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2-Hydroxy-5-nitrobenzyl as a Diazeniumdiolate Protecting Group: Application in NO-Releasing Polymers with Enhanced Biocompatibility

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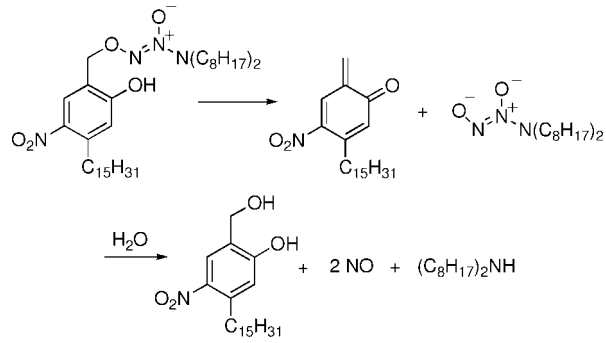
ABSTRACT



The 2-hydroxy-5-nitrobenzyl group is shown to be an effective protecting group for diazeniumdiolates. *O*^{2}-(2-Hydroxy-5-nitrobenzyl)-substituted diazeniumdiolates display enhanced thermal stability, but efficiently release nitric oxide (NO) in pH 7.4 aqueous solutions. A lipophilic analogue incorporated into hydrophobic polymers shows NO surface flux rates comparable to that of the natural endothelium. Importantly, these polymer formulations also show significantly enhanced biocompatibility in vivo with use of a porcine implant model.

One of the most significant problems concerning the use of synthetic biomaterials in blood-contacting applications is the rapid onset of thrombosis (i.e., the formation of a blood clot). Recent research in the field has focused on the development of more thromboresistive surfaces.[1] Strategies range from design of more biocompatible hydrophobic surfaces to grafting of drugs and biomolecules to polymer surfaces. A completely thromboresistive surface must exhibit a range of biological activity including inhibition of both fibrinogen adsorption and its subsequent conversion to fibrin and a reduction of adherence, aggregation, and release reactions of platelets at the surface. The most thromboresistive material known is the natural endothelium, which regulates the above responses by a variety of mechanisms, including prostacyclin release and nitric oxide (NO) generation. Many new biomaterials are designed to mimic the properties of the endothelium.

NO is continually released by the endothelium at a flux of approximately 1×10^{-10} mol cm^{-2} min^{-1}.[2] This naturally controlled-release has been shown to regulate vascular tone

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(1) For a recent review, see: Wu, Y.; Meyerhoff, M. E. *Talanta* **2008**, *75*, 642–650.

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and also to inhibit platelet adhesion and aggregation.[3−6] Much research has focused on the development of biomaterials that mimic the NO-releasing properties of the endothelium. Indeed, a variety of synthetic hydrophobic surfaces that release NO at approximately the desired endothelium flux all show reduced platelet adhesion and thus enhanced thromboresistivity compared to NO-free analogues.[1]

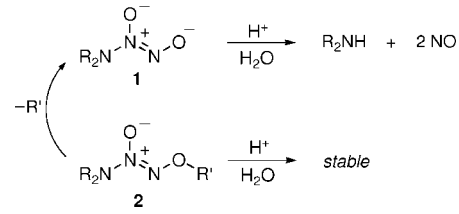
Many NO-releasing biomaterials incorporate an NO donor based on the diazeniumdiolate $[N(O)=NO]^-$ functional group. Compounds containing this functional group have proven useful as research tools in a variety of applications requiring spontaneous release of NO.[7] Anions such as 1-(*N*,*N*-dialkylamino)diazen-1-ium-1,2-diolates **1** are stable as solid salts, but release up to 2 molar equiv of NO when dissolved in aqueous solution at physiologically relevant conditions in an acid-catalyzed dissociation reaction.[8]

Smith, Keefer, and co-workers first reported the preparation and study of several NO-releasing polymers containing diazeniumdiolates in 1996.[9] In one case, diazeniumdiolates were noncovalently dispersed through water-soluble polyethylene glycol (PEG) or biodegradable polycaprolactone (PCL) matrices by simple mechanical mixing of the melted polymer and a variety of different diazeniumdiolates. Although diazeniumdiolates survived the blending process with PEG (at 46 °C) with no appreciable decomposition, those blended into PCL (at 60 °C) suffered 50% decomposition. This observation highlights one of the major problems associated with the development of NO-releasing biomaterials of this kind: free diazeniumdiolate anions decompose readily even at only slightly elevated temperatures making them impractical for use in standard polymer manufacturing techniques such as extrusion and injection molding.

Several chemical strategies have been developed for the protection of dialkylamino-substituted diazeniumdiolates **1**.[10] These approaches convert **1** to the stable prodrug (O^2-substituted diazeniumdiolate **2**) by reaction with a variety of alkylating or arylating agents that affix electrophilic groups (R′ in Scheme 1) to the terminal oxygen. These "protected" diazeniumdiolates can then be converted back to the NO-releaser **1**.

2-Hydroxy-5-nitrobenzyl bromide was first introduced by Koshland et al. as a protein-modification reagent with selectivity for tryptophan residues in 1964.[11] Such modification introduces the pH sensitive 4-nitrophenolic functionality

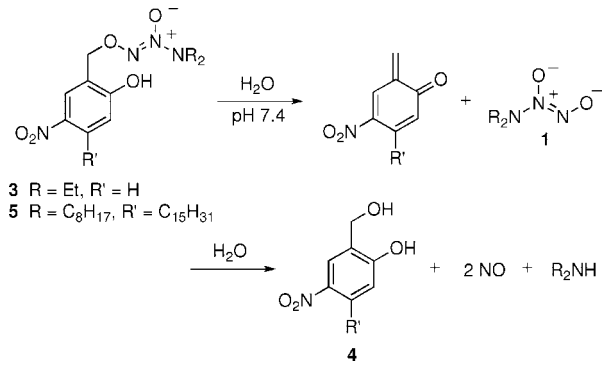
Scheme 1. The Dissociation of Diazeniumdiolate **1** and Its Protection As Prodrug **2**



into protein and peptide structures. Since the acid form (λ_{max} = ca. 320 nm) can easily be distinguished from the basic form (λ_{max} = ca. 410 nm) by absorption spectroscopy, this[11,12] and related[13,14] reagents have served as environmentally sensitive probes. Reaction with nucleophiles is initiated by deprotonation of the 2-hydroxy group (pK_a = ca. 7.4) to form a quinone methide intermediate that is subsequently trapped by the nucleophile.

On the basis of this work, we reasoned that a modified Koshland's reagent could serve as a diazeniumdiolate protecting group that could be removed at pH 7.4 in aqueous solution. Thus, we synthesized precursor **3** (see the Supporting Information) and examined its aqueous chemistry. The pH dependence of the absorption spectra of NO precursor **3**, with λ_{max} = 310 nm for the protonated form and 405 nm for the deprotonated form, is analogous to that previously reported for 2-hydroxy-5-nitrobenzyl alcohol (see the Supporting Information).[11] As expected, at pH 7.4 **3** decomposes cleanly to benzyl alcohol **4**, amine, and NO as shown in Scheme 2. The half-life (determined by UV−vis

Scheme 2. Decomposition of NO Precursor **3** and Its Lipophilic Analogue **5**



absorption spectroscopy) of **3** in pH 7.4 solutions is 25 min at room temperature and 5 min at 37 °C. For comparison,

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the half-life of 2-hydroxy-5-nitrobenzyl bromide in pH 7.4 solutions is 5.5 min at room temperature and 1.5 min at 37 °C, consistent with the expectation that the diazeniumdiolate is a poorer leaving group than bromide.[15] At pH 11.6 decomposition is much more rapid; however, NO is not observed (diazeniumdiolate **1** is stable under these basic conditions) until the solution is acidified. Encouraged by these promising results, we also synthesized (see the Supporting Information) the lipophilic analogue **5** that could be incorporated into hydrophobic polymers.

Because the protection of diazeniumdiolates is meant to impart thermal stability to the NO-releasing functionality, we performed qualitative thermal decomposition experiments under conditions intended to mimic a typical polymer extrusion process. The thermal stability of **5** was examined by thermogravimetric analysis (TGA). Precursor **5** is stable up to 90 °C, but lost ca. 9% of its weight over 2 h at 100 °C; heating from 100 to 170 °C over 2 min resulted in another 3% weight loss. (For comparison, diazeniumdiolate **1** (R = C_8H_{17}) is not stable above 60 °C.) These results suggest that this diazeniumdiolate protection strategy should hold up to extrusion conditions where high temperatures are maintained for only a few minutes.

To demonstrate the feasibility of 2-hydroxy-5-nitrobenzyl as a diazeniumdiolate protecting group that can be used in a commercially viable NO-releasing polymer, we have examined formulations of the medical-grade polyurethane (PU) Tecoflex blended with precursor **5**. (Tecoflex is currently used in the manufacturing of many blood and tissue contact catheters and sensors.) Precursor **5** (2 wt %) was blended into Tecoflex EG-80A by dissolving in THF. The resulting polymer cocktail solution was cast inside 2.5 cm i.d. Teflon rings and cured overnight under ambient conditions; the resulting film thicknesses ranged from 150 to 200 μm.

Films were placed in pH 7.4 PBS solution at 37 °C and NO surface flux was analyzed over the course of approximately 20 h, using a chemiluminescence analyzer. Results are shown in Figure 1. Disappointingly, following a bolus of NO production over the first 30 min, very little NO was observed, i.e., only ca. 1% of the maximum possible (NO release profile **I**, Figure 1a).[16] However, when analogous films were prepared that also incorporated 8 wt % of the lipophilic salt, potassium tetrakis-4-chlorophenyl borate (KTpClPB),[17] the extremely promising NO release profile **II** (Figure 1b) was observed. The amount of NO observed is now approximately 60% of the maximum possible, demonstrating the paramount importance of the added lipophilic salt as has been reported previously.[17] In addition, an NO flux rate above 1×10^{-10} mol cm^{-2} min^{-1} is maintained for over 20 h thus mimicking the NO-releasing properties of the natural endothelium.

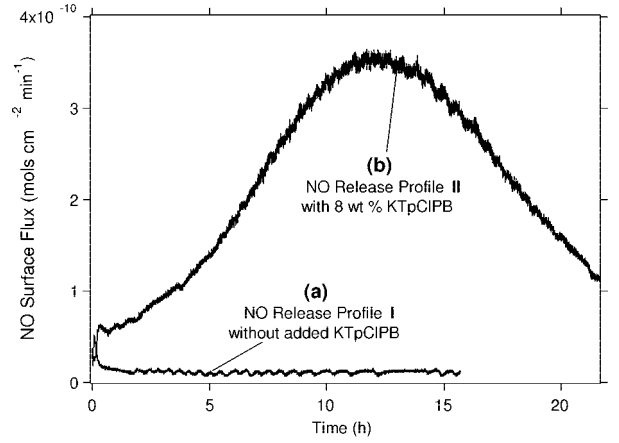

Figure 1. Nitric oxide surface flux observed at pH 7.4 and 37 °C for a polymer containing (a) 2 wt % NO precursor **5** blended with Tecoflex EG-80A and (b) 2 wt % NO precursor **5** and 8 wt % KTpClPB blended with Tecoflex EG-80A.

The origin of the effect of added lipophilic salt has been proposed to be related to a buffering effect.[17] Proton-induced decomposition of diazeniumdiolates within a polymer matrix occurs as water diffuses in, leading to the formation of secondary amines, which effectively raise the pH of the polymer environment due to the formation of secondary ammonium hydroxide moieties. In the case of lipophilic diazeniumdiolates such as **1** (R = C_8H_{17}), the secondary ammonium hydroxide will be unable to diffuse out of the polymer bulk due to hydrophobic interactions. This ultimately leads to a retardation of the decomposition of diazeniumdiolates and an inability to recover all of the NO from deprotected diazeniumdiolates in a reasonable amount of time. By incorporating lipophilic anions into the polymer, the alkali cations are able to pair with the hydroxide (and likewise the lipophilic borate anions with the lipophilic ammonium cations), and now the hydroxide is able to diffuse out of the polymeric matrix and into the surrounding aqueous buffer.

We also examined NO release profiles from polyvinyl chloride (PVC) rods coated with PU containing our newly developed NO donor **5**. PVC tubing (0.027″ i.d., 0.045″ o.d.) was capped with PU and smoothed before coating. Several 2-cm ends of 10-cm lengths of these rods were dip-coated six times with a homogeneous polymer cocktail solution containing Tecoflex SG-80A, NO-releasing agent **5**, and KTpClPB in THF. An additional thin layer of Tecoflex SG-80A was applied as an overcoat. Coating integrity studies were performed to ensure good adherence of the coating to the substrate by soaking the rods in PBS buffer for 24 h with a high velocity stir rate. The coatings were visually examined pre- and postbuffer exposure. No difference in the coating appearance was observed following this treatment.

Three different polymer cocktail solutions were used for the dip-coating procedure to determine the effect of the relative amounts of NO donor and lipophilic salt on NO

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(16) The lack of NO production over the course of 15 h (Figure 1a) strongly suggests that NO precursor **5** does not leach from the polymer into the aqueous solution. Further leaching studies are ongoing.

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release profiles. These profiles, measured in pH 7.4 PBS solutions at 37 °C, are shown in Figure 2. Comparison of

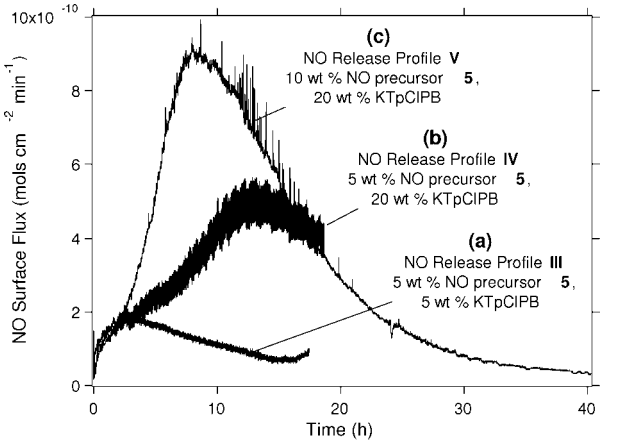

Figure 2. Nitric oxide surface flux observed at pH 7.4 and 37 °C for PVC rods coated with (a) 5 wt % NO precursor **5** and 5 wt % KTpClPB blended with Tecoflex EG-80A, (b) 5 wt % NO precursor **5** and 20 wt % KTpClPB blended with Tecoflex EG-80A, and (c) 10 wt % NO precursor **5** and 20 wt % KTpClPB blended with Tecoflex EG-80A.

profiles **III** and **IV** (Figure 2, panels a and b), where the concentration of NO precursor **5** was kept constant and that of KTpClPB was increased 4-fold, demonstrates that an excess of KTpClPB is required to maximize NO release. Comparison of profiles **IV** and **V** (Figure 2, panels b and c), where the concentration of KTpClPB was kept constant and that of NO precursor **5** was doubled, suggests that a polymer matrix formulation with 20 wt % KTpClPB may be optimal for NO release (i.e., doubling the amount of **5** doubles the amount of NO produced).

To test the biocompatibility of materials incorporating NO precursor **5**, we examined polymer formulations using a porcine implant model to evaluate thrombus formation.[18] Four Tecoflex SG-80A-coated rods were prepared (two sets of two, with each set containing a control rod without any NO donor and another rod incorporating NO precursor **5**). The rods were prepared as described above by dip-coating in polymer cocktail solutions containing (1) 20 wt % KTpClPB without any NO precursor **5** and (2) 20 wt % KTpClPB with 5 wt % NO precursor **5**. One set was then implanted in the femoral arteries and the other in the carotid arteries of a 25 kg farm swine (see the Supporting Information).

Given the NO release profiles shown in Figure 2, the rods were soaked for 7 h in pH 7.4 PBS solution at 37 °C prior to implantation to ensure that the NO surface flux was in an optimal range during the in vivo experiment. The rods remained implanted for 8 h. Following removal, each rod was rinsed gently with pH 7.4 PBS and examined visually; digital images are shown in Figure 3 where a significant

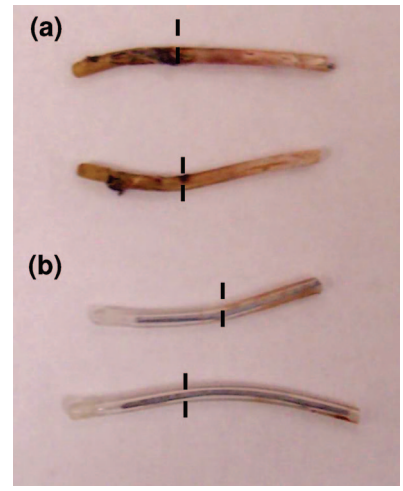

Figure 3. Digital images of coated PVC rods after implantation for 8 h. The portion of the rods to the left of the dotted lines was exposed to flowing blood. The rods were prepared by dip-coating in Tecoflex EG-80A cocktail solutions containing (a) 20 wt % KTpClPB without any NO precursor **5** and (b) 20 wt % KTpClPB with 5 wt % NO precursor **5**.

difference in thrombus formation can be seen. (Note that only the portion of the rods to the left of the dotted lines in Figure 3 was exposed to flowing blood.) The rods that released NO (Figure 3b) have no observable thrombus formation on their surfaces, whereas the control rods that contained no NO precursor (Figure 3a) possess significant areas of clots on their surfaces. These results, taken together with the enhanced thermal stability imparted to the diazeniumdiolate functionality by 2-hydroxy-5-nitrobenzyl substitution, suggest that lipophilic NO precursor **5** may be useful as a general additive to hydrophobic polymers to enhance their thromboresistivity in blood-contacting biomedical applications.

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Supporting Information Available: General experimental and synthetic procedures, spectra, and details concerning the porcine implantation procedure. This material is available free of charge via the Internet at http://pubs.acs.org.

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