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COMMUNICATION

Preparation and characterization of an improved Cu^{2+}–cyclen polyurethane material that catalyzes generation of nitric oxide from *S*-nitrosothiols†

Kun Liu and Mark E. Meyerhoff*

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A new, stable and highly efficient Cu^{2+}–cyclen–polyurethane material is described and shown to exhibit an improved performance compared to that of prior materials for the catalytic decomposition of *S*-nitrosothiols to physiologically active nitric oxide.

Nitric oxide (NO) generating polymeric materials have been investigated recently and demonstrated to produce NO locally from *S*-nitrosothiols (RSNOs), such as *S*-nitrosoglutathione (GSNO), *S*-nitrosocysteine (CySNO) and *S*-nitrosoalbumin (AlbSNO).[1] These materials may continuously produce NO under physiological conditions from the pool of endogenous *S*-nitrosothiols present in blood, and thereby prevent the activation of platelets and concomitant formation of thrombus on blood contacting medical devices coated with such materials. Indeed, it is known that certain metal ions (Cu^{2+}, Fe^{2+}, Hg^{2+}, and Ag^{+})[2] as well as organometallic species (organodiselenides (RSeSeR)[3,4] and organoditellurides (RTeTeR)[5]) can catalyze the decomposition of RSNOs to NO. Hence, new biomaterials being developed, incorporating such catalysts on their surfaces, have the potential to sustain NO generation utilizing endogenous RSNOs as substrates or by infusing additional amounts of these species[6] into blood.

Among materials reported to date, Cu^{2+}-mediated NO generating polymers are attractive because Cu^{2+} is a well-known and very effective catalyst for RSNO decomposition.[7] The mechanism involves Cu^{2+} reduction by a thiolate anion (RS^{-}) to yield Cu^{+} and a disulfide (RSSR), and Cu^{+} then binds and transfers an electron to the RSNO species, yielding NO and a thiolate anion along with Cu^{2+}. The thiolate anion can then reduce Cu^{2+} to the active Cu^{+} species, creating a catalytic cycle.

In recent work, a NO generating polymer containing copper nanoparticles coupled with RSNO infusion[6] was successfully applied to a rabbit model for extracorporeal circulation (ECC) (with the NO generating coating on the inner walls of the ECC tubing). The results suggested that significantly reduced thrombosis and prevention of platelet aggregation after 4 h of blood exposure can be achieved *via* the NO generation chemistry. In this work, a new NO generating polymer that possesses a covalently bound Cu^{2+}–ligand complex (**1**, Fig. 1) is developed as an alternative to the nanoparticle-based system to avoid potential cytotoxicity due to copper leaching from the polymer. Although two analogous polymers (**2** and **3**, Fig. 1)[8,9] were previously reported to exhibit catalytic decomposition activity toward RSNOs, these materials had limitations with respect to their future biomedical application. For example, the preparation of polymer **2** is quite complicated and the catalyst content is difficult to control. Moreover, the amide bond between the catalyst and the modified polymer is not stable and therefore is susceptible to acid, base, or enzymatic degradation. The structure of polymer **3** is too hydrophobic due to the absence of a hydrophilic linker when tethering the Cu(II)–cyclen catalyst to the polymer backbone. Indeed, the hydrophobicity of polymer **3** might compromise its true biocompatibility, owing to a higher degree of protein adsorption.

In this communication, the optimized preparation, stability and catalytic activity of the new Cu^{2+}–cyclen mediated NO generating material are examined. Compared to the published polymers, polymer **1** is stable and potentially more thromboresistant because of an established covalent bonding (C–C) and the hydrophilic PEG (tetraethylene glycol) linker that attaches the catalytic complex to the polyurethane backbone. To synthesize polymer **1**, a short amino-terminated PEG linker (**4**) was first prepared in good yield following a modified Gabriel synthesis[10] (ESI, Scheme S1†). This LMW PEG is soluble in non-polar organic solvents (*e.g.*, chloroform, methylene chloride, ether) and therefore it is easy to wash away unreacted materials during the workup. In contrast, the HMW

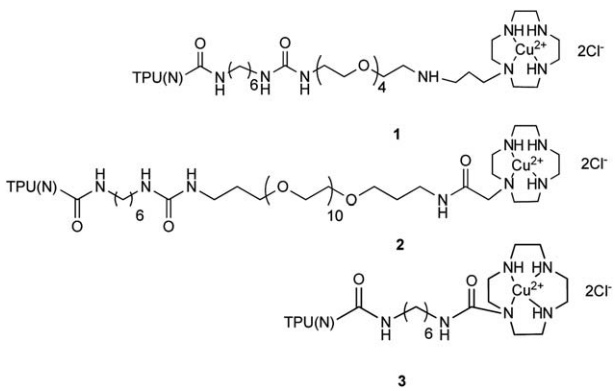


Fig. 1 Comparison of the newly synthesized (**1**) with the published (**2** and **3**) Cu^{2+}-mediated NO generating polymers.

Department of Chemistry, University of Michigan, 930 N. University Ave, Ann Arbor, MI, 48109, USA. E-mail: mmeyerho@umich.edu; Fax: +1-734-6474865; Tel: +1-734-7642169

† Electronic supplementary information (ESI) available: Experimental details for the synthesis of polymer **1**, characterization spectra and additional chemiluminescence data. See DOI: 10.1039/c2jm32726k

diamino-terminated polyethylene glycol (dipropylamine-PEO, DPA-400E)[8] used in the preparation of polymer **2** is difficult to remove from the reaction mixture using a Soxhlet extraction owing to its high polarity. Secondly, a stable cyclen derivative (**5**) was synthesized by covalently binding cyclen and the new PEG linker (**4**) through a three-step synthesis (ESI, Scheme S2†). Finally, compound **5** was applied to prepare the target polymer, TPU-PEG–cyclen–Cu^{II} (**1**) (Scheme 1), in accordance with previous methods.[8,11–13] IR and NMR spectroscopy were employed to monitor and characterize each step of the syntheses (ESI, Fig. S1 and S2†). Finally, it was found that the final polymer **1** contained 0.2–0.5 wt% copper, as quantitated by ICP-AES analysis. The presence of the Cu(II)–cyclen complex within the isolated polymer was also characterized by UV-Vis spectroscopy *via* the absorbance band with a λ at 674 nm (ESI, Fig. S3†).

Cu(II)-catalyzed decomposition of RSNO has previously been investigated, including the mechanism, proposed structures of active intermediates, rate constants and rate limiting steps.[7,14,15] All of these factors have an influence on the NO generation observed with polymers possessing tethered Cu^{2+}–ligand complexes. When films of the new polymer were cast (using THF as solvent) and the solvent removed, the dried films were tested for NO generation from RSNOs in a solution of PBS (10 mM, pH 7.4) containing 3 μM EDTA (used to chelate trace metal ion contaminates in the buffer) using real-time detection of NO by chemiluminescence. The new polymer (**1**) exhibited excellent NO generating activity, even when using a low concentration of GSNO (5 μM), with apparent fluxes of 6–10 × 10^{-10} mol cm^{-2} min^{-1} (see Fig. 2(a)). This is higher than the biologically relevant NO fluxes (*ca.* 0.5–4.0 × 10^{-10} mol cm^{-2} min^{-1}) known to occur from the surface of endothelial cells that line the inner walls of healthy blood vessels.[16,17] Repeated reinsertion/removal of the film from the test solution indicated that a relatively constant NO flux of 6 × 10^{-10} mol cm^{-2} min^{-1} can be achieved when the polymer is placed into the GSNO solution. When the film is removed from the buffer, the NO signal gradually decreases to the baseline, suggesting that the copper ion coordinated within cyclen does not significantly leach out of the polymer after participating in the redox cycle that generates NO from the RSNO species in solution (also, see the results below from a copper leaching study in blood). This reaction likely takes place at the polymer film/solution interface, although it is possible that the test RSNO species (GSNO) may have some capability to diffuse into the polyurethane polymer, given the hydrophilic nature of the base polymer employed in this work (*i.e.*, water uptake value of 100 wt%).

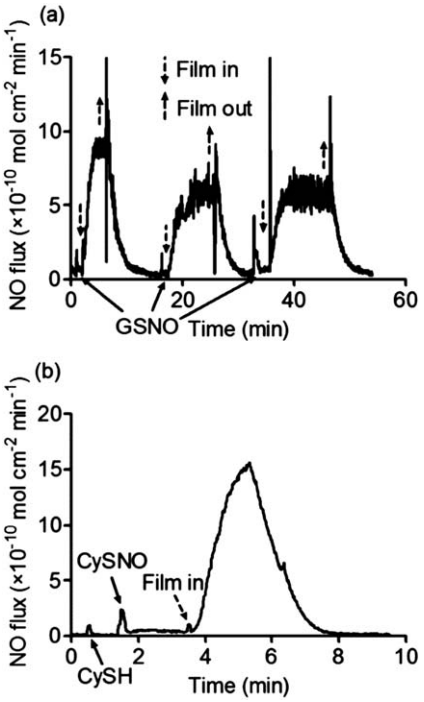
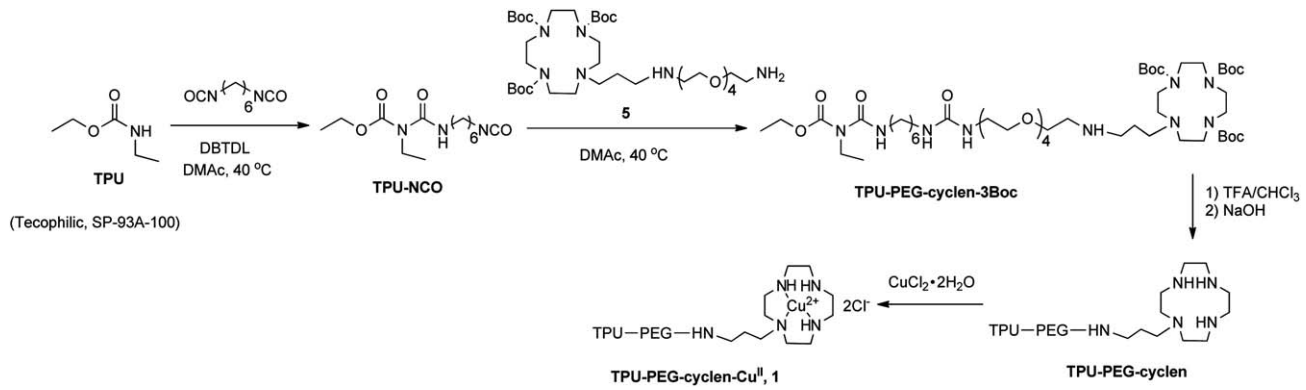


Fig. 2 The measurements of catalytic NO generation by the small films of TPU-PEG–cyclen–Cu^{II} (**1**) (thickness: 30 μm, 0.47 wt% copper) in a solution of (a) 5 μM GSNO/GSH (area = 0.13 cm^2) or (b) 0.5 μM CySNO/CySH (area = 0.31 cm^2) in PBS buffer (10 mM, pH 7.4) containing 3 μM EDTA *via* chemiluminescence NOA at RT. Arrows indicate addition or removal of the given species into the mixture. All spikes in figures are artifacts due to opening the reaction vessel.

To confirm the catalytic mechanism of NO generation from GSNO by the new polymer appended Cu(II)–cyclen complex, the decomposition of RSNOs was studied using a much higher concentration of GSNO (1 mM). After incubation with a small piece of the polymer film, a 24-fold greater amount of NO (1.5 × 10^{-6} moles) was generated by the new polymer containing 6.3 × 10^{-8} moles of Cu(II) sites in the GSNO solution prepared in 10 mM PBS (pH 7.4) with 1 mM reducing agent present. In this experiment, the catalytic amount of Cu(II) present was only 3% of the total GSNO used. In contrast, when no Cu(II) was incorporated into the polyurethane material, very little NO (7.89 × 10^{-9} moles) was observed using the same concentration of GSNO with a 0.4% conversion rate



Scheme 1 Synthesis of the new Cu^{2+}-mediated NO generating polymer: TPU-PEG–cyclen–Cu^{II} (**1**).

(see ESI, Fig. S4†). These data clearly demonstrate that the Cu(II)–cyclen–PU material can catalytically decompose RSNO to generate NO, and the polymer alone cannot produce a significant amount of NO without the presence of the catalytic Cu(II) sites.

A much higher surface flux of NO, 16×10^{-10} mol cm^{-2} min^{-1} (Fig. 2(b)), was observed when a much lower concentration of CySNO (0.5 μM) (rather than GSNO) was used in the test solution. Askew *et al.*[14] studied the rate constants of Cu(II)-catalyzed decomposition of a variety of RSNOs by forming a five or six-membered ring intermediate *via* the amino or carboxyl group of the RSNOs. The rate constant for free Cu(II) for decomposing CySNO is 24 500 ± 500 mol^{-1} dm^3 s^{-1}, which may explain why the polymer (**1**) could produce more NO even when a 10-fold lower concentration of CySNO than that of GSNO was used.

A NO generation study was also conducted under physiological conditions[18] at a temperature of 37 °C with 1 μM GSNO and 30 μM GSH present in the test solution. A flux of 5×10^{-10} mol cm^{-2} min^{-1} of NO was generated from a small film of the new polymer (see ESI, Fig. S5†). The total generated NO over 12 min was found to be 9×10^{-10} mol, equivalent to a 45% conversion rate of the GSNO species. This low catalytic efficiency is assumed to be due to a low second-order rate constant of Cu(II)-catalyzed decomposition of GSNO,[14] and also to the inefficient reducing ability of GSH (CySH is regarded as a more favorable reducing agent at physiological pH than GSH[8]). Depending on the structure (and hence reactivity) of the given RSNO, either Cu(I) formation or its reaction with RSNO can be rate-limiting.[7]

Beyond testing in PBS buffer, it is critical to also assess the NO generating ability of the new polymer after contacting with blood. Therefore, small films of the polymer were soaked in whole sheep blood or platelet-rich sheep plasma for 24 h. The levels of NO generated per cm^2 of film were detected *via* chemiluminescence both prior to and after soaking in the blood/plasma. Both GSNO and CySNO were employed as substrates to evaluate any changes in activity owing to the exposure to blood. As shown in Fig. 3(a) and S6,† a significant portion of the NO generating activity could be preserved after contacting whole blood (30–50%) or platelet-rich plasma (50–70%). A surface flux of 2–3 $\times 10^{-10}$ mol cm^{-2} min^{-1} of NO is generated in the presence of 5 μM GSNO (ESI, Fig. S6(a)†). More NO is produced (5–10 $\times 10^{-10}$ mol cm^{-2} min^{-1} NO flux) from 0.5 μM CySNO (Fig. 3(a)). Moreover, the ability of the polymer film to generate NO from CySNO after exposure to blood is reproducible

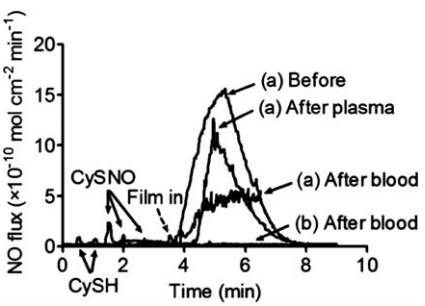


Fig. 3 The evaluation of NO generation catalyzed by the same sized films (area = 0.23 cm^2) of (a) TPU-PEG–cyclen–Cu^{II} (**1**) (thickness = 30 μm, Cu content = 0.47 wt%) and (b) polymer **2** (0.11 wt% Cu) in a solution of 0.5 μM CySNO/CySH in 10 mM PBS buffer (pH 7.4, 3 μM EDTA) before and after contacting with whole sheep blood or platelet-rich sheep plasma at 4 °C for 24 h *via* a chemiluminescence NOA.

at a constant NO level with repeated insertion/removal of the film from the test solution (see ESI, Fig. S6(b)†).

Another blood-contact study was performed with the previously published polymer (**2**). Unfortunately, it loses nearly all the NO generating activity under the same experimental conditions (see Fig. 3(b) and S7†), clearly suggesting that polymer (**1**) is a more stable catalyst that is less poisoned by the presence of species in blood. Regardless of what type of RSNO species serves as the NO source, 1–5 $\times 10^{-10}$ mol cm^{-2} min^{-1} NO flux can be generated from the new polymer (**1**) even after contacting with blood/plasma for 24 h.

A prolonged copper leaching study of the new material as well as polymer **2** was conducted by soaking polymer films in fresh whole sheep blood for 2 days. The copper concentrations in the plasma phase before and after contacting with the materials were analyzed *via* ICP-AES (see ESI, Fig. S8†). Results indicated that 25% of the copper leached from the new polymer **1** and that 49% was lost from the previously reported polymer **2** material. This result is consistent with the above *in vitro* evaluation of the NO generating activities of the two different polymers after contact with whole blood, and provides a reasonable explanation for the above results.

The *in vivo* stability of the new NO generating material was also investigated using a rabbit model or extracorporeal circulation (ECC) in which the NO generating material is coated on the inner walls of PVC tubing, as described previously.[6] The polymer **1** material still preserved its NO generating ability after 4 h of blood exposure with a surface flux of 13×10^{-10} mol cm^{-2} min^{-1} using SNAP as the test RSNO species (see ESI, Fig. S9†). In addition, the plasma copper assay prior to and post-ECC exposure did not show a significant amount of copper leaching (ESI, Fig. S10†). These data clearly demonstrate that the new NO generating material is relatively stable in a physiological environment.

Protein adsorption is the first of a complex series of events triggered by material–blood interactions. Adsorption of proteins leads to activation of platelets and leukocyte adhesion, and finally coagulation, which normally occurs within seconds of material–blood contact.[19] Thus, it is also important to assess whether new materials can cause increased protein adsorption when in contact with blood. A control blood-contact study with the unmodified TPU (SP-93A-100) was carried out using the same blood samples as described above. The NOA data for the control films did not show any obvious change before and after blood exposure, with all the measurements only detecting baseline NO signals (see ESI, Fig. S11†). This result suggests all the generated NO after contact with blood/plasma (shown in Fig. 3(a) and S6†) is derived from decomposition of RSNO catalyzed by the Cu(II)–cyclen structure bound to the new polymer (**1**), not from the adsorbed metallo-proteins on the surface of the material.

Another method to determine whether the new NO generating material exhibits appreciable protein adsorption involves assessing, *in vitro*, fibrinogen adsorption.[20] Fibrinogen is a crucial clotting factor within the blood-coagulation cascade and it plays a major role in hemostatic plug formation by acting as a cell adhesion molecule for platelets[21] and leukocytes.[22] It participates in platelet aggregation *via* specific inducible receptors (glycoprotein IIb–IIIa, Gp IIb–IIIa)[23,24] on the surface of platelets.[25] Platelets have also shown an increase in adhesion on fibrinogen-immobilized surfaces.[26,27] A fluorescent assay takes advantage of a fluorescein-labeled goat anti-fibrinogen IgG (polyclonal) antibody to quantitatively detect surface-adsorbed fibrinogen by fluorescence measurements. Using such an assay, the

Cu(II)–cyclen modified polyurethane (**1**) does not exhibit any significant increase in fibrinogen adsorption compared to that of the unmodified TPU ($P > 0.1$, see ESI, Fig. S12†). This result suggests that the new derivatization chemistry to tether the Cu^{2+}–cyclen catalyst to the TPU material does not alter the fundamental protein adsorption properties of the material compared to those of the base polymer. Hence, with the added NO generation activity, the new polymer could further enhance thromboresistance of this biomedical grade polyurethane.

Conclusions

In conclusion, a new NO generating polymer, Cu(II)–cyclen–PEG-TPU (**1**), was successfully prepared. This material exhibits greatly improved catalytic efficiency for generating NO from RSNO species compared to those of analogous materials reported previously. This improvement is achieved by optimizing the synthesis and tethering a more active catalyst to a commercial PU structure. Based on the pendant isocyanate sites created on the isocyanated polymer (TPU-NCO) and the final copper content, a mean yield of 50% for each step was obtained during the entire synthesis of the polymer. The new polymer is able to generate 6–10 $\times$ 10^{-10} mol cm^{-2} min^{-1} surface flux of NO in the presence of 5 μM GSNO and 16 $\times$ 10^{-10} mol cm^{-2} min^{-1} NO flux from 0.5 μM CySNO in PBS buffer (10 mM, pH 7.4). No evidence of significant leaching of copper ions is observed. Exposure to whole blood or plasma reduces the rate of NO generation, but physiological fluxes still remain (1–5 $\times$ 10^{-10} mol cm^{-2} min^{-1}). This value is much higher than that which can be obtained from an earlier polymer (**2**) that utilizes different linking chemistry. The new polymer reported here is a very attractive material for future studies to assess the improved thromboresistance achieved with and without supplementation of exogenous RSNOs to the bloodstream of the animals, and potentially, in humans.

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