

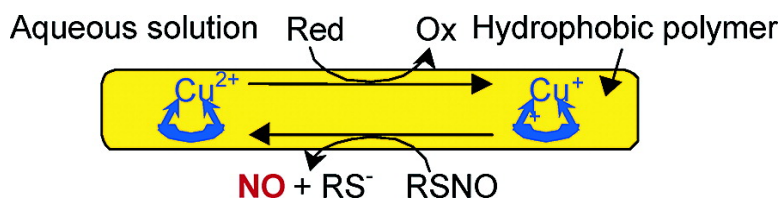
Communication

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Bong Kyun Oh, and Mark E. Meyerhoff

J. Am. Chem. Soc., **2003**, 125 (32), 9552-9553 • DOI: 10.1021/ja035775x • Publication Date (Web): 18 July 2003

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Spontaneous Catalytic Generation of Nitric Oxide from *S*-Nitrosothiols at the Surface of Polymer Films Doped with Lipophilic Copper(II) Complex

Bong Kyun Oh and Mark E. Meyerhoff*

Department of Chemistry, University of Michigan, 930 North University Avenue, Ann Arbor, Michigan 48109-1055

Received April 24, 2003; E-mail: mmeyerho@umich.edu

Recent research in this laboratory^{1–5} and elsewhere^{6–9} has demonstrated a significant improvement in the *in vivo* thrombo-resistivity of hydrophobic polymeric films (e.g., silicone rubber, poly(vinyl chloride), etc.) that are capable of continuously releasing low levels of nitric oxide (NO). Indeed, NO is known to be a potent, naturally occurring inhibitor of platelet adhesion and activation, as well as smooth muscle cell proliferation.^{10–12} Ongoing studies have shown that biomedically useful NO release polymers can be created by incorporating novel NO adducts (e.g., diazeniumdiolates at 1–10 wt %) within films of various polymeric materials. Improved blood compatibility has been observed when the NO fluxes from the surface of such films are greater than that of normal endothelial cells that line the walls of all healthy blood vessels (1×10^{-10} mol $\text{cm}^{-2} \text{min}^{-1}$).^{1,3,13} However, use of existing NO release polymers for long-term biomedical implants (e.g., as coatings on shunts, grafts, stents, etc.) is limited by the relatively small reservoir of NO adducts that can be loaded within thin coatings of the polymeric materials. In contrast, normal blood already possesses a substantial reservoir of NO precursors in the form of nitrite and *S*-nitrosothiols (RSNOs) (e.g., *S*-nitrosogluthathione (GSNO), *S*-nitrosocysteine (CysNO), etc.) present at near μM levels (0.02–0.2 μM GSNO, 0.2–0.3 μM CysNO).¹⁴ The RSNO species are generated *in vivo* via reactions of thiols with oxidation products of endogenous NO produced by nitric oxide synthase (NOS) in endothelial as well as other cells.¹⁵ It is believed that these species can serve as substrates to generate locally enhanced NO levels for extended time periods *in vivo* at the interface of polymers possessing the appropriate catalytic sites. Indeed, it will be shown herein that poly(vinyl chloride) (PVC) and polyurethane films doped with a lipophilic Cu(II) complex (see Figure 1A), are capable of catalytically decomposing endogenous RSNO species to NO. This work adds substantially to our very recent finding that similar Cu(II) complex-doped polymer films can catalyze the generation of NO from inorganic nitrite in the presence of ascorbate via a catalytic redox mechanism.¹⁶

It is well-known that free Cu(II) ions in aqueous solution efficiently decompose RSNOs to NO catalytically.¹⁷ The Cu(II) is reduced to Cu(I) by appropriate reducing equivalents (e.g., free thiols, ascorbate) in solution. It is hypothesized that the resulting Cu(I) can then form either a five- or a six-membered intermediate ring structure¹⁷ that facilitates the electron exchange to generate NO and thiolate anion. The latter product can reduce additional Cu(II) species to Cu(I), and the cycle continues until all the RSNO species in solution are consumed. This copper ion-mediated catalytic NO generation is known to be completely inhibited by hexacoordinate EDTA,¹⁷ which chelates Cu(II) and prevents its reduction and subsequent reaction with RSNO species. In preliminary studies for the present work, however, it was found that among a wide range of soluble four-coordinate Cu(II) ligands examined, a Cu(II)–cyclen complex, with a reported complexation constant of 10^{26}M^{-1} ,¹⁸ can efficiently carry out this same catalytic chemistry in aqueous solution (data not shown).

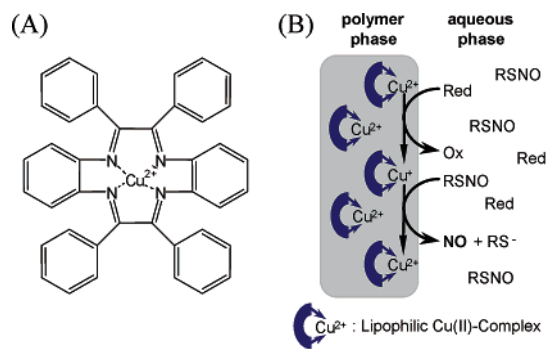


Figure 1. Structure of a lipophilic analogue of cyclen, Cu(II)–dibenzo[e,k]-2,3,8,9-tetraphenyl-1,4,7,10-tetraaza-cyclododeca-1,3,7,9-tetraene (Cu(II)–DTTCT) (A), and schematic diagram illustrating the concept of NO generation at the surface of Cu(II)–DTTCT-doped polymer film via catalytic reduction of *S*-nitrosothiols in solution phase also containing reducing equivalents (Red) (B).

To explore whether this catalytic chemistry can be immobilized within organic polymers, the more lipophilic Cu(II) complex (analogue to cyclen) shown in Figure 1A (Cu(II)–dibenzo[e,k]-2,3,8,9-tetraphenyl-1,4,7,10-tetraaza-cyclododeca-1,3,7,9-tetraene (Cu(II)–DTTCT)) was synthesized via a previously reported method.¹⁶ Incorporation of this complex at 2 wt % into either plasticized PVC (65.5 wt % PVC and 32.5 wt % *o*-nitrophenyloctyl ether (*o*-NPOE)) or polyurethane (PU) (SG-80A, Thermedics, Inc.) films cast from THF on a glass plate yielded homogeneous coatings, with a thickness of ca. 200 μm . Small disks (0.4 cm diameter, 0.25 cm^2 surface area) from the parent film were punched out and employed to test their catalytic activity in generating NO from RSNOs under various conditions. The RSNOs examined in this work, including *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) and *S*-nitrosogluthathione (GSNO), were prepared as described elsewhere¹⁷ and stored in the dark to prevent light-induced decomposition. The NO generated in an amber reaction cell containing 2 mL of PBS buffer (pH 7.4) with given concentrations of these nitrosothiol substrates was detected via on-line chemiluminescence measurements with a nitric oxide analyzer (NOA) (in the presence and absence of a small piece of the polymer film). The buffered sample solution was continuously purged with a stream of nitrogen to transport the generated NO in solution to the headspace and then directly into the NOA.

Figure 1B illustrates the overall concept of generating NO from RSNOs via a heterogeneous catalytic reaction at the surface of the Cu(II)–DTTCT-doped polymeric films, where reducing equivalents convert Cu(II) to Cu(I) in the complex, and then the complexed Cu(I) decomposes the RSNO species to NO, concomitant with oxidation of Cu(I) back to Cu(II). Figure 2A illustrates the catalytic NO generation of a small piece (0.25 cm^2) of the Cu(II)–DTTCT-doped PVC film when sequentially placed in and removed from 2 mL of a PBS solution containing 200 μM of SNAP, a well-known and stable RSNO species. As can be seen, a very low baseline of NO is observed in the absence of the Cu(II)–DTTCT-doped PVC

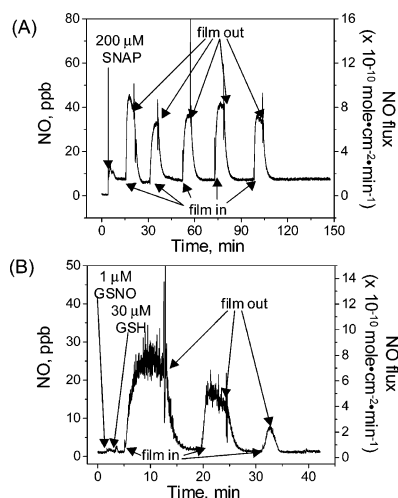


Figure 2. Catalytic NO generation by a small piece of Cu(II)-DTTCT-doped PVC film in (A) a high concentration (200 μM) of SNAP in PBS buffer and (B) a low concentration (1 μM) of endogenous GSNO and 30 μM GSH in PBS buffer. NO detected continuously by purging the solution with nitrogen and monitoring the NO content of nitrogen stream with chemiluminescence NOA.

film. When the PVC film is placed into the solution, a large increase in the NO level is detected by the NOA, reaching a steady-state value equivalent to a flux of ca. $8 \times 10^{-10} \text{ mol cm}^{-2} \text{ min}^{-1}$ from the surface of the film. This NO response quickly returns to baseline levels when the film is removed. This process can be repeated until all the SNAP in solution is depleted.

It should be noted that in the initial studies with SNAP shown in Figure 2A, exogenous reducing equivalents were not necessary to convert Cu(II) to Cu(I) at the polymer surface, since a small percentage of free thiol (*N*-acetyl-DL-penicillamine) is always present as an impurity¹⁹ in such high concentrations of SNAP solution. The NO generation in this example also does not appear to result from any leaching of free copper ions and/or the Cu(II)-DTTCT complex from the polymeric film. Indeed, if this were the case, a continuous increase in the background level of detected NO, via decomposition of SNAP to NO in the solution phase, would be expected. Furthermore, the same generation of NO is also observed when the PBS solution contains a high level of EDTA (100 μM), known to prevent any catalytic reaction by even trace levels of free copper ions in solutions containing RSNO species.¹⁷

Similar NO generation is observed when either PVC or PU films possessing the Cu(II)-DTTCT complex are placed in and removed from a PBS solution containing relatively low, physiological levels (μM range) of RSNOs. For example, Figure 2B illustrates the catalytic generation of NO by a Cu(II)-DTTCT-doped PVC film placed in PBS solution containing only 1 μM GSNO. In this case, to observe measurable NO generation, the solution must contain an adequate level of free thiols in the form of glutathione (GSH) (30 μM) to serve as the reducing agent to more efficiently generate the required Cu(I) complex intermediate species at the polymer/solution interface. In addition, the amount of NO generated from each successive immersion of the film decreases substantially in this experiment due to the relatively rapid consumption of the low concentration of GSNO originally present. Indeed, integration of the signal for the total NO generated for three discrete immersions with the same film (for ca. 7 min each) indicates ca. 100% conversion of the GSNO to NO.

While the data presented in Figure 2 were obtained by flushing the PBS buffer/RSNO solution with nitrogen (for the purposes of chemiluminescence detection of NO), it was found that the presence of ambient oxygen does not significantly alter the ability of the

polymer films to generate NO from RSNO species. This was confirmed by monitoring the local NO generated near the surface of the Cu(II) complex-doped films under ambient conditions with a miniaturized amperometric NO gas sensor²⁰ (see Figure 1S in Supporting Information). Hence, the presence of oxygen does not react with the reduced Cu(I) complex in the polymer faster than the electron-transfer reaction with the RSNO species.

The catalytic activity demonstrated above has also been observed for low levels of *S*-nitrosocysteine (CysNO), another naturally occurring RSNO species found in blood.¹⁴ In addition, it has been found that the films containing Cu(II)-DTTCT can be soaked in buffer or undiluted plasma for several days, after which they still exhibit essentially the same catalytic function toward RSNO substrates (see Figure 2S in Supporting Information for NO generation after soaking in plasma for 12 h at 37 °C). This suggests that adsorption of proteins, etc., on the surface of the polymer films does not significantly influence the interfacial redox chemistry observed. Moreover, as shown in Figure 2B, the “apparent surface fluxes” of NO generated from 1 μM of GSNO can be well above the $1 \times 10^{-10} \text{ mol cm}^{-2} \text{ min}^{-1}$ produced by a healthy endothelium. Hence, while the exact interfacial chemistry responsible for this catalytic function remains to be examined in detail, the in vitro data obtained thus far strongly support the likelihood that spontaneous generation of NO at physiologically relevant levels will occur when such Cu(II) complex-doped polymers are in contact with fresh flowing blood, known to contain a number of different of RSNO species.¹⁴ If so, then such polymers are expected to exhibit greatly enhanced thromboresistivity when employed to coat or prepare a variety of implantable or other blood contacting devices (e.g., extracorporeal circuits used in kidney dialysis, coronary bypass surgery, plasmapheresis, etc.).

Acknowledgment. We thank the National Institutes of Health for supporting this work (EB 00783).

Supporting Information Available: Experimental results illustrating local generation of NO from SNAP and GSNO at the surface of PVC film doped with Cu(II)-DTTCT, in the presence of ambient oxygen, as measured with amperometric NO gas sensor, as well as NO generation of film before and after exposure to plasma (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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JA035775X