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Bacteria responsive antibacterial surfaces for indwelling device infections

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ABSTRACT

Indwelling device infections now represent life-threatening circumstances as a result of the biofilms' tolerance to antibiotic treatments. Current antibiotic impregnation approaches through sustained antibiotic release have some unsolved problems which include short life-span, narrowed antibacterial spectrum, ineffectiveness towards resistant mutants, and the potential to hasten the antibiotic resistance process. In this study, bacteria responsive anti-biofilm surfaces were developed using bioactive peptides with proved activity to antibiotic resistant bacteria and biofilms. Resulting surfaces were stable under physiological conditions and in the presence of high concentrations of salts (0.5 M NaCl) and biomacromolcules (1.0% DNA and 2.0% alginate), and thus showed good biocompatibility to various tissue cells. However, lytic peptide immobilized surfaces could sense bacteria adhesion and kill attached bacteria effectively and specifically, so biofilms were unable to develop on the lytic peptide immobilized surfaces. Bacteria responsive catheters remained biofilm free for up to a week. Therefore, the bacteria responsive antibacterial surfaces developed in this study represent new opportunities for indwelling device infections.

1. Introduction

Bacteria prefer to attach and grow on any solid surfaces to develop into biofilms, an assemblage of microbial cells that is associated with a surface. It is estimated that over 95% of bacteria existing in nature are found in biofilms. Bacterial cells of the same microbial species can exhibit extremely different physiological states in biofilms [1]. One of the most important features of biofilms is their tolerance towards antibiotic treatments. Bacteria living in biofilms can exhibit antibiotic tolerance up to several hundred times greater than planktonic bacteria [1–3]. As the wide use of biomedical devices and implants such as vascular grafts and catheters, biofilm infections pose potential life-threatening circumstances for patients [4–7]. Currently, the most effective strategy is antibiotic impregnation, which is accomplished by introducing antimicrobial agents onto biomedical device surfaces. Because a high local concentration of antibiotics is provided through sustained release, bacteria are killed before they grow in biofilms [8–10]. However, some unsolved problems are associated with current antibiotic impregnations: 1) since antibiotics for resistant bacteria such as MRSA are very limited at the present time [11,12], antibiotic impregnations do not work in resistant mutant involved infections; 2) antibiotic impregnations usually have narrowed antibacterial spectrum and short life-spans because only limited amounts and types of antibiotics can be loaded; 3) the sustained antibiotic release feature of antibiotic impregnations has a fatal drawback with respect to the genetic feedback and can hasten the antibiotic resistance process.

Lytic peptides are a group of recently discovered membrane-acting antibacterial peptides. Besides hardly developed resistance and a wide antibacterial spectrum, lytic peptides are active to antibiotic resistant bacteria (including MRSA) [13] and demonstrate good activity to preformed biofilms [14]. In this study, bacteria responsive antibacterial surfaces designed for biofilms associated with bio-fouling and biomedical device infections were constructed using lytic peptides. Resulting surfaces were stable under physiological conditions but could sense bacteria adhesion and biofilm formation to release bactericidal peptides at the contamination sites to kill attached bacteria specifically and effectively. Modified catheters with such responsive antibacterial surfaces maintained bio-fouling free after being continuously exposed to bacteria for a week.

2. Materials and methods

2.1. Materials and bacterial strains

MTT (methylthiazolydiphenyl-tetrazolium bromide), glucose, tryptic soy broth acrylic acid, acetic acid, and Toluidine Blue O were supplied by Sigma-Aldrich (St. Louis, MO). A LIVE/DEAD staining kit was purchased from Invitrogen Life Technologies (Carlsbad, CA). Peptides including fluorescent labeled peptides were synthesized by GenScript (Piscataway, NJ). The purity (>90%) of the synthesized peptide was confirmed by mass spectrometry and HPLC. *Staphylococcus aureus* (ATCC 25923, penicillin and methicillin sensitive, MSSA; ATCC 29213,

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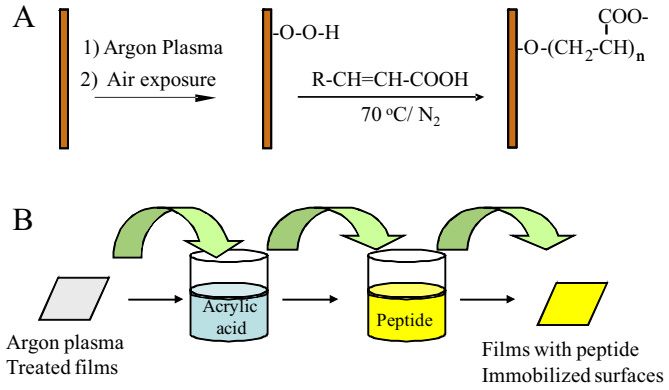

Fig. 1. A) Chemistry of plasma-mediated surface graft polymerization; B) A schematic illustration of lytic peptide immobilization on material surfaces.

penicillin resistant but methicillin sensitive, MSSA; ATCC 44300, penicillin and methicillin resistant, MRSA) and *Staphylococcus epidermidis* (ATCC 146), the two most common bacteria found in indwelling device infections, were purchased from the American Type Culture Collection (Manassas, VA). Human fetal osteoblasts (HfOB 1.19), human lung cells (A549), and mouse osteoblast precursor cells (MC-3T3) were also purchased from the American Type Culture Collection (Manassas, VA).

2.2. Lytic peptide characterization

The secondary structures of peptides were analyzed using CDPRO software based on the circular dichroism (CD) spectra of peptides recorded on a Jasco J-710 spectropolarimeter [15]. Peptide self-assembly in solution to form peptide aggregates was estimated by using 1-anilinonaphthalene-8-sulfonic acid (1, 8-ANS) as a fluorescence probe [15]. The ANS (20 μM) fluorescence emission spectrum changes associated with peptide aggregation were recorded on the fluorescence microplate reader (Biotek Inc.) by setting excitation wavelength at 369 nm.

2.3. Preparation of lytic peptides immobilized surfaces

The preparation process involved two steps: 1) plasma mediated surface modification (Fig. 1A); and 2) lytic peptide immobilization (Fig. 1B). Material samples (polyethylene terephthalate, PET, films and silicon wafers) were first activated using argon plasma produced in a Plasma Prep III device (SPI Supplies) [15] and then exposed to the air to generate hydroperoxide reactive centers (Fig. 1A). The amount of hydroperoxides was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) [16]. Surface polymerization was carried out at 70 °C under a nitrogen atmosphere in the presence of acrylic acid. Total poly(acrylic acid) grafted the surfaces was estimated through Toluidine Blue O staining [17]. Lytic peptide immobilization was done by immersing polymer grafted samples in lytic peptide solutions at room temperature for 1.0 h (Fig. 1B). After washing with distilled water to remove unbound peptides, samples were characterized and tested for peptide immobilization efficiency, stability, antibacterial activity, and biocompatibility.

2.4. Surface characterization

Graft polymerization and lytic peptide immobilization were analyzed using attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) (JASCO FT/IR-460Plus) and atomic force microscopy (AFM). ATR-FTIR was conducted in the percent transmittance mode in the range of 400–4000 cm^{-1} with a KRS-5 prism and an incident angle of 45°. AFM measurements were operated in air on a NSCRIPTOR dip pen nanolithography system (Pacific Nanotechnology, Inc.) using P-MAN-SICC-0 AFM cantilevers with a nominal force constant of 40 N/m [15].

2.5. Peptide release tests using fluorescent labeled peptides

Impregnations prepared from fluorescent labeled peptides were immersed in PBS solutions containing NaCl (0–5.0 M), DNA (1%), alginate (2%), or with varied pHs (pH = 4.5–7.0) and incubated at room temperature for 60 min. Peptides released into the solutions were monitored by measuring solution fluorescence intensity changes at 535 nm (λ_{ex} = 485 nm). Since pHs affected the fluorescence intensity of fluorescein, peptide release measured at different pHs was calibrated using fluorescein standard curves prepared at various pHs.

2.6. Biological stability in human plasma

Peptide immobilized samples were incubated with 1.0 mL pooled human sera and incubated at 37 °C for 4 h. At the end of incubation, trifluoroacetic acid was added (0.05%, final concentration) to precipitate plasma protein and release peptides from sample surfaces. Peptides in supernatants were then purified through $ZipTip_{C\text{-}18}$ column (Millipore). The amount of intact peptide was determined using MALDI-TOF mass spectrometry in the matrix containing α-cyano-4-hydrocinnamic acid (10 mg/mL in 50% acetonitrile with 0.05% trifluoroacetic acid). Measurements were made with Bruker UltraFlextreme mass spectrometer and the data analyzed with Flex analysis version 3.3 software (Bruker Daltonik GmbH).

2.7. Biocompatibility tests

Peptide immobilized samples (2.5 × 2.5 cm) were placed in 6-well plates. Human tissue cells, MC-3T3, in alpha MEM medium supplemented with 10% fetal bovine serum were seeded and incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h. At the end of incubation, samples were washed with saline and then stained with Live/Dead kit. Cell images were recorded using a Zeiss LSM510 Confocal Microscope by setting the excitation wavelength at 488 nm and the emission wavelengths at 505–530 nm (for the live cells) and 560 nm (for the dead cells). The percentage of green pixels out of total green and red pixels in captured cell images was calculated to estimate live/dead cell ratios [18].

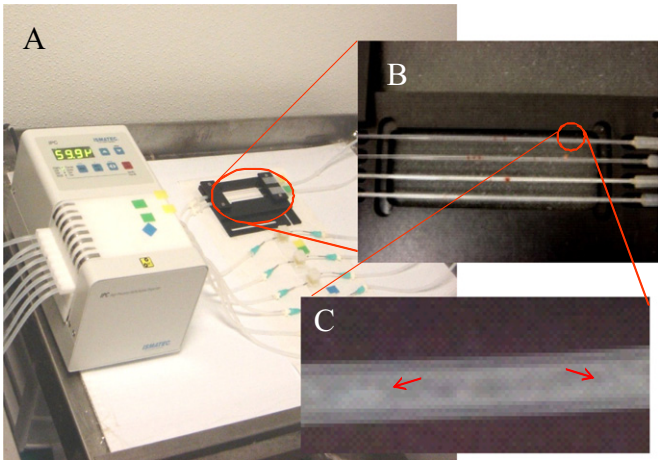

Fig. 2. A) Micro-fluidic biofilm cultivation system; B) Silicone tubes, 1.0 mm in diameter, mounted on multi-channel base; C) Images of *S. aureus* biofilms formed inside of silicone tubes after 24 h cultivation. Biofilms (white color clusters) of various sizes are indicated by arrows.