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Substrate independent silver nanoparticle based antibacterial coatings

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ABSTRACT

Infections arising from bacterial adhesion and colonization on medical device surfaces are a significant healthcare problem. Silver based antibacterial coatings have attracted a great deal of attention as a potential solution. This paper reports on the development of a silver nanoparticles based antibacterial surface that can be applied to any type of material surface. The silver nanoparticles were surface engineered with a monolayer of 2-mercaptosuccinic acid, which facilitates the immobilization of the nanoparticles to the solid surface, and also reduces the rate of oxidation of the nanoparticles, extending the lifetime of the coatings. The coatings had excellent antibacterial efficacy against three clinically significant pathogenic bacteria i.e. *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Studies with primary human fibroblast cells showed that the coatings had no cytotoxicity *in vitro*. Innate immune studies in cultures of primary macrophages demonstrated that the coatings do not significantly alter the level of expression of pro-inflammatory cytokines or the adhesion and viability of these cells. Collectively, these coatings have an optimal combination of properties that make them attractive for deposition on medical device surfaces such as wound dressings, catheters and implants.

1. Introduction

Today, medical devices are an important part of medical practice, improving patient wellbeing and saving millions of lives each year. Despite all these benefits, infections associated with medical devices constitute a significant healthcare burden [1,2]. These types of complications account for more than half of all hospital acquired infections (HAI) and are the most complex and costly to treat [3,4]. Hospital acquired infections annually affect more than two million patients in the USA and cause more than 100,000 deaths [5,6]. Infection rates in the case of biomedical implants can be as high as 4% for some devices and can cost in excess of $50,000 per patient to rectify the problem if revision of the implanted site is required [7–10]. Catheters are another type of medical device that are often the cause of infections. For example, central venous catheters cause an estimated 80,000 catheter-related bloodstream infections in the USA, resulting in 28,000 deaths per year [11,12]. Likewise, urinary catheters are estimated to be associated with 80% of all urinary tract infections [13,14].

Medical device-associated infections are mainly caused by bacterial attachment to, and colonization of the device surface [15–19]. For this reason, it is well accepted that preventing bacterial adhesion to the device surface through the application of an antibacterial coating is a potential solution [20,21]. Amongst the various strategies for generating antibacterial surfaces are those that are based on silver compounds and silver nanoparticles [14,22–24]. Silver (Ag) is precious metal that has been known to humans for more than seven millennia and has been appreciated not only because of its beauty but also because of its antibacterial properties [25–29]. The historical use of silver and silver formulations was mainly based on empirical observations that liquids did not spoil and wounds healed better. Over time, the medical use of silver became more sophisticated and by the end of the 19th century a number commercial products were available [28]. Silver was almost replaced by other antibacterial compounds after the penicillin revolution of the 1930s. However, the development of antibiotic resistance led to the resurgence of silver in medicine in the 1970s [28,29]. Currently, there is

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substantial research in silver coated medical devices [21,23,24,30–33]. This is mainly driven by the fact that silver is active against both Gram-positive and Gram-negative bacteria and resistance has not been yet been convincingly demonstrated for clinically-relevant pathogens. For more on the extensive efforts on development of silver based antibacterial coatings, we refer the reader to several instructive recent reviews on the topic [21,23,24,31–33].

When an antibacterial coating is being developed, several factors need to be taken into consideration in order to ensure efficacy against bacteria and successful wound healing. Among them are the capacity to kill bacteria, the need to eliminate the coating's cytotoxicity toward eukaryotic cells and the ability to avoid any undesired inflammatory response that may occur [34]. However, most reports are confined to presenting efficacy against one or more bacterial strains but fall short of reporting biocompatibility with mammalian tissues and cells and even more rarely deal with possible adverse innate immune inflammatory consequences. In the case of silver, it is generally accepted that mammalian cells are capable of tolerating higher concentrations compared to bacteria. However, it is evident that the concentration of silver which is demonstrably cytotoxic varies greatly for different mammalian cells [27]. In addition, the studies were not always conducted with the same silver compound and when nanoparticles were investigated, they had various sizes and surface functionalities. Nanocrystalline silver has been reported to reduce inflammation [35–37]. However, silver nitrate has been reported to increase inflammatory responses, and silver nanoparticles may cause the necrosis of immune cells which would also result in exacerbated inflammation [35]. It has also been reported that metallic silver surfaces induced cell death and a pro-inflammatory response in cultured J774 macrophages [38]. These contradictions clearly highlight the need for the full characterization of any new coating that contains silver. Without a doubt, the ultimate tests are *in vivo* studies. However, these types of studies are expensive and animal welfare and ethical processes require *in vitro* assessment prior to the use of an *in vivo* model.

Here, silver nanoparticle-based antibacterial surfaces were generated, such that deposition onto any type of substrate material was possible, for the purpose of coating a wide variety of medical devices. To achieve this goal, silver nanoparticles with appropriate surface functionality were synthesized in solution and adsorbed to model substrata pre-modified with a functional plasma polymer film. Plasma polymers were used because they have been demonstrated to grow independently from the substrate material except in the very early stages of deposition [39–41]. This approach also provides a ready control over the amount of immobilized silver nanoparticles. The efficacy of the coatings against both Gram-positive and Gram-negative bacteria was determined. The cytotoxicity was evaluated using primary human dermal fibroblast cells. Finally, evaluation of the inflammatory response potential was assessed by culture of primary macrophages and analysis of the cell viability and levels of expression of pro-inflammatory cytokines.

2. Experimental

2.1. Materials

2.1.1. Substrate preparation

All chemicals were used as received. Allylamine (AA) (98%), silver nitrate (99.99%), sodium borohydride, 2-mercaptosuccinic acid (97%), nitric acid (70%) were supplied by Aldrich (Australia). Hydrochloric acid (36%) by Ajax Finechem Pty. Ltd (Australia), Safranin O, acetic acid glacial and sodium hydroxide pellet were purchased from Chem Supply(Australia). All solution preparation and glassware cleaning procedures were performed using ultrapure (Milli-Q) water (resistivity 18.2 Ω). All glassware and magnet stirrer were soaked in aqua regia solution (3:1 conc.HCl: conc.HNO_3) and then rinsed thoroughly with the Milli-Q water before nanoparticle synthesis.

2.1.2. Microorganisms

Staphylococcus epidermidis strain ATCC 35984, *Staphylococcus aureus* ATCC 4330 (Methicillin-resistant *Staphylococcus aureus* (MRSA)), *Pseudomonas aeruginosa* ATCC 27853 were cultured in tryptone soya broth (TSB, Oxoid, UK) in incubator at 37 °C and 60% humidity.

2.1.3. Fibroblasts cell cultures

Human dermal fibroblasts (HDFs) were harvested and grown as described previously [42] from split thickness skin grafts (STSG's) obtained from scavenged tissue specimens following routine breast reductions and abdominoplasties. All patients gave informed consent for skin to be used for research through a protocol approved by the Ethical Committee at the Queen Elisabeth Hospital and the University of South Australia Human Ethics Committee. Briefly fibroblasts were grown in fibroblast culture medium (FCM) consisting of Dulbecco's Modified Eagle Medium (DMEM) high glucose (Gibco, Life Technologies, Australia), 10% v/v fetal calf serum (FCS, Ausgenex, Australia), 0.625 μg/mL amphotericin B (Sigma–Aldrich, Australia), 100 IU/mL penicillin and 100 mg/mL streptomycin (Gibco, Life Technologies, Australia) in an incubator at 37 °C, 5% CO_2 in a humidified atmosphere. The medium was changed every 3–4 days until the cells were 80% confluent. HDFs between passages 3 and 9 were used. Cells were stained with the Phallotoxin "Alexa Fluor 488® phalloidin" (a filamentous actin probe from Life) and DAPI- dilactate were purchased from Life Technologies, Invitrogen.

2.1.4. Immune response study

Roswell Park Memorial Institute (RPMI; Sigma Aldrich) medium or DMEM (Sigma Aldrich) was supplemented with 10% fetal calf serum, penicillin (100 U/ml), gentamycin (100 μg/ml), β-mercaptoethanol (2 mM), L-glutamine (2 mM), and HEPES (10 mM) to produce complete RPMI (cRPMI) or complete DMEM (cDMEM), respectively. The L-929 conditioned media was prepared by culturing L-929 cells in culture flasks to >95% confluency and cultured in cDMEM until the media was exhausted. The conditioned media containing the required macrophage colony stimulating factor was removed, sterilely filtered and aliquoted for storage at −20 °C until use.

2.2. Silver nanoparticle-based antibacterial coating preparation

2.2.1. Synthesis of silver nanoparticles capped with mercaptosuccinic acid (AgNPs@MSA)

Mercaptosuccinic acid (MSA) modified silver nanoparticles (AgNPs) were synthesized by mixing 12 mL of 2 mM silver nitrate ($AgNO_3$) with 5 mL of 2 mM MSA under ice-cold condition and vigorous stirring followed by drop-wise addition of 0.5 mL 0.05 M sodium borohydride ($NaBH_4$). The color of the solution changed from colorless to dark red-brownish within a few seconds indicating formation of silver nanoparticles. The functionalized AgNPs colloidal solutions were sealed and stored in darkness. These nanoparticles have been shown to be stable up to twelve months (data not shown).

2.2.2. Deposition of allylamine plasma polymerized (AApp) thin film

Plasma polymerization was carried out in a custom-built reactor described previously [43,44] using a 13.56 MHz plasma generator and a matching network (Advanced Energy, USA). The 13 mm Thermanox cover slips substrates (Thermo Fisher Scientific, USA) were cleaned with ethanol and Milli-Q water before placing in the reactor chamber. Samples were air cleaned by exposure to air plasma for 2 min at pressure of 2.5×10^{-2} mbar using a power of 20 W. The deposition of allylamine (AA) was carried out at a pressure of 2×10^{-3} mbar and an input radio frequency (RF) power of 10 W for 5 min at a monomer flow rate of 10 sccm. These conditions result in nitrogen-rich films with a thickness of about 24 nm. Allylamine plasma polymer (AApp) coated substrates are kept sealed overnight at room temperature any further treatment. The nanoparticles were deposited on silicon wafers for SEM and AFM analysis, and on Thermanox coverslips for bacterial and cell culture studies.

2.2.3. Immobilization of AgNPs@MSA onto the AApp surface

AApp coated substrates were immersed in the solution of AgNPs@MSA for defined time intervals ranging from 1 h to 24 h at 23 °C temperature. The samples were rinsed thoroughly with Milli-Q water, dried with nitrogen and kept sealed.

2.3. Characterization of nanoparticles

2.3.1. X-ray photoelectron spectroscopy (XPS)

XPS spectra were recorded on a SPECS SAGE spectrometer with an Mg Kα radiation source (hν 1253.6 eV) operating at 10 kV and 10 mA. The hemispherical analyzer was a Phoibos 150, with an MCD-9 detector. The elements present were identified from a survey spectrum recorded over the energy range 0–1000 eV at pass energy of 100 eV and a resolution of 0.5 eV. The areas under the photoelectron peaks in the spectrum were used to calculate the percentage atomic concentrations. High resolution (0.1 eV) spectra were then recorded for pertinent photoelectron peaks at pass energy of 20 eV to identify the chemical states of each element. All binding energies (BEs) were referenced to the C 1s neutral carbon peak at 285 eV to compensate for the effect of surface charging. The analysis area was circular and 5 mm in diameter. Processing and component fitting of high resolution spectra were performed with CasaXPS software (Casa Software Ltd).