Biomaterials 85 (2016) 142–151

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

# Antimicrobial peptide melimine coating for titanium and its *in vivo* antibacterial activity in rodent subcutaneous infection models

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ARTICLE INFO

*Article history:*
Received 1 October 2015
Received in revised form
22 January 2016
Accepted 27 January 2016
Available online 29 January 2016

*Keywords:*
Antimicrobial peptides
Biomaterial
*In vivo* infection model
*Pseudomonas*
*Staphylococcus*

ABSTRACT

Implant-associated infections represent a significant health problem and financial burden on healthcare systems. Current strategies for the treatment or prevention of such infections are still inadequate and new strategies are needed in this era of antibiotic resistance. Melimine, a synthetic antimicrobial peptide with broad spectrum activity against bacteria, fungi and protozoa, has been shown to be a promising candidate for development as antimicrobial coating for biomedical devices and implants. In this study, the *in vitro* and *in vivo* antimicrobial activity of melimine-coated titanium was tested. The titanium surface was amine-functionalised with 3-aminopropyltriethoxysilane (APTES) followed by reaction with a bifunctional linker 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylic 3-sulfo-n-hydroxysuccinimide ester (Sulfo-SMCC) to yield a maleimide functionalised surface. Melimine was then tethered to the surface via a thioether linkage through a Michael addition reaction of the cysteine at its N-terminus with the maleimide moiety. Melimine coating significantly reduced *in vitro* adhesion and biofilm formation of *Pseudomonas aeruginosa* by up to 62% and *Staphylococcus aureus* by up to 84% on the titanium substrates compared to the blank ($p < 0.05$). The activity was maintained after ethylene oxide gas sterilisation. The coating was also challenged in both mouse and rat subcutaneous infection models and was able to reduce the bacterial load by up to 2 $\log_{10}$ compared to the uncoated surface ($p < 0.05$). Melimine coating is a promising candidate for development as a surface antimicrobial that can withstand industrial sterilisation while showing good biocompatibility.



## 1. Introduction

Implant-associated infections are a significant economic burden to society. The treatment costs for biomedical device-related infections in just the United States are estimated to be as high as $27 billion per annum [1]. Infections associated with surgical implants are difficult to manage as they require long periods of antibiotic therapy and repeated surgical intervention [2,3]. Coupled with this, antibiotic resistance is emerging as a major challenge to our ability to control and treat implant-associated infections. It is estimated that by 2050, drug-resistant bacteria will kill 10 million people a year worldwide, which is more people than currently die from cancer [4]. The development of novel strategies for the prevention of bacterial colonisation and biofilm formation is urgently needed to reduce the extensive burden of disease in terms of both financial and human cost.

Current strategies for preventing device–associated infections [5], apart from improving sterile technique, involve coating with antibiotics (i.e. minocycline-rifampin), antimicrobial polymers [6], silver [7] and antiseptics (i.e. chlorhexidine and silver-sulfadiazine) [8]. The effectiveness of each strategy is often dependent on the clinical application and device configuration. Catheters are the most studied device configuration and have a long history of clinical use and trial data for device-associated infections. In a recent review of existing published clinical data by Ramritu et al. [9], minocycline-rifampin coated catheters were found to significantly outperform antiseptic catheters. However in randomized controlled trials, silver coating on catheters show no clinical advantage [8,10] and, in an Australian study, minocycline-rifampin central venous catheters showed no advantage over silver-eluting catheters for rates of bloodstream infections [11]. Other problems facing the development of antimicrobial coatings include loss of

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http://dx.doi.org/10.1016/j.biomaterials.2016.01.063
0142-9612/

activity after covalent attachment, and rapid loss of non-covalently bound antimicrobials due to out-diffusion [12]. This latter effect can lead to microbes being exposed to sub-lethal concentrations of the antimicrobials, potentially leading to the development of resistance *in-situ* [1]. Furthermore, drug releasing strategies or silver impregnated biomaterials can be cytotoxic to mammalian cells [13,14].

Antimicrobial peptides are another class of molecules that have been investigated for prevention of device-infections [15]. We have developed a coating based on a cationic peptide, melimine, which prevents microbial colonisation of biomaterials *in vitro* [16,17] and is active and safe to use when coated onto contact lenses [18] and worn by human volunteers [19]. Melimine has broad spectrum activity against a range of Gram-positive and Gram-negative bacteria (including multi-drug resistant strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*), fungi and the protozoan *Acanthamoeba* [17,20]. Importantly, when bacteria were cultured for 30 times at sub-inhibitory concentrations of melimine they did not gain resistance [17]. Moreover, melimine is not cytotoxic at well above active concentrations [17,20]. Furthermore, melimine coated surfaces can be sterilised by autoclaving [17], and their antimicrobial activity can be modulated by the method of coating to the biomaterial surface [16,21]. We have also shown melimine-coated contact lenses helps prevent and reduce the pathology of contact lens acute red eye and contact lens-induced peripheral ulcer in rabbit and guinea pig models [18]. Melimine acts on bacterial cell surfaces in solution by disrupting bacterial membranes [17], especially the integrity of the cytoplasmic membranes both for *P. aeruginosa* and *S. aureus* albeit with slightly different kinetics for the two bacterial types [22]. Once bound to a surface, melimine has also been found to disrupt bacterial surfaces [16] and reduce adhesion of bacteria (including antibiotic resistant strains) and other microbes [16,17,20]. This makes melimine an excellent candidate for development as an antimicrobial surface for biomaterials.

In this study, the effectiveness of tethered melimine on titanium surfaces against bacterial colonisation before and after ethylene oxide treatment was evaluated. The efficacy of melimine-coated titanium surfaces against *S. aureus* in two rodent subcutaneous implant infection models was also determined.

## 2. Materials and methods

### 2.1. Substrates

Titanium (Ti) disks or hollow round casings (Cochlear Ltd, Australia, 13 mm diameter) were immersed in 1:1 ratio of concentrated $H_2SO_4$ and HCl for 2 min, followed by sonication (Unisonics, FXP 10M, Australia) in distilled $H_2O$ for 3 × 15 min. The cleaned Ti substrates were stored in absolute ethanol.

### 2.2. Synthesis of peptides

Cys-Melimine (C T L I S W I K N K R K Q R P R V S R R R R R R G G R R R R) was synthesized by conventional solid-phase peptide synthesis protocols and was obtained from American Peptide Company (CA, USA). Peptides with >80% purity were used in experiments.

### 2.3. 3-Aminopropyltriethoxysilane (APTES) attachment to titanium

Amine functionalization was achieved using silanisation of the Ti substrates through vapour deposition previously described [23]. Briefly, cleaned Ti substrates (disks or casings) were placed on steel mesh within a glass vessel. APTES (Sigma–Aldrich, MO, USA) solution (10% w/v in dry toluene) was transferred into a small container underneath the steel mesh. The glass vessel was sealed and placed in an oven at 140 °C for 18 h. The substrates were then removed and cleaned by rinsing with dry toluene (3×).

### 2.4. Attachment of peptides

Peptide surface attachment through a cysteine residue has been described in our previous study [21]. Briefly, a 2 mg/mL crosslinker solution of 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylic 3-sulfo-*N*-hydroxysuccinimide ester (crosslinker; Sulfo-SMCC, ProteoChem, Inc., CO, USA) was freshly prepared in phosphate-buffered saline-ethylenediaminetetraacetic acid solution (PBS-EDTA, 50 mM phosphate, 0.15 M NaCl, 10 mM EDTA, pH7.2). The APTES treated surfaces were then immersed in the crosslinker solution and incubated at room temperature for 1 h. The surfaces (designated MAL) were then rinsed with PBS-EDTA and subsequently air-dried. The MAL surfaces were then immersed in cys-melimine solution (2 mg/mL in PBS-EDTA, 10 mM tris(2-carboxyethyl)phosphine) for 24 h and then rinsed 3× with PBS-EDTA. The samples were air-dried and then stored at room temperature until further use.

### 2.5. Preparation of dummy implant

Unmodified and melimine-coated hollow Ti casings (13 mm diameter) prepared as described above were encased in a molded medical-grade silicone rubber pocket to simulate a device configuration. The silicone rubber pocket had a 6 mm diameter opening centred on the top of the device as pictured in Fig. 1.

### 2.6. Estimation of peptide concentration on the modified surfaces

The quantity of peptide attached to the surfaces was estimated by a direct dye binding method [16,24]. Measurements were made in triplicate with at least two repeats. Briefly, coated and uncoated Titanium were immersed in Bradford reagent (Biorad, CA, USA) and shaken for 15 min. The supernatant was removed and its absorbance measured at 465 nm. A standard curve was constructed from a solution of melimine according to the manufacturer's directions but measured at 465 nm instead of 595 nm in order to determine the levels of remaining unbound dye at each concentration.

### 2.7. X-ray photoelectron spectroscopy (XPS)

XPS measurements were performed on an ESCALAB 220iXL. Monochromatic Al Kα X-rays (1486.6 eV) incident at 58° to the analyser lens were used to excite electrons from the sample. Emitted photoelectrons were collected on a hemispherical analyser with a multichannel detector at a take-off angle of 90° from the plane of the sample surface. The analysing chamber operated below $10^{-8}$ Torr, and the spot size was approximately 1 $mm^2$. The resolution of the spectrometer was ~0.6 eV. All energies are reported as

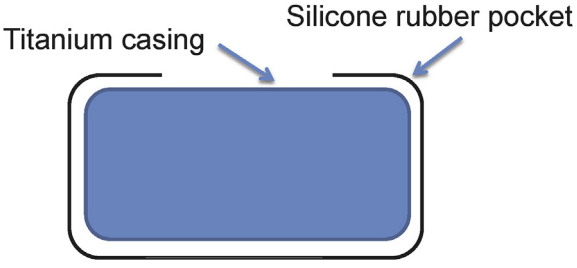


**Fig. 1.** Dummy implants of silicone rubber pocket with 13 mm titanium casing insert.