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Polymethacrylates with a Covalently Linked Cu^{II}–Cyclen Complex for the In Situ Generation of Nitric Oxide from Nitrosothiols in Blood**

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Nitric oxide produced by endothelial cells (EC) which line the walls of all blood vessels plays an important role in preventing platelet activation, adhesion, and, ultimately, thrombosis at the EC/blood interface.[1,2] We have reported previously that

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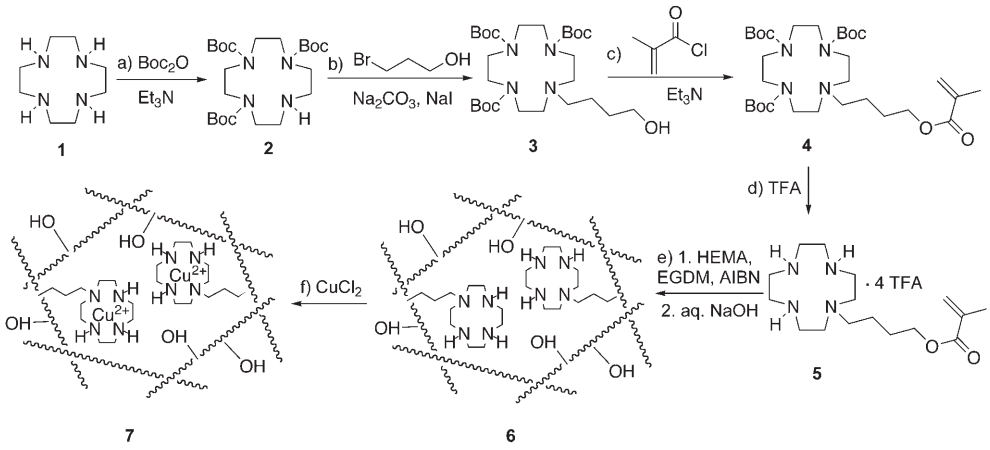
plasticized polymeric films doped with a lipophilic copper(II) complex decompose *S*-nitrosothiols (RSNO), such as *S*-nitrosoglutathione (GSNO), to NO at their interface in the presence of naturally occurring reducing equivalents (e.g., glutathione (GSH)).[3] Indeed, it is well known that Cu^{II}/Cu^{I} ions can catalyze the generation of NO from RSNO species (see the Supporting Information).[4,5] Furthermore, it was reported that peptide- and protein-bound Cu^{II} can be reduced to Cu^{I} by a thiol to generate NO from RSNO.[6] It now appears that appropriate Cu^{II}/Cu^{I} organic ligand complexes can carry out similar redox chemistry.[3] Hence, it should be possible to utilize endogenous reducing equivalents (e.g., ascorbate, GSH, and cysteine (CySH)) and the endogenous NO precursors (e.g., GSNO, S-nitrosocysteine (CysNO), and S-nitrosoalbumin (AlbSNO)) in blood to generate locally enhanced NO levels at a polymer/blood interface by immobilizing catalytic Cu^{II}/Cu^{I} sites within the polymeric material. Although the exact concentrations of RSNOs in normal human blood are in debate,[7,8] the levels of RSNOs and required reducing equivalents are thought to be in the nanomolar to micromolar range.[9,10] As very low levels of NO (< 1 nM) can be effective in inhibiting platelet function,[11] even a small fractional conversion of endogenous RSNO species into NO at a polymer/blood interface could be beneficial in lowering the risk of thrombus formation at such interfaces.



Scheme 1. The synthesis of the cross-linked hydrogel Cu^{II}–cyclen–pHEMA polymer **7**. a) Boc_2O (3 equiv), Et_3N (3 equiv), $CHCl_3$, RT, 12 h (76%); b) 3-bromopropanol (3.4 equiv), Na_2CO_3 (1.2 equiv), NaI (1.3 equiv), CH_3CN, 80 °C, 12 h (88%); c) methacryloyl chloride (5.4 equiv), Et_3N (9.5 equiv), THF, −20 °C, RT, 1 h (59%); d) trifluoroacetic acid (TFA), CH_2Cl_2, 0 °C, RT, 12 h (quantitative yield); e) 1. HEMA (66.0 wt%), ethyleneglycol dimethacrylate (EGDM; 2.1 wt%), azobisisobutyronitrile (AIBN; 0.4 wt%), 65 °C, 12 h; 2. soaked and washed with a solution of aqueous NaOH/EtOH (1:10, v/v, pH 11–12); f) $CuCl_2 \cdot 2H_2O$ (1 equiv vs **5**) in EtOH, 65 °C, 4 h.

Herein, we report the synthesis of a modified Cu^{II}–cyclen (cyclen = 1,4,7,10-tetraazacyclododecane) complex covalently attached to a cross-linked poly(2-hydroxyethyl methacrylate) (pHEMA) and demonstrate that this new material can catalytically generate NO from naturally occurring RSNOs at the physiological pH value. Further, it will be shown that spontaneous NO generation from RSNOs which exist in fresh sheep's blood can be achieved with this novel material.

Cyclen is known to have very high binding constant with Cu^{II} ($10^{23}\,M^{-1}$)[12] and square-pyramidal structure,[12,13] thus allowing this complex to possess an open site(s) at which interactions with reducing equivalents and RSNO species are possible. Herein, a propylmethacrylate derivative of cyclen was prepared for copolymerization with 2-hydroxyethyl methacrylate (HEMA; see Scheme 1). pHEMA is a high-water-uptake polymer network into which endogenous reducing agents (CySH, GSH, ascorbate, etc.) and low-molecular-weight RSNOs (CySNO and GSNO) can readily penetrate. Thus, in contrast to the system described previously,[3] potential NO generation by the resulting polymeric material could take place within the bulk of a polymer coating, not merely at the polymer/solution interface.

The film of Cu^{II}–cyclen–pHEMA **7** displayed a λ_{max} value in the range 621–644 nm in the visible region of the UV/Vis spectrum after being fully hydrated with deionized water or phosphate-buffered saline (PBS) (10 mM, pH 7.4). This absorption is within the range known for square-pyramidal Cu^{II} complexes (550–670 nm).[14] In addition, electron paramagnetic resonance (EPR) spectra of **7** in both frozen aqueous and PBS-buffered solutions exhibited the typical four-line patterns expected for a Cu^{II}–cyclen complex (see the Supporting Information).[15] Moreover, the extent of the blue color of **7** and the corresponding copper content, as determined by atomic absorption (AA) spectrophotometric analysis after dissolution of a given mass of film in a solution of sulfuric acid, correlated very closely with the molar ratio of the modified cyclen monomer **5**/HEMA used in the polymerization reaction and the theoretical amount of copper expected (see the Supporting Information). Taken together, the data suggest that most cyclen sites covalently linked to the polymer are complexed with Cu^{II} ions in the final cross-linked pHEMA films.

Figure 1 illustrates the NO generation that is achieved when a small piece of the Cu^{II}–cyclen–pHEMA film is placed in and then removed from a solution of GSNO and GSH in deoxygenated PBS buffer. The NO generated is monitored continuously with a chemiluminescence NO analyzer (NOA). As shown in Figure 1a, a blank experiment in which a hydrogel film of **6** (not treated with copper) was placed in the test solution did not generate any detectable NO. This experiment demonstrates that, as expected, copper ions are key for NO generation. In a second blank experiment, a cross-linked pHEMA film of the same size was prepared by the reaction of HEMA, EGDM, and AIBN, but without copolymerization of **5**, and further treated with copper ions by using

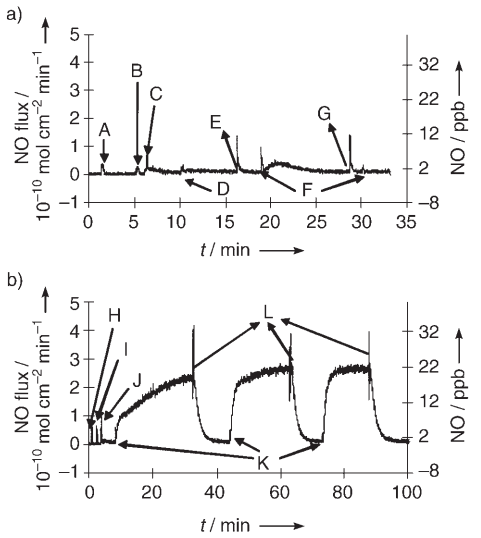


Figure 1. The profiles of NO generation for the following disk polymers (thickness: 0.3 mm; radius: 2.0 mm) in deoxgenated PBS buffer (2 mL, 10 mM; pH 7.4), monitored with a chemiluminescence NOA at room temperature after the following steps: a) A: Addition of EDTA (10 μM); B: addition of GSH (30 μM); C: addition of GSNO (10 μM); D: insertion of the cross-linked film **6**; E: removal of the cross-linked film **6**; F: insertion of pHEMA; G: removal of pHEMA. b) H: Addition of EDTA (10 μM); I: addition of GSH (5 μM); J: addition of GSNO (5 μM); K: insertion of Cu^{II}–cyclen–pHEMA **7**; L: removal of **7**.

the same method employed for the preparation of **7**; this film yielded only a small NO flux upon the very first immersion in the solution of GSNO. This behavior is due to nonspecifically adsorbed Cu^{II} ions adhered to the pHEMA matrix. Indeed, little or no NO generation was observed for subsequent immersions of the same blank films (see Figure 1a). In contrast, the first immersion of a similarly sized film of **7** into the solution of GSNO provided a slow increase in the NO flux which reached a steady state (see Figure 1b). The NO flux decreased to the baseline quickly after this film was removed from the reaction cell. Subsequent immersion/removal cycles of the same film showed that **7** could reversibly achieve nearly the same steady-state NO flux on sequential immersions into fresh solutions of GSNO/GSH. When CysNO was examined as the RSNO substrate, higher fluxes of NO generation were achieved over a relatively short time periods (see the Supporting Information), but the NO generation decreased quickly, as the bulk CysNO substrate was consumed by a rapid catalytic reaction. These observations might relate to the relative reaction rates of the two species with immobilized Cu^{II} sites. As the concentration of CySNO in blood is higher than GSNO,[10] CySNO is more likely to contribute toward a larger fraction of the potential NO-generating capability by the new Cu^{II}–cyclen–pHEMA polymers when they are in contact with fresh blood.

Several soaking experiments with the new polymeric films were conducted in the presence of excess amounts of solutions of GSH and/or GSNO in PBS buffer to investigate the potential leaching of copper ions and the deactivation of **7**. The extraction solutions were shaken continuously, and replaced with fresh solution daily. There was substantial leaching of copper ions, as measured by AA spectrophotometric analysis (the films soaked in the solutions still retained more than 64% of their original copper content after 15 days at 37.5°C; see the Supporting Information), and this leaching resulted in a decrease in the apparent level of NO generation (25–50% decrease) relative to a control of the same film (not soaked in solutions of GSH and/or GSNO). However, the λ_{max} value of the film, as shown by UV/Vis spectroscopic analysis, did not change in location before and after such soaking, thus suggesting that GSH (a reducing agent) and glutathione disulfide (GSSG, a by-product of the NO-generation reaction), both strong ligands for copper ions,[16,17] do not bind irreversibly as ligands for the covalently linked complex. A more critical test was the examination of NO generation by films of the new Cu^{II}–cyclen–pHEMA **7** material (radius: 2.8 mm; thickness: 0.2 mm; type: disk) from GSNO before and after the exposure to animal blood for a finite period. It was found that NO generation from GSNO is maintained after soaking such films in 10 mL of the sheep plasma for 3 days at 4°C or whole blood for 1 day at 4°C (see the Supporting Information). These results indicate that the deactivation of **7** caused by endogenous free thiols, proteins, and other blood components may not be a significant issue. Clearly, a large number of in vivo studies over varying time periods will be required to fully assess the true NO-generation ability and resultant thromboresistance of the new polymer under physiological conditions.

To further demonstrate that the Cu^{II}–cyclen–pHEMA polymer can generate NO spontaneously in fresh whole blood, an amperometric RSNO sensor was constructed using an interpenetrating network (IPN) film of **7** and hydrophilic polyurethane (HPU, Tecophilic, SP-93A-100) as an outer film on a NO-selective electrochemical sensor (see the Supporting Information). The response of a control NO sensor without the polymer containing the Cu^{II}–cyclen complex (but with an HPU outer membrane) was also monitored in the same blood samples. Each sensor was precalibrated for an intrinsic direct amperometric response to NO (see the Supporting Information), and then the NO and RSNO levels were measured, respectively, in the fresh sheep's blood diluted with PBS buffer at 37°C. Figure 2 shows that the NO sensor without the catalytic Cu^{II}–cyclen–pHEMA polymer site in the outer membrane has relatively little amperometric response when placed in the blood sample, whereas the difference in NO responses between the RSNO and NO sensors (equivalent to ≈25 nM NO, calculated from calibration curves) appears to be due to the decomposition of RSNO species in the sheep's blood by the IPN film of **7** and HPU. Therefore, this experiment confirms that the Cu^{II}–cyclen complex covalently attached to pHEMA generates NO from endogenous substrates in the sheep blood in vitro.

In conclusion, a Cu^{II}–cyclen complex was covalently to attached pHMEA, and the resulting material is capable of generating NO from naturally occurring RSNO species, both in buffer solution and directly when in contact with fresh whole blood. The presence of endogenous reducing equivalents is required for such catalytic chemistry to take place. Assessment of the stability of the new Cu^{II}–cyclen–pHEMA **7** material suggests that the slow leaching of copper ions from

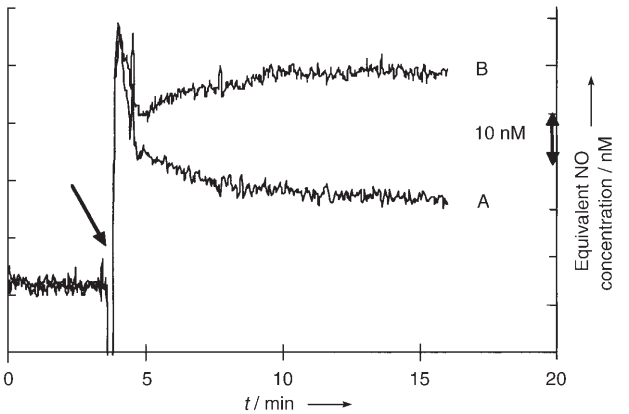


Figure 2. The direct amperometric detection of endogenous RSNOs in fresh sheep's blood by using a NO sensor A) as a control and a RSNO sensor and B) upon injection of 6 mL of fresh sheep's blood (→) into PBS buffer (30 mL, 10 mM; pH 7.4) at 37°C under a stream of nitrogen.

the polymer film may cause a measurable decrease in NO generation with time, but copper deactivation is not a significant problem. Although fundamental questions with respect to the exact mechanism by which **7** generates NO remain, the data presented herein suggest that this new material may prove beneficial as a potentially new thromboresistant blood-contacting material for biomedical applications with contact times of hours to several days. Experiments in vivo to demonstrate the effectiveness of this material in reducing thrombus formation will begin shortly.

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