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Long-term nitric oxide release and elevated temperature stability with *S*-nitroso-*N*-acetylpenicillamine (SNAP)-doped Elast-eon E2As polymer

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ABSTRACT

Nitric oxide (NO) is known to be a potent inhibitor of platelet activation and adhesion. Healthy endothelial cells that line the inner walls of all blood vessels exhibit a NO flux of 0.5–4 $\times$ 10^{-10} mol cm^{-2} min^{-1} that helps prevent thrombosis. Materials with a NO flux that is equivalent to this level are expected to exhibit similar anti-thrombotic properties. In this study, five biomedical grade polymers doped with *S*-nitroso-*N*-acetylpenicillamine (SNAP) were investigated for their potential to control the release of NO from the SNAP within the polymers, and further control the release of SNAP itself. SNAP in the Elast-eon E2As polymer creates an inexpensive, homogeneous coating that can locally deliver NO (via thermal and photochemical reactions) as well slowly release SNAP. Furthermore, SNAP is surprisingly stable in the E2As polymer, retaining 82% of the initial SNAP after 2 months storage at 37 °C. The E2As polymer containing SNAP was coated on the walls of extracorporeal circulation (ECC) circuits and exposed to 4 h blood flow in a rabbit model of extracorporeal circulation to examine the effects on platelet count, platelet function, clot area, and fibrinogen adsorption. After 4 h, platelet count was preserved at 100 $\pm$ 7% of baseline for the SNAP/E2As coated loops, compared to 60 $\pm$ 6% for E2As control circuits (n = 4). The SNAP/E2As coating also reduced the thrombus area when compared to the control (2.3 $\pm$ 0.6 and 3.4 $\pm$ 1.1 pixels/cm^2, respectively). The results suggest that the new SNAP/E2As coating has potential to improve the thromboresistance of intravascular catheters, grafts, and other blood-contacting medical devices, and exhibits excellent storage stability compared to previously reported NO release polymeric materials.

1. Introduction

Nitric oxide (NO) is an endogenous gas molecule that plays several key physiological roles, including prevention of platelet adhesion and activation, inhibiting bacterial adhesion and proliferation, enhancing vasodilation, promoting angiogenesis, and aiding in wound healing [1–10]. The effects of NO are highly dependent on the location and its concentration in the physiological system [11]. For example, endothelial cells that line the inner walls of healthy blood vessels produce an estimated NO surface flux of 0.5–4.0 $\times$ 10^{-10} mol cm^{-2} min^{-1} [12]. The function of many blood-contacting devices, including vascular grafts, stents, intravascular sensors, intravascular catheters, and extracorporeal life support circuits, can be impaired due to platelet activation and thrombus formation [13,14]. One approach to improve the hemocompatibility of such devices is the use of coating materials that mimic the endothelial cells with respect to NO release. Indeed, in recent years there has been considerable interest in developing NO-release and NO-generating materials that can be used to improve the biocompatibility of such devices [15–23].

Nitric oxide is highly reactive under physiological conditions and thus a wide range of NO donor molecules, with functional groups that can store and release NO, have been studied for potential biomedical applications. Such molecules include organic nitrates, metal-NO complexes, *N*-diazeniumdiolates, and *S*-nitrosothiols (RSNOs) [4,24]. Physiological RSNOs, such as *S*-nitrosohemoglobin and *S*-nitrosoglutathione (GSNO), are considered an endogenous reservoir of NO *in vivo* [4,25–27]. Other synthetic RSNOs, such as *S*-nitroso-*N*-acetyl-*L*-cysteine (SNAC) and *S*-nitroso-*N*-acetylpenicillamine (SNAP, Fig. 1A) have been shown to exhibit significant

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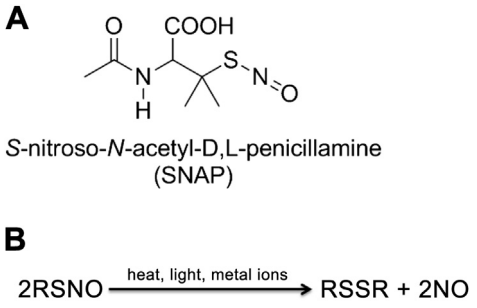

Fig. 1. Structure of (A) *S*-nitroso-*N*-acetylpenicillamine (SNAP) and (B) scheme of *S*-nitrosothiol (RSNO) decomposition, which can be catalyzed by metal ions (e.g., Cu^{+}), light, and heat, yielding the disulfide (RSSR) product and nitric oxide (NO).

antimicrobial and antithrombotic effects [28–31]. It has also been demonstrated that RSNOs are both vasodilators and potent inhibitors of platelet aggregation [32,33]. RSNOs undergo thermal decomposition releasing NO and producing a corresponding disulfide species (RSSR), as shown in Fig. 1B. The NO release from RSNOs can be catalyzed by metal ions (e.g., Cu^{+}) [34] and by light, through the irradiation at energies that correspond to the *S*-nitroso absorption bands at 340 and/or 590 nm [35–37]. It has been suggested that the more potent activity of RSNOs vs. NO as antiplatelet agents arises from the enhanced stability of RSNOs vs. NO, and generation of NO from RSNOs locally at the surface of platelets by membrane proteins that contain catalytic sites to convert RSNOs to NO [38].

Incorporation of RSNOs into polymers can extend the utility of these NO donors to be applicable as coatings in biomedical devices, providing localized NO release at the blood/device interface. Several NO-release polymers consisting of small-molecule RSNOs dispersed in various polymer matrices, including polyethylene glycol (PEG), poly(vinyl alcohol), poly(vinyl pyrrolidone), and Pluronic F127 hydrogel, have been reported [22,23,39–42]. These materials have potential applications for topical NO delivery on wounds via the diffusion of the hydrophilic RSNOs from the polymer to the tissue. In fact, daily application of a GSNO-containing hydrogel has been shown to accelerate the wound healing process [42]. However, the rapid leaching of the RSNOs from such polymers can significantly shorten the NO/RSNO release lifetime, lasting only several hours [22,39,40]. An alternate approach has been to synthesize RSNO-modified materials, where the RSNO functionality is covalently bound to the matrix. Fumed silica particles [18], dendrimers [43], polyurethanes [16], polyesters [15,44–46], poly(dimethylsiloxane) (PDMS) [19], xerogels [47,48], self-assembled monolayers [49], and poly(vinyl methyl ether-*co*-maleic anhydride) (PVMMA) [50] have all been modified with RSNO functionalities. Ricco et al. reported RSNO-modified xerogels that release NO for up to 14 d and exhibit reduced platelet and bacterial adhesion [47,48]. However, such RSNO-modified xerogels suffer from synthesis complications leading to cracking and non-uniform films, low RSNO conversion efficiency (maximum of 40% for the tertiary RSNO-modified xerogels), and thermal instability at room temperature that would limit their shelf-life. Many of the other RSNO modified materials reported to date exhibit both thermal and photoinitiated NO release, but these materials have not proven clinically useful due to their limited NO release lifetimes, low conversion to RSNO during synthesis, or lack of RSNO stability during storage This lack of stability of most NO release materials reported to date could pose a significant hurdle with regard commercializing medical devices that employ such materials, owing to the increased shipping costs to protect products from thermal degradation, etc. This could prevent the application of NO release materials in the biomedical market regardless of their potential benefits.

Another approach reported to achieve localized NO delivery at a polymer/blood interface is to use NO-generating coatings, in which immobilized catalysts (Cu(I/II) or organoselenium species) can generate NO from endogenous RSNOs [20,51–53]. Recently, an NO generating coating containing Cu^{0} nanoparticles was evaluated using a rabbit model for extracorporeal circulation (ECC) [20]. However, to achieve good efficacy in reducing thrombus formation, continuous infusion of SNAP was required to supplement the endogenous RSNO levels.

As an alternative to the continuous infusion of RSNO species, in this study we investigate several biomedical polymers that are capable of storing RSNO species. Such RSNO-doped coatings can release NO as well as potentially supplement the endogenous RSNO levels, if NO generating catalysts are also employed. Five biomedical polymers are examined for their potential to act as a storage reservoir for SNAP. These include: silicone rubber (poly(dimethylsiloxane)); Elast-eon E2As (a copolymer with a mixed soft segment of poly(-dimethylsiloxane) and poly(hexamethylene oxide) with a methylene diphenyl isocyanate (MDI) hard segment); CarboSil (a thermoplastic urethane copolymer with a mixed soft segment of poly(-dimethylsiloxane) and hydroxyl-terminated polycarbonate with a hard segment of an aromatic diisocyanate, MDI); Tecoflex SG80A (a poly(tetramethylene glycol) polyurethane capped with diisocyanatodicyclohexylmethane); and Tecophillic SP-60D-60 (an aliphatic, hydrophilic polyether-based polyurethane). Each of the SNAP-doped polymers are examined as films or coatings that can release NO thermally (at physiological temperature) and/or can serve as a reservoir to supplement endogenous RSNO levels (by SNAP diffusion into blood from the polymer). The SNAP-doped polymers are characterized for their *in vitro* NO/SNAP release, where the more hydrophobic polymers are expected to have slower SNAP/NO release under physiological conditions. The Elast-eon polymer has been reported to have excellent intrinsic biocompatibility and biostability properties, and exhibits low levels of blood protein adhesion [54,55]. Therefore, the SNAP/E2As polymer is further tested for the stability of SNAP during a 2-month storage period, in order to ascertain any self-life concerns. The new SNAP/E2As polymer is also examined for potential biomedical applications via an ECC rabbit model of thrombogenicity to assess preservation of platelet count and function, and thrombus area after 4 h of ECC.

2. Materials and methods

2.1. Materials

N-Acetyl-DL-penicillamine (NAP), sodium chloride, potassium chloride, sodium phosphate dibasic, potassium phosphate monobasic, ethylenediaminetetraacetic acid (EDTA), tetrahydrofuran (THF), sulfuric acid and *N*,*N*-dimethylacetamide (DMAc) were purchased from Sigma–Aldrich (St. Louis, MO). Methanol, hydrochloric acid and sulfuric acid were obtained from Fisher Scientific (Pittsburgh, PA). Tecophilic SP-60D-60 and Tecoflex SG-80A were products of Lubrizol Advanced Materials Inc. (Cleveland, OH). Dow Corning RTV 3140 Silicone Rubber (SR) was purchased from Ellsworth Adhesives (Germantown, WI). CarboSil 20 90A was from the Polymer Technology Group (Berkeley, CA). Elast-eon™ E2As was obtained from AorTech International, plc (Scoresby, Victoria, Australia). Human plasma fibrinogen containing ≥90% clottable proteins was a product of Calbiochem (La Jolla, CA) and fluorescein-labeled goat IgG (polyclonal) against denatured human fibrinogen was purchased from MP Biomedicals, LLC (Solon, OH). Black, polypropylene 96-well microtiter plates used for fluorescence measurements were obtained from Nalge Nunc International (Rochester, NY). All aqueous solutions were prepared with 18.2 MΩ deionized water using a Milli-Q filter (Millipore Corp., Billerica, MA). Phosphate buffered saline (PBS), pH 7.4, containing 138 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate, 100 μM EDTA was used for all *in vitro* experiments.

2.2. Synthesis of SNAP

SNAP was synthesized using a modified version of a previously reported method [56]. Briefly, an equimolar ratio of NAP and sodium nitrite was added to a 1:1 mixture of water and methanol containing 2 M HCl and 2 M H_2SO_4. After 30 min of stirring, the reaction vessel was cooled in an ice bath to precipitate the green SNAP