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Conversion of a Polysaccharide to Nitric Oxide-Releasing Form. Dual-Mechanism Anticoagulant Activity of Diazeniumdiolated Heparin

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Abstract—We describe heparin/diazeniumdiolate conjugates that generate nitric oxide (NO) at physiological pH. Like the heparin from which they were prepared, they inhibit thrombin-induced blood coagulation. Unlike heparin, they can also inhibit and reverse ADP-induced platelet aggregation (as expected for an NO-releasing agent), suggesting potential utility as dual-action antithrombotics. Published by Elsevier Science Ltd.

With the ultimate aim of exploiting the receptor-mediated interactions of saccharides[1] for delivering nitric oxide (NO) to physiological sites of need, we are devising means of converting these natural products to NO-releasing form. As a first example, we show how covalent attachment of [N(O)=NOR] groups to heparin can improve its utility as an anticoagulant.

Preparation of Diazeniumdiolated Heparin

Thrombus is a solid mass composed of fibrin, platelets, and other blood constituents that, when inappropriately deposited in the circulatory system, can lead to a variety of clinical disorders. Heparin is a widely used inhibitor of this coagulation cascade; its binary complex with antithrombin III binds and inhibits both Factor Xa and thrombin, two proteases that are essential to the ultimate conversion of fibrinogen to fibrin.[2,3] NO also has potent anticoagulant activity, expressed by inhibiting the adhesion and aggregation of platelets via a cyclic guanosine 3′,5′-monophosphate (cGMP)-dependent mechanism.[4]

We speculated that conjugating NO-releasing functional groups to heparin might beneficially combine these two mechanisms of anticoagulant activity. To test this hypothesis, we chose to work initially with MOM-PIPERAZI/NO (**1**), a diazeniumdiolate with a half-life for NO release of 17 days in physiological buffer whose nucleophilic secondary amino group was shown previously to displace chloride from poly(vinyl chloride), converting the polymer to an NO-releasing form.[5,6] Reasoning that similar displacement might occur on reaction with a sulfated polysaccharide, we stirred MOM-PIPERAZI/NO (113 mg, 0.60 mmol in 1 mL of 95% ethanol) with 400 mg of bovine lung heparin sodium salt (Sigma catalogue number H 4898, $\geqslant$140 USP units/mg, approximate molecular weight range 9–10 kDa) in 5 mL of 10 mM sodium hydroxide until the starting diazeniumdiolate was consumed. This produced an adduct that was purified by elution from a Bio-Rad Econo-Pak 10DG desalting column (6-kDa exclusion limit) with deionized water. The resulting white powder (226 mg) had an absorbance at 231 nm of 3.5 L cm^{-1} g^{-1}. Assuming an extinction coefficient for the [N(O)=NOR] group of 8.2 mM^{-1} cm^{-1}, as was observed for **1**,[5] we calculate an equivalent weight of 8.2/3.5 = 2.3 kDa/diazeniumdiolate group. This value is nearly identical to that expected (2.4 kDa) if there were

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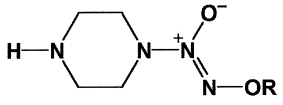
on average one MOM-PIPERAZI/NO moiety and 20 water molecules for every three of the disaccharide units shown in Scheme 1. Elemental analysis offered strong support for this empirical formula, with found values for all elements being within 0.3% of calculated except that of sodium, which was 0.44% high. A somewhat greater degree of loading was indicated by the ^{1}H NMR spectrum. Comparing the integrals of the protons at C-1 of the pyranose rings (broad singlets at δ 5.22 and 5.42, taken as totalling 1 H) with those for the MOM-PIPERAZI/NO moiety (singlets at δ 5.35 and 3.52, multiplets at δ 3.54–3.50 and 3.12–3.09, average integral per proton of 0.22) suggested that there were 0.44 diazeniumdiolate groups per disaccharide unit. We conclude that the true value lies in the range of 0.33–0.44 [N(O)=NOR] groups per disaccharide for this product.[7]



1 (MOM-PIPERAZI/NO): R = CH_2OCH_3
2 (PIPERAZI/NO): R = Na

This preparation, designated **3** in the following, is expected to mimic MOM-PIPERAZI/NO in generating not only NO but also methanol and formaldehyde on hydrolysis. For use in biomedical applications in which these one-carbon by-products might be viewed as undesirable, an alternative conjugate in which the diazeniumdiolate groups are ionic rather than MOM-protected was prepared using PIPERAZI/NO[8] (**2**) in place of **1** as starting material, as separately described.[6]

NO Release Characteristics

Both **3** and this latter preparation (**4**) bear diazeniumdiolate groups that, by analogy with starting materials **1** and **2**, are expected to generate NO on hydrolysis. This was confirmed using an established chemiluminescence method.[9] Integrating the NO release profile of **3**, shown in Figure 1, pointed to a cumulative recovery of 0.82 mmols of NO per gram over the total 8-week observa-

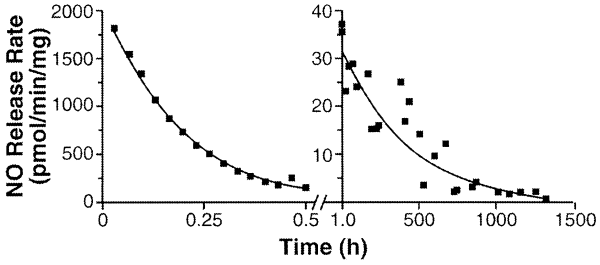
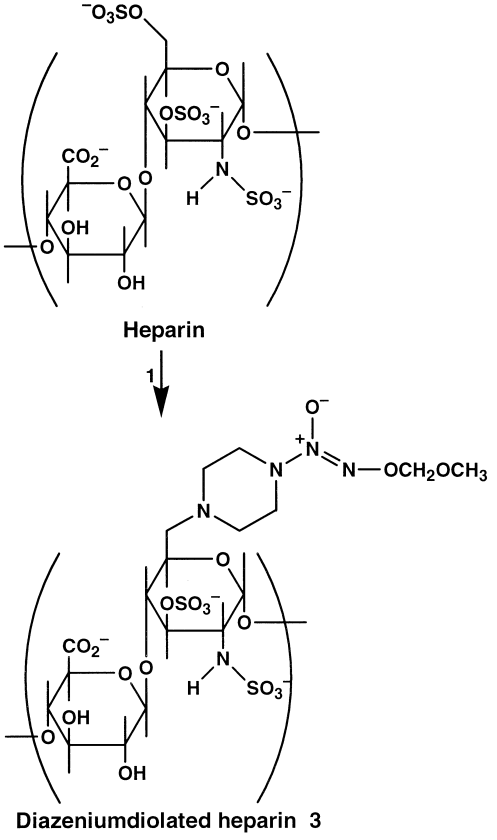
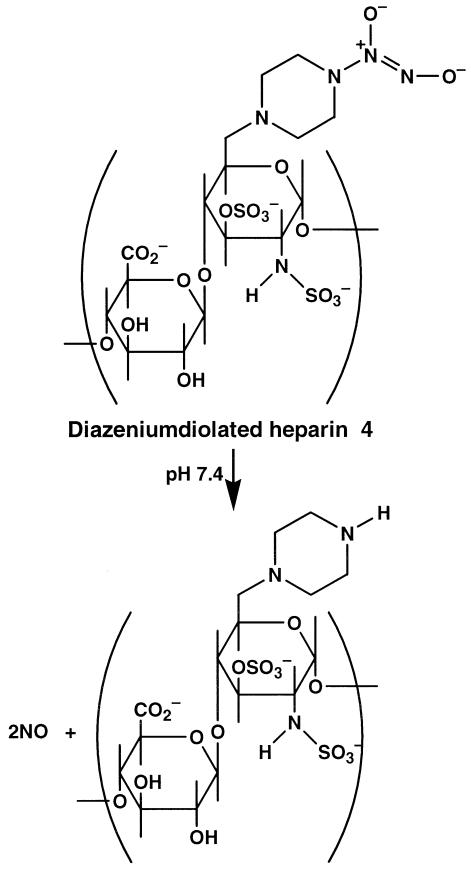


Figure 1. NO release profile of covalently bonded diazeniumdiolate **3**.



Scheme 1. Synthesis of diazeniumdiolated heparin **3**. Substitution of **1** at C-6 of a sulfated glucosamine ring is shown for illustrative purposes, but similar substitutions at other positions in the heparin molecule are also likely.



Scheme 2. Hydrolytic generation of NO from diazeniumdiolated heparin derivative **4**.

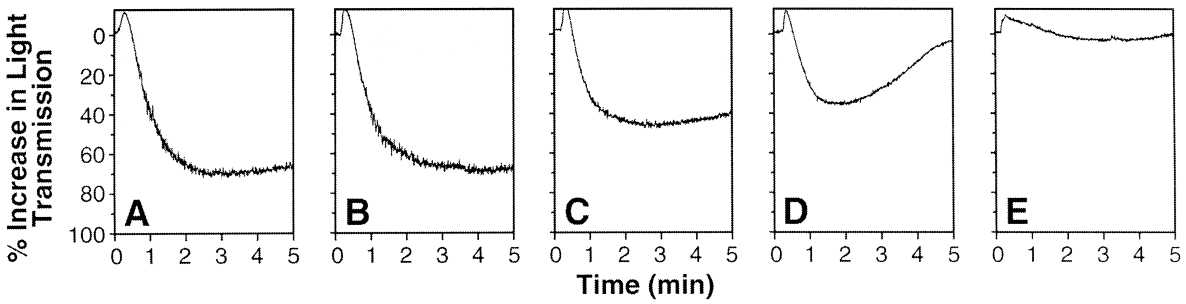


Figure 2. Effect on platelet aggregation of: A, citrate/dextrose (control); B, commercial heparin; C and D, NO-releasing heparin **3**; E, NO-releasing heparin **4**. Blood was drawn into tubes containing citrate/dextrose, whereupon standard procedures[12] were used to prepare platelet-rich plasma (PRP). PRP was incubated with the various heparin preparations for 5 min prior to addition at time zero of the platelet agonist, 5 μM ADP. The heparin concentrations were 25 μg/mL for B, C, and E, but 100 μg/mL for D.

tion period. This represents 98% of the theoretical yield based on the equivalent weight inferred from the elemental analysis data.

Preparation **4**, on the other hand, having no protecting group to cleave prior to protolytic NO release, dissociated NO much more rapidly under these conditions (Scheme 2). This first order reaction was characterized by a half-life of 8.4 min at pH 7.4 and 37 °C, comparable to the 5.3-min half-life seen for **2**. The total amount of NO eventually recovered was 0.51 mmols of NO per gram of **4**.[6]

Antithrombotic Activity

Compounds **3** and **4** showed significant heparin-like anticoagulant activity, with thrombin times of 80 (±8 sd) s and 31 (±5) s, respectively, versus 5.4 (±0.2) s with no added anticoagulant and 92 (±6) s for underivatized heparin.[10] Thus, while **3** and **4** were less active than the starting material from which they were prepared, they retained a significant degree of heparin-mediated thrombin inhibitory activity.

Preparations **3** and **4** nevertheless differed considerably from native heparin in their ability to inhibit platelet aggregation. Figure 2A shows the normal aggregation of platelet-rich human plasma (PRP) when treated with 5 μM adenosine 5′-diphosphate (ADP) in citrate/dextrose solution. ADP-induced aggregation was not affected by preincubating for 5 min with 25 μg/mL of control heparin (Fig. 2B), but similar preincubation with **3** inhibited aggregation in a concentration-dependent manner (Fig. 2C and D). With its much faster NO release rate, freshly prepared solutions of diazeniumdiolated heparin **4** (25 μg/mL) completely prevented platelet aggregation (Fig. 2E).

In addition to its initial retarding activity on platelet aggregation, **3** at the higher concentration induced nearly complete disaggregation during the 5-min observation period, as shown in Figure 2D. The latter effect is consistent with the report of Mellion et al. that NO, but not heparin, is able to reverse ADP-induced platelet aggregation.[11]

The observation of both thrombin inhibitory activity and antiaggregatory action is consistent with our hypothesis that diazeniumdiolated heparin is capable of acting by two complementary antithrombotic mechanisms, one NO-dependent, the other the result of retained heparin-like anticoagulant activity.

Acknowledgements

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