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Improved Hemocompatibility of Silicone Rubber Extracorporeal Tubing via Solvent Swelling-Impregnation of *S*-Nitroso-*N*-acetylpenicillamine (SNAP) and Evaluation in Rabbit Thrombogenicity Model

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Abstract

Blood-contacting devices, including extracorporeal circulation (ECC) circuits, can suffer from complications due to platelet activation and thrombus formation. Development of nitric oxide (NO) releasing polymers is one method to improve hemocompatibility, taking advantage of the ability of low levels of NO to prevent platelet activation/adhesion. In this study a novel solvent swelling method is used to load the walls of silicone rubber tubing with the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP). This SNAP-silicone rubber tubing exhibits an NO flux of ca. 1×10^{-10} mol cm⁻² min⁻¹, which mimics the range of NO release from the normal endothelium, which is stable for at least 4 h. Images of the tubing before and after swelling, obtained via scanning electron microscopy, demonstrate that this swelling method has little effect on the surface properties of the tubing. The SNAP-loaded silicone rubber and silicone rubber control tubing are used to fabricate ECC circuits that are evaluated in a rabbit model of thrombogenicity. After 4 h of blood flow, the SNAP-loaded silicone rubber circuits were able to preserve the blood platelet count at 64% of baseline (vs. 12% for silicone rubber control). A 67% reduction in the degree of thrombus formation within the thrombogenicity chamber was also observed. This study demonstrates the ability to improve the hemocompatibility of existing/commercial silicone rubber tubing via a simple solvent swelling-impregnation technique, which may also be applicable to other silicone-based blood-contacting devices.

Graphical Abstract

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Silicone rubber (SR) tubing is soaked in a swelling solution to impregnate the entire tubing wall with *S*-nitroso-*N*-acetylpenicillamine (SNAP). The SNAP-silicone rubber tubing is used to fabricate extracorporeal circulation (ECC) loops that delivers nitric oxide (NO), a potent inhibitor of platelet adhesion/activation, and can significantly reduce thrombosis in a rabbit model of thrombogenicity.

Keywords

blood compatibility; extracorporeal circulation; nitric oxide delivery; *S*-nitrosothiols; thrombosis

1. Introduction

Extracorporeal circulation (ECC) circuits are critical to several medical procedures including hemodialysis, open heart surgery, and extracorporeal membrane oxygenation. During these medical procedures blood is pumped through tubing and is also exposed to devices for gas exchange or filtration. Exposure of blood to these foreign surfaces results in a complex response that initiates the coagulation cascade, surface fouling, and inflammation (involving neutrophils, monocytes, and complements) that can potentially lead to adverse medical outcomes. In the coagulation cascade proteins, such as fibrinogen and fibronectin, rapidly adsorb to the foreign surface [1]. These adsorbed proteins interact with platelets leading to the exposure of the platelet glycoprotein GPIIb/IIIa receptor that binds platelets to fibrinogen, as well as conformational changes that further induce the activation and aggregation of more platelets (e.g., excretion of P-selectin) [2]. This surface contact (intrinsic pathway) converges with the extrinsic pathway at the common pathway of coagulation where thrombin converts fibrinogen to fibrin, which ultimately results the entrapment of platelets/red blood cells forming thrombus on the surface [2]. Systemic anticoagulation is necessary to preserve the patency of the ECC circuit; however, platelet consumption is still observed. The platelet count typically drops to < 40 % of normal during the initial 1 – 2 h of blood flow through ECC circuits [3]. According to a report by the Extracorporeal Life Support Organization (ELSO), bleeding and thrombosis still occur with a significant number of patients (7-34%) on extracorporeal life support (ECLS) devices [4]. Despite systemic anticoagulation, thrombus formation, hemorrhage, and infection are some of the most adverse side effects that occur during these ECC procedures which can lead to catastrophic complications and mortality [5-7].

The development of nitric oxide (NO) releasing polymers is an approach to improve hemocompatibility of ECC and other blood-contacting devices that has been reported in the literature [8-10]. Nitric oxide has a number of important physiological roles, including its

ability to attenuate platelet activation. The normal endothelium releases NO at a flux in the range of $0.5 - 4 \times 10^{-10} \text{ mol cm}^{-2} \text{ min}^{-1}$ that prevents the activation of platelets that come in close proximity of this surface [11]. In addition, NO serves other critical physiological roles including prevention of infection, due to its broad spectrum antimicrobial properties [12], which is also important for ECC applications. Therapeutic delivery of NO from polymer surfaces is a promising approach to improve hemocompatibility and reduce device-related infections for ECCs [13-16], as well as other application (e.g., in vivo biosensors [17-21], catheters [22-25] grafts [26]).

To date, NO releasing polymers have been prepared utilizing common NO donor molecules such as *N*-diazoniumdiolates [13, 16, 18, 26-28] and *S*-nitrosothiols (RSNOs) [15, 23, 29-32], including *S*-nitrosoglutathione (GSNO) and *S*-nitroso-*N*-acetylpenicillamine (SNAP). These NO donor molecules have been incorporated into biomedical polymers via synthetic modifications [31-33] and non-covalent dispersal [14, 15, 29], where ultimate exposure to physiological conditions can initiate the release of their NO payload. The advantage of this approach is that the NO delivery can be adjusted for a specific medical application by modifying variables such as the concentration of NO donor within the polymer matrix or utilizing polymer topcoats (which also can serve to reduce leaching of the NO donor). Heat, light, and metal ions (e.g., Cu^+) are the major catalysts for RSNO species that initiate NO release [34]. For RSNO-based polymers, the major limitations have been low synthetic yields, leaching of the RSNO (which delocalizes the NO delivery and can cause effects downstream), and instability of the RSNO functionality during storage. Incorporating the NO donor SNAP into hydrophobic polymers, such as silicone rubber, has been reported to reduce leaching, prolong NO release, and retain NO functionality during storage [15]. Despite the promising *in vitro* and *in vivo* biocompatibility results reported for these NO releasing polymers, many of these materials may face challenges in being translated to clinical applications, especially in the areas of polymer processing and manufacturing.

Silicone rubber has been widely used in various biomedical applications including intravascular and urinary (Foley) catheters, drains, and insulators to pacemaker leads as well as in ECC procedures (e.g., tubing, blood oxygenators, peristaltic pump chambers, etc.) [35]. Most catheters and medical grade tubing are manufactured using an extrusion process. For example, silicone rubber tubing formed using liquid injection molding undergoes temperatures of $150 - 200^\circ\text{C}$ during typical extrusion to ensure proper curing of the polymer [36]. Many of the NO donor compounds currently being studied are heat sensitive and their NO functionality could be severely affected by these extreme temperatures. Bainbridge et al. reported that clean decomposition of SNAP and GSNO occurs at 148°C and results in the release of NO [37]. This suggests that a significant loss of the NO payload could occur during the silicone rubber extrusion process if the NO donors are present in the extrusion mixture. Therefore, alternative methods are needed in order to incorporate NO donors within silicone-based biomedical devices. Recently, it was demonstrated that SNAP could be loaded into pre-existing silicone rubber urinary catheters by a solvent swelling-impregnation technique to reduce risk of infection [23]. In this study, we investigate this novel solvent swelling-impregnation approach to incorporate SNAP into silicone rubber ECC circuits. It is known that NO has excellent diffusion properties in silicone rubber [38]

and also the leaching of SNAP from silicone rubber can be reduced because of silicone rubber's low water uptake and cross-linked polymer network [15, 23]. Hence, for this work, SNAP-loaded silicone rubber tubing is investigated to prepare extracorporeal circulation (ECC) loops, and the resulting loops are evaluated for their effects on hemocompatibility in an ECC rabbit model of thrombogenicity.

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). Saint-Gobain™ Tygon™ Formula 3350 Silicone Tubing was purchased from Fisher Scientific (Pittsburgh, PA). Dow Corning RTV 3140 silicone rubber sealant was purchased from Ellsworth Adhesives (Germantown, WI). 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 SNAP Synthesis Protocol

SNAP was synthesized using a modified version of a previously reported method [39]. Briefly, an equimolar ratio of NAP and sodium nitrite was dissolved in a 1:1 mixture of water and methanol containing 2 M HCl and 2 M H₂SO₄. After stirring, the reaction vessel was cooled in an ice bath to precipitate the green SNAP crystals. The crystals were collected by filtration, rinsed with water, and dried under ambient conditions. The reaction mixture and resulting crystals were protected from light at all times.

2.3 Preparation of SNAP Impregnated Tubing and ECC Loops

The SNAP swelling solution was prepared by dissolving SNAP in THF using concentrations of 15, 25, and 35 mg/mL. The Saint-Gobain™ Tygon™ silicone rubber tubing was soaked in the SNAP swelling solution for 24 h. The tubing was removed, briefly rinsed with PBS, and dried for 48 h under ambient conditions to allow the excess THF to evaporate. The tubing and swelling solutions were protected from light throughout the swelling process. Small sections of tubing (~1 cm lengths) were used for the NO release and SEM experiments.

The ECC loop configuration was used as previously described [13-16, 40]. Briefly, the ECC loops consisted of 16-gauge and 14-gauge IV polyurethane angi catheters (Kendall Monoject Tyco Healthcare Mansfield, MA), two 16 cm lengths of ¼ inch inner diameter (ID) silicone rubber tubing, and one 8 cm length of 3/8 inch silicone rubber tubing to create a thrombogenicity chamber to expose blood to an area of flow disturbance and recirculation. For SNAP-loaded silicone rubber ECC loops, all the silicone rubber tubing used to create the circuit was swelled in a THF solution containing 25 mg/mL SNAP. The angi catheters were

coated with 10 wt% SNAP in silicone rubber (using a solution containing 15 mg SNAP, 135 mg RTV sealant, and 3 mL THF) followed by 1 top coat of silicone rubber (150 mg RTV sealant in 3 mL THF). The silicone rubber control ECC loops consisted of the silicone rubber tubing (no SNAP) and angiocatheters coated with only silicone rubber (150 mg RTV sealant in 3 mL THF; no SNAP added). All ECC loop pieces were assembled together using a solution of 200 mg/mL RTV sealant in THF. The ECC loops were dried under ambient conditions for 48 h followed by vacuum drying for 24 h. All ECC loops were soaked in saline solution for 1 h and this solution was discarded immediately prior to each rabbit experiment.

2.4 NO Release Measurements

Nitric oxide release from the tubing was measured using a Sievers chemiluminescence Nitric Oxide Analyzer (NOA), model 280i (Boulder, CO). All SNAP-loaded silicone rubber tubing was soaked in PBS/EDTA at room temperature for 1 h prior to the NO release testing. A sample of SNAP-loaded silicone rubber ECC tubing (1 cm) was placed in 4 mL PBS buffer with EDTA at 37 °C. Nitric oxide liberated from the sample was continuously swept from the solution and headspace of the sample cell by purging the buffer with a nitrogen sweep gas stream and bubbler, and then this stream flowed into the chemiluminescence detection chamber. The nitrogen flow rate was set to 200 mL/min with a chamber pressure of 6 Torr and an oxygen pressure of 6.2 psi. The NO release from test tubing samples was normalized by the surface area to obtain a flux unit for NO release rate ($\times 10^{-10}$ mol cm⁻² min⁻¹). After the 4 h rabbit studies, 1 cm sections of the ECC tubing were tested for NO release post-blood exposure.

2.5 Scanning Electron Microscopy

Scanning electron microscopy images were recorded using an AMRAY 1910 Field Emission Scanning Electron Microscope at a working distance of 7 mm at 5.0 kV. Tubing samples were glued to stubs and sputter-coated with gold for 120 s prior to imaging.

2.6 Rabbit Model of Thrombogenicity

A previously reported rabbit model was used to evaluate the hemocompatibility of the ECC tubing [13-16, 40-42]. The animal handling and surgical procedures were approved by the University Committee on the Use and Care of Animals in accordance with university and federal regulations. A total of 8 New Zealand white rabbits (Myrtle's Rabbitry, Thompson's Station, TN) were used in this study. Initially, all rabbits (2.5–3.5 kg) were anesthetized with intramuscular injections of 5 mg kg⁻¹ xylazine injectable (AnaSed® Lloyd Laboratories Shenandoah, Iowa) and 30 mg kg⁻¹ ketamine hydrochloride (Hospira, Inc., Lake Forest, IL).

Isoflurane gas (maintenance anesthesia) was administered via inhalation at a rate of 1.5–3% via mechanical ventilation which was done via a tracheotomy and using an A.D.S. 2000 Ventilator (Engler Engineering Corp. Hialeah, FL). Peak inspiratory pressure was set to 15 cm of H₂O and the ventilator flow rate set to 8 L min⁻¹. In order to aid in maintenance of blood pressure stability, IV fluids of Lactated Ringer's were given at a rate of 10 mL kg⁻¹ h⁻¹. For monitoring blood pressure and collecting blood samples, the rabbits' right carotid artery was cannulated using a 16-gauge IV angiocatheter (Jelco®, Johnson & Johnson,

Cincinnati, OH). Blood pressure and derived heart rate were recorded with a Series 7000 Monitor (Marquette Electronics Milwaukee, WI). Body temperature was monitored with a rectal probe and maintained at 37 °C using a water-jacketed heating blanket.

Prior to placement of the arteriovenous (A-V) custom-built ECC loops, the rabbit's left carotid artery and right external jugular vein were isolated and baseline hemodynamics as well as arterial blood pH, PCO_2 , PO_2 , and total hemoglobin were measured using an ABL 800 blood-gas analyzer. The right carotid artery was cannulated and used for all blood sampling. In addition, baseline blood samples were collected for platelet and total white blood cell (WBC) counts which were measured on a Coulter Counter Z1 (Coulter Electronics, Hialeah, FL). Activated clotting times (ACT) were determined using a Hemochron Blood Coagulation System Model 801 (International Technidyne Corp., Edison, NJ). Platelet function was assessed using a Chrono-Log optical aggregometer model 490 (Havertown, PA). After baseline blood measurements, the custom-built ECC was placed into position by cannulating the left carotid artery for ECC in flow and the right external jugular vein for ECC out flow. Blood flow through the ECC was initiated by unclamping the arterial and venous sides of the ECC and blood flow was monitored with an ultrasonic flow probe and flow meter (Transonic HT207, Ithaca, NY). Animals were not systemically anticoagulated during the experiments.

Rabbit whole blood samples were collected in non-anticoagulated 1 mL syringes for ACT measurements, 3.2% sodium citrate vacutainers (Becton, Dickinson, Franklin Lakes, NJ) in 3 mL volumes for cell counts and aggregometry, and 1 mL syringes containing 40 U/mL of sodium heparin (APP Pharmaceuticals, LLC Schaumburg, IL) for blood-gas analysis. Following the initiation of ECC blood flow, blood samples were collected each hour during the 4 h experiments. Samples were tested within 2 h of collection to avoid any activation of platelets, monocytes or plasma fibrinogen, except for blood-gas measurements which were run immediately after drawing the samples. To correct for hemodilution due to added fluids during the experiment, the following formula was used:

$$\begin{aligned} &\text{Corrected Platelet Count } (\times 10^8 \text{ platelets/mL}) \\ &= \text{Platelet Count } (\times 10^8 \text{ platelets/mL})_t * \frac{\text{Hemoglobin } (g/dL)_{t=0}}{\text{Hemoglobin } (g/dL)_t} \end{aligned}$$

Rabbit platelet aggregation was assayed based on the Born's turbidimetric method using a Chrono-Log optical aggregometer. Briefly, 6 mL citrated blood (1:10 blood to 3.8% sodium citrate) was collected and platelet-rich plasma (PRP) was obtained by centrifugation at $110 \times g$ for 15 min. Platelet-poor plasma (PPP) was obtained by another centrifugation of the PRP-removed blood sample at $2730 \times g$ for 15 min and was used as the blank for aggregation. A solution of normalized PRP (with concentration of 2×10^8 platelets/mL) was obtained by mixing the appropriate ratio of PPP and PRP. This normalized PRP solution was incubated for 10 min at 37 °C and then 25 mg mL⁻¹ collagen (Chrono-PAR #385, Havertown, PA) was added. The percentage of aggregation was determined 3 min after the addition of collagen using Chrono-Log Aggrolink software.

After 4 h of blood flow, the ECC loops were clamped, removed from the rabbit, and rinsed with 60 mL of saline and drained. Due to the opacity of the ECC tubing, the thrombogenicity chamber was longitudinally cut open to observe the extent of thrombus formation within the lumen. The thrombogenicity chamber was photographed and the thrombus was gently transferred to a weighing boat for quantification. The rabbits were euthanized using a dose of Fatal Plus (130 mg kg⁻¹ sodium pentobarbital) (Vortech Pharmaceuticals, Dearborn, MI).

2.7 Statistical Analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM). The results between the silicone rubber control and SNAP-loaded silicone rubber ECCs were analyzed by a comparison of means using Student's *t*-test. Values of $p < 0.05$ were considered statistically significant for all tests.

3. Results and Discussion

3.1 In Vitro NO Release from SNAP-loaded Silicone Rubber Tubing

Blood-contacting devices used in clinical applications can suffer from complications due to the activation of the coagulation cascade, fouling of the surface, and inflammatory response [2]. Various NO-releasing polymers have been reported to improve the hemocompatibility of blood-contacting devices such as catheters [22-25], vascular grafts [26], implantable sensors [17-21], and extracorporeal circuits [13-16, 40]. Due to the heat sensitivity of many NO donor molecules used in these studies, typical extrusion processes may not be feasible. Therefore alternative methods to incorporate NO donors in polymers are under investigation. Recent efforts have shown that incorporating SNAP (Fig. 1A) within hydrophobic polymer matrices create polymers that have excellent properties in terms of NO release and shelf-life stability [15, 25]. In this study, a new solvent swelling technique was used to impregnate the walls of existing silicone rubber tubing with SNAP (Fig. 1B). Tetrahydrofuran was employed for this study because of its high vapor pressure, which allows for rapid evaporation after loading with SNAP, and SNAP's high solubility in this solvent. Test samples of the silicone rubber tubing were swelled in THF containing SNAP concentrations between 15, 25, and 35 mg/mL. In previous studies SNAP concentration of 125 mg/mL were used to impregnate Foley urinary catheters for long-term antimicrobial studies [23]. However, in this study the lower concentration range was selected due to the short-term of NO release required for the ECC studies (at least 4 h) as well as the large volumes of solution required to impregnate the large ECC circuits for the animal studies. Tetrahydrofuran is an excellent swelling solvent for silicone rubber [43], where the tubing increases ca. 1.3 times its original size. After 24 h soaking in the SNAP solution, the SNAP-loaded silicone rubber tubing was rinsed to remove excess SNAP on the surface and dried completely. During the drying process the tubing returned to its original dimensions. Samples of the tubing were also examined by SEM before and after the swelling process. The original silicone rubber tubing was manufactured by an extrusion process which yields a very smooth surface. No significant differences in the surface characteristics of the original silicone rubber tubing, silicone rubber tubing post-swelling in THF without SNAP, and SNAP-loaded silicone rubber tubing were observed (Fig. 2). During the impregnation process the polymer swells, but after drying the tubing

was thoroughly rinsed to remove any excess SNAP crystals on the surface. This further demonstrates that the swelling method has no adverse effects on the surface properties of the tubing due to the fact that the tubing returns to its original dimensions and excess surface SNAP is rinsed away post-swelling.

Prior to NO release measurements, the tubing samples were soaked in PBS buffer for 1 h to ensure that any excess SNAP that might remain on the surface was washed away. The NO release from samples of SNAP-loaded silicone rubber tubing was measured under physiological conditions (in PBS buffer with EDTA at 37 °C) using a chemiluminescence NO analyzer. All of the samples had consistent NO release of ca. $1 \times 10^{-10} \text{ mol cm}^{-2} \text{ min}^{-1}$ during the 4 h test period without any significant initial burst of NO release, which mimic the endothelial range of NO surface flux. Previous NO-releasing polymers based on diazeniumdiolates have had a more dramatic “burst” release of NO which rapidly consumes the NO donor [26]; however, the SNAP-loaded silicone in this study did not exhibit any significant burst release of NO. A representative NO release profile for tubing prepared with 15, 25, or 35 mg/mL SNAP in the swelling solution is shown in Fig. 3A. The average NO release during this 4 h period was 0.98 ± 0.06 , 1.04 ± 0.01 , and $1.02 \pm 0.02 \times 10^{-10} \text{ mol cm}^{-2} \text{ min}^{-1}$ for the tubing soaked in 15, 25, or 35 mg/mL SNAP, respectively (Fig. 3B). The NO release from this SNAP-loaded silicone mimics the lower range of endothelial NO surface flux ($0.5\text{--}4 \times 10^{-10} \text{ mol cm}^{-2} \text{ min}^{-1}$) and is also very similar to the previously reported SNAP-doped Elast-eon E2As circuits [15].

3.2 SNAP-Impregnated ECC Loops and Effects on Rabbit Hemodynamics

The custom-built ECC loops were prepared using the SNAP-loaded or silicone rubber control tubing as described in Section 2.3 (Fig. 4). All loops were soaked in saline solution for 1 h prior to rabbit experiments in order to wash away any initial burst associated with SNAP leaching from the surface of the tubing. The NO release from the SNAP-loaded silicone rubber ECC loops was measured using a chemiluminescence analyzer and had an average flux of ca. $0.9 \times 10^{-10} \text{ mol cm}^{-2} \text{ min}^{-1}$ for a time period that exceeds the 4 h duration of the rabbit experiment. This NO release level approaches the lower end of the range of the physiological levels of NO produced by the healthy endothelium, $0.5\text{--}4 \times 10^{-10} \text{ mol cm}^{-2} \text{ min}^{-1}$. The NO release from a 1 cm section of the ECC tubing after the 4 h of blood exposure in the rabbit model did not significantly decrease and was measured to be $0.8 \pm 0.1 \times 10^{-10} \text{ mol cm}^{-2} \text{ min}^{-1}$. It has previously been demonstrated that blood exposure does not alter the NO release kinetics from NO-releasing polymers [14-16].

As shown in Table 1, no significant differences in the rabbit hemodynamics were observed between the SNAP-loaded and silicone rubber control circuits. The mean arterial pressure (MAP) dropped to 31-41 mmHg after initiating blood flow through the ECC circuits. As thrombus began to form in the silicone rubber control ECCs, the blood flow (ECC BF) gradually decreased until the circuit was completely occluded (no flow). In contrast, the SNAP-silicone rubber circuits had consistent blood flow for the duration of the experiment. Intravascular fluids were administered at $10 \text{ mL kg}^{-1} \text{ h}^{-1}$ to maintain flow through all ECC circuits. The activated clotting time (ACT) during the 4 h experiment increased for both the SNAP-loaded and silicone rubber control circuits, which can be attributed to the

administration of IV fluids and hemodilution effects. No significant differences in the total white blood count (WBC) were observed between the SNAP-loaded and silicone control ECCs as these cells were likely trapped within the thrombus that formed within these tubing sets.

3.3 Effects of SNAP-Impregnated ECCs on Rabbit Platelet Function and Thrombus Formation

Platelet activation and function throughout the 4 h ECC experiments were assessed by recording the platelet count, plasma fibrinogen levels, and platelet aggregation, which were corrected for hemodilution due to the added IV fluids. The baseline platelet counts ($\times 10^8$ platelets/mL) were 3.7 ± 0.7 for the SNAP-loaded silicone rubber circuits and 4.0 ± 0.9 for silicone rubber control circuits. The platelet count was maintained near baseline levels during the initial 2 h of blood flow for the SNAP-loaded silicone rubber circuits, and then gradually decreased to 64% of baseline at 4 h (Fig. 5). In contrast, the platelet count for silicone rubber control circuits exhibited a much more dramatic time-dependent loss in platelets, dropping to 12% of baseline after 4 h of blood flow. The platelet functionality was also assessed each hour via *ex vivo* collagen stimulation of PRP that was measured by optical turbidity (Fig. 6). In an ideal situation, prevention of platelet activation should be a localized effect at the ECC surface to allow platelets to function normally downstream from the device. The platelets sampled from the SNAP-loaded silicone rubber circuits showed similar response to collagen stimulated platelet aggregation during the 4 h blood exposure, maintaining $47 \pm 15\%$ aggregation (with $62 \pm 11\%$ at baseline). This indicates that NO released from the SNAP-loaded silicone rubber polymer did not have any adverse effect on the ability of the platelets to maintain their functionality and aggregate downstream from the ECC circuit. The preservation of platelet functionality and significant reduction in thrombus formation observed with the SNAP-loaded ECCs demonstrates the localized effects of NO release. For the silicone rubber control circuits, platelets exhibited a significant decrease in their functionality during the 4 h of blood exposure. Platelets were already activated by the foreign silicone rubber surface, and therefore could not be further activated upon addition of collagen.

The initial plasma fibrinogen levels were 230 ± 10 and 221 ± 3 mg/dL for the silicone rubber control and SNAP-loaded silicone rubber circuits, respectively, and were corrected for hemodilution due to the addition of IV fluids during the ECC experiment. During the 4 h of blood flow, the plasma fibrinogen levels exhibited a time-dependent decrease resulting in 80% of baseline at 4 h for the SNAP-loaded silicone rubber circuits. For the silicone rubber control circuits, the plasma fibrinogen levels exhibited a less dramatic loss (~10% of baseline), which is consistent with previous reports that NO releasing surfaces bind more fibrinogen [44]. The decrease in plasma fibrinogen levels can be attributed to the fact that fibrinogen is involved with thrombus formation and can adsorb to foreign surfaces as part of the coagulation cascade [15, 16, 44]. The NO release from the SNAP-loaded silicone rubber circuits is able to overcome the fibrinogen adsorption on the surface that normally plays a key role in activation of platelets.

Finally, the extent of thrombus formation was assessed by weighing the clot that had formed in the thrombogenicity chamber of the ECC circuits after 4 h of blood flow. The thrombogenicity chamber for each circuit was cut open, photographed, and the clot was weighed. The thrombogenicity chamber of the silicone rubber control circuits were completely filled with clots, while the SNAP-loaded silicone rubber circuits had some smaller clots that formed that still allowed for patency of the circuit and continuous blood flow (Fig. 7A and B). The SNAP-loaded silicone rubber circuits had significantly reduced amount of thrombus formation (67% reduction) as compared to the silicone rubber control circuits (Fig. 7C).

This study demonstrates the ability to convert existing commercial tubing into NO releasing ECC tubing via a simple solvent swelling technique. Some potential effects of hypotension and hyperglycemia have been reported with applications of SNAP [15, 40, 45, 46]. However, in this study, presoaking the ECC circuits to wash away excess SNAP on the surface as well as administration of IV fluids were able to prevent any significant hypotensive effects. It should also be noted that NAP, the parent thiol, is also used clinically to treat heavy metal poisoning and cystinuria without any significant side effects [47, 48]. As previously reported, polymers can have widely varying intrinsic hemocompatibility properties [13]. In this work the silicone rubber control polymer had worse hemocompatibility properties, in terms of rapid platelet consumption and occlusion of the circuits, than other control polymers tested in a similar rabbit ECC model [13-16, 40]. The poor hemocompatibility properties of silicone rubber have been attributed to the surface microstructure (e.g., exposed silica particles) which can induce clotting [49]. The silicone rubber control tubing used in this study has poor intrinsic hemocompatibility properties alone; however, the NO released from the SNAP-loaded silicone rubber ECCs is able to significantly improve its hemocompatibility by preserving the plasma platelet and fibrinogen levels, as well as preserving platelet functionality, and ultimately reducing clot formation on the surface. This swelling technique has the potential to improve the hemocompatibility of other silicone-based polymers used in biomedical devices. The solvent swelling method also has the advantage of loading SNAP throughout the entire wall thickness of the tubing, thereby increasing the SNAP reservoir which will allow for more long-term *in vivo* studies. Further, the same impregnation methodology should also be possible with other biomedical grade polymers, although the choice of solvent will need to be optimized for each, in order to fabricate other NO-releasing devices for medical applications. Indeed, utilizing polymers with better intrinsic hemocompatibility properties than silicone rubber may prove beneficial in terms of improving the overall hemocompatibility and effectiveness of the NO release [13].

4. Conclusions

This study demonstrates that the solvent swelling technique can be used to load SNAP within the walls of commercially available silicone rubber tubing, resulting in tubing that can release physiologically relevant levels of NO. Scanning electron microscopy images revealed that this solvent swelling techniques has little effect on the surface characteristics of the tubing. Nitric oxide release from the SNAP-loaded silicone rubber tubing was shown to attenuate the activation of platelets and maintain their functionality, while significantly

reducing the extent of thrombus formation during 4 h of blood flow in the rabbit model of thrombogenicity. Results of this study suggest the potential of improving the hemocompatibility of other polymeric biomedical devices via the solvent swelling SNAP-impregnation technique.

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A **B**

Fig. 1. Structure of *S*-nitroso-*N*-acetylpenicillamine (A). Schematic of the solvent swelling method used to load the walls of silicone rubber (SR) tubing with SNAP (B).

Fig. 2.

Representative scanning electron microscopy images of original silicone rubber tubing (A), silicone rubber control tubing swelled in THF without SNAP (B), and SNAP-loaded silicone rubber tubing (C). The images demonstrate that the swelling process used for impregnating SNAP within the tubing wall does not significantly change the surface characteristics of the tubing, and is able to maintain a smooth surface similar to the original extruded tubing.

Fig. 3.

Representative NO release profile for SR tubing prepared with swelling solutions containing 15, 25, or 35 mg/mL SNAP (A). Average NO flux from n=3 samples of tubing prepared with 15-35 mg/mL SNAP in the swelling solution (B). Real-time NO release was measured from tubing samples submerged in PBS at 37 °C by chemiluminescence.

Fig. 4.
Schematic of the assembled ECC loop configuration used in an A-V shunt rabbit model of thrombogenicity.

Fig. 5.

Comparison of time-dependent effects of SNAP-SR and SR control tubing on platelet consumption (% of baseline, n=4 of each circuit). # = $p < 0.05$ for Baseline vs. SR Control.

* = $p < 0.05$, SNAP-loaded silicone rubber (SNAP-SR) vs. silicone rubber control (SR). § = Control (n=1) and SNAP-SR (n=3).

Fig. 6.

Time-dependent effects of SNAP-SR vs. SR control tubing on platelet function, as measured by aggregometry (n=4 circuits for each). * = $p < 0.05$, SNAP-loaded silicone rubber (SNAP-SR) vs. silicone rubber control (SR). § = Control (n=1) and SNAP-SR (n=3).

Fig. 7. Representative images of clot formation within the thrombogenicity chamber after 4 h of blood flow for the SNAP-loaded silicone rubber tubing (SNAP-SR) (A) and silicone rubber (SR) control (B) ECC loops. (C) Quantitation of clot weight within the thrombogenicity chamber after 4 h of ECC blood flow (n=4). * = $p < 0.05$, SNAP-loaded silicone rubber (SNAP-SR) vs. silicone rubber control (SR).

Table 1

Effects of the SNAP-SR and SR control ECC circuits on rabbit hemodynamic parameters during 4 h of ECC blood flow: mean arterial pressure (MAP), heart rate (HR), extracorporeal circuit blood flow (ECC BF), activated clotting time (ACT), and white blood count (WBC).

ECC	Parameter	Time on ECC (h)				
		Baseline	1	2	3 [§]	4 [§]
SR Control	MAP (mmHg)	65 ± 6	40 ± 4	38 ± 2	41	38
	HR (beats/min)	214 ± 7	224 ± 9	217 ± 8	220	225
	ECC BF (mL/min)	85 ± 11	41 ± 22	45 ± 44	39 *	0 *
	ACT (sec)	157 ± 6	163 ± 9	182 ± 24	193	200
	WBC (×10 ³ WBC/mL)	5.8 ± 0.6	2.7 ± 0.5	2.8 ± 0.2	2.5	2.0
SNAP-SR	MAP (mmHg)	62 ± 6	44 ± 7	34 ± 2	32 ± 1	31 ± 2
	HR (beats/min)	229 ± 14	219 ± 16	216 ± 14	209 ± 10	209 ± 4
	ECC BF (mL/min)	59 ± 3	70 ± 22	63 ± 213	65 ± 22 *	62 ± 22 *
	ACT (sec)	164 ± 11	153 ± 7	171 ± 5	198 ± 9	191 ± 9
	WBC (×10 ³ WBC/mL)	5.6 ± 0.4	3.6 ± 0.3	3.2 ± 0.6	3.3 ± 0.6	3.0 ± 0.8

[§] = SR Control (n=1) and SNAP-SR (n=3)

* = p < 0.05, SNAP-loaded silicone rubber (SNAP-SR) vs. silicone rubber control (SR).