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ScienceDirect
Biomaterials 29 (2008) 2443–2452
Biomaterials
www.elsevier.com/locate/biomaterials

Polyurethane with tethered copper(II)–cyclen complex: Preparation, characterization and catalytic generation of nitric oxide from *S*-nitrosothiols

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Received 2 November 2007; accepted 7 February 2008
Available online 7 March 2008

Abstract

The preparation and characterization of a commercial biomedical-grade polyurethane (Tecophilic®, SP-93A-100) material possessing covalently linked copper(II)–cyclen moieties as a nitric oxide (NO) generating polymer are described. Chemiluminescence NO measurements demonstrate that the prepared polymer can decompose endogenous *S*-nitrosothiols (RSNOs) such as *S*-nitrosoglutathione and *S*-nitrosocysteine to NO in the presence of thiol reducing agents (RSHs; e.g., glutathione and cysteine) at physiological pH. Since such RSNO and RSH species already exist in blood, the proposed polymer is capable of spontaneously generating NO when in contact with fresh blood. This is demonstrated by utilizing the polymer as an outer coating at the distal end of an amperometric NO sensor to create a device that generates response toward the RSNO species in the blood. This polymer possesses the combined benefits of a commercial biomedical-grade polyurethane with the ability to generate biologically active NO when in contact with blood, and thus may serve as a useful coating to improve the hemocompatibility of various medical devices.

Keywords: Copper; Hemocompatibility; Nitric oxide; Polyurethane; S-nitrosothiols

1. Introduction

Nitric oxide (NO) has a wide variety of vasoprotective/physiological activities including serving as a potent antiplatelet agent [1–3], inhibiting smooth muscle cell proliferation [4], preventing microbial growth [5], and enhancing wound healing [6–8]. Hence, the development of novel NO releasing materials using NO donors (e.g., diazeniumdiolates or *S*-nitrosothiols (RSNOs)) either embedded within or covalently linked to polymers has been pursued for application as coatings on various biomedical devices to improve their biocompatibility. Such materials have been shown to greatly reduce platelet adhesion and thrombus formation *in vitro* and *in vivo* using several animal models [9,10], and also to inhibit restenosis following angioplasty [11–13]. The ultimate biomedical applications of such polymers may, however, be limited to short-term use (i.e., on the order of a few days) due to the relatively small reservoir of NO donors that can be loaded within thin polymeric coatings. Another obstacle for practical implementation of NO releasing polymers is the highly labile nature of most NO donors (heat, light and moisture sensitive), making the manufacturing and shipping of NO releasing polymeric coatings quite difficult and costly. Hence, even short-term biomedical applications of NO production at the surface of blood contacting medical devices (e.g., catheters, extracorporeal circuits, etc.) require solution of these problems associated with polymers possessing NO donor type chemistries.

In order to overcome these limitations, different strategies to create more biocompatible polymeric materials that are more robust, less costly, and release/generate NO for prolonged time periods once implanted *in vivo* have recently

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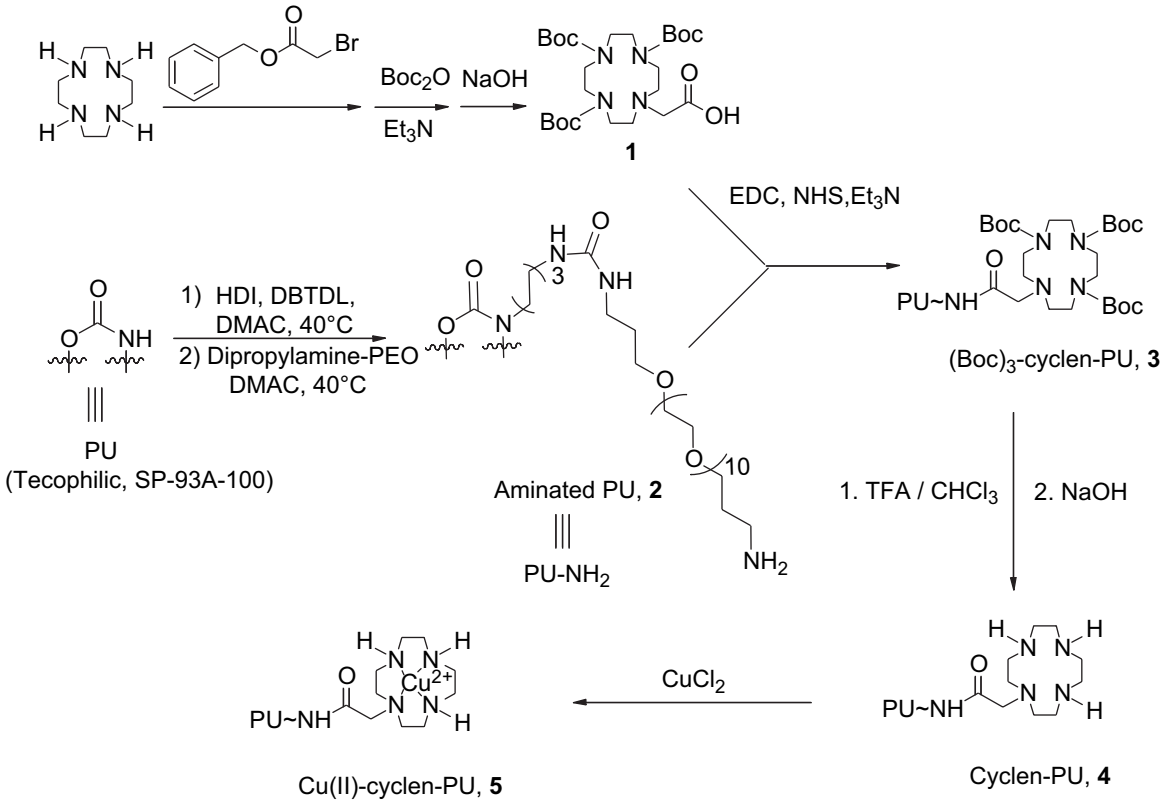
doi:10.1016/j.biomaterials.2008.02.004

been suggested. For example, Duan and Lewis proposed polymers with appended cysteine moieties that can gain NO from endogenous *S*-nitrosothiols in blood (e.g., *S*-nitrosoglutathione (GSNO), *S*-nitrosocysteine (CySNO), and *S*-nitrosoalbumin (AlbSNO)) via transnitrosation reactions [14,15]. The polymer accumulated CySNO can also release NO, and this was shown to reduce platelet adhesion using a radiolabeled platelet model *in vitro* [14,15]. Another related approach introduced in this laboratory is based on immobilizing catalytic sites within polymeric materials that can locally generate NO from endogenous RSNOs at the polymer/blood interface (so-called NO generating polymers (NOGPs)). NOGPs may be broadly classified into two types; those based on immobilized metal-ion complexes and those using immobilized organodichalcogenides as catalysts. For example, a Cu(II)–cyclen complex [16] or organodichalcogenides (RSeSeR [17] and RTeTeR [18]) can be tethered to polymeric materials to serve as redox catalytic sites. The oxidized form of catalytic site (Cu(II), RSeSeR, or RTeTeR) is reduced to Cu(I), RSe^-, or RTe^- species by common endogenous reducing agents such as free thiolates (RS^-) in blood. The reduced form of catalyst then reacts with endogenous RSNOs to liberate NO, and reverts the catalytic site to its original oxidized form. NOGPs have been shown to exhibit catalytic NO generation from endogenous RSNOs at physiological pH, in addition to the spontaneous NO production when in contact with fresh animal whole blood *in vitro* [16,19].

The specific polymeric matrix used to prepare NOGPs will dictate the physical properties of the material, which is also a critical factor in determining the biocompatibility of any implanted device coated with NOGPs. In general, thermoplastic polyurethanes (PUs) are considered to offer relatively good hemocompatibility and mechanical properties when compared to the other synthetic materials (e.g., poly(vinyl chloride) and silicone rubber) [20–22]. Indeed, they have been used to prepare a variety of medical devices including blood bags, catheters, vascular grafts, and portions of artificial hearts [23,24]. However, PUs are not completely thromboresistant, as platelet adhesion/activation can still occur when in contact with blood for extended periods of time [21,25–27].

Herein, we report a different NOGP in which a NO generating catalyst, Cu(II)–cyclen, is covalently linked to a medical-grade hydrophilic thermoplastic PU (Tecophilic®, SP-93A-100). The synthesis and characterization of this NOGP, Cu(II)–cyclen–PU (**5**), are described in detail (see Scheme 1). Further, catalytic NO generation properties of this biomaterial are examined using various RSNOs and RSHs as substrates and reducing agents, respectively. Leaching of Cu(II) from the polymer is also studied and found not to be a major problem. Finally, the spontaneous NO generation when this polymer in contact with fresh animal whole blood is demonstrated via use of the polymer to construct an amperometric RSNO sensor.



Scheme 1. The synthesis of a polyurethane possessing covalently appended Cu(II)–cyclen complex (Cu(II)–cyclen–PU (**5**)) from a modified cyclen derivative (**1**) and aminated PU (**2**) (see Section 2 for details).

2. Materials and methods

2.1. Materials

1,4,7,10-Tetraazacyclododecane (cyclen) was purchased from Strem Chemicals (Newburyport, MA). *N*-(3-Dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), glutathione (GSH) and cysteine (CySH) were products of Sigma (St. Louis, MO). Thermoplastic polyurethane (Tecophilic®, SP-93A-100) was a gift from Lubrizol Advanced Materials, Inc. (formerly Noveon Inc.) (Cleveland, OH). Dipropylamine-PEO (DPA-400E, F.W. = 573.73) was donated by Tomah products (Milton, WI). Other chemical reagents from Aldrich (Milwaukee, WI), all solvents from Fisher Scientific (Fair Lawn, NJ), and NMR reagents from Cambridge Isotope Laboratories, Inc. (Andover, MA) were used without further purification unless otherwise noted. Distillation was employed for the purification of hexamethylene diisocyanate (HMDI), *N*,*N′*-dimethylacetamide (DMAC), triethylamine (Et_3N), acetonitrile (CH_3CN) and tetrahydrofuran (THF) prior to use. Deionized water was provided by a Milli-Q filter system (18 MΩ cm^{-1}; Millipore Corp., Billerica, MA, USA).

2.2. Measurements

^1H and ^{13}C NMR spectra were recorded using either a Varian 400 or 500 MHz spectrometer. High-resolution (HR) mass spectra were obtained on a Waters Autospec Ultima Magnetic Sector mass spectrometer with an electrospray interface/ionization source. FTIR spectra were collected with a Perkin–Elmer spectrum BX FTIR system. UV spectra were recorded using a Perkin–Elmer Lambda 35 UV/VIS spectrometer. EPR spectra were taken with a Bruker EMX (center field, 3300 G; sweep width, 2000 G; frequency, 9.248 GHz). The copper content of the polymers was determined by inductively coupled plasma high-resolution mass spectrometry (ICP-HRMS), using a Thermo Finnigan Element. Nitric oxide generation measurements were conducted using a Sievers Nitric Oxide Analyzer (NOA), model 280. The current responses of amperometric NO/RSNO sensors were monitored via a microchemical sensor analyzer (Diamond General, Ann Arbor, MI).

2.3. Synthesis

2.3.1. $(Boc)_3$–cyclen–*N*-acetic acid (*1*)

$(Boc)_3$–cyclen–*N*-acetic acid (**1**) (see Scheme 1), was synthesized by modifying a reported procedure [28]. In brief, to a stirred solution of cyclen (3.0 g, 17.4 mmol) in anhydrous CH_3CN (40 mL) was slowly added a solution of benzyl 2-bromoacetate (1.5 mL, 9.2 mmol) in anhydrous CH_3CN (50 mL) over 6.5 h at 85 °C under a nitrogen atmosphere. After additional stirring for 1 h, the mixture was cooled to room temperature (RT). Without further purification, an excess amount of Et_3N (10 mL) was poured into the mixture at 0 °C, followed by the addition of di-*tert*-butyl-dicarbonate (Boc_2O, 14.8 g, 65.8 mmol), and then the reaction mixture was stirred overnight at RT. The solution was then concentrated and the residue was extracted with Et_2O/water. The separated organic layer was washed with dilute HCl, water, satd $NaHCO_3$, and water sequentially. The organic layer was dried with anhydrous Na_2SO_4, filtered, and washed with Et_2O. After the filtrate was concentrated, the crude material was dissolved in MeOH (20 mL) and stirred at RT, and then a solution of 2 N NaOH (10 mL) was added dropwise into the reaction solution over a 2 h period. The solvent was then evaporated under reduced pressure, and the residue was extracted with Et_2O/water. The separated water layer was acidified with citric acid to a pH between 3 and 4. After the extraction with ethyl acetate (EA) three times, the combined organic layers were dried with Na_2SO_4, filtered, and washed with EA. After the filtrate was concentrated, the crude product was purified by column chromatography using silica gel (EA, then MeOH/CH_2Cl_2 = 1/8) to afford the desired product, an amorphous white solid (1.4 g, overall yield = 15%).

^1H NMR (500 MHz, $CDCl_3$): $\delta = 3.90–3.06$ (m, 14H, $7NCH_2$), 2.86 (bs, 4H, $(CH_2)NCH_2CO_2$), 1.40 (s, 9H, $3CH_3$), 1.37 (s, 18H, $6CH_3$); ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 155.92$, 155.34, 79.77, 79.43, 53.77, 51.34, 49.68, 47.30, 28.59, 28.39; HRMS (EI) *m/z* $[M + Na]^+$ calcd. for $C_{25}H_{46}N_4O_8Na$: 553.3213, found: 553.3207.

2.3.2. Aminated PU (*2*)

A procedure adapted from previous reports [29,30] was employed to prepare the aminated PU polymer (see Scheme 1). Thermoplastic PU (Tecophilic®, SP-93A-100) was purified by Soxhlet extraction with methyl *tert*-butyl ether (MTBE) for 3 d prior to use and then dried thoroughly. The purified PU (20 g, ca. 80 mmol of urethane group) was dissolved in anhydrous DMAC (400 mL). This solution was added dropwise into a stirred solution of HMDI (39 mL, 241 mmol) and dibutyltin dilaurate (DBTDL, 0.72 mL, 1.2 mmol) in DMAC (40 mL) at 40 °C over a 3.5 h period, and then stirred for 1.5 d. The mixture was cooled to RT and slowly added into anhydrous Et_2O (4 L) under a nitrogen atmosphere. The resultant solid was filtered and washed with anhydrous Et_2O (600 mL). After the filter cake was dried by with N_2, it was further vacuum dried to yield a colorless polymer. This polymer (19.3 g) was again dissolved in anhydrous DMAC (400 mL) and slowly added into a stirred solution of dipropylamine-PEO (15.5 g) in anhydrous DMAC (40 mL) at 40 °C over 3 h. The mixture was stirred for 4 d at 40 °C, and then slowly added into Et_2O (4 L) to form a solid, which was filtered and washed with Et_2O (600 mL). The filter cake was Soxhlet extracted with MTBE for 3 d and then cooled to RT. The remaining polymer was dried under a vacuum pump for 4 d to yield the desired aminated PU (**2**) (19.4 g). The amount of free amine sites created in the aminated PU (**2**) was analyzed by a colorimetric method [31] and found to vary depending on the reaction scale and time used (see Section 2.4). The given method described above afforded ca. 80 μmol of amine sites per one gram of the polymer.

IR (neat) = 3323 (N–H), 2916, 2857 (CH_2), 1715 (C=O), 1614 (HNCONH), 1529 (C–N, N–H), 1102 cm^{-1} (CH_2–O–CH_2).

2.3.3. Cu(II)–cyclen–PU (*5*)

EDC (0.4 g, 2.1 mmol), Et_3N (0.8 mL, 5.7 mmol), and NHS (0.26 g, 2.3 mmol) were sequentially added into a stirred solution of $(Boc)_3$–cyclen–*N*-acetic acid (**1**) (0.8 g, 1.5 mmol) in THF/water (10 mL/10 mL). After 30 min, this mixture was poured into a clear solution of aminated PU (**2**) (19.1 g, ca. 1.53 mmol of amine sites) in THF (500 mL). The reaction mixture was stirred for 5 d at 30 °C, and then concentrated to one-fifth volume under a reduced pressure. The gradual addition of the residue into stirred solvents of DI water/Et_2O (0.25 L/3.75 L) yielded a solid polymer. The polymer was sieved and washed with DI water. The residual polymer was immersed and stirred in Et_2O (1 L) for 1 d at RT. The resulting polymer was filtered and washed with Et_2O, and then dried via a vacuum pump to yield the desired polymer, $(Boc)_3$–cyclen–PU (**3**) (18.5 g, see Scheme 1).

To remove the Boc groups from the appended cyclen moieties, TFA (10 mL) was added into a polymer (**3**) (17 g) solution in $CHCl_3$ (500 mL) at 0 °C, and the mixture was stirred for 3 d at RT. The reaction mixture was then concentrated using a rotary evaporator and the residue was diluted with $CHCl_3$. The same procedure was repeated three times to remove any remaining TFA. The solid polymer formed in Et_2O was filtered and washed with Et_2O. The filter cake was soaked in dilute NaOH (pH ~ 12) for 6 h at RT, and then filtered and washed with DI water until the pH of the filtrate reached approximately 7. The polymer was then washed with Et_2O and MTBE and Soxhlet extracted with MTBE for 3 d to afford a slightly yellowish polymer, cyclen–PU (**4**) (16 g, see Scheme 1), obtained after thorough drying via a vacuum pump.

To incorporate Cu(II) into the deprotected cyclen–PU, to a stirred solution of cyclen–PU (**4**) (15 g) in THF (500 mL) was added a clear solution of cupric chloride dihydrate (2.9 g) in EtOH (100 mL) at 50 °C. The mixture was stirred overnight and cooled to RT. The solvents of the mixture were evaporated to one-third the original volume under reduced pressure, and then the residue was slowly added into a mixed solvent of EtOH/Et_2O (0.5 L/2 L) to form a green-colored solid polymer. After this polymer was filtered and washed with EtOH/Et_2O (0.5 L/2 L), the filter cake was fully immersed and stirred in EtOH, and then filtered and washed with EtOH/Et_2O (0.5 L/2 L). The filter cake was then soaked and stirred in DI water for 1 d, and the residual solid was filtered again and washed with DI water and MTBE, sequentially. Finally, the polymer was purified by Soxhlet extraction with MTBE and dried under vacuum for 3 d to give a slightly blue-tinted polymer, Cu(II)–cyclen–PU (**5**) (10 g, see Scheme 1).

IR (neat) = 3325 (N–H), 2919, 2863 (CH_2), 1715 (C=O), 1530 (C–N, N–H), 1109 cm^{-1} (CH_2–O–CH_2).

2.4. Determination of free amine equivalents in the aminated PU (2)

Amine equivalents in the aminated PU (**2**) were determined by a conventional colorimetric titration method as a reported elsewhere [18,31]. Three different solutions were prepared as follows; (i) colorimetric indicator: 0.1 N NaOH solution was slowly dropped into a solution of bromophenol blue (0.1 g) in MeOH (100 mL) until the solution color changed from pale purple to blue; (ii) samples: three solutions of aminated PU (**2**) (73.1, 73.6, and 71.6 mg, respectively) dissolved in DMAC (5 mL), and a blank solution having only the solvent; and (iii) titrating agent: 1.0 mM *p*-toluenesulfonic acid monohydrate dissolved in isopropanol. Each sample solution (including the blank sample) was diluted with isopropanol (5 mL) and mixed with one drop of the indicator solution. Finally, this mixture was titrated by a solution of *p*-toluenesulfonic acid until the color disappeared. The average value of free amine sites in the polymer was obtained after correction for the endpoint of the blank solution.

2.5. Measurements of copper content in the polymers

To assess the copper content of the various PU–Cu(II)–cyclen polymers, a method described elsewhere [16] was followed. In brief, a given small piece of polymer (a few mg) was dissolved in conc. sulfuric acid (0.5 mL) overnight at RT and then diluted with DI water (4.5 mL). The resultant clear mixture was passed through a syringe filter (0.45 μm PTFE, a National Scientific Company product), and then the copper content in the filtrate solution was analyzed by ICP-HRMS.

2.6. NO measurements via a chemiluminescence NO analyzer (NOA)

All NO generation measurements from RSNO species by the new PU-based NOGP were carried out using an NOA via the same methodology described in several previous papers [16–18]. Various RSNOs (GSNO, CySNO, and macromolecular BSA-NO) were freshly prepared by methods reported previously [17,19,32].

2.7. Catalyst leaching studies

2.7.1. Soaking in a GSH/GSNO solution

Prior to soaking, the NO generation flux of a small disk-shaped film of Cu(II)–cyclen–PU (**5**) ($r = 3.2$ mm, $\theta = 65$ μm) was determined in the presence of 10 μM GSH/GSNO solution dissolved in 2 mL of 10 mM PBS buffer, pH 7.4, containing 3 μM EDTA at RT via the NOA. This film was then fully bathed (with agitation) in a high concentration of GSH/GSNO (0.1 mM) in 2 mL of PBS buffer, pH 6.6, overnight at RT. The film was removed from the mixture and washed with DI water and PBS buffer, and then placed into 10 mL of a fresh PBS buffer, pH 7.4. The same washing procedure was carried out several times using fresh PBS buffer. The resulting film was stored in fresh PBS buffer to ensure that the film was in a hydrated state immediately before use in the NOA experiments. Finally, this film was retested to assess whether this soaking treatment changed the levels of NO generation that could be achieved (compared to the initial test, before extensive conditioning) using the same test reaction conditions (10 μM GSH/GSNO). For a longer-term leaching study, polymer **5** (~1 cm × 1 cm, $\theta = 65$ μm, $W_d = 8.86$ mg, 0.08 wt % copper) was fully soaked and shaken in 10 μM GSH/GSNO in 5 mL of PBS buffer, pH 7.3, at RT. The solution was replaced daily with a fresh solution over a 7 d period. Then, the copper content in each solution (1, 2, 3, 5, and 7 d) was analyzed via inductively coupled plasma high-resolution mass spectrometry (ICP-HRMS).

2.7.2. Soaking in sheep plasma

The NO generation from a small disk of Cu(II)–cyclen–PU (**5**) ($r = 2.6$ mm, $\theta = 30$ μm) was also monitored in a solution of 0.5 μM CySH/CySNO in 2 mL of 10 mM PBS buffer, pH 7.4, containing 3 μM EDTA. Simultaneously, several films (same size) were separately immersed in platelet-rich sheep plasma obtained by centrifuging (1300 rpm for 18 min at 4 °C) whole blood from sheep (purchased from Lampire Biological Lab, Pipersville, PA), and then storing the films in plasma at 4 °C. After a given time interval, the typical washing procedure with DI water and PBS buffer (see Section 2.7.1) was carried out. Then, the NO generation capability of each film was examined using the same reaction conditions (0.5 μM CySH/CySNO) as the film that had not been soaked in sheep plasma.

2.8. Fabrication of amperometric NO/RSNO sensors

Each amperometric sensor was constructed in a manner analogous to that reported elsewhere [19,33]. In brief, a platinized Pt working electrode (Pt disk with 250 μm o.d.) sealed in glass wall tubing was surrounded by a coiled Ag/AgCl wire reference/counter electrode, and these two electrodes were integrated behind a polytetrafluoroethylene (PTFE) gas-permeable membrane (GPM) treated with 0.5 μL of 1% Teflon AF solution (Dupont Fluoroproducts, Wilmington, DE) [34]. A thermoplastic PU film (Tecophilic®, SP-93A-100) was used as a control layer for one sensor (control sensor with no NO generation) by mounting it on the GPM of a NO sensor using an O-ring. To prepare the RSNO sensor, a piece of Cu(II)–cyclen–PU (**5**) was mounted over the GPM of the NO sensor. Finally, both sensors were polarized at +0.75 V vs Ag/AgCl for at least 12 h prior to use. All amperometric measurements were performed using the same applied potential.

2.9. Amperometric NO detection in blood

The amperometric NO measurements in blood using both the NO and RSNO sensors were carried out using the procedures described previously [16,19,33]. In these experiments, 70 mL of PBS buffer, pH 7.4, containing 5 μM EDTA and 10 μM GSH was used to dilute 30 mL of fresh sheep whole blood (obtained from the ECMO research lab at the University of Michigan Medical School).

3. Results and discussion

3.1. Synthesis and characterization

In order to conjugate the Cu(II)–cyclen complex to an existing medical-grade hydrophilic thermoplastic PU (Tecophilic®, SP-93A-100), the two key precursors, $(Boc)_3$–cyclen–*N*-acetic acid (**1**) and aminated PU (**2**), were prepared by methods analogous to those reported elsewhere [28–30] (see Scheme 1). To maintain the strong Cu(II) complexation ability with the cyclen ligand after covalent linkage to the polymer (without any loss of basicity in the cyclen compound), a carboxylic group was introduced on the cyclen moiety via *N*-alkylation of cyclen using 2-bromoacetic acid and *in situ* Boc-protection to yield $(Boc)_3$–cyclen–*N*-acetic acid (**1**). To link this species to the polymer, free amine sites were created within the thermoplastic PU by an isocyanation reaction, followed by amination in dilute conditions to form the aminated PU (**2**). The total amount of free amines in the resultant aminated PU (**2**) was found to be 80 μmol/g of the polymer, as determined by the colorimetric titration method [31]. It should be noted that the amount of amine sites created in the polymer can be varied by controlling the reaction conditions (i.e., such as scales, reagent equivalents and times) and ranged from 80 to 200 μmol/g polymer.

Infrared spectra of the polymers clearly show the expected characteristic absorption band patterns [35], in which the newly formed isocyanate and urea bands appear in the

isocyanated PU-intermediate. The isocyanate band disappears after free amine sites are formed in the final aminated PU (**2**) while the urea group still remains (see Fig. 1). After the conjugation of $(Boc)_3$–cyclen–*N*-acetic acid (**1**) with the aminated PU (**2**) using EDC coupling chemistry, the Boc groups were deprotected with TFA and the mixture was treated with dilute NaOH to remove TFA. Copper ions were then incorporated into the polymer. The extensive washing procedures with various solvents to remove the non-specifically bound copper ions in the polymer backbone ultimately afforded the desired polymer, Cu(II)–cyclen–PU (**5**).

Generally, when the aminated polymer (**2**) containing a higher level of free amine sites is employed in the reaction, the resulting polymer **5** has somewhat increased copper content, as determined by ICP-HRMS (in the range from 0.08 to 0.4 wt %). However, this relatively low copper content in all polymer **5** formulations suggests that only a very low percentage of the cyclen derivative added to the reaction mixture is actually covalently attached to PU. Consequently, identifying the cyclen groups within the isolated polymers using NMR or IR is difficult. Nonetheless, the UV and EPR spectra, as well as the NO detection experiments (see below) support the fact that Cu(II)–cyclen complexes are in fact linked to the hydrophilic thermoplastic PU. Indeed, the Cu(II)–cyclen complex is known to have a square-pyramidal structure with an absorption band in the region 550–670 nm in the visible range of the spectrum [36]. While the λ_{max} of Cu(II)(cyclen)Cl_2 has been reported to be 594 nm in water [36], a film of Cu(II)–cyclen–PU (**5**) has an absorption maximum at 617 nm when in a fully hydrated state (see Fig. 2(A)). Such a bathochromic shift in λ_{max} has been observed in a previous study [16], where the Cu(II)–cyclen complex was covalently attached within a crosslinked poly(2-hydroxyethyl methacrylate) (pHEMA) film. This change in UV–vis absorption may be caused by the surrounding polymer matrix, a possible structural distortion of the Cu(II)–cyclen complex in the polymer matrix, or the substitution of chloride ion for hydroxide ion as the axial ligand for the Cu(II)–cyclen–PU (**5**) during the preparation process. Nonetheless, the EPR spectrum of Cu(II)–cyclen–PU (**5**) matches quite well with that of the small molecule Cu(II)–cyclen complex, showing the typical four lines observed for a square-pyramidal structure of Cu(II)-N4 macrocyclic ligand (the values of $g_\perp$ (2.05) and $g_\parallel$ (2.13) were measured to be identical with those values of Cu(II)–cyclen as shown in Fig. 2(B)) [37], suggesting successful copper ion complexation within the cyclen moieties appended to the polymer.

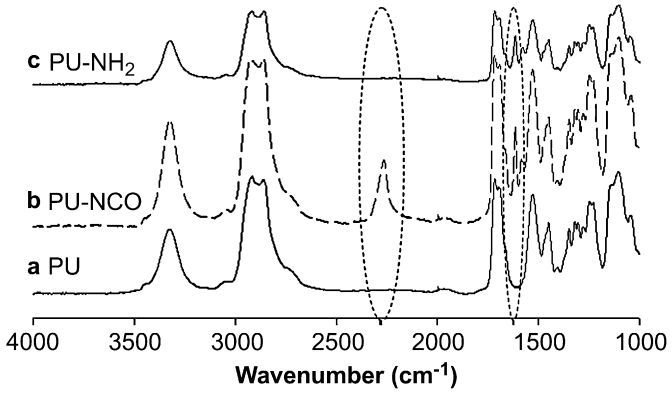


Fig. 1. IR spectra of (a) thermoplastic PU (Tecophilic®, SP-93A-100), (b) isocyanated intermediate, and (c) aminated PU (**2**), where two distinguishable peaks corresponding to the newly formed isocyanate group (2265 cm^{-1}) and urea group (1615 cm^{-1}) appeared in the (b) spectrum, while the band at 2265 cm^{-1} disappeared in the (c) spectrum; these spectra were recorded using the polymers analyzed to have the highest amine sites (200 μmol/g polymer).

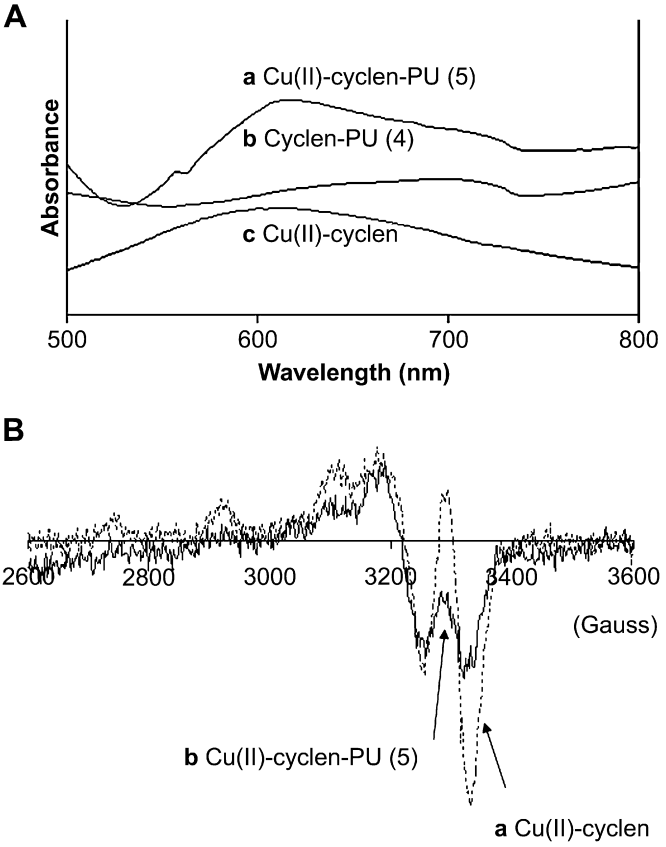


Fig. 2. (A) UV spectra of (a) Cu(II)–cyclen–PU (**5**), (b) cyclen–PU (**4**) in fully hydrated states, and (c) a small molecular Cu(II)–cyclen in PBS; *Y*-axis (absorbance) is relative, not absolute. (B) EPR spectra of (a) Cu(II)–cyclen complex and (b) Cu(II)–cyclen–PU (**5**) in PBS buffer, pH, 7.4, at 77 K: Center field, 3300 G; sweep width, 2000 G; frequency, 9.248 GHz.

3.2. Catalytic S-nitrosothiol decomposition to NO via Cu(II)–cyclen–PU (5)

The estimated NO flux of healthy endothelium cells that line all blood vessels is approximately 10^{-10} mol/cm^2 min [38]. In fact, only a very low concentration of NO (<1 nM) is known to be effective in reducing thrombus formation on the surface of a foreign material [39–42]. Endogenous RSNOs are known to exist in blood in the range of nM to μM levels, although their exact concentrations are still in question [43]. Low molecular weight (LMW) endogenous RSHs such as CySH and GSH are also present in human plasma at various concentrations (10–200 μM for CySH, 5–20 μM for GSH)

[44,45]. Therefore, even a small fractional decomposition of endogenous RSNOs to locally produce biologically relevant NO levels at the polymer/blood boundary should be effective to improve a polymer's hemocompatibility.

When a small piece (radius = 2.5 mm, thickness = 30 μm, copper content = 13 nmol (0.14 wt %)) of the newly modified PU material is placed into a solution of 10 μM GSNO/GSH of a phosphate buffered saline (PBS), pH 7.4, containing 3 μM EDTA (2 mL), biologically relevant levels of NO are produced (see Fig. 3). The amount of NO generated from a given reaction solution by the new Cu(II)–cyclen–PU (**5**) material can be continuously monitored by the NOA. A burst of NO signal is initially detected (see Fig. 3(A)) and then the NO flux slowly decreases to reach a steady-state NO level (approximately 1×10^{-10} mol/cm^2 min). When the film is then removed from the solution, the NO signal returns to the original baseline, implying that the presence of this polymer initiates the NO liberation from GSNO. The continual reinsertion/removal of the film demonstrates that the polymer can generate a similar steady-state NO flux after each immersion and removal from the test solution (see Fig. 3(A)). The same film **5** was also tested in a solution of 0.5 μM CySNO/CySH in PBS buffer. Upon addition of the film into this solution, the NO signal increases to reach given NO flux (approximately 1×10^{-10} mol/cm^2 min), and then gradually decreases to the baseline until all the CySNO is completely consumed (see Fig. 3(B)).

To demonstrate that immobilized copper ions are the active species to denitrosate the RSNO species, a similar sized film of cyclen–PU (**4**) that was not treated with copper ions was tested for NO generation ability. When this blank film is soaked in a solution of 20 μM GSNO/GSH in the PBS buffer, the NO baseline increases slightly and soon decreases to essentially the original baseline (see Fig. 3(C)). To prove whether free copper ion can non-specifically bind to the polymeric backbone, a film of 3-Boc–cyclen–PU (**3**), which was treated with copper ions by the same method employed to prepare polymer **5**, was immersed into the 20 μM GSNO/GSH solution. Similar to the previous control film, the baseline is initially elevated, but then quickly returns to the original baseline (see Fig. 3(D)). This data supports the fact that the copper ions complexed with the cyclen moieties in Cu(II)–cyclen–PU are responsible for the NO generation observed with this new polymeric material.

Ascorbate is known to be a good reducing agent for the copper ion-mediated RSNO decomposition [46]. The reducing ability of ascorbate using this new NOGP (Cu(II)–cyclen–PU (**5**)) is observed to be equivalent to GSH (see Fig. 1s in Supplementary data). When the different species at equal concentrations were tested as the reducing agent for NO generation

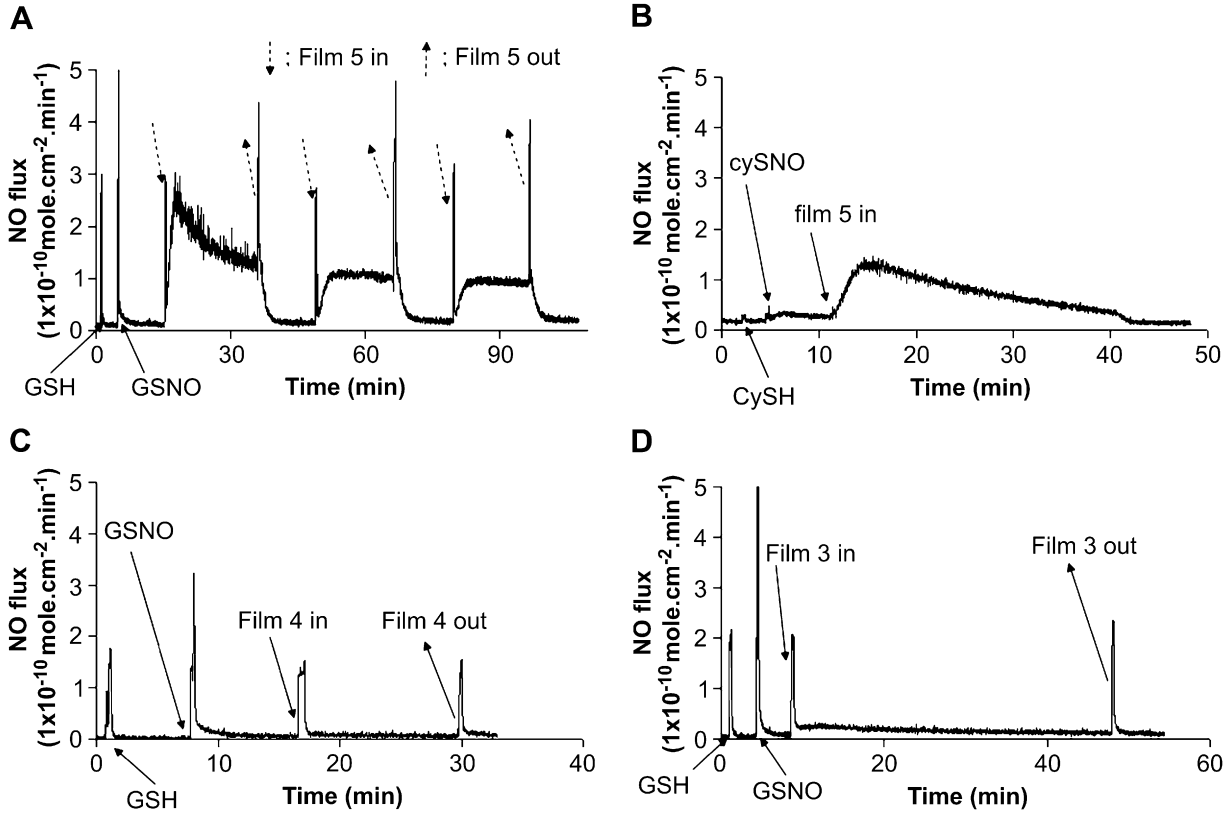


Fig. 3. The catalytic NO production mediated by a small disk film of Cu(II)–cyclen–PU (**5**) (radius = 2.6 mm, thickness = 30 μm, W_d (weight at a dried state) = 0.6 mg, Cu content = 0.15 wt %) in the solution of (A) 10 μM GSNO/GSH, and (B) 0.5 μM CySNO/CySH in PBS buffer, pH 7.4, containing 3 μM EDTA; a similar sized film of (C) cyclen–PU (**4**) and (D) copper ion-treated $(Boc)_3$–cyclen–PU (**3**) in 10 μM GSNO/GSH solution in PBS buffer, pH 7.4, containing 3 μM EDTA; arrows indicate the moment when a given species is added or removed, and all NO measurements were recorded by a chemiluminescence NO analyzer. All spikes in figures are artifacts due to opening the reaction vessel.

by polymer **5**, the highest NO flux is achieved in the presence of CySH. In addition, the reaction rate of CySNO with this NOGP is greater than that of GSNO at the same concentration of reducing agent (GSH and/or CySH (GSNO/GSH < GSNO/CySH < CySNO/GSH, see Fig. 2s in Supplementary data)). This confirms why the lower concentration of CySNO/CySH (0.5 μM) could achieve a similar NO flux as higher concentrations of GSNO/GSH (10 μM) (see Fig. 3(A) and (B)). Further, when BSA-NO (10 μM) was employed as a high molecular weight (HMW) RSNO test species in the presence of GSH (10 μM), the NO flux exhibited by films of polymer **5** is slightly lower than that observed for the GSNO/GSH (see Fig. 3s in Supplementary data).

Given that the rate determining step for free copper ion-mediated RSNO decomposition is known to be the reduction step in which RSH species reduces Cu(II) to Cu(I) [47], and the pK_a value of CySH (8.3) is lower than GSH (8.7) [48], it is reasonable that CySH is likely a more favorable reducing agent at physiological pH. Therefore, the reaction rates observed at steady state in the experiments described above might be anticipated. However, Noble and Williams have recently reported unexpected results in which the decomposition rates of GSNO and CySNO are equivalent in the presence of a low concentration (μM) of free copper ions [49]. Based on the pK_a value of the RSHs and the RSNO decomposition rate at μM concentrations of copper(II), the reason why the NO flux derived from the CySNO/GSH mixture is greater than that for GSNO/CySH becomes less clear. However, if we consider that because reaction with the polymer film is a heterogeneous process, the mass transfer rate of substrates (RSNO and reducing agent) could also come into play and dictate the observed reaction rate in these experiments. Indeed, since GSH is a much larger molecule than CySH, and GSNO is similarly much larger than CySNO, the observed order of NO generation level in our experiments (GSNO/GSH < GSNO/CySH < CySNO/GSH < CySNO/CySH) seems to fit well with a mass transport/diffusion limited model. Since the diffusion of macromolecular BSA-NO to and within the polymeric phase is not likely to be very high, the reason why the NO flux observed for the BSA-NO/GSH experiment is comparable with GSNO/GSH must also be explained. Our speculation is that a bulk solution phase transnitrosation reaction between BSA-NO and GSH occurs [50–52] to create smaller more diffusible GSNO species, and it is the LMW GSNO formed by transnitrosation that likely is the molecule that is yielding most of the NO generated in these experiments with macromolecular RSNOs.

3.3. Copper leaching studies

Ideally, a useful NOGP must be designed to generate NO for prolonged periods of time once implanted. To achieve this goal, the catalytic NO generation activity of the Cu(II)–cyclen–PU should be maintained under the physiological conditions for an extended time period. The stability of Cu(II)–cyclen complex within the polymer is the primary concern because any blood components such as free thiols and proteins that possess high binding affinities with copper ions may facilitate demetalation and/or deactivation of the complex. For example, low molecular weight endogenous RSHs as well as their oxidized forms (disulfides, RSSRs) are known to bind copper ions tightly [53,54]. In fact, previous studies [16] suggested that free thiols, such as GSH, can influence the copper ion leaching from the Cu(II)–cyclen complex immobilized in a crosslinked poly(2-hydroxyethyl methacrylate) matrix, but does not deactivate the copper ion sites of Cu(II)–cyclen complex in the polymer.

Although the NOGP developed here (Cu(II)–cyclen–PU (**5**)) is a different polymer matrix, the potential leaching of copper ions from the polymer remains a concern and was examined using the method previously reported [16]. First, a film of Cu(II)–cyclen–PU (**5**) was repeatedly tested for its NO generation using an RSH/RSNO solution under typical test conditions, showing that the maximum steady-state NO level continuously decreases with every trial until it reaches a given steady-state NO level after approximately 12 sequential uses (ca. 50% reduction compared to the original NO flux; (see Fig. 4(A)). Additional experiments were carried out to examine whether the Cu(II)–cyclen–PU polymer is still able to liberate NO from GSNO after soaking in a high concentration of GSH/GSNO solution (0.1 mM) and a low pH (pH = 6.6) overnight at RT, conditions that are expected to accelerate the demetalation and/or deactivation reactions. However, after such soaking and subsequent washing, the polymer is still able to generate NO from GSNO, albeit at a reduced flux (see Fig. 4s in Supplementary data). In order to confirm the extent of demetalation of polymer **5**, a 7 d leaching study was performed. A small sample of polymer **5** was fully soaked and shaken in the 10 μM GSNO/GSH solution in 5 mL of PBS buffer, pH 7.3, at RT. The soaking solution was replaced daily. The analyzed copper content in each solution as determined by ICP-HRMS showed that 25% of Cu(II) was lost after only 1 d of soaking, and another 25% was lost by the end of the 7th day (total of 50% decrease) (see Fig. 4(B)). Therefore, once pretreatment of the polymer occurs with RSH/RSNO solution prior to use which removes weakly bound copper(II) ions (including non-specifically bound to backbone and to amine sites), then the copper leaching rate is minimized. Analogous experiments, carried out with a similar Cu(II)–cyclen–polymethacrylate material described previously, yielded somewhat similar leaching of Cu(II), but still exhibited more than 50% of its original NO generating ability after 15 d [16]. Taken together, the demetalation of Cu(II)–cyclen–PU (**5**) can occur slowly under physiological conditions; however, the deactivation of the new NOGP caused by the strong coordination of small molecular RSH and/or RSSR species onto the metal-ion center does not appear to be a serious issue.

To evaluate how the NO generation profiles of Cu(II)–cyclen–PU in a given RSNO/RSH solution can be changed before and after contact with sheep plasma for a finite time period, films of Cu(II)–cyclen–PU were separately immersed into the platelet-rich sheep plasma for a given time interval at 4 °C. Then, the NO production profiles mediated

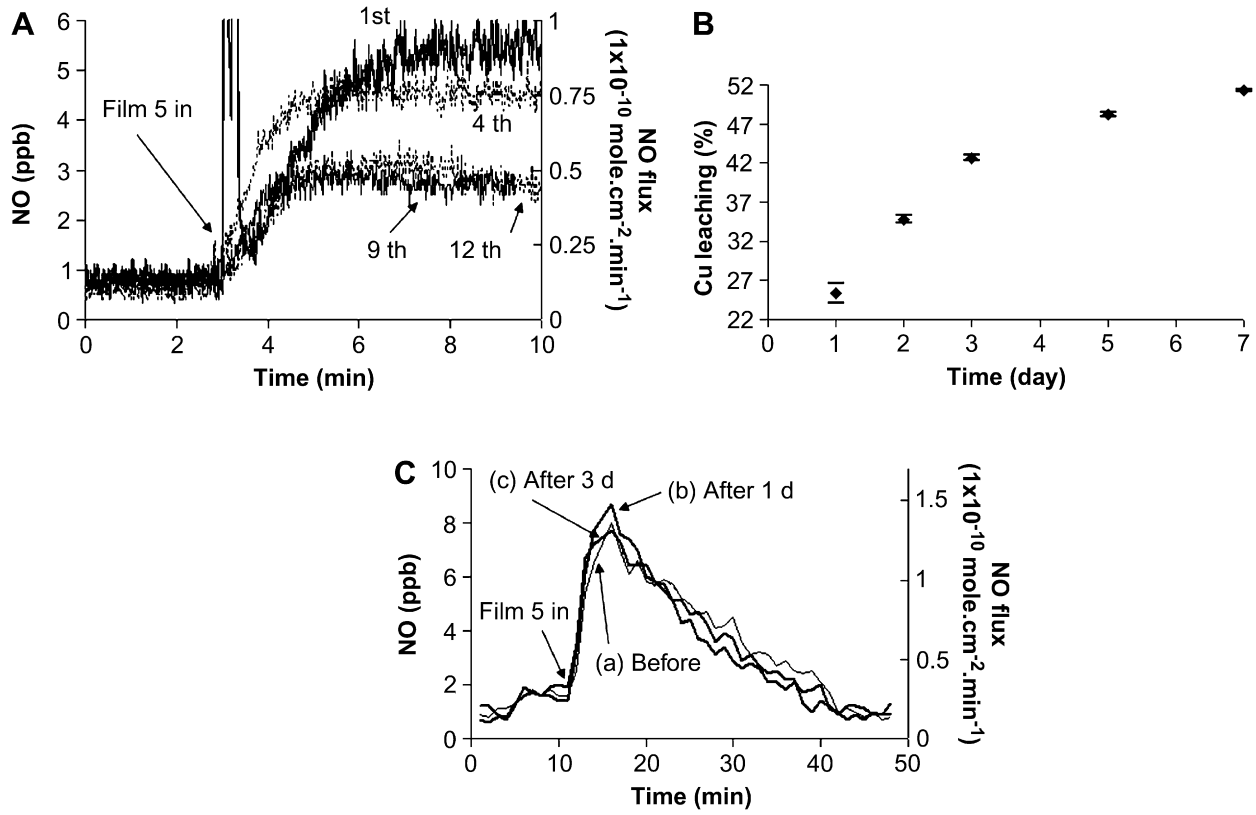


Fig. 4. (A) Cu(II)–cyclen–PU (**5**) (the same film used in the experiments as shown Fig. 3) was sequentially tested with various substrates (also see Fig. 1s and 2s in Supplementary data), where the number of times used in the experiments is indicated in the graph, then its NO flux at a steady-state in a solution of 10 μM GSNO/GSH in PBS buffer, pH 7.4, containing 3 μM EDTA was independently recorded and collectively plotted. (B) The analysis of copper amount (%, standard deviation = ± 0.4%) in a soaking solution with time (for 7 d) as measured via ICP-HRMS after a new film (Cu(II)–cyclen–PU (**5**), size = ~1 cm × 1 cm, thickness = 65 μm, W_d = 8.86 mg, 0.08 wt % copper) was fully soaked and shaken in 10 μM GSH/GSNO in 5 mL of PBS buffer, pH 7.3, at RT. The soaking solution was replaced daily with a fresh solution over one week. (C) The comparisons of the NO generation profiles using the same sized films of Cu(II)–cyclen–PU (**5**) in a solution of 0.5 μM CySNO/CySH in the PBS buffer before (a) and after soaked in a platelet-rich sheep plasma at 4 °C for 1 d (b) and 3 d (c); all NO measurements were monitored by a chemiluminescence NO analyzer.

by these films from an CySNO/CySH solution were measured by the NOA. As shown in Fig. 4(C), the NO generation by these films exhibit very similar behavior regardless of the exposure time to sheep plasma. This result suggests that the blood components do not have a significant influence on copper ion leaching and/or deactivation of the Cu(II)–cyclen sites in polymer **5**, at least during the time period tested.

The results from the various soaking experiments carried out here with polymer **5** are consistent with the prior study where Cu(II)–cyclen was appended to pHEMA [16], and indicate that the demetalation and/or deactivation of the Cu(II)–cyclen complex in the polymeric matrix by various blood components is not a serious concern, although the presence of LMW endogenous RSH and/or RSSR species likely can affect the rate of copper ion demetalation in this new polymer. More intensive *in vivo* studies for the various time periods are required to fully assess whether copper leaching from the new NOGP (Cu(II)–cyclen–PU (**5**)) will be a problem for practical biomedical applications of this new material.

3.4. Spontaneous S-nitrosothiol decomposition to NO in whole blood

To further assess the potential biomedical utility of new Cu(II)–cyclen–PU, it is essential to prove that the polymer can generate NO in the presence of fresh whole blood. One experimental strategy to demonstrate this NO generation capability is to measure the increase in NO levels produced by the Cu(II)–cyclen–PU polymer in blood when a polymer film of this material is coated over the distal end of an amperometric NO sensor. This approach has been utilized in the previous study [16] to prove that the Cu(II)–cyclen–pHEMA material can generate NO when in contact with fresh blood. To conduct such experiments, two sensors need to be constructed. One is a control NO sensor where a thin film of plain unmodified thermoplastic PU (Tecophilic®, SP-93A-100) is used as an outer membrane over the distal tip of the NO selective sensor. The second sensor is an RSNO sensor where a similar sized film of Cu(II)–cyclen–PU (**5**) is employed as an outer membrane over the tip of the device (see Section 2.8 for details). Prior to contact with fresh blood, the inherent

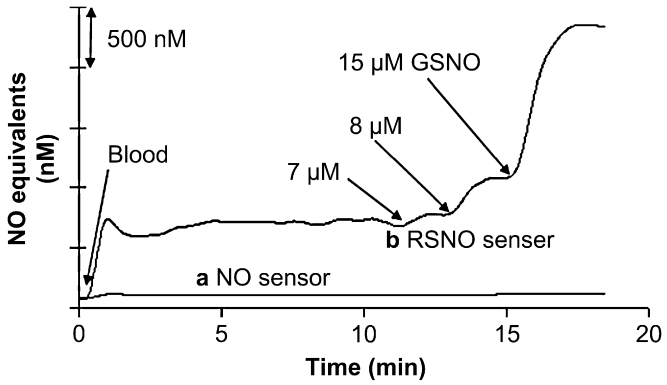


Fig. 5. The direct detection of endogenous RSNOs in fresh sheep blood using the amperometric (a) NO sensor and (b) RSNO sensor by injecting 30 mL of fresh sheep blood into 70 mL of 10 mM PBS buffer (pH 7.4) containing 5 μM EDTA at 36 °C; the intrinsic amperometric responses of two sensors toward the exogenous RSNO were also demonstrated by adding the GSNO solution into the same blood sample; arrows indicate when a given species was added.

amperometric responses of both sensors toward NO are determined using pure NO gas standard solution (see Fig. 5s in Supplementary data).

The two sensors are then simultaneously placed into PBS buffer at 36 °C and fresh sheep whole blood is then added. As shown in Fig. 5, the amperometric responses of each sensor are recorded in real time. While the control NO sensor exhibits a small background signal when the fresh whole blood is added (little or no free NO in blood), the RSNO sensor exhibits a significantly higher NO response signal (see Fig. 5), indicating that the presence of the Cu(II)−cyclen−PU causes an increase in NO levels locally at the polymer/blood interface. Subsequent addition of a standard amount of GSNO to the same diluted fresh blood sample clearly confirms that the RSNO sensor (with the Cu(II)−cyclen−PU polymer on the surface) responds to the exogenous GSNO while the NO sensor does not (see Fig. 5). Consequently, *in situ* NO generation in the fresh sheep whole blood by the Cu(II)−cyclen complex tethered PU (Cu(II)−cyclen−PU) is confirmed by such simple measurements with the modified NO sensor coated with a film of this new polymer.

4. Conclusions

In summary, the newly prepared Cu(II)−cyclen−PU (**5**) has been shown to exhibit significant catalytic denitrosation of various RSNOs including GSNO, CySNO and BSA-NO in the presence of LMW RSHs or ascorbate at physiological pH. A variety of soaking experiments using excess amounts of GSNO/GSH or the platelet-rich sheep plasma indicate that high levels of LMW endogenous RSHs and/or RSSRs are likely to accelerate the copper ion leaching from the polymeric film. Most importantly, it was shown that the Cu(II)−cyclen−PU material can generate significant levels of NO when in contact with fresh animal whole blood, as determined via use of the polymer to construct an amperometric RSNO sensor. Thus, this polymer is a potential candidate for *in vivo* animal testing as a coating material for a wide range of blood contacting biomedical devices, including implantable needle-type of glucose and other chemical sensors, as well as catheters and extracorporeal circuits.

Acknowledgements

The authors acknowledge Dr. Zhengrong Zhou, a former student in this laboratory, for advising on the Boc-deprotection step, Nathan Lafayette in the ECMO research lab at the University of Michigan Medical School, for providing the fresh sheep blood, and Dr. Ted Huston in the Department of Geology at the University of Michigan, for providing the copper analysis using ICP-HRMS. This research was funded by NIH (EB-000783 and EB-004527) and the U.S. Army (W81XWH-05-1-0602).

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.biomaterials.2008.02.004.

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