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A multi-defense strategy: Enhancing bactericidal activity of a medical grade polymer with a nitric oxide donor and surface-immobilized quaternary ammonium compound

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ABSTRACT

Although the use of biomedical devices in hospital-based care is inevitable, unfortunately, it is also one of the leading causes of the nosocomial infections, and thus demands development of novel antimicrobial materials for medical device fabrication. In the current study, a multi-defense mechanism against Gram-positive and Gram-negative bacteria is demonstrated by combining a nitric oxide (NO) releasing agent with a quaternary ammonium antimicrobial that can be covalently grafted to medical devices. Antibacterial polymeric composites were fabricated by incorporating an NO donor, *S*-nitroso-*N*-acetyl-penicillamine (SNAP) in CarboSil® polymer and top coated with surface immobilized benzophenone based quaternary ammonium antimicrobial (BPAM) small molecule. The results suggest that SNAP and BPAM individually have a different degree of toxicity towards Gram-positive and Gram-negative bacteria, while the SNAP-BPAM combination is effective in reducing both types of adhered viable bacteria equally well. SNAP-BPAM combinations reduced the adhered viable *Pseudomonas aeruginosa* by 99.0% and *Staphylococcus aureus* by 99.98% as compared to the control CarboSil films. Agar diffusion tests demonstrate that the diffusive nature of NO kills bacteria beyond the direct point of contact which the non-leaching BPAM cannot achieve alone. This is important for potential application in biofilm eradication. The live-dead bacteria staining shows that the SNAP-BPAM combination has more attached dead bacteria (than live) as compared to the controls. The SNAP-BPAM films have increased hydrophilicity and higher NO flux as compared to the SNAP films useful for preventing blood protein and bacterial adhesion. Overall the combination of SNAP and BPAM imparts different attributes to the polymeric composite that can be used in the fabrication of antimicrobial surfaces for various medical device applications.

Statement of significance

A significant increase in the biomedical device related infections (BDRIs), inability of the currently existing antimicrobial strategies to combat them and a proportional rise in the associated morbidity demands development of novel antimicrobial surfaces. Some of the major challenges associated with the currently used therapeutics are: antibiotic resistance and cytotoxicity. In the current study, engineered polymeric composites with multi-defense mechanism were fabricated to kill bacteria via both active and passive mode. This was done by incorporating a nitric oxide (NO) donor *S*-nitroso-*N*-acetypenicillamine (SNAP) in a medical grade polymer (CarboSil®) and a benzophenone based quaternary ammonium antimicrobial small molecule (BPAM) was surface immobilized as the top layer. The developed biomaterial was tested with Gram-positive and Gram-negative strains and was found to be effective against both the strains resulting in up to 99.98% reduction in viable bacterial count. This preventative strategy can be used to fabricate implantable biomedical devices (such as catheters, stents, extracorporeal circuits) to not only significantly limit biofilm formation but also to reduce the antibiotic dose which are usually given post infections.

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1. Introduction

Invasive medical devices predispose patients to more than 850,000 biomedical device-related infections (BDRIs) annually [1]. Out of various biomedical devices that are frequently used in clinical practices, intravenous infusion devices, and urinary catheters represent a major source of nosocomial septicemia [1,2]. For instance, the incidences of catheter-related bloodstream infections in the United States are approximately 80,000 cases/year in intensive care units alone and up to 250,000 cases/year in total with an attributable mortality of up to 35% [3]. Medical devices or implants create high risks for infections through several modes including (i) infecting patients directly by serving as a substrate for microorganisms' growth and colonization (biofilm), and/or (ii) damaging or invading epithelial layer in the host, which is a barrier to infection. It is evident that incorporation of microbicides in the medical devices would prevent BDRIs and ultimately bring down the cost associated with prolonged hospital stays. Presently, antibiotics and silver nanoparticles-based approaches are used to kill microorganisms; however, the emergence of antibiotic resistance and the issue of cytotoxicity and genotoxicity raises alarming concerns [4–7]. Most of the BDRIs involve multiple strains of microorganisms, and hence treatment with a single antibiotic is not effective [8]. It has been shown that tolerance level for antibiotics is 1000 times higher for biofilms when compared to bacterial suspensions [9]. This demands immediate attention as the use of multiple antibiotics and high doses only exacerbate the existing issues of antibiotic resistance and cytotoxicity. These challenges necessitate the exploration of alternative approaches to overcome the challenges of BDRIs caused by a wide variety of bacteria. In this regard, the combination of novel bactericidal agents such as surface-bound poly quaternary ammonium cations and nitric oxide (NO) donors can be advantageous due to their distinctive biocidal actions. This combination would not only assure high bactericidal efficiency but will also minimize the emergence of antibiotic resistance in pathogens due to their non-specific actions.

Membrane-disrupting poly "-onium" (quaternary ammonium) cations with various alkyl chain lengths have drawn considerable interest as a class of antimicrobial reagents because of their facile synthesis, broad application, outstanding antimicrobial activity, low cost, and low bacterial resistance [10–14]. Designing surfaces containing covalently bound poly "oniums" is one of the most successful strategies to date used to overcome surface microbial infections [15–18]. Among these polycations, the benzophenone chromophore has been utilized to develop photochemically grafted quaternary ammonium coatings [19,20]. Benzophenone based quaternary ammonium cations (BPAM) has been shown to exhibit instant contact killing and high biocidal activity against both Gram-positive and Gram-negative bacteria. BPAM also exhibits rapid surface attachment (within 1 min) to the polymer with mild UV irradiation and good mechanical durability (survives Taber abrasion testing) due to the high photochemical efficiency of benzophenone and cross-linked network structure with polymer post irradiation [20].

The generally accepted hypothesis for the biocidal mechanism of surface-immobilized quaternary ammoniums suggests that the positively charged "-onium" replaces the bacteria's natural counterions (Mg^{2+} and Ca^{2+}) and disrupt the ionic integrity of the membrane [21,22]. In addition, the alkyl chains of polycations intercalate into the phospholipid bilayer structure which disturbs its organization, forming holes in the membrane [23,24]. An alternative mechanism bacteria killing by quaternary ammoniums hypothesized that phospholipids are drawn out of bacterial lipid bilayer where they can permeate into cationic films [25,26]. Klibanov et al. have reported that bacteria fail to develop resistance to the lethal action of surface-bound quaternary ammonium because such surface acting antibacterial agent permeates bacterial membranes non-selectively via a 'brute-force' mechanism [11]. Although widely accepted as a highly efficient antibacterial agent, BPAM has several disadvantages that can limit its applications. The charge density of surface-bound quaternary ammonium might be eliminated by neutralization with anionic cellular components in the cytoplasm that is expelled out of the dead bacteria or screened by the layer of negatively charged dead bacterial cells covering the material's surface [27,28]. Moreover, due to its inability to act on bacteria that are not in intimate contact, BPAM cannot act on bacteria deeply embedded in a matrix of biofilm on a polymer surface. Furthermore, in the case of blood-contacting materials, fouling of the surface through the adsorption of protein can potentially hinder the surface-contact effect of quaternary ammonium cations such as BPAM on adhered bacteria. These limitations of the BPAM can be overcome by combining it with a nitric oxide (NO) donor as NO can diffuse through the biofilm matrix. Owing to its antimicrobial and antithrombotic potential, NO can provide antibacterial activity even if the surface of the material is compromised due to aforementioned reasons [29,30]. Schoenfisch group has done multiple studies demonstrating the combination of NO donors with other antimicrobials agents resulting in a significant improvement in the overall bactericidal activity of the material [31–33].

In nature, nitric oxide (NO) is an endogenously produced (macrophages, endothelial cells, neurons) free radical gas with a very short half-life of fewer than 5 s [34–39]. Healthy endothelium lining in the inner wall of blood vessels releases an estimated NO flux of $0.5–4.0 \times 10^{-10}$ mol min^{-1} cm^{-2} [40–43]. Endogenous NO is catalytically released by nitric oxide synthase in mammals and plays an important role in the immune response to infections caused by bacteria, fungus or viruses [44,45]. The NO released within the sinus cavities and macrophages functions as a natural antimicrobial agent to non-specifically combat pathogen invasion in mammals including humans [37]. Over the past two decades, NO-based therapies have emerged as a potential bactericidal agent to kill even the most prevalent pathogens causing hospital-acquired infection such as methicillin-resistant *Staphylococcus aureus* (MRSA) *and Pseudomonas aeruginosa,* and other bacteria including *Escherichia coli*, *Acinetobacter baumanii*, *Listeria monocytogenes,* and *Enterococcus faecalis* [29,46–54]. The nonspecific innate immune response of NO results from lipid peroxidation and tyrosine nitration in the cell wall, nitrosation of amines and thiols in the extracellular matrix, and DNA cleavage in the cellular matrix [55]. Due to the non-specific action of NO and rapid reduction of bacteria load at the infection locale, the possibility of NO resistant strains remains limited [48,52,56–58]. In addition, NO based material can be used in blood-contacting device applications because it temporarily inhibits the activation of platelets on the polymer's surface which BPAM cannot [59]. The realization of the immense potential of NO in creating biomimetic materials has encouraged researchers to synthesize several NO donor molecules to allow the storage and local delivery of NO at the material surface. *S*-nitroso-*N* acetylpenicillamine (SNAP) is one such NO donor that has widely been used in developing NO-releasing materials [43,52,60–63]. In the past, Worley et al., have demonstrated the (NO)-releasing quaternary ammonium (QA)-functionalized generation 1 (G1) and generation 4 (G4) poly(amidoamine) (PAMAM) dendrimers using *N*-diazonium diolate NO donors [32]. The present study demonstrates the enhanced bactericidal effect by permanent photocrosslinking and surface immobilization of BPAM on a CarboSil based polymeric composite with SNAP embedded as an NO donor.

This study investigated, the combined effect of the NO-releasing donor (SNAP) and quaternary ammonium (BPAM) to prevent the