cells were attached to silicone coated with Ag NPs. Moreover the silicone surface coated with 5 mg l^{-1} Ag NPs formed aggregations of nanoparticles (Fig 13 a b), however when silicone was coated with 50 mg l^{-1} Ag NPs, bigger aggregates were formed compared to the concentration of 5 mg l^{-1}. Furthermore the presence of Ag was confirmed as a coating by EDS measurements for both 5 and 50 mg l^{-1} concentrations of Ag NPs (Fig 13 c, d).

When the silicone surface was coated with $AgNO_3$, sterilisation with chlorhexidine and PBS showed a different distribution of silver compared with unsterilised silicone (Fig 14). Silicone coated with 5 and 50 mg l^{-1} $AgNO_3$ showed silver chloride formation on the surface when silicone was sterilised (Fig 14a b), whereas when the surface was not sterilised less silver was detected (Fig 14 e), indicating that PBS had an effect on AgCl formation on the surface.

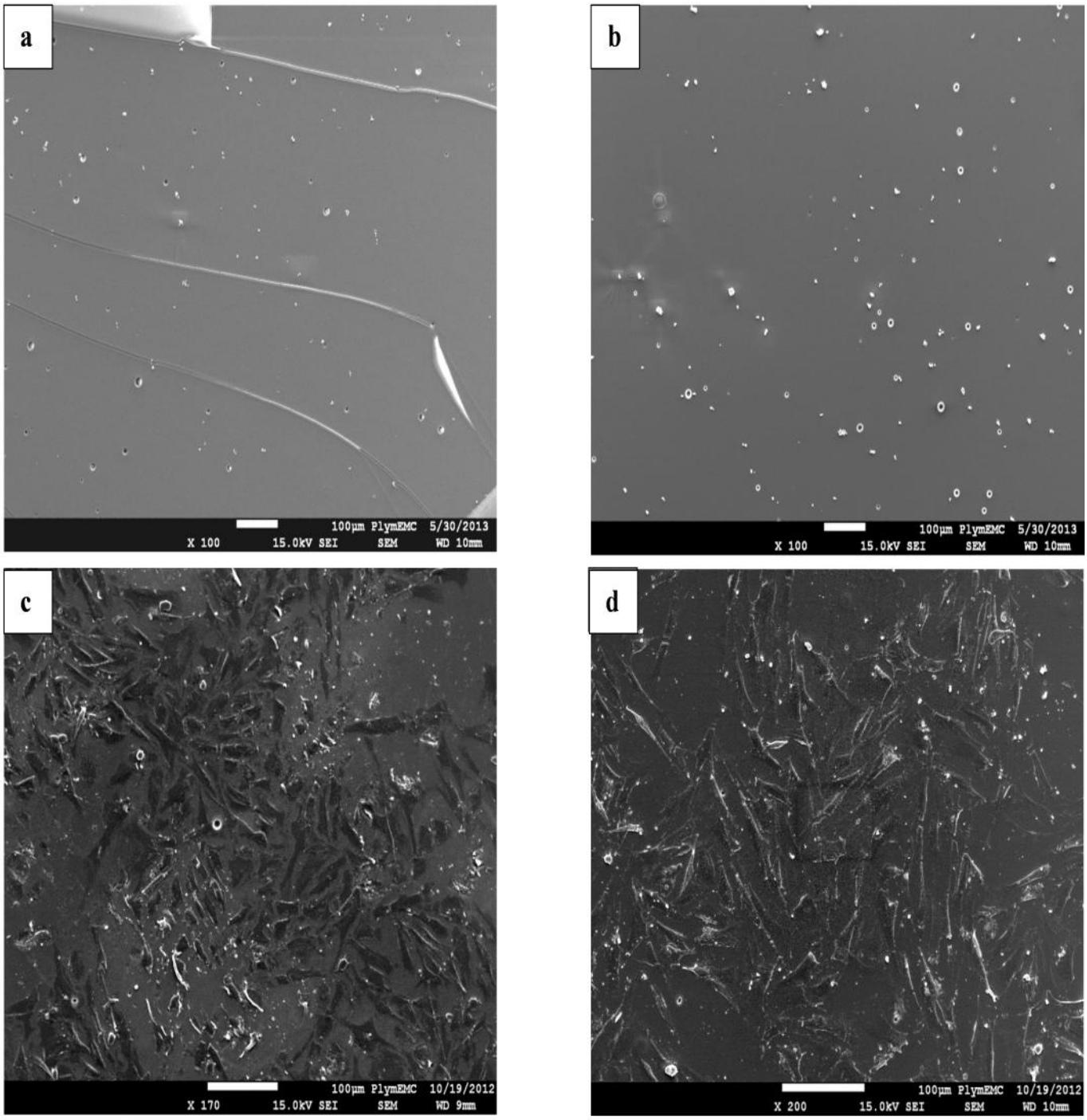


Figure 12 SEM images of silicone coated with Ag NPs: a) unsterilised silicone surface coated with 5 mg l^{-1} Ag NPs; b) unsterilised silicone surface coated with 50 mg l^{-1} Ag NPs; c) Sterilised silicone surface coated with 5mg l^{-1} Ag NPs with fibroblast cells grown for 72h; d) Sterilised silicone surface coated with 50 mg l^{-1} Ag NPs with fibroblast cells grown for 72h (scale bar represents 100 µm).

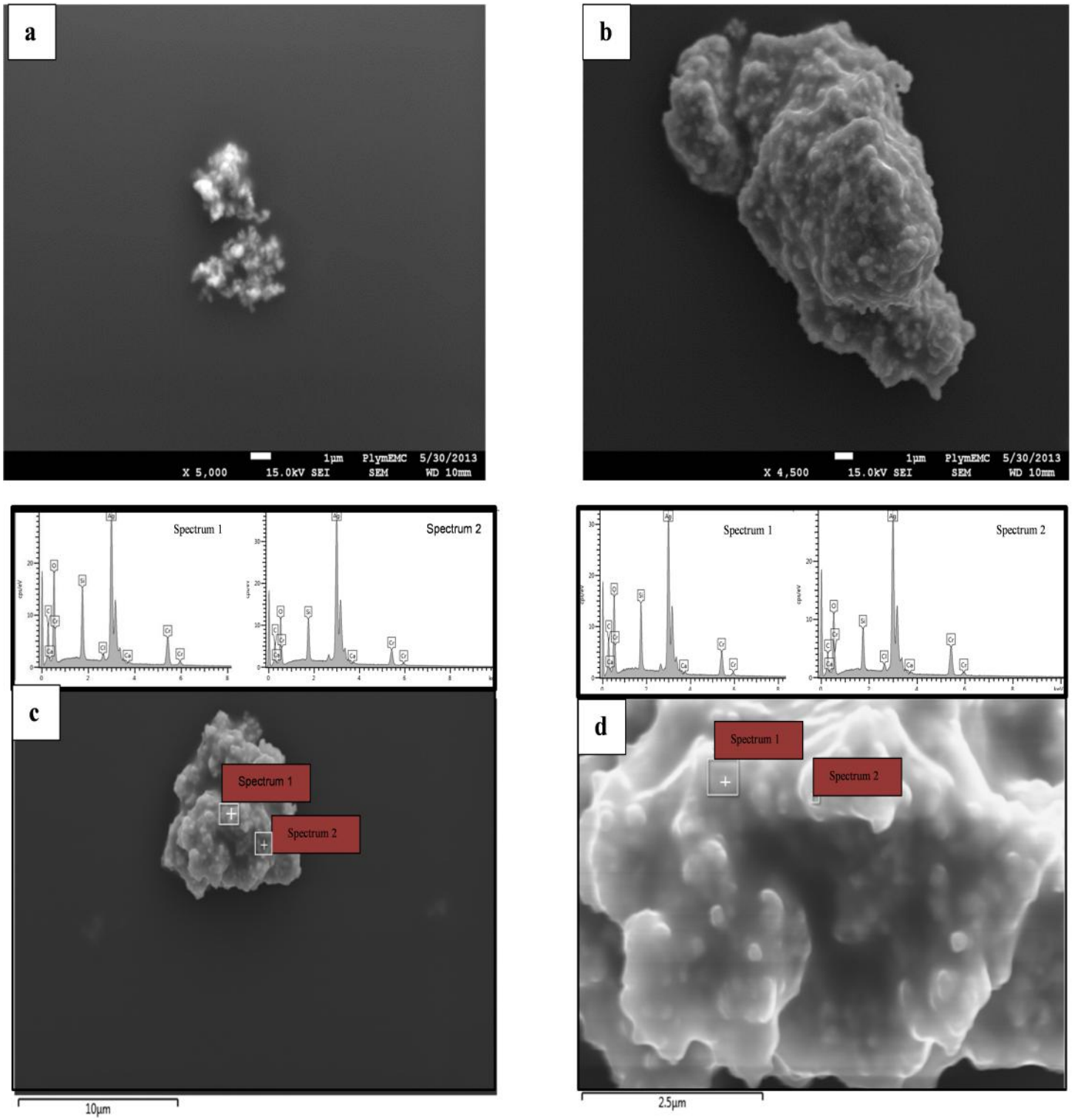


Figure 13 SEM images of unsterilised silicone coated with Ag NPs, a) silicone surface coated with 5 mg l^{-1} Ag NPs (scale bar represents 1µm); b) Silicone surface coated with 50 mg l^{-1} Ag NPs (scale bar represents 1 µm); c) silicone surface coated with 5 mg l^{-1} Ag NPs confirming the presence of silver (scale bar represents 10µm); d) Silicone surface coated with 50 mgl^{-1} Ag NPs confirming the presence of silver (scale bar represents 2.5µm).

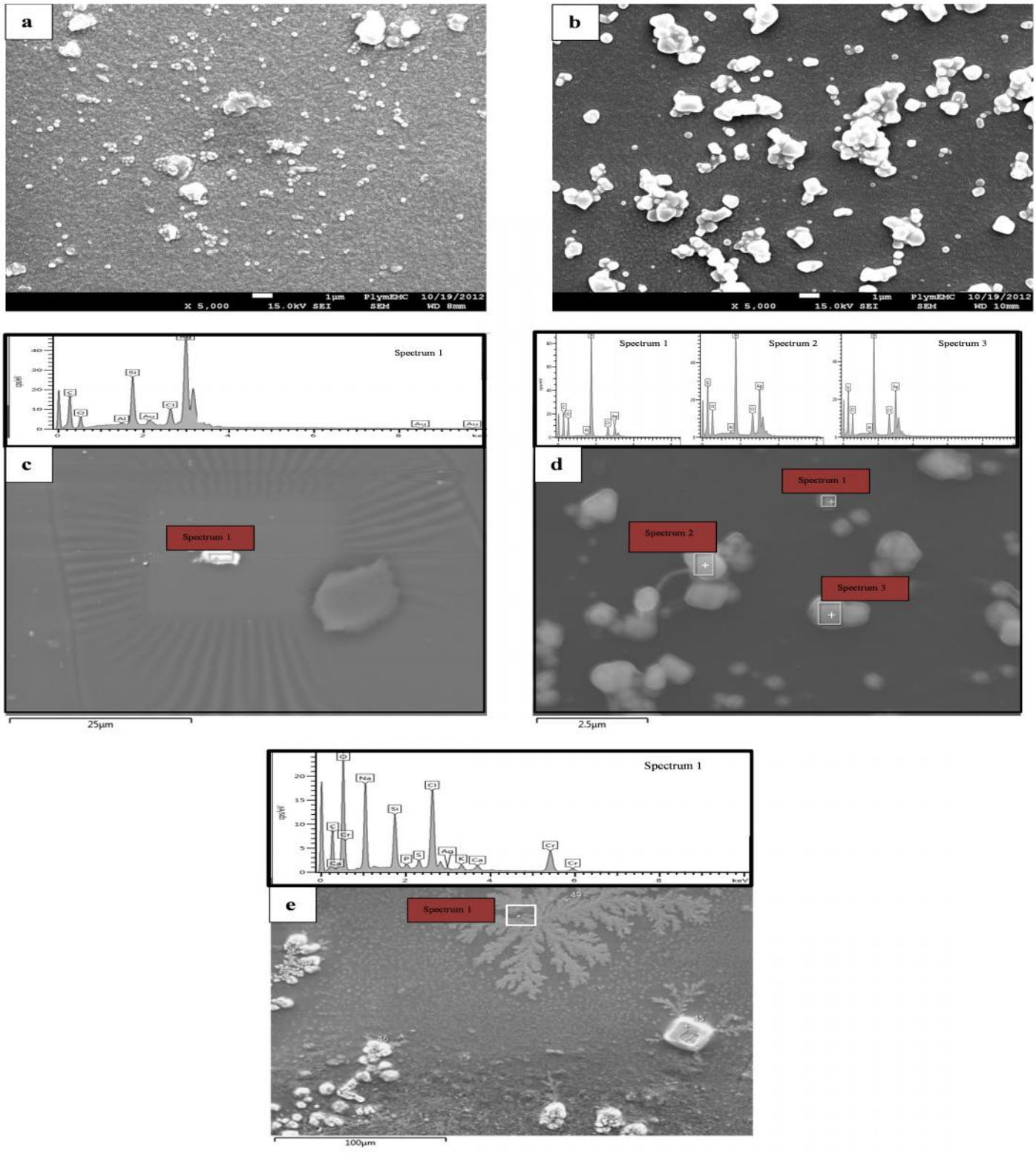


Figure 14 SEM images of silicone coated with $AgNO_3$; a) Sterilised silicone surface coated with 5 mg l^{-1} $AgNO_3$; b) Sterilised silicone coated with 50 mg l^{-1} $AgNO_3$; c) Sterilised silicone surface coated with 5 mg l^{-1} $AgNO_3$ confirming presence of AgCl; d) Sterilised silicone coated with 50 mg l^{-1} $AgNO_3$ confirming presence of AgCl; e) Unsterilised silicone surface coated with 50 mg l^{-1} $AgNO_3$, while no silver was found at concentration 5mgl^{-1}.

Investigating the antifungal properties of new silver coated silicone against *C. albicans*.

Silver was again measured in the media when the cells were exposed to treatments for 96h (Fibroblast cells were grown for 72h and then inoculated with *C. albicans* for another 24h). From Table 2, the same trends as the previous 72h exposure were seen. Furthermore, silver accumulation in the cells was also measured and showed the highest silver accumulation when cells were grown over silicone coated $AgNO_3$ compared to other treatments (Table 5). Cell electrolytes were also measured in cell homogenate after 96h exposure to silicone coated prostheses, and showed an increases of K^+ content when silicone was coated with Ag NPs, while Na^+ content increased when cells were exposed to $AgNO_3$ (Table 5).

Morphology of fibroblast cells grown on silicone for 96h and inoculated with *C.albicans* in the last 24h of the experiment (Fig 15) showed that *C.albicans* were attached to the uncoated silicone and the fibroblast cells were dead. In contrast, the fibroblast cells were viable and protected when the silicone was coated with Ag NPs (more cells were attached when silicone was coated with 50 mg l^{-l} than 5 mg l^{-l}). Whereas silicone coated with $AgNO_3$ showed no fibroblast cells growing on the surface, as the cells had been already detached from silicone, in addition to that few yeast cells were observed when the silicone was coated with 5 mg l^{-l} $AgNO_3$ compared to 50 mg l^{-l} $AgNO_3$.

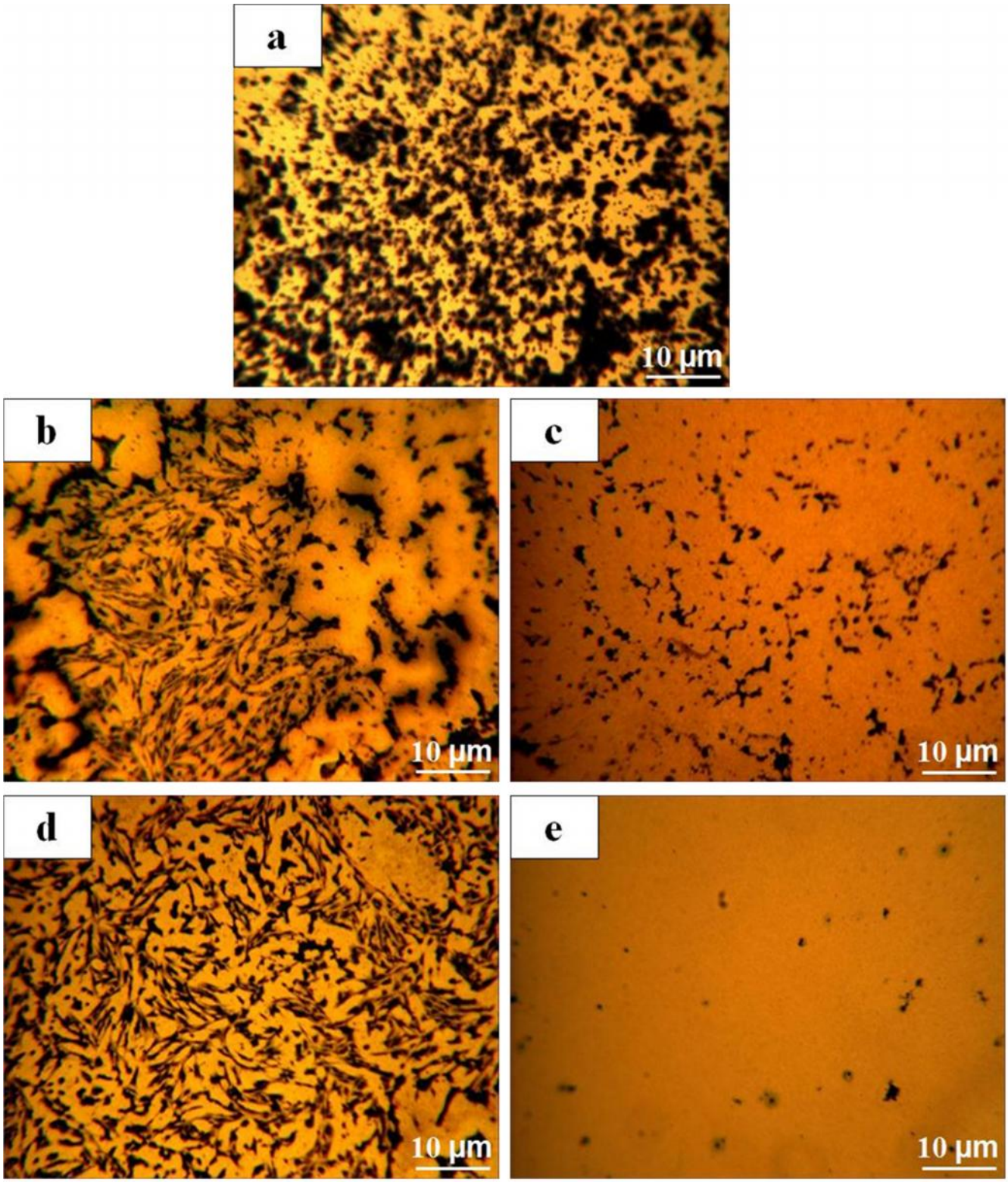


Figure 15 Morphology of fibroblast cells after 96hand in last 24h cells were inoculated with *C. albicans* (10^6 cell ml $^{-1}$) stained with methylene blue, exposed to different concentrations of Ag NPs and $AgNO_3$. Olympus microscope SZ-1145 CHI with transmitted light magnification 40x and camera Scope Tek MDC 560 with magnification 0.6 x, was used (n = 6) scale bar = 10 µm.(a) control uncoated (b) silicone coated with 5mg l^{-l} Ag NPs (c) silicone coated with 5mg l^{-1} $AgNO_3$ (d) silicone coated with 50 mg l^{-l} Ag NPs. (e) silicone coated with 50mg l^{-1} $AgNO_3$.

Figure 16 A shows cumulative LDH leak to the external media after 24 inoculations with *C. albicans*. LDH leak of cells grown on uncoated silicone was 6.8 % . Whereas total LDH leak of cells grown on silicone coated with Ag NPs were 6.2 and 4.2 % of cell LDH at concentrations 5 and 50 mg l^{-l} respectively, although more cells were attached to silicone than unexposed cells. While LDH leak of cells grown on silicone coated with $AgNO_3$ was around 12.1 and 51 % at concentrations 5 and 50 mg l^{-l} respectively. After 96 h exposure to 5 and 50 mg l^{-1} Ag NPs and $AgNO_3$, the adherent cells were washed and lysed same as second experiment to measure LDH from cell homogenate (Fig 16B). Table 8 shows total LDH activity leaked into media every day µmol min^{-1} ml^{-1} and LDH from cell homogenate.

Table 8 LDH activity in the media from fibroblast cells every day for 96h grown on silicone coated with different concentrations of Ag NPs and $AgNO_3$, in last 24h the *C. albicans* were inoculated into culture media.

Treatment		**Total LDH activity**			
		Total LDH activity after 24h	Total LDH activity after 48h	Total LDH activity after 72h	Total LDH activity after 96h
Media LDH activity (µmol min^{-1} ml^{-1})	control	0.05 ± 0.01 a	0.04 ± 0.00 a	0.04 ± 0.00 a	0.18 ± 0.00 b
	control-MQ	0.03 ± 0.00 a	0.03 ± 0.00 a	0.03 ± 0.00 a	0.18 ± 0.00 b
	$AgNO_3$ 5	0.11 ± 0.03 a	0.01 ± 0.00 b	0.01 ± 0.00 b	0.03 ± 0.01 b
	$AgNO_3$ 50	0.23 ± 0.03 a	0.01 ± 0.00 b	0.01 ± 0.00 b	0.04 ± 0.00 b
	AgNPs 5	0.11 ± 0.01 a	0.09 ± 0.00 ab	0.08 ± 0.00 b	0.11 ± 0.01 ac
	AgNPs 50	0.09 ± 0.01 a	0.08 ± 0.00 a	0.04 ± 0.00 b	0.10 ± 0.01 a
Cell homogenate LDH activity after 96 h (µmol min^{-1} mg^{-1} protein)	control	0.54 ± 0.09 a			
	control-MQ	0.79 ± 0.08 b			
	$AgNO_3$ 5	0.32 ± 0.12 c			
	$AgNO_3$ 50	0.33 ± 0.03 c			
	AgNPs 5	0.42 ± 0.06 c			
	AgNPs 50	0.42 ± 0.03 c			

Media LDH activity: The effect of Ag NPs and $AgNO_3$ exposure on total LDH activity in the media after 96 h from fibroblast cells. Values are means ± S.E (*n*=6). Different letters are statistically different from each other within row (One way ANOVA or Kruskal-Wallis test, $p<0.05$). Cell homogenate LDH activity, Total LDH in fibroblast cells after 96h incubation. Values are means ± S.E (n = 6). Different letters are statistically different from each other (One way ANOVA t, $p<0.05$)

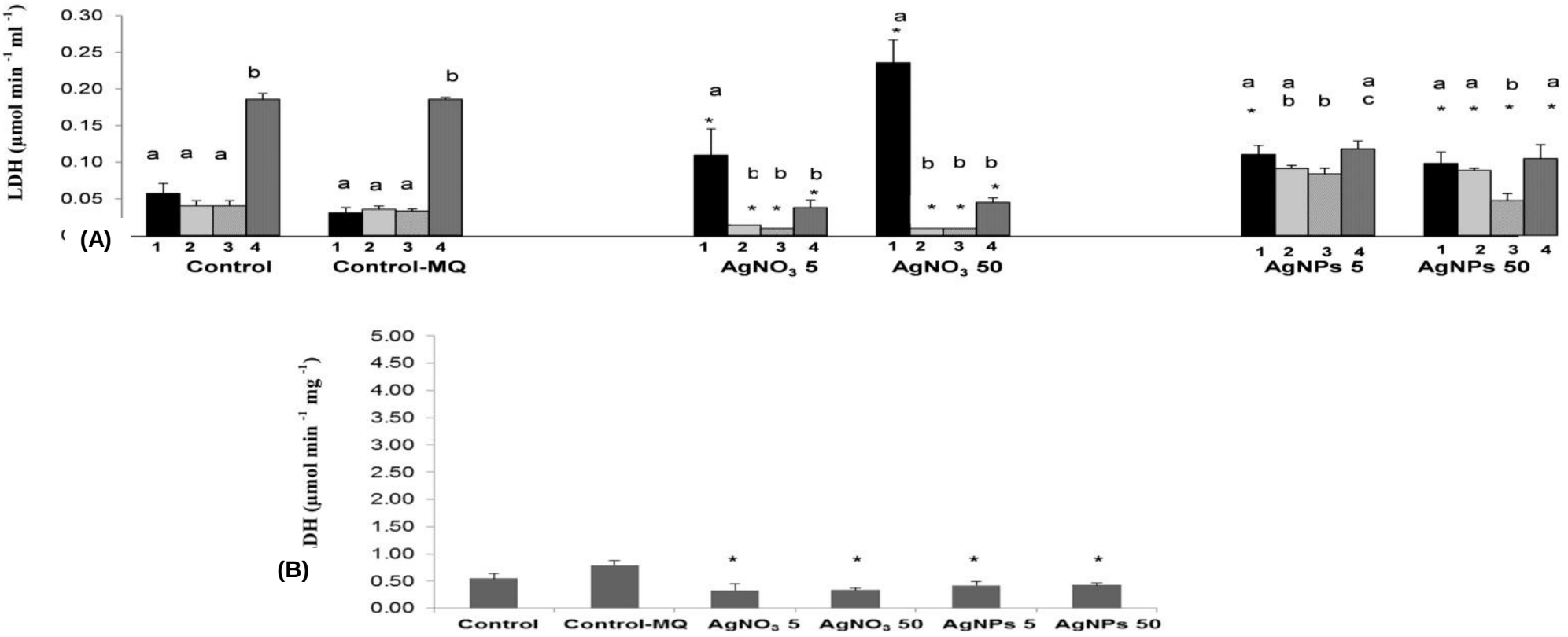


Figure 16 LDH activity after 96h exposure to Ag NPs and $AgNO_3$. (A) The effect of Ag NPs and $AgNO_3$ exposure on LDH activity leaked to external media per 2 ml over 24 h (B) LDH activity from cell homogenate after 96 h exposure. Data are means ± S.E (*n* = 6).* difference between control and treatment with in day, different letters with in treatment indicates a time effect, # concentration effect within day. (One way ANOVA or Kruskal-Wallis test, $p<0.05$).

The protein assay was measured, and showed that the protein content of viable cells that were left after 96 h exposure was highest when cells were grown over silicone coated with Ag NPs compared to silicone coated with $AgNO_3$ (Fig 17). Protein content in the controls were 0.078 ± 0.020 and when the cells were exposed to 5 and 50 mg l^{-1} Ag NPs were 0.152 ± 0.026, 0.188 ± 0.010 mg ml^{-1} respectively, and protein content were 0.042 ± 0.023, 0.017 ± 0.004 when cells where exposed to 5 and 50 mg l^{-1} $AgNO_3$.

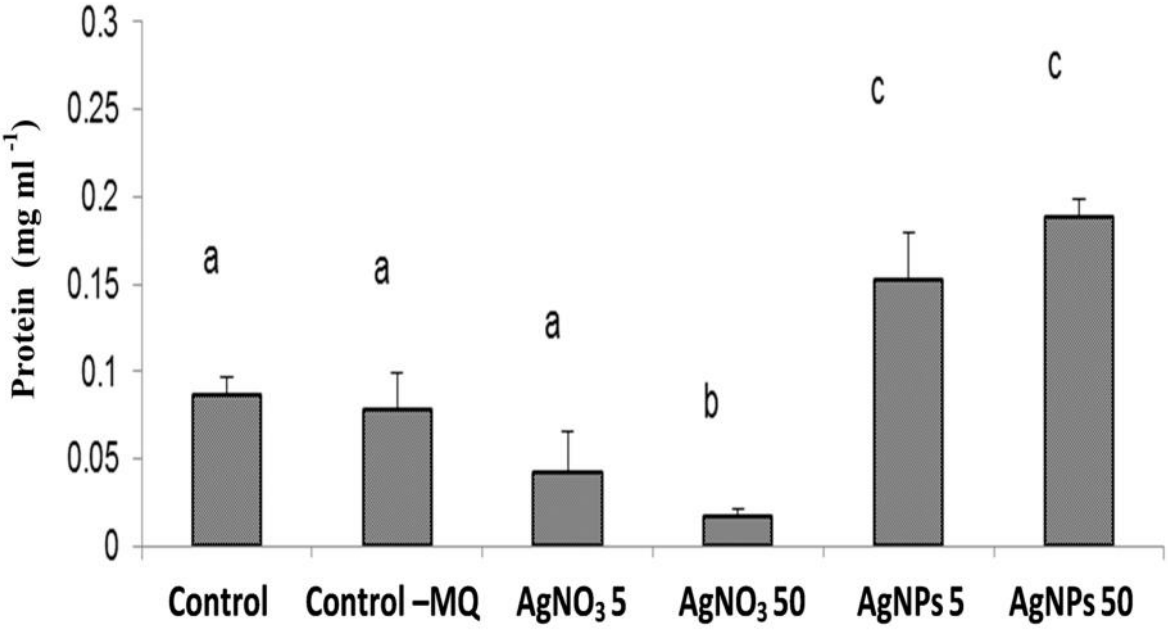


Figure 17 Protein content of fibroblast cells after 96h exposure to Ag NPs and $AgNO_3$.and inoculated with *C. albicans* in last 24 h. Bars with different letters are statistically different from each other (One way ANOVA, $p<0.05$).

Extracellular ethanol produced by *C. albicans* was also measured to investigate whether the yeast cells were capable of aerobic metabolism (no ethanol production) or if they used fermentation to make ATP and therefore produce ethanol (Fig 18). The main findings were that ethanol production before incubating the plates (mean ± S.E) was about 0.07 ± 0.02 µmols ml^{-1}, and after 24h incubations with the yeast, the ethanol production was highest when the fibroblasts were grown on uncoated silicone 43.2 ± 25.02 µmols ml^{-1}, since *Candida* grew more than the on the other treatments. However when the silicone was coated with 5 and 50 mg l^{-l} Ag NPs, ethanol production was less at 1.8 ± 1.08 and 1.1 ± 0.50 µmols ml^{-1} respectively. Whereas ethanol production was 3.6 ± 1.19 and 6.7± 4.98 µmols ml^{-1} when silicone were coated with 5 and 50 mg l^{-l} $AgNO_3$ respectively.

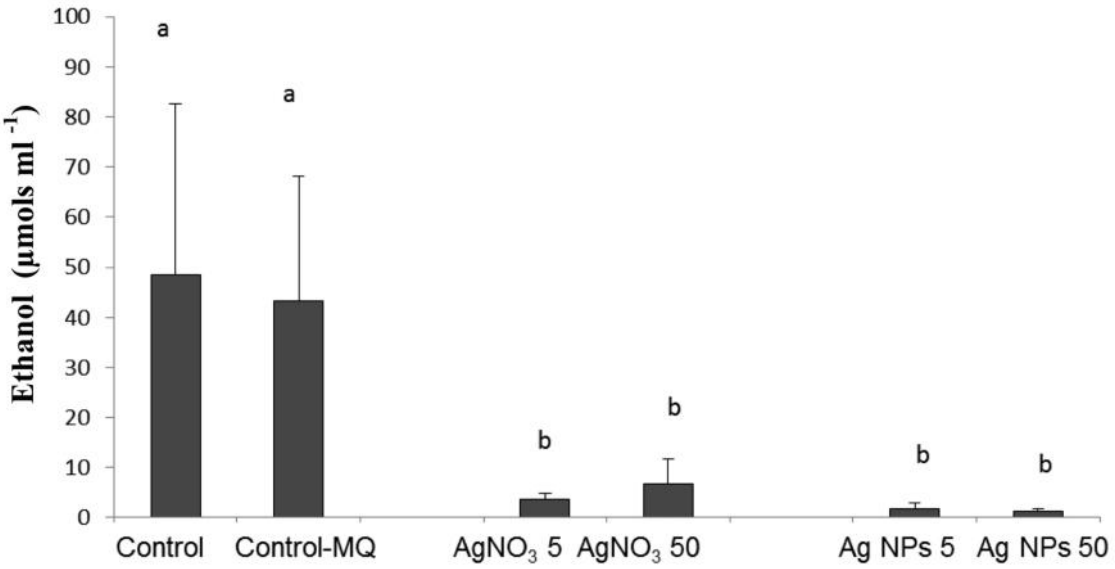


Figure 18 Ethanol productions by *C. albicans* into external media after 24h. (*n* = 4). Data are expressed as (Mean ± S.E) numbers with different letters are statistically different from each other (Kruskal-Wallis test, $p<0.05$). Ethanol production before incubating the plates was 0.07 ± 0.023 µmols ml^{-1}. After 24h incubations ethanol production of the cells grown on uncoated silicone was higher compared to both 5 and 50 mg l^{-l} Ag NPs and $AgNO_3$.

Discussion

C. albicans infection remains one of most significant problems of facial prostheses made of silicone. This study revealed that facial prostheses coated with Ag NPs preserved fibroblast cells from fungal infection compared to uncoated silicone prosthesis; Ag NPs also inhibited the production of ethanol by *C.albicans*. Furthermore, Ag NPs as a coating over silicone had no toxic effect on fibroblast cells compared to $AgNO_3$ which was toxic as a coating. Similar observations were made when fibroblasts were exposed via Ag additions to the cell culture media. Overall, this study shows that coating the silicone material used for facial prosthesis with 50 mg l^{-1} Ag NPs is biocompatible with dermal fibroblast cells and was able to prevent fungal infection. Coating of silicone prosthesis with Ag NPs could therefore be a clinically relevant method to prevent fungal infection in patients with maxillofacial prostheses.

Ag NPs and $AgNO_3$ characterisation

The hydrodynamic diameter of aggregates was measured by nanoparticle tracking analysis (NTA) shown in Figures 3 and 4. The main findings were that control Milli-Q water showed few/no particles which could be due to background noise of the instrument, while culture media control (without silver) showed a hydrodynamic diameter of aggregates (189 ± 19 nm). The latter is likely due to NaCl crystals and colloidal materials such as the protein and FBS in the culture media. This high

background of existing particulate material in the culture media prevented any reliable detection of the particle distributions due to the Ag NPs or AgCl crystals formed from $AgNO_3$. This masking of the Ag NP distribution due to organic material or salt crystals in biological media is to be expected and has been reported by Besinis et al. (2012) for salines and bacterial broth mixtures for the same nanomaterials used here.

The hydrodynamic diameter of aggregates in Milli-Q water, increased with increasing concentration, while the behaviour of Ag NPs and $AgNO_3$ in culture media was dissimilar, as it is difficult to visualise individual particles and track their Brownian motion when Ag NPs and $AgNO_3$ added into media. The increase in mean hydrodynamic of aggregates with increasing Ag NP concentration could simply be a function of particle collision rate;with more particles more collisions may form aggregates.

Effect of silver nanoparticles on fibroblast cells

To confirm Ag exposure to fibroblast cells, Ag accumulation in/on the fibroblast cells were measured (Table 2), as well as Ag concentration in the media (Table 5). When Ag was added into media after 24 h 20 % of the expected total Ag concentration was measured from the Ag NP treatment at the nominal concentration of 5 mg l^{-1}; and 27 % of the expected value at a nominal concentration of 50 mg l^{-1}. This effect was probably mainly due to particle settling from the media, although there are also inefficiencies in detecting Ag from Ag NPs by ICP-OES. Interestingly, despite very reliable detection of Ag from $AgNO_3$ by ICP-OES, the values in the media were also lower than the expected nominal concentrations, but not as low as those for Ag NPs.

For $AgNO_3$ 53% of the total Ag was measured at the nominal concentration of 5 mg l^{-1} and 46 % at the nominal concentration of 50 mg l^{-1}, indicating that most of silver had been either up taken by cells or combined to culture media and settled very quickly. Furthermore when the silver was measured every day for three days, it revealed that there was a constant release of silver from silicone coated with $AgNO_3$ to the media, compared to silicone coated with Ag NPs. There are two explanations for this: (i) the culture media contained some debris of fibroblast cells that picked up silver nitrate, or (ii) distressing the plates every day to change culture media might cause damage to the coating, the former seems most likely explanation.

The results of silver accumulation revealed that, silver accumulation after washing the cells was higher when cells were exposed for 24h to Ag NPs and $AgNO_3$ prepared in the media compared to silver accumulation in cells grown on silver coated silicone. Although the cells were exposed for a longer duration with direct contact with silver in the coating experiment, the total Ag accumulation was less in fibroblasts compared to those in the previous experiment exposed via additions to the cell culture media. This indicates that the silver was not bioavailable as a coating and/or was less easily removed from the surface of silicone even after washing the cells twice. The latter seems likely since the SEM images showed particles adhered to the surface of the silicone (see below). Studies showed that fibroblast cells can take up silver particles, for instance, a study revealed when primary fibroblasts cells were exposed to 30 µg l^{-1} Ag NPs in the culture media for 24 h, Ag were accumulated in the mitochondria when visualized by transmission electron microscope (Arora et al., 2009). Furthermore, another study by Lu et al, (2010) found that Ag NPs at 100 µg ml^{-1} were able to aggregate inside the keratinocyte which was determined by high-resolution ICP-OES and ICP-MS.

When cells were exposed to $AgNO_3$ as a coating, or and when added in to the media, the treatments caused the cells to detach from the dishes at both concentrations. Thus, in the present study the lethal concentrations of $AgNO_3$ against human dermal fibroblast cells were below 5 mg l^{-l}. Similarly, Hidalgo et al. (1998) determined that $AgNO_3$ at concentrations 4.12 and 82.4 µmol l^{-1} produced inhibition in human dermal fibroblast proliferation using (Eagle's Minimal Essential Medium). In contrast, in the present study when cells were exposed to Ag NPs as a coating and when added in to media, the cells were viable and well attached to the dishes, with apparently limited toxicity. Panáček et al. (2009) also found limited effects of using 30 mg l^{-1} Ag NPs against human fibroblast cells. Furthermore, Wen et al, (2007) reported that when Ag NPs, at concentrations 30 and 50 % *w/v* inoculated into confluent human fibroblasts (Ag NPs diluted in culture broth DMEM), inhibited the viability of fission of human fibroblasts, but without causing internal damage.

The low toxicity of nanoparticles may be due to the presence of biomolecules like proteins in culture media (10% FBS) reducing the bioavailability of any silver ions released, or by protein adsorbing to the surface of the particles, thus preventing the reactive surface of the particles from coming into direct contact with the cell membranes. Greulich et al. (2012) has reported that the release of silver ions from Ag NPs may be decreased by the presence of proteins. Additionally, silver may precipitate and form poorly soluble salts like silver chloride due to the presence of chloride in the culture media. Besinis et al, (2012) found that $AgNO_3$ rapidly forms insoluble AgCl crystals, but with a small fraction of apparently dissolved silver (0.01%) being likely responsible for toxicity,, while most of the Ag NPs had settled

very quickly and only about 10% of Ag NPs remained in the media (similar to the findings here), which was responsible for its toxicity against bacteria.

AgNPs and $AgNO_3$ as a coating on the silicone surface

This study showed that silver coating of the prostheses with Ag NPs or $AgNO_3$ was not easily removed from the surface of silicone. SEM images showed that when the surface of silicone was coated with Ag NPs (with and without sterilisation), the particles were attached to the surface, even after undergoing of several stages of media changing and washings for SEM preparation (Fig 12). Interestingly, when the surface was coated with $AgNO_3$, the PBS washing step appeared to play a vital role in attaching the silver to the surface of silicone compared to unwashed silicone (Fig 14). This could be due to the presence of chloride in PBS which might be responsible for formation of insoluble AgCl on the surface of silicone. The EDS measurements showed that the material was rich in both Ag and Cl. It may also be possible that during the formation of AgCl the crystal became annealed to the surface of the silicone.

The insoluble AgCl form is regarded as having low/negligible toxicity. Contreras et al. (2010) demonstrated that more than one hour of contact of human gingival fibroblasts cells with AgCl was necessary to induce irreversible cell death, but still the type of cell death was not clarified and free silver ions may have been present in the media. Additionally, Ratte (1999) predicted that the presence of ionic silver (the toxic form) will be decreased with increasing chloride concentration, and this explains the low toxicity of Ag in our culture conditions. However, Ramstedt et al. (2009) demonstrated that even in the presence of 10% FBS plus the media

containing likely mmolar amounts of chloride, that cytotoxic effects were seen on bronchial epithelial cells, alveolar epithelial cells and mouse fibroblast cells.

Effect of silver nanoparticles on electrolytes in fibroblast cells and *C. albicans*

Silver is well known for its ability to inhibit Na^+,K^+-ATPase membrane bound transport system (Hussain et al., 1994). This study measured cell homogenate concentrations of K^+ and Na^+ as well as in the media (Tables 3, 4 and 5). The main findings were that exposure of fibroblast cells to Ag NPs and $AgNO_3$ caused significant change in cellular electrolytes. The cells exposed for 24 h to Ag NPs prepared in the media caused a decrease in cell K^+ content (normalised to protein) which could be related to the inhibitory effect of silver on the Na^+ K^+-ATPase which would result in the normal outward K^+ gradient slowly depleting the cell of K^+. However, when the cells were exposed at the same concentrations to Ag NPs and $AgNO_3$ coated on silicone prostheses for longer duration (72h), they showed no K^+ depletion (Table 5). This indicates that the Ag NPs remained attached to silicone and did not release Ag ions to block the Na^+ pump or cause K^+ leak across the membrane. In keeping with this explanation, when cells were exposed to $AgNO_3$ the cell Na^+ content increased (diffusional influx of Na^+ down the electrochemical gradient). High concentrations of silver ions may block epithelial Na^+ channels (Bury., et al 1999), but in the media used here this would prevent Na^+ entry into cells, and so the most likely explanation of the silver-effect is on the Na^+ pump. Whereas when the cells were exposed to Ag for 96 h and in the last 24h inoculated with *C. albicans*,

Ag NPs caused an increase in cell K^+ content well as Na^+ content at both concentrations (normalised to protein).

In *C. albicans*, Na^+/H^+ antiporters play an important role in the maintenance and regulation of intracellular Na^+ and H^+ homeostasis (Soong et al., 2000), and like many organisms, the inward Na^+ gradient is used to drive the export of metabolic acid from the cell. Unsually, the Na^+/H^+ exchanger in *C. albicans* is not as specific as in higher organisms, and may also transport K^+ (Kinclová et al., 2001; Kinclová, et al., 2002). Fungi expend energy to accumulate large amounts of K^+, which serves as an important cofactor in many biosynthetic processes and is required for various biological functions, e.g., regulation of cell volume and intracellular pH (Kinclova-Zimmermannova et al., 2007). Few studies have been done on the effect of Ag on membrane permeability or osmoregulation in *C. albicans*. For instance, a study by Kim et al, (2009) indicated that nano-Ag exhibited potent antifungal effects on *C. albicans*, through destruction of membrane integrity by breaking down the membrane permeability barrier causing the leakage of ions and other materials, as well as forming pores and dissipating the electrical potential of the membrane. Furthermore Das et al, (2012) also revealed that Ag NPs were potent cytotoxic agents on the yeast cells wall (*Saccharomyces cerevisiae*) leading to altered membranes permeability and cell death. In this study, the change in K^+ and Na^+ content could not be due to block of the Na^+/H^+ antiporter alone by Ag. Block of the Na^+/H^+ would prevent Na^+ influx (less Na^+ in the cell), and membrane damage from Ag might increase the leak of K^+ from *C. albicans*. However, given that both fibroblast cells and *C. albicans* were present in the media, discerning the precise contributions of Ag interference with the different Na^+ tranporters in the cells requires further work.

Effect of silver nanoparticles on ethanol production of *C. albicans*

The main findings were that fibroblast cells grown over uncoated prostheses were detached from prosthesis when the cells were inoculated with *C. albicans* after 24 h exposure. While when the surface was coated with $AgNO_3$, the fibroblast cells were already detached due to toxic effect of $AgNO_3$, but equally few candida were observed when silicone was coated with 5 mg l^{-1} compared to 50 mg l^{-1} coating indicating that $AgNO_3$ was toxic to both fibroblasts and *C. albicans*. Whereas when fibroblast cells grown over Ag NPs-coated prostheses, at both concentrations of 5 and 50 mg l^{-1}, the fibroblasts were viable (though more fibroblast cells were attached at concentration 50 mg l^{-1} compared with 5 mg l^{-1}) when inoculated with *C. albicans* (Fig 15), which could be due to antifungal properties of Ag NPs against *C. albicans*. Ramstedt et al. (2009) showed that medium inoculated with AgCl were too low in concentrations to inhibit growth of *P. aeruginosa* but, was toxic against bronchial epithelial cells. In this study, Ag NPs silicone coated prostheses were more fungicidal against *C. albicans* than those coated with $AgNO_3$, as few candida cells were seen on the surface of silicone coated with $AgNO_3$ compared to Ag NPs; although $AgNO_3$ was more cytotoxic to fibroblast cells than Ag NPs.

The antifungal effect of Ag NPs has received only marginal attention. One study suggests that 2 mg l^{-1} nano-Ag induces programmed cell death through the accumulation of reactive oxygen species (ROS), in the early apoptosis phase (Hwang et al., 2012). In present study, Ag NPs at a concentration of 50 mg l^{-1} as a coating over silicone prosthesis, inhibited the growth of *C. albicans*, and at the same time preserved the growth of fibroblast cells (Fig 15). This suggests that a biocompatible coating concentration for Ag NPs was found, whilst also being

antifungal. Monteiro et al. (2011) 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. This indicates that *C. albicans* is at least sensitive to Ag NP additions to the external media. In the present study, Ag NP coating were used instead, but also showed fungicidal activity against the growth of *C. albicans* at both Ag NP concentrations but more at 50 mg l^{-1} compared to 5 mg l^{-1} suggesting that direct silver ion toxicity (contact toxicity) could play a part in the toxicity to *C. albicans* in this study. This suggests that the yeast was penetrating through the fibroblast layer to have direct contact with the Ag NP coating, or that the coating somehow prevented the attachment of the yeast to the fibroblast culture.

Furthermore, Ag NPs also altered ethanol production by *C. albicas* (Fig 18). The ethanol production by *C. albicans* was higher after 24 h incubation on the uncoated silicone compared to that coated with Ag NPs or $AgNO_3$. Yeast have the capability of aerobic metabolism, and can switch to anaerobic fermentation in less favourable conditions. In the present study, the ethanol production by yeast in the uncoated silicone treatment represents 43.2 µmols ml^{-1}. Similarly, Ogasawara et al, (2008) measured ethanol production in aerobic conditions for 2 h and was about 4 and 6 µg/10^5 cells at pH 7 and pH5 respectively. Ogasawara et al. (2006) stated that during aerobic conditions, fermentation dominates during the lag phase of growth and respiration takes over in the log phase, this explains high ethanol production in controls in our study. Additionally *C. albicans* can produce ethanol from a variety of substrates and glucose is one of the best substrates (high glucose in culture media) (Yajima et al., 2006). Furthermore, 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 pyruvate to alcohol. 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 (Hwang et al., 2012). Functioning mitochondria can also alter metabolism in response to changes in the external media. In this aspect, Ogasawara et al, (2008) revealed that ethanol production was inhibited when *C. albicans* was under anaerobic conditions (additions of sodium sulphite) at pH 5. Conditions in our experiment were not anaerobic (culture dishes were incubated with CO_2 at 37^oC, aerobic growth), but low pH alone may also have an effect on ethanol production, as these findings indicates that under anaerobic conditions, and in acidic conditions, *C. albicans* are unable to produce ethanol. After 24 h incubation with silicone coated with Ag NPs the media had a pH of about 5.9 to 6. The lowering of pH suggest some metabolic acidic production (e.g., by fermenting to lactate), but the absence of ethanol suggests that the fermentation was incomplete (not all the way to alcohol). Ag NP was confirmed not to interfere with the ethanol assay at the concentrations used in this experiment, so the likely explanation is that Ag NPs prevented the conversion of lactate to ethanol. Moreover, ethanol is produced by converting acetaldehyde into ethanol using the alcohol dehydrogenase enzyme (Strijbis et al., 2010). The absence of ethanol production in the present experiment could therefore be due to some Ag-dependent inhibition of the alcohol dehydrogenase. This is a new finding, and this requires further investigation.

Conclusion

Ag NP-coated prosthesis can prevent the attachment of *C. albicans* to silicone prostheses surface without any toxic effect to human dermal fibroblast cells at concentrations of 50 mg l^{-1}. The current experiments used high doses of the yeast to challenge the fibroblast cultures (10^6 yeast cells ml^{-1}), and yet the coatings 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, and perhaps a lower concentration of Ag NPs could be used. This study also revealed that silver was able to block of Na pump of both *C. albicans* and fibroblast cells, causing change and leakage of ions which will lead to alteration in membrane permeability. Furthermore, silver prevented the production of ethanol by *C.albicans*, possibly by inhibiting alcohol dehydrogenase enzyme. Overall, this coating could be of great use to prevent fungal infection for the patient, and appears to be biocompatible with human cells. However, the edges of the prosthesis may have prolonged contact with other cells in the dermis, as well as wandering immune cells, and further studies are required to increase the certainty of safe use of Ag NPs in prosthetics.

References

Arora, S., Jain, J., Rajwade, J. and Paknikar, K. (2009) Interactions of silver nanoparticles with primary mouse fibroblasts and liver cells. Toxicology and Applied Pharmacology, 236 (3), 310-318.

Aitken, R. J., Chaudhry, M. Q., Boxall, A. B. A. and Hull, M. (2006) Manufacture and use of nanomaterials: current status in the UK and global trends. Occupational Medicine, 56 (5). pp 300-306.

Atiyeh, B. S., Costagliola, M., Hayek, S. N. and Dibo, S. A. (2007) Effect of silver on burn wound infection control and healing: Review of the literature. Burns, 33 (2). pp 139-148.

Auffan, M., Rose, J., Bottero, J. Y., Lowry, G. V., Jolivet, J. P. and Wiesner, M. R. (2009) Towards a definition of inorganic nanoparticles from an environmental, health and safety perspective. Nature Nanotechnology, 4 (10). pp 634-641.

Aziz, T., Waters, M. and Jagger, R. (2003) Analysis of the properties of silicone rubber maxillofacial prosthetic materials. Journal of Dentistry, 31 (1). pp 67-74.

Beyth, N., Yudovin-Farber, I., Bahir, R., Domb, A. J. and Weiss, E. I. (2006) Antibacterial activity of dental composites containing quaternary ammonium polyethylenimine nanoparticles against *Streptococcus mutans*. Biomaterials, 27 (21). pp 3995-4002.

Bengt, K. (2002) Biological surface science. Surface Science, 500 (1–3). pp 656-677.

Benson, D. E., Burns, G. L. and Mohammad, S. F. (1996) Effects of plasma on adhesion of biofilm forming *Pseudomonas aeruginosa* and *Staphylococcus* epidermidis to fibrin substrate. ASAIO Journal, 42 (5). pp M655-660.

Besinis, A., De Peralta, T. and Handy, R. D. (2012) 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, in press.

Bell, E., Ivarsson, B. and Merrill, C. (1979) Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. Proceedings of the National Academy of Sciences, 76 (3). pp 1274-1278.

Borghi, E., Sciota, R., Biassoni, C., Cirasola, D., Cappelletti, L., Vizzini, L., Boracchi, P. and Morace, G. (2011) Cell surface hydrophobicity: a predictor of biofilm

production in Candida isolates. Journal of Medical Microbiology, 60 (5). pp 689-690.

Busscher, H. J., de Boer, C. E., Verkerke, G. J., Kalicharan, R., Schutte, H. K. and van der Mei, H. C. (1994) In Vitro ingrowth of yeasts into medical grade silicone rubber. International Biodeterioration and Biodegradation, 33 (4). pp 383-390.

Bury, N. R. and Wood, C. M. (1999) Mechanism of branchial apical silver uptake by rainbow trout is via the proton-coupled Na^+ channel. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 277 (5). pp R1385-R1391.

Burrell, R. E. (2003) A scientific perspective on the use of topical silver preparations. Ostomy Wound Management, 49 (5; SUPP). pp 19-24.

Chang, P. P., Hansen, N. A., Phoenix, R. D. and Schneid, T. R. (2009) The Effects of primers and surface bonding characteristics on the adhesion of polyurethane to . Journal of Prosthodontics, 18 (1). pp 23-31.

Chen, Y., Huang, L., Chan, C., Su, C., Chang, S., Chang, Y., Chen, M., Hung, C., Chen, W., Lin, F. and Lee, Y. (2004) SARS in hospital emergency room. Emerging Infectious Diseases, 10 pp 782 - 788.

Coenye, T., De Prijck, K., Nailis, H. and Nelis, H. (2011) Prevention of *Candida albicans* biofilm formation. Open Mycology Journal, 5 pp 9-20.

Contreras, R. G., Sakagami, H., Nakajlama, H. and Shimada, J. (2010) Type of cell death induced by various metal cations in cultured human gingival fibroblasts. In Vivo, 24 (4). pp 513-517.

Christian, P., Von der Kammer, F., Baalousha, M. and Hofmann, T. (2008) Nanoparticles: structure, properties, preparation and behaviour in environmental media. Ecotoxicology, 17 (5). pp 326-343.

Das, D. and Ahmed, G. (2012) Silver nanoparticles damage yeast cell wall. Journal of Biotechnology Vol. 3(2), pp.37-39

Davies, R. L. and Etris, S. F. (1997) The development and functions of silver in water purification and disease control. Catalysis Today, 36 (1). pp 107-114.

Deng, H.-Y., Zwetchkenbaum, S. and Noone, A.-M. (2004) Bond strength of silicone to polyurethane following immersion of silicone in cleaning solutions. The Journal of Prosthetic Dentistry, 91 (6). pp 582-585.

De Prijck, K., De Smet, N., Honraet, K., Christiaen, S., Coenye, T., Schacht, E. and Nelis, H. J. (2010) Inhibition of *Candida albicans* biofilm formation by antimycotics released from modified polydimethyl siloxane. Mycopathologia, 169 (3). pp 167-174.

Dominic, R., Shenoy, S. and Baliga, S. (2007) *Candida* biofilms in medical devices: evolving trends. Kathmandu University Medical Journal Vol. 5, No. 3, Issue 19, 431-436.

Engel, E., Michiardi, A., Navarro, M., Lacroix, D. and Planell, J. A. (2008) Nanotechnology in regenerative medicine: the materials side. Trends in Biotechnology, 26 (1). pp 39-47.

Estivill, D., Arias, A., Torres-Lana, A., Carrillo-Muñoz, A. J. and Arévalo, M. P. (2011) Biofilm formation by five species of Candida on three clinical materials. Journal of Microbiological Methods, 86 (2). pp 238-242.

Fadeel, B. and Garcia-Bennett, A. E. (2010) Better safe than sorry: Understanding the toxicological properties of inorganic nanoparticles manufactured for biomedical applications. Advanced Drug Delivery Reviews, 62 (3). pp 362-374.

Feng, Q., Wu, J., Chen, G., Cui, F., Kim, T. and Kim, J. (2000) A mechanistic study of the antibacterial effect of silver ions on Escherichia coli and Staphylococcus aureus. Journal of biomedical materials research, 52 (4). pp 662-668.

Ghosh, S., Kebaara, B. W., Atkin, A. L. and Nickerson, K. W. (2008) Regulation of aromatic alcohol production in *Candida albicans*. Applied and Environmental Microbiology, 74 (23). pp 7211-7218.

Goiato, M. C., Pesqueira, A. A., Santos, D. M. d., Zavanelli, A. C. and Ribeiro, P. d. P. (2009) Color stability comparison of silicone facial prostheses following disinfection. Journal of Prosthodontics, 18 (3). pp 242-244.

Graf, P., Mantion, A., Foelske, A., Shkilnyy, A., Mašić, A., Thünemann, A. F. and Taubert, A. (2009) Peptide‐coated silver nanoparticles: synthesis, surface chemistry, and pH‐triggered, reversible assembly into particle assemblies . Chemistry-A European Journal, 15 (23). pp 5831-5844.

Green, H., MacDougall, J., Tarnopolsky, M. and Melissa, N. (1999) Downregulation of Na^+-K^+-ATPase pumps in skeletal muscle with training in normobaric hypoxia . Journal of Applied Physiology, 86 (5). pp 1745-1748.

Greulich, C., Braun, D., Peetsch, A., Diendorf, J., Siebers, B., Epple, M. and Koller, M. (2012) The toxic effect of silver ions and silver nanoparticles towards bacteria and human cells occurs in the same concentration range . RSC Advances, 2 (17). pp 6981-6987.

Handy, R., von der Kammer, F., Lead, J., Hassellöv, M., Owen, R. and Crane, M. (2008) The ecotoxicology and chemistry of manufactured nanoparticles. Ecotoxicology, 17 (4). pp 287-314.

Handy, R. D. and Shaw, B. J. (2007) Toxic effects of nanoparticles and nanomaterials: Implications for public health, risk assessment and the public perception of nanotechnology . Health, Risk and Society, 9 (2). pp 125-144.

Hellstrand, E., Lynch, I., Andersson, A., Drakenberg, T., Dahlbäck, B., Dawson, K. A., Linse, S. and Cedervall, T. (2009) Complete high‐density lipoproteins in nanoparticle corona . FEBS Journal, 276 (12). pp 3372-3381.

Haro, R., Garciadeblas, B.and Rodriguez-Navarro, A. (1991) A novel P-type ATPase from yeast involved in sodium transport. FEBS Letters, 291 (2). pp 189-191.

Hidalgo, E., Bartolome, R., Barroso, C., n, o., Moreno, A. and Dominguez, C. (1998) Silver nitrate: antimicrobial activity related to cytotoxicity in cultured human fibroblasts . Skin Pharmacology and Physiology, 11 (3). pp 140-151.

Hooper, S. M., Westcott, T., Evans, P. L. L., Bocca, A. P. and Jagger, D. C. (2005) Implant-Supported Facial Prostheses Provided by a Maxillofacial Unit in a U.K. Regional Hospital: Longevity and Patient Opinions. Journal of Prosthodontics, 14 (1). pp 32-38.

Hussain, S., Meneghini, E., Moosmayer, M., Lacotte, D. and Anner, B. M. (1994) Potent and reversible interaction of silver with pure Na,K-ATPase and Na,K-ATPase-liposomes . Biochimica et Biophysica Acta (BBA) - Biomembranes, 1190 (2). pp 402-408.

Hwang, I. s., Lee, J., Hwang, J. H., Kim, K. J. and Lee, D. G. (2012) Silver nanoparticles induce apoptotic cell death in *Candida albicans* through the increase of hydroxyl radicals. FEBS Journal, 279 (7). pp 1327-1338.

Ilmén, M., Koivuranta, K., Ruohonen, L., Rajgarhia, V., Suominen, P. and Penttilä, M. (2013) Production of L-lactic acid by the yeast Candida sonorensis expressing heterologous bacterial and fungal lactate dehydrogenases. Microbial Cell Factories, 12 (1). pp 1-15.

Jani, R. M. and Schaaf, N. G. (1978) An evaluation of facial prostheses. The Journal of Prosthetic Dentistry, 39 (5). pp 546-550.

Kanaparthy, R. and Kanaparthy, A. (2011) The changing face of dentistry: nanotechnology. International Journal of Nanomedicine, 6 pp 2799.

Katsikogianni, M. and Missirlis, Y. (2004) Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria–material interactions. Eur. Cell Mater, 8 pp 37-57.

Kawata, K., Osawa, M. and Okabe, S. (2009) In vitro toxicity of silver nanoparticles at noncytotoxic doses to HepG2 human hepatoma cells. Environmental Science and Technology, 43 (15). pp 6046-6051.

Keck, C. M. and Müller, R. H. 'Nanotoxicological classification system (NCS) – A guide for the risk-benefit assessment of nanoparticulate drug delivery systems'. European Journal of Pharmaceutics and Biopharmaceutics, 84 (3). pp 445-448.

Khorasani, M., MoemenBellah, S., Mirzadeh, H. and Sadatnia, B. (2006) Effect of surface charge and hydrophobicity of polyurethanes and silicone rubbers on L929 cells response. Colloids and Surfaces B: Biointerfaces, 51 (2). pp 112-119.

Korting, H. C. and Braun-Falco, O. (1996) The effect of detergents on skin pH and its consequences. Clinics in dermatology, 14 (1). pp 23-28.

Kiat-amnuay, S., Johnston, D. A., Powers, J. M. and Jacob, R. F. (2005) Color Stability of Dry Earth Pigmented Maxillofacial Silicone A-2186 Subjected to Microwave Energy Exposure . Journal of Prosthodontics, 14 (2). pp 91-96.

Kim, K.-J., Sung, W. S., Suh, B. K., Moon, S.-K., Choi, J.-S., Kim, J. G. and Lee, D. G. (2009) Antifungal activity and mode of action of silver nano-particles on *Candida albicans* . Biometals, 22 (2). pp 235-242.

Kinclova-Zimmermannova, O.and Sychrová, H. (2007) Plasma-membrane Cnh1 Na^+/H^+ antiporter regulates potassium homeostasis in *Candida albicans*. *Microbiology*, 153 (8). pp 2603-2612.

Kinclová, O., Potier, S. and Sychrová, H. (2001) The Candida albicans Na+/H+ antiporter exports potassium and rubidium. FEBS Letters, 504 (1–2). pp 11-15.

Kinclová, O., Potier, S. and Sychrová, H. (2002) Difference in substrate specificity divides the yeast alkali-metal-cation/H^+ antiporters into two subfamilies. *Microbiology*, 148 (4). pp 1225-1232.

Kurtulmus, H., Kumbuloglu, O., Özcan, M., Ozdemir, G. and Vural, C. (2010) *Candida albicans* adherence on silicone elastomers: Effect of polymerisation duration and exposure to simulated saliva and nasal secretion. Dental Materials, 26 (1). pp 76-82.

Lemon, J. C., Chambers, M. S., Jacobsen, M. L. and and Powers, J. M. (1995) Color stability of facial prostheses. The Journal of Prosthetic Dentistry, 74 (6). pp 613-618.

Leow, M., Kour, A., Inglis, T., Kumarasinghe, G. and Pho, R. (1997) Fungal colonisation in digital silicone rubber prostheses. Prosthetics and Orthotics International, 21 (3). pp 195-198.

Lewis, D. H. and Castleberry, D. J. (1980) An assessment of recent advances in external maxillofacial materials. The Journal of Prosthetic Dentistry, 43 (4). pp 426-432.

Liau, S., Read, D., Pugh, W., Furr, J. and Russell, A. (1997) Interaction of silver nitrate with readily identifiable groups: relationship to the antibacterialaction of silver ions. Letters in Applied Microbiology, 25 (4). pp 279-283.

Lu, W., Senapati, D., Wang, S., Tovmachenko, O., Singh, A. K., Yu, H. and Ray, P. C. (2010) Effect of surface coating on the toxicity of silver nanomaterials on human skin keratinocytes. Chemical Physics Letters, 487 (1–3). pp 92-96.

Maller, Udita S., K. S. Karthik, and Sudhakara V. Maller. Maxillofacial prosthetic materials—past and present trends. Journal of Indian Academy of Dental Specialists 1.2 (2010), 25-30.

Mandracci, P., Mussano, F., Ricciardi, C., Ceruti, P., Pirri, F. and Carossa, S. (2008) Low temperature growth of thin film coatings for the surface modification of dental prostheses. Surface and Coatings Technology, 202 (11). pp 2477-2481.

Monteiro, D. R., Gorup, L. F., Silva, S., Negri, M., de Camargo, E. R., Oliveira, R., Barbosa, D. B. and Henriques, M. (2011) Silver colloidal nanoparticles: antifungal effect against adhered cells and biofilms of *Candida albicans* and *Candida glabrata. Biofouling*, 27 (7). pp 711-719.

Montgomery, P. C. and Kiat-Amnuay, S. (2010) Survey of Currently Used Materials for Fabrication of Extraoral Maxillofacial Prostheses in North America, Europe, Asia, and Australia. Journal of Prosthodontics-Implant Esthetic and Reconstructive Dentistry, 19 (6). pp 482-490.

Oei, J. D., Zhao, W. W., Chu, L., DeSilva, M. N., Ghimire, A., Rawls, H. R. and Whang, K. (2012) Antimicrobial acrylic materials with in situ generated silver nanoparticles. Journal of Biomedical Materials Research Part B: Applied Biomaterials, 100B (2). pp 409-415.

Ogasawara, A., Iino, Y., Sato, K., Nakajima, Y., Bessho, S., Watanabe, T., Mikami, T. and Matsumoto, T. (2008) Anti-Candida Activity of Sodium Sulfite. Biological and Pharmaceutical Bulletin, 31 (6). pp 1101-1103

Ogasawara, A., Odahara, K., Toume, M., Watanabe, T., Mikami, T. and Matsumoto, T. (2006) Change in the respiration system of *Candida albicans* in the lag and log growth phase. Biological and Pharmaceutical Bulletin, 29 (3). pp 448-450.

Padial-Molina, M., Galindo-Moreno, P., Fernández-Barbero, J. E., O'Valle, F., Jódar-Reyes, A. B., Ortega-Vinuesa, J. L. and Ramón-Torregrosa, P. J. (2011) Role of wettability and nanoroughness on interactions between osteoblast and modified silicon surfaces. Acta Biomaterialia, 7 (2). pp 771-778.

Panáček, A., Kolář, M., Večeřová, R., Prucek, R., Soukupová, J., Kryštof, V., Hamal, P., Zbořil, R. and Kvítek, L. (2009) Antifungal activity of silver nanoparticles against *Candida* spp. Biomaterials, 30 (31). pp 6333-6340

Pompilio, A., Piccolomini, R., Picciani, C., D Antonio, D., Savini, V. and Di Bonaventura, G. (2008) Factors associated with adherence to and biofilm formation on polystyrene by *Stenotrophomonas maltophilia*: the role of cell surface hydrophobicity and motility. FEMS Microbiology Letters, 287 (1). pp 41-47.

Plummer DT (1971) An Introduction to Practical Biochemistry. McGraw Hill, London, pp. 288–289.

Rai, M., Yadav, A. and Gade, A. (2009) Silver nanoparticles as a new generation of antimicrobials. Biotechnology Advances, 27 (1). pp 76-83.

Ramstedt, M., Ekstrand-Hammarström, B., Shchukarev, A. V., Bucht, A., Österlund, L., Welch, M. and Huck, W. T. S. (2009) Bacterial and mammalian cell response to poly(3-sulfopropyl methacrylate) brushes loaded with silver halide salts. Biomaterials, 30 (8). pp 1524-1531.

Ramage, G., Martínez, J. P. and López‐Ribot, J. L. (2006) Candida biofilms on implanted biomaterials: a clinically significant problem. FEMS Yeast Research, 6 (7). pp 979-986.

Roe, D., Karandikar, B., Bonn-Savage, N., Gibbins, B.and Roullet, J.-B. (2008) Antimicrobial surface functionalization of plastic catheters by silver nanoparticles. Journal of Antimicrobial Chemotherapy, 61 (4). pp 869-876.

Rothen-Rutishauser, B. M., Schürch, S., Haenni, B., Kapp, N. and Gehr, P. (2006) Interaction of fine particles and nanoparticles with red blood cells visualized with advanced microscopic techniques. Environmental Science and Technology, 40 (14). pp 4353-4359.

Schultz, A. G., Ong, K. J., MacCormack, T., Ma, G., Veinot, J. G. and Goss, G. G. (2012) Silver nanoparticles inhibit sodium uptake in juvenile rainbow trout (*Oncorhynchus mykiss*). Environmental Science and Technology, 46 (18). pp 10295-10301.

Schierholz, J. M. (1997) Physico-chemical properties of a rifampicin-releasing polydimethyl-siloxane shunt. Biomaterials, 18 (8). pp 635-641.

Soustre, J., Rodier, M.-H., Imbert-Bouyer, S., Daniault, G. and Imbert, C. (2004) Caspofungin modulates in vitro adherence of *Candida albicans* to plastic coated with extracellular matrix proteins. Journal of Antimicrobial Chemotherapy, 53 (3). pp 522-525.

Soong, T.-W., Yong, T.-F., Ramanan, N. and Wang, Y. (2000) The *Candida albicans* antiporter gene CNH1 has a role in Na^+ and H^+ transport, salt tolerance, and morphogenesis . Microbiology, 146 (5). pp 1035-1044.

Sozer, N. and Kokini, J. L. (2012) The applications of nanotechnology. Chemical analysis of food-Techniques and applications. Academic, Elsevier, pp 145-176.

Sobolewska, E., Fraczak, B., Czarnomysy-Furowicz, D., Ey-Chmielewska, H. and Karakulska, J. (2007) Bacteria adhesion to the surface of various prosthetics materials, Annales Academiae Medicae Stetinensis. pp. 68-71.

Spielmann, N. and Wong, D. T. (2011) Saliva: diagnostics and therapeutic perspectives. Oral Diseases, 17 (4). pp 345-354.

Subramani, Karthikeyan, Waqar Ahmed, and James K. Hartsfield, eds. *Nanobiomaterials in Clinical Dentistry*. William Andrew, 2012, pp1-14

Strijbis, K. and Distel, B. (2010) Intracellular acetyl unit transport in fungal carbon metabolism. Eukaryotic Cell, 9 (12). pp 1809-1815.

Tian, J., Wong, K. K., Ho, C. M., Lok, C. N., Yu, W. Y., Che, C. M., Chiu, J. F. and Tam, P. K. (2007) Topical delivery of silver nanoparticles promotes wound healing. Chem Med Chem, 2 (1). pp 129-136.

Tweden, K. S., Cameron, J. D., Razzouk, A. J., Holmberg, W. R.,and Kelly, S. J. (1997). Biocompatibility of silver-modified polyester for antimicrobial protection of prosthetic valves. The Journal of heart valve disease, 6(5),pp 553-561.

von Eiff, C., Jansen, B., Kohnen, W. and Becker, K. (2005) Infections associated with medical devices. Drugs, 65 (2). pp 179-214.

Warriner, R. and Burrell, R. (2005) Infection and the chronic wound: a focus on silver. Advances in Skin and Wound Care, 18 (8). pp 2-12.

Wen, H.-C., Lin, Y.-N., Jian, S.-R., Tseng, S.-C., Weng, M.-X., Liu, Y.-P., Lee, P.-T., Chen, P.-Y., Hsu, R.-Q. and Wu, W.-F. (2007) Observation of growth of human

fibroblasts on silver nanoparticles, Journal of Physics: Conference Series. IOP Publishing, pp. 445

Xiu, Z.-m., Zhang, Q.-b., Puppala, H. L., Colvin, V. L. and Alvarez, P. J. (2012) Negligible particle-specific antibacterial activity of silver nanoparticles. Nano letters, 12 (8). pp 4271-4275.

Yajima, D., Motani, H., Kamei, K., Sato, Y., Hayakawa, M. and Iwase, H. (2006) Ethanol production by *Candida albicans* in postmortem human blood samples: Effects of blood glucose level and dilution. Forensic Science International, 164 (2–3). pp 116-121.

Zortuk, M., Kesim, S., Kaya, E., Özbilge, H., Kiliç, K. and Çölgeçen, Ö. (2010) Bacterial adhesion of porphyromonas gingivalis on provisional fixed prosthetic materials. Dental Research Journal, 7 (1). pp 35.

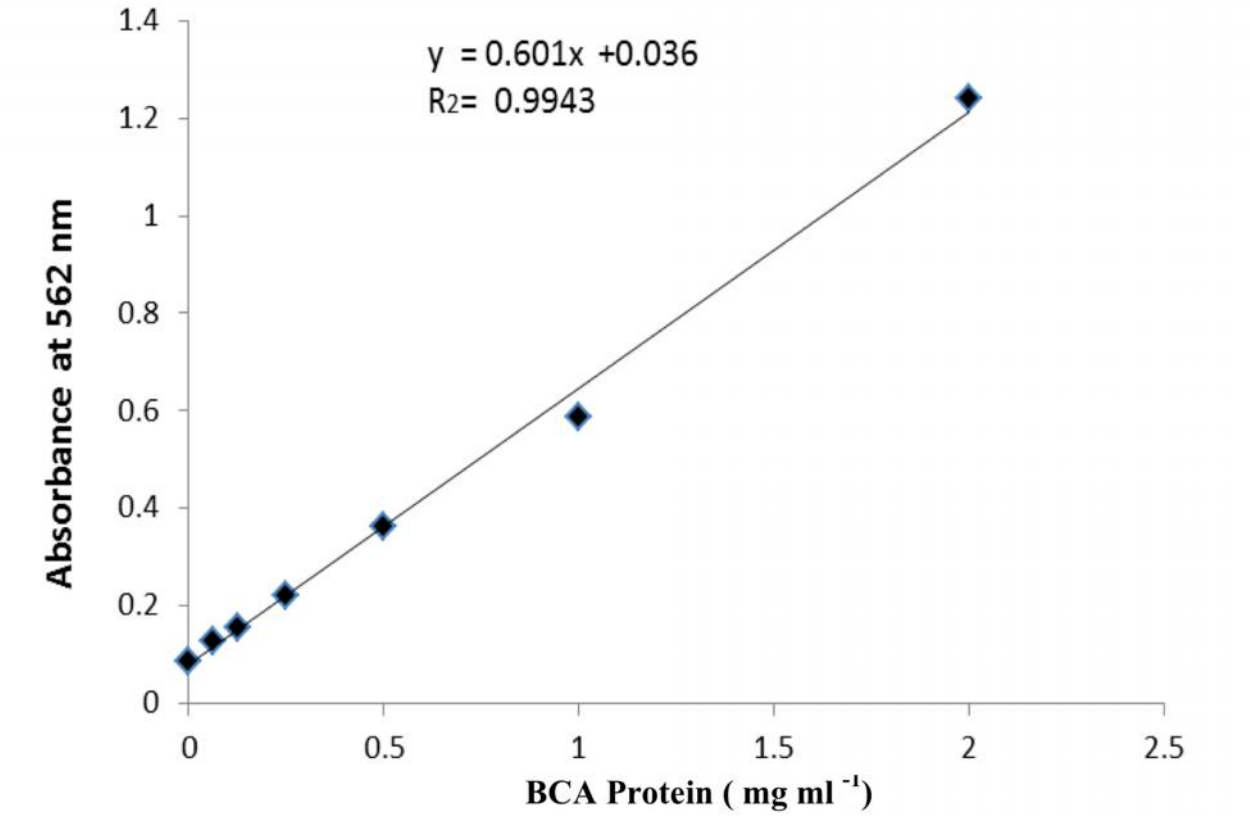
Appendix

Contain list of figures and tables that provides supplemental information for all experiments

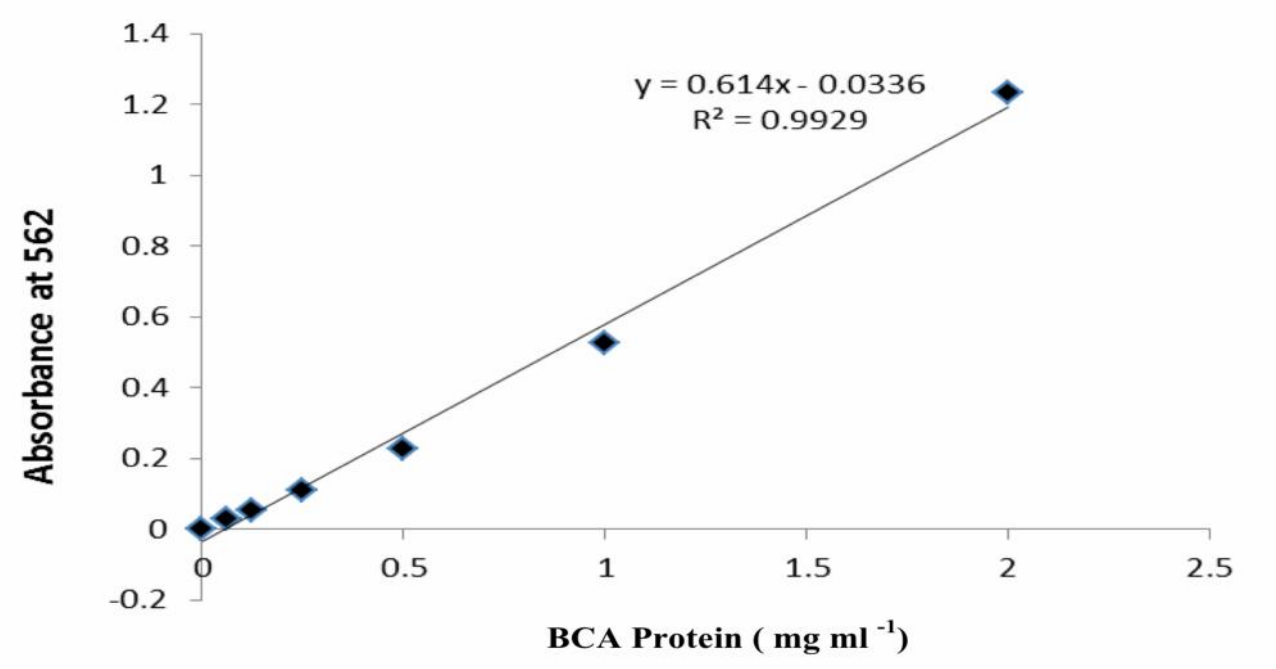
Appendix A: Protein

Protein assay standards from bovine serum albumin.

Standards	BCA Protein mg/ml (means ± S.E) *n* = 3		
	1st Experiment **(Fig a)**	2nd Experiment **(Fig b)**	3rd Experiment **(Fig c)**
0	0.086 ± 0.002	0.001 ± 0.000	0.107 ± 0.017
0.0625	0.129 ± 0.011	0.029 ± 0.000	0.114 ± 0.003
0.125	0.156 ± 0.002	0.054 ± 0.000	0.131 ± 0.010
0.25	0.221 ± 0.015	0.110 ± 0.001	0.181 ± 0.002
0.5	0.361 ± 0.035	0.229 ± 0.009	0.298 ± 0.018
1	0.586 ± 0.082	0.525 ± 0.033	0.441 ± 0.048
2	1.239 ± 0.036	1.232 ± 0.134	0.808 ± 0.019



a



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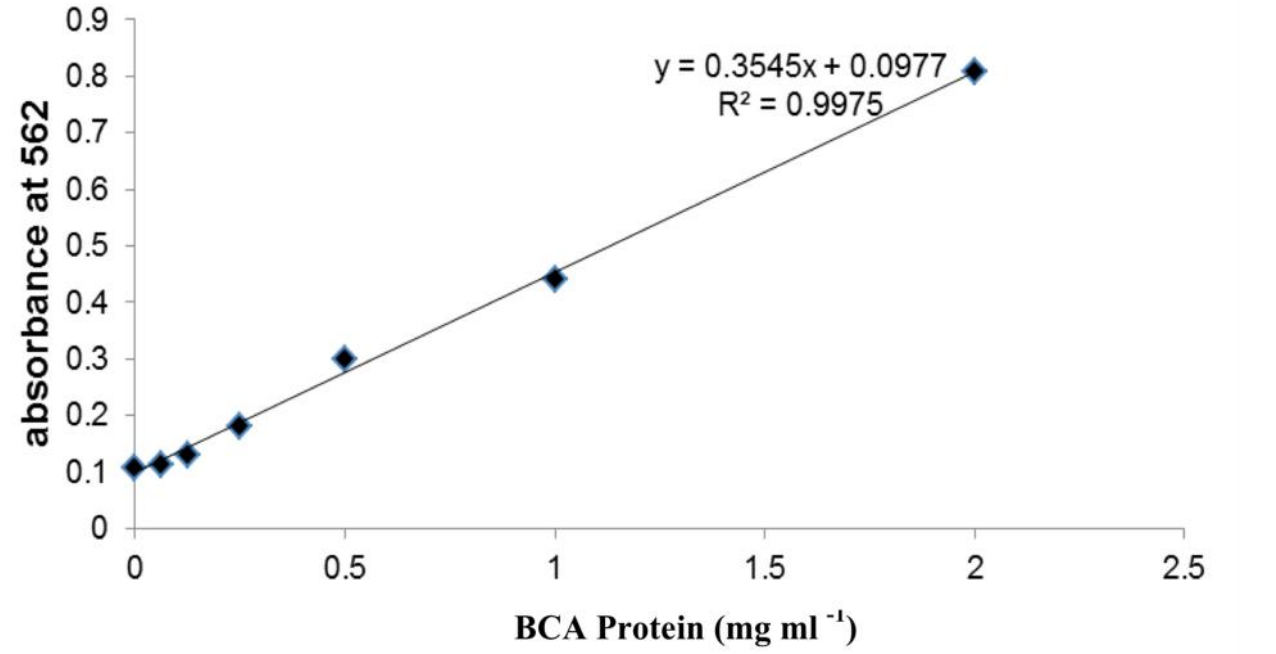

c

Figure: Protein assay standards from bovine serum albumin.a) first experiment standards; b) Second experiment standards; c) Third experiment standards.

Protein content in cell homogenate after 24h exposure to Ag NPs and $AgNO_{3.}$

Treatment mg per ml)	Protein content
control	0.168 ± 0.009 a
control-MQ	0.168 ± 0.013 a
$AgNO_3$ 5	0.083 ± 0.007 b
$AgNO_3$ 50	0.081 ± 0.010 b
AgNPs 5	0.226 ± 0.054 a
AgNPs 50	0.188 ± 0.009 a

*Values were measured by BCA assay. Data are means ± S.E(*n* = 6). Different letters are statistically different from each other (Kruskal-Wallis test, p<0.05).

Protein content in cell homogenate after 72h exposure to Ag NPs and $AgNO_{3.}$

Treatment (mg per ml)	Protein content
control	0.181 ± 0.026 a
control-MQ	0.166 ± 0.007 a
AgNO3 5	0.111 ± 0.035 b
AgNO3 50	0.110 ± 0.005 b
AgNPs 5	0.145 ± 0.023 a
AgNPs 50	0.151 ± 0.013 a

*Values were measured by BCA assay. Data are means ± S.E(*n* = 6).Different letters are statistically different from each other (One way ANOVA, *p*<0.05).

Protein content in cell homogenate after 96h exposure to Ag NPs and $AgNO_{3.}$

Treatment (mg per ml)	Protein content
control	0.086 ± 0.010 a
control-MQ	0.078 ± 0.020 a
AgNO3 5	0.042 ± 0.023 a
AgNO3 50	0.017 ± 0.004 b
AgNPs 5	0.152 ± 0.026 c
AgNPs 50	0.188 ± 0.010 c

*Values were measured by BCA assay. Data are means ± S.E(*n* = 6). Different letters are statistically different from each other (One way ANOVA, *p*<0.05).

Appendix B: LDH

LDH leak from fibroblast cells into media every day for 72h, cells were grown over silicone coated with different concentrations of Ag NPs and $AgNO_3$.

Treatment	Total LDH activity µmol min^{-1} ml^{-1}		
	Cumulative LDH after 24h	Cumulative LDH leak after 48h	Cumulative LDH leak after 72h
control	0.181 ± 0.007 a	0.310 ± 0.016 b	0.437 ± 0.023 b
control-$_{MQ}$	0.229 ± 0.034 a	0.505 ± 0.123 a	0.694 ± 0.122 a
$AgNO_3$ 5	0.335 ± 0.047 a	0.343 ± 0.048 b	0.343 ± 0.048 b
$AgNO_3$ 50	0.403 ± 0.079 a	0.403 ± 0.079 b	0.403 ± 0.079 b
AgNPs 5	0.146 ± 0.013 a	0.308 ± 0.014 a	0.487 ± 0.015 a
AgNPs 50	0.284 ± 0.028 a	0.411 ± 0.025 b	0.616 ± 0.020 c

*The effect of Ag NPs and $AgNO_3$ exposure on total LDH activity release (cumulative LDH each day) after 72h in fibroblast cells. Values are means ± S.E. (*n* = 6). Bars with different letters are statistically different from each other (One way ANOVA or Kruskal-Wallis test, $p<0.05$).

LDH leak from fibroblast cells into media every day for 96h, cells were grown over silicone coated with different concentrations of Ag NPs and $AgNO_3$.

Treatment	Total LDH activity µmol min^{-1} ml^{-1}			
	Cumulative LDH after 24h	Cumulative LDH leak after 48h	Cumulative LDH leak after 72h	Cumulative LDH leak after 96h
control	0.05 ±0.014 a	0.09 ±0.015 b	0.13 ±0.011 c	0.32 ±0.009 d
control-$_{MQ}$	0.031 ± 0.007 a	0.067 ± 0.006 b	0.100 ± 0.009 c	0.286 ± 0.006 d
$AgNO_3$ 5	0.110 ± 0.035 a	0.124 ± 0.033 b	0.133 ± 0.033 c	0.171 ± 0.039 d
$AgNO_3$ 50	0.236 ± 0.031 a	0.245 ± 0.031 a	0.255 ± 0.031 a	0.300 ± 0.034 a
AgNPs 5	0.110 ± 0.012 a	0.202 ± 0.010 a	0.286 ± 0.014 a	0.404 ± 0.023 a
AgNPs 50	0.098 ± 0.015 a	0.187 ±0.018 b	0.235 ± 0.027 c	0.340 ± 0.042 d

*The effect of Ag NPs and $AgNO_3$ exposure on total LDH activity release (cumulative LDH each day) after 96h in fibroblast cells. Values are means ± S.E. (*n* = 6). Bars with different letters are statistically different from each other (One way ANOVA or Kruskal-Wallis test, $p<0.05$).

Appendix C: Ethanol

Ethanol production by *C. albicans* into external media after 24h.

Treatment	Ethanol production by *C. albicans* micromole ml^{-1}
Time 0	0.07 ± 0.023 a
control	48.41 ± 34.179 b
control-MQ	43.24 ± 25.021 b
$AgNO_3$ 5	3.63 ± 1.191 c
$AgNO_3$ 50	6.73 ± 4.985 c
AgNPs 5	1.80 ± 1.086 c
AgNPs 50	1.1 ± 80.507 c

*Total ethanol production by *C. albicans* after 24h incubation. Values are means ± S.E(*n* = 4). Bars with different letters are statistically different from each other (Kruskal-Wallis test, $p<0.05$). Ethanol production before incubating the plates was 0.07 ± 0.023 µmols ml^{-1}. After 24h incubations ethanol production of the cells grown on uncoated silicone was higher compared to both 5 and 50 mg l^{-1} Ag NPs and $AgNO_3$.

Ethanol assay standards.

Standards	Ethanol µg ml^{-1} (means ± S.E.) *n* = 7 **(Fig d)**
0	0.025 ± 0.006
0.5	0.214 ± 0.098
5	0.762 ±0.129
50	1.480 ± 0.102
500	2.386 ± 0.110
5000	3.524 ± 0.233

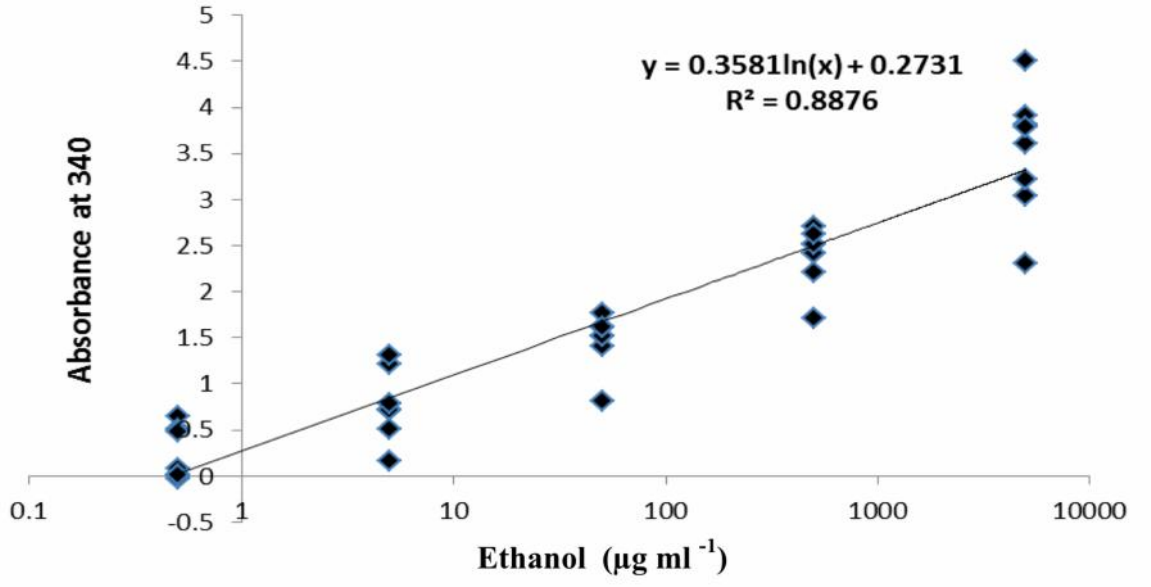

Figure d: Ethanol assay standards.

Appendix D: pH

Ph of the external media every day for 72 h. cells were grown over silicone coated with different concentrations of Ag NPs and $AgNO_3$.

Treatment	Ph of external media after 24	Ph of external media after 48	Ph of external media after 72
control	7.62 ± 0.042	7.61 ± 0.075	7.67 ± 0.049
control-MQ	7.69 ± 0.023	7.62 ± 0.034	7.67 ± 0.033
$AgNO_3$ 5	7.60 ± 0.024	7.67 ± 0.017	7.65 ± 0.021
$AgNO_3$ 50	7.60 ± 0.022	7.67 ± 0.019	7.66 ± 0.019
AgNPs 5	7.66 ± 0.049	7.60 ± 0.029	7.68 ± 0.025
AgNPs 50	7.68 ± 0.028	7.67 ± 0.018	7.64 ± 0.016

pH of the external media every day for 96h. cells were grown over silicone coated with different concentrations of Ag NPs and $AgNO_3$.

Treatment	pH of external media after 24	pH of external media after 48	pH of external media after 72	pH of external media after 72 (Incubated with *C. albicans* for 24h).
control	7.62 ± 0.047	7.70 ± 0.053	7.54 ± 0.126	6.68 ± 0.082
control-MQ	7.37 ± 0.233	7.63 ± 0.030	7.62 ± 0.049	6.74 ± 0.148
$AgNO_3$ 5	7.19 ± 0.275	7.59 ± 0.031	7.61 ± 0.037	6.15 ± 0.092
$AgNO_3$ 50	7.64 ± 0.050	7.60 ± 0.017	7.61 ± 0.023	6.13 ± 0.066
AgNPs 5	7.56 ± 0.015	7.61 ± 0.018	7.67 ± 0.035	5.90 ± 0.114
AgNPs 50	7.65 ± 0.033	7.65 ± 0.038	7.64 ± 0.037	6.06 ± 0.048