

A Nitric Oxide-Releasing Heparin Conjugate for Delivery of a Combined Antiplatelet/Anticoagulant Agent

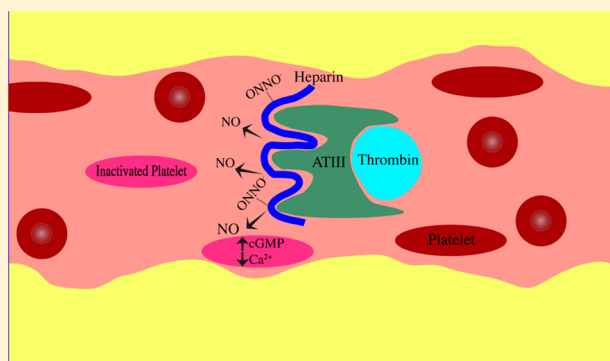
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ABSTRACT: Heparin is a widely used anticoagulant due to its ability to inhibit key components in the coagulation cascade such as Factor Xa and thrombin (Factor IIa). Its potential to preferentially bind to antithrombin (ATIII) results in a conformational change and activation that leads to the prevention of fibrin formation from fibrinogen and ultimately obstructs a hemostatic plug from forming. Nitric oxide (NO) exhibits potent antiplatelet activity attributed to its capacity to increase the amount of cyclic guanosine monophosphate (cGMP) within platelets, which decreases the Ca^{2+} concentration required for platelet activation. Currently there is no single agent that combines the functions of both antiplatelet and anticoagulant (anti-Xa and anti-IIa) activities to effectively block both the extrinsic and the intrinsic coagulation pathways. The research reported herein demonstrates the ability to combine the physiological capabilities of both heparin and NO into one functional compound via use of a spermine derivative of heparin, thus enabling formation of a novel diazeniumdiolate (NONOate). The heparin–spermine NONOate has a half-life of 85 min at 25 °C (pH 7.4). The heparin backbone of the conjugate maintains its anticoagulant activity as demonstrated via an anti-Xa assay, providing an anticoagulant conversion of 3.6 $\mu\text{g}/\text{mL}$ of the heparin–spermine–NONO conjugate being equivalent to 2.5 $\mu\text{g}/\text{mL}$ (0.50 IU/mL) of underivatized heparin in terms of anti-Xa activity. Using standard platelet aggregometry, it is shown that the functionality of the NO release portion of the heparin conjugate prevents (nearly 100%) platelet aggregation in the presence of adenosine diphosphate (ADP, platelet agonist).

KEYWORDS: nitric oxide release, diazeniumdiolated heparin, combined antiplatelet/anticoagulant agent



INTRODUCTION

Approximately 795 000 people each year in the United States experience a new or recurrent stroke, and on average, someone has a stroke every 40 s.¹ Strokes account for one out of every 18 deaths in the United States.¹ Similar high frequencies of events occur annually for other significant thrombotic events, such as pulmonary embolism, heart attack, and deep vein thrombosis. Physicians often prescribe anticoagulant medications to help curtail the risk of clotting events for patients at higher risk to have such thrombosis induced episodes. At the same time, when patients present with symptoms that may be signs of an impending or already occurring thrombotic event, the immediate use of anticoagulant therapy is usually initiated. There are many anticoagulants in the market already, such as warfarin, heparin, and low-molecular-weight heparins (LMWH), as well as direct oral anticoagulants such as pradaxa, apixaban, and rivaroxaban. Current antiplatelet drugs consist of aspirin and clopidrel, along with glycoprotein receptor antagonists such as abciximab and tirofiban.

Existing antithrombotic agents are classified into two main categories: antiplatelets (those that inhibit platelet function; e.g., clopidrel and aspirin) and antithrombins (those that prevent the formation of fibrin and ultimately a stable

hemostatic plug from forming). Nitric oxide (NO) is an endogenous and very potent antiplatelet agent due to its ability to increase cyclic guanosine monophosphate (cGMP) levels within platelets, thus lowering intracellular Ca^{2+} levels that are required for the activation of the common pathway in the coagulation cascade. Seminal work using some existing NO donors has supported this mechanism of action.^{2–5} Further, the use of exogenous NO release agents as drugs to achieve antiplatelet activity *in vivo* is attractive since it mimics the natural role of NO release from the endothelial cells that line the inner walls of all healthy blood vessels.

Heparin has been thoroughly studied for its antithrombotic properties. Heparin functions by binding rapidly to antithrombin III (ATIII), disrupting the salt bridges in the protein structure, and inducing a conformational change that allows it to bind up to 2 orders of magnitude faster with thrombin (as well as to Factor Xa).⁶ This ultimately inhibits thrombin formation (from prothrombin via Factor Xa) thereby hindering

Received: August 21, 2013

Revised: December 31, 2013

Accepted: January 14, 2014

Published: January 14, 2014

fibrin development, from fibrinogen, and ultimately preventing a secure, cross-linked clot from forming.^{6,7}

In most cases, patients require only one type of anticoagulant, but in some cases when vascular prostheses (i.e., stent, vascular graft) are involved, or when a deep vein thrombosis coupled with a myocardial infarction (MI) presents itself, both antithrombins and antiplatelets are required.⁸ Initially, the physician must prescribe a dosage of each of the drugs, and based on the reaction of the patient, the dosage of each is then tailored. Being able to have antiplatelet and antithrombin activities within one functional drug would be quite attractive for a number of situations in the hospital setting. Earlier work by Keefer and co-workers suggested the possibility of combining NO release with heparin. However, the secondary amine donor that was attached to heparin was a piperazine group.^{9,10} The use of this sterically hindering moiety caused a large decrease (66%) in heparin's functional activity as an anticoagulant, thus eliminating a large fraction of the dual-action capabilities of the conjugate. Further, without protecting the diazeniumdiolated piperazine moiety with a methoxymethyl group, the heparin agent previously prepared had a half-life of NO release of only 8.4 min under physiological conditions (pH 7.4 and 37 °C), providing limited functional lifetime for therapeutic use. The research herein aims to synthesize a more suitable dual acting heparin/NO release agent that still maintains its anticoagulant and antiplatelet activities and has a significantly longer half-life at pH 7.4 than the agent reported earlier.

■ EXPERIMENTAL SECTION

The heparin that was utilized here was in the sodium salt form, extracted from porcine intestinal mucosa, with an average chain length of 17 000–19 000 Da. Heparin and all other chemicals used for the synthesis of the heparin–spermine conjugate were purchased from Sigma-Aldrich (St. Louis, MO). Adenosine diphosphate (ADP) used for platelet aggregometry was obtained from Chrono-Log Corporation (Havertown, PA).

Preparation of Heparin–Spermine Conjugate. The heparin–spermine conjugate was synthesized using the standard reaction of *N*-hydroxysuccinimide (NHS) with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to activate free carboxyl groups on the heparin polymer chain for subsequent amide formation with the terminal primary amines of spermine.^{11–13} Heparin, NHS, and EDC were dissolved in 0.05 M MES buffer (pH 5.54) at a molar ratio of 1:2:2, respectively. Preactivation ensued for 10 min with continuous agitation. Then, a 5:1 molar ratio of spermine to heparin (assuming avg. M.W. of heparin = 18 kD) was added, and the reaction was allowed to proceed for 2 h. Afterward, a 10 000 MWCO centrifuge filter-tube was used to isolate the product. Three centrifugation cycles for 20 min at 3990 rpm, with water washings in between, were run. The final product solution was put into the lyophilizer for 2 d to obtain a white solid.

Heparin–Spermine Conjugate Diazeniumdiolation. The diazeniumdiolate of the heparin–spermine conjugate was formed via the use of an NO reactor, as typically employed to make diazeniumdiolate NO donors.^{14,15} The necessary base was provided in the form of 145 μ L of sodium methoxide in methanol added to the bulk MeOH, followed by the heparin–spermine conjugate, thus forming a heterogeneous solution. The NO reactor cell/solution was purged with argon gas, followed by the introduction of NO gas. The reactor was held at 70 psi for 5 days with continuous stirring. Afterward, the

solution was filtered and washed using methanol. The resulting white solid was left to dry for 30 min over a Hirsch funnel.

NO Release Measurements. Measurement of NO release per mass of the heparin–spermine NONOate was determined from the acidification technique through the use of a highly sensitive and selective Sievers nitric oxide analyzer (NOA). A given volume of a 1 mg/mL solution of the heparin–spermine–NONO conjugate was injected into 0.183 M H₂SO₄. A review on the use of chemiluminescence to detect NO from various NO donors is available.¹⁶ Briefly, the NO gas liberated from the conjugate is purged out of solution and delivered to the NOA reaction chamber by a nitrogen carrier gas stream, where it then reacts with O₃ to produce NO₂ in its excited state. Upon relaxation, a photon is emitted and detected via a photomultiplier tube. The photon flux correlates to the concentration of NO in the chamber and thus in the gas phase. Integration of the NO signal after addition of the conjugate to the acid solution provides the total moles of NO capable of being released by the heparin–spermine–NONO conjugate.

Half-Life Measurements of Heparin–Spermine–NONO. To establish the half-life of the new polymeric NONOate, a standard protocol was used as previously described.¹⁷ In brief, a 0.42 mg/mL solution of the heparin–spermine–NONO species was prepared in 10 mM PBS (pH 7.4), and the decrease in absorbance of the characteristic NONOate absorbance band at 248 nm was monitored over time using a Shimadzu UV–vis spectrophotometer. Spectra were fit to two Gaussian functions, centered at 210 and 248 nm, to remove the contribution of the background to the absorption band of interest (248 nm). The heights of the fitted Gaussian functions centered at 248 nm were used to generate an absorbance vs time plot. Using a first-order kinetics reaction scheme (which is standard for NONOates¹⁷), the plot was fitted to an exponential curve from which the $t_{1/2}$ could be obtained.

Anti-Xa Assay. The anti-Xa assay was obtained in the form of a kit from Aniaira (West Chester, OH) and included all necessary reagents except ATIII, which was purchased separately from Haematologic Technologies Incorporated (Essex Junction, VT). Preparation of control proceeded as follows: 12 μ L of a 0.816 mg/mL ATIII solution dissolved in 10 mM PBS buffer (pH 7.4) was added to 36 μ L of 18.3 M Ω Millipore water and preincubated with 70 μ L of Sxa-11 substrate at 37 °C for 3 min after proper agitation. Then, 50 μ L of Factor Xa was added and mixed thoroughly with the solution for exactly 90 s at 37 °C. The solution gradually turned yellow over this time period. The reaction was then stopped by adding 100 μ L of 20% acetic acid. The absorbance at 405 nm was measured within 30 min using a LabSystems Multiskan RC Type 351 microtiter plate reader (color change is stable for 2 h). Every reagent and vial was preincubated at 37 °C before the assay was performed. A blank containing no anticoagulant was run in parallel with each sample, and the respective absorbance was subtracted from the sample containing the anticoagulant. Preparation of samples proceeded as follows: The 12 μ L of solution that is mixed with the substrate and water was allocated properly to allow for addition of the varying concentrations of the anticoagulant, while still maintaining the final ATIII concentration of 0.51 mg/mL. As an example, to create the 0.25 IU/mL concentration of heparin, a 0.66 IU/mL stock was created in 10 mM PBS. Upon dilution of 4.5 μ L of the stock with 7.5 μ L of the 0.816 mg/mL ATIII stock, a final concentration of 0.25 IU/mL of heparin was achieved, along

with a solution containing 0.51 mg/mL ATIII. The initial stock concentration of heparin was increased to compensate for dilution with ATIII for the remaining heparin samples. The % inhibition of Factor Xa activity was calculated according to eq 1:

$$\text{inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\% \quad (1)$$

where the values for the control and sample were the absorbance at 405 nm, and the control contained no anticoagulant.

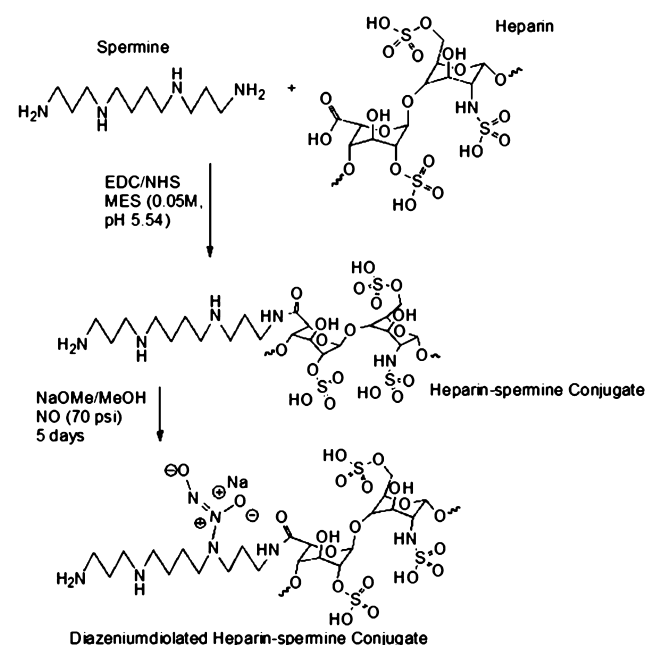
Platelet Aggregometry. Fresh sheep blood (40 mL) was collected in a 60 mL syringe containing 1:10 citrate to blood solution. Sheep blood is often employed as a good model for the thrombotic behavior of human blood.¹⁸ The blood was centrifuged at 110g for 15 min to obtain platelet rich plasma (PRP). The PRP was removed, and another centrifugation was performed at 2730g for 15 min to obtain platelet poor plasma (PPP). Platelets were counted in the PRP using a Coulter Counter Z1 (Coulter Electronics Hialeah, FL) and diluted accordingly with PPP to create a normalized 2×10^8 platelets/mL solution. PPP was employed as the blank, and when necessary, the heparin–spermine–NONO conjugate was added. Samples were preincubated at 37 °C for 10 min (350 μ L PRP and varying amounts of heparin–spermine–NONO and saline to yield a total volume of 400 μ L). Two microliters of 2 mM ADP was determined to be the optimal volume to activate (aggregate) platelets. Upon adding ADP, a light transmission (%) vs time plot was generated to determine the rate of platelet aggregation using a Chrono-Log optical aggregometer.

RESULTS AND DISCUSSION

Characterization of Heparin–Spermine Conjugate. To create a drug with combined antithrombin and antiplatelet activities, heparin was modified at its carboxylic acid sites using a standard EDC/NHS reaction in the presence of spermine (Scheme 1). Once the reaction was performed and the product isolated, the heparin–spermine conjugate was analyzed for elemental nitrogen content by Atlantic Microlab Inc. (Norcross, GA). The theoretical value for nitrogen content in the starting heparin preparation is 2.41 wt %, and in the conjugate, if all carboxyl sites were derivatized with spermine, would be 9.19 wt % (assuming average molecular weight of the repeating heparin disaccharide unit is 577.3 g/mol and the conjugate containing bound spermine is 761.6 g/mol). The experimental values were found to be 1.85 wt % and 10.52 wt %, respectively. This disparity is most likely due to heparin having a high polydispersity, causing a skew in the % nitrogen, as has been reported previously.¹⁹ Thus, from the elemental analysis data, spermine was successfully attached to heparin via an amide bond at one of spermine's two primary amine sites yielding, on average, one spermine molecule per disaccharide unit of heparin.

Using a polyamine such as spermine to derivatize heparin is advantageous because of its structure and presence as an endogenous physiological species at picomolar to millimolar concentrations depending on location.^{20–22} When diazeniumdiolated at one of its secondary amine sites, the resulting derivative of native spermine is reported to exhibit an average half-life of 230 min at 25 °C (pH 7.4).²³ Hence, such a lifetime of NO release would certainly be more than sufficient for

Scheme 1. Synthesis of the Diazeniumdiolated Heparin–Spermine Conjugate



interaction with circulating platelets for *in vivo* drug applications. Indeed, it is envisioned that after being injected into the bloodstream, the heparin–spermine conjugate, once all of the NO has been released, would be guided to the liver, pancreas, and gall bladder where it is systematically broken down into elements that can be safely excreted renally or recycled by the body. With the aid of polyamine oxidase, which is most prominent in the pancreas, but is also found in the liver and various other organs in the gastrointestinal tract, spermine can be oxidized ultimately to putrescine where it begins the polyamine cycle once again.²⁴ The cytotoxicity of the NO-depleted heparin–spermine conjugate to healthy cells is therefore likely to be minimal.

Nitric Oxide Releasing Properties of the Diazeniumdiolated Heparin–Spermine Conjugate. Once the spermine was attached to heparin, the heparin–spermine conjugate was diazeniumdiolated (Scheme 1). Upon isolating the diazeniumdiolate of the polymeric species, it was analyzed for its NO release capability (Figure 1): a recovery of 94 ± 6 μ mol of NO per μ g of product was obtained, along with a diazeniumdiolation value (percentage of diazeniumdiolated secondary amines divided by total possible secondary amines on the heparin–spermine conjugate) of 2%. This low value is most likely attributable to the insoluble nature of the heparin–spermine conjugate in methanol, which forces a heterogeneous reaction to occur, and thus leads to poor reactivity and low diazeniumdiolate formation between the loaded NO and the conjugate. Employing different solvents or reaction conditions are possible solutions to this problem.

Figure 2 shows the UV spectrum of the heparin–spermine–NONO conjugate, illustrating a significant absorption shoulder at 248 nm. Through standard spectroscopic procedures and deconvolution using Matlab (see Figure 2), it is possible to monitor the change in UV absorption at 248 nm with time to determine the half-life of the new NO donor. Using this approach, the half-life of the macromolecular NONOate was determined to be ~85 min in pH 7.4 PBS buffer at 25 °C. This

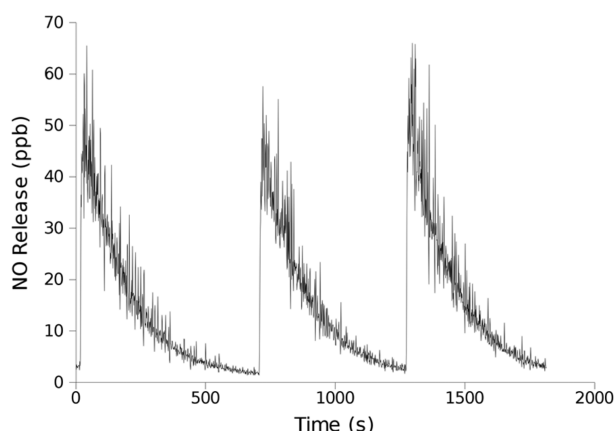


Figure 1. Nitric oxide release measurements via chemiluminescence from 10 μ L injections of a 1 mg/mL heparin–spermine NONOate solution into 0.183 M H_2SO_4 . Integration of these NO release signals can be used to obtain the number of moles of NO produced from the injected NONOate.

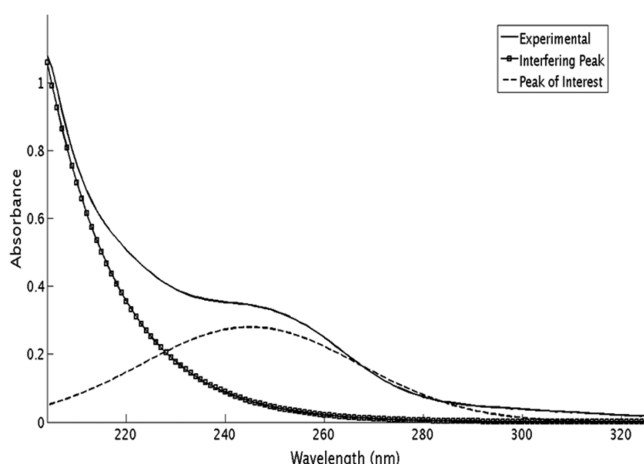
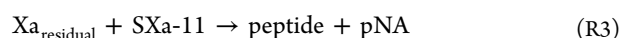
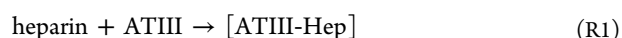


Figure 2. Deconvolution of the experimental spectrum (heparin–spermine–NONO) with Gaussian fits to background absorbance as well as to the characteristic NONOate peak at 248 nm. The concentration of conjugate was 0.42 mg/mL in 10 mM PBS (pH 7.4).

is considerably shorter than the half-life of the native spermine NONOate ($t_{1/2} = 230$ min). This is most likely due to the sterics of the heparin backbone that inhibit dimerization of the spermine molecules.^{23,25} In parallel with sterics, a change in the structure of the entire NONOate also can result in significant changes in NO release kinetics. For example, diazeniumdiolated diethylenetriamine ($t_{1/2} = 20$ h) and diazeniumdiolated dipropyltriamine NONOate ($t_{1/2} = 30$ min) vary only by the addition of two carbons, yet their respective half-lives are substantially different.²⁶ Thus, adding the heparin moiety to spermine provides a plausible rationale for such a drastic change in half-lives of the two NONOates. Although the $t_{1/2}$ of the heparin–spermine NONOate is only 85 min, this lifetime is still quite useful for potential medical applications, including as a combined systemic agent to prevent clotting and platelet loss during extracorporeal circulation (ECC) procedures (i.e., coronary artery bypass, kidney dialysis, etc.) as well as for balloon angioplasty, and other less invasive procedures where activation of platelets and thrombosis are possible. Indeed, the fastest circulatory time of blood is about 30 s, and the mean time is ca. 60 s.²⁷ Thus, a half-life on the order of several

minutes under physiological conditions would be of practical value for a systemic anticoagulant.

Anti-Xa Assay Evaluation. Although heparin was modified with spermine, this conjugation did not significantly affect the anticoagulant properties of the heparin backbone based on anti-Xa assay activity. This reaction monitors the binding efficiency of ATIII to Factor Xa. When heparin is absent, ATIII binding to Factor Xa is minimal, and the protein binds rapidly to the chromogenic substrate (SXA-11), producing *p*-nitroaniline (pNA) with a strong absorbance at 405 nm. However, when the glycosaminoglycan is in solution, it binds to ATIII, thus increasing the reactivity of ATIII, causing Factor Xa to become bound, which results in much unreacted SXA-11 and ultimately a decrease in absorbance. The sequence of reactions in this assay is illustrated below:



An experiment was conducted with varying concentrations of pure heparin, along with an approximately equimolar disaccharide solution of the heparin–spermine conjugate equivalent to 0.50 IU/mL heparin. A true overall conversion factor of International Units (IU) of heparin activity to the conjugate is difficult to obtain because the number of IUs is generated after evaluating the compounds activity in a myriad of assays (i.e., thrombin time, activated partial thromboplastin time).²⁸ The conjugation of spermine to heparin may have altered the original efficacy, even if in just one of the assays, so it would be difficult to correlate the IU activity, without performing the classical assay on determining the extension of the clotting time of fresh sheep blood. Unfortunately, we did not have access to fresh sheep blood to conduct such experiments. Thus a theoretical calculation was performed to equate the molar concentration of the disaccharide unit (active species in this assay) of the two compounds. Figure 3 provides the results of the anti-Xa activity experiments for both the pure heparin standards and the heparin–spermine conjugate, as well as the diazeniumdiolated conjugate. As shown, the conjugate both with NO and without NO (at the same molar concentrations of the heparin disaccharide in the assay mixture; 4.4 μ M) exhibited significant anti-Xa activity, comparable to the values of the 0.50 IU/mL heparin standard (Figure 3). The % inhibition of the conjugate containing NO is higher because the ONNO[−] moiety increases the net negative charge on the compound.²⁹ This result supports the notion that strong binding between heparin–spermine/heparin–spermine–NONO and ATIII still occurs. As the NO dissociates from the diazeniumdiolated conjugate, the overall charge on the conjugate becomes more positive, leading to a slight decrease of factor Xa inhibition. Hence, it is possible to state that 3.6 μ g/mL of the heparin–spermine–NONO conjugate is equivalent in anti-Xa activity to 0.50 IU/mL heparin.

Assessment of Platelet Aggregation. Standard platelet aggregometry experiments provided evidence that the nitric oxide bound to the heparin–spermine conjugate still maintained its antiplatelet activity. High levels of Ca^{2+} are required for the specific tenase complex to form on an activated platelet. Forming this complex concludes the intrinsic coagulation cascade and initiates the common pathway.³⁰ However, NO is capable of increasing the concentration of

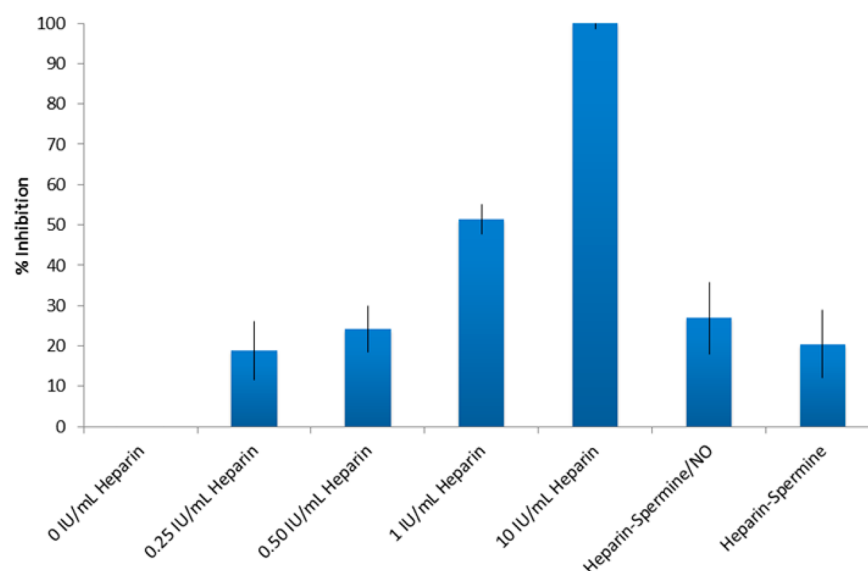


Figure 3. Factor Xa inhibition (%) by various concentrations of heparin and spermine derivatives. The concentration of the heparin–spermine conjugate both with and without NO were made to be 0.50 IU/mL based on molar concentrations of disaccharide units of heparin.

cyclic guanosine monophosphate (cGMP) within platelets, resulting in decreased intracellular Ca^{2+} levels, and thereby preventing the activation of platelets. This intricate function of NO makes it a potent mediator in interrupting the intrinsic coagulation pathway. Figure 4a provides evidence to support this. Upon the addition of the heparin–spermine–NONO (0.21 mg/mL), even at low volumes (i.e., 10 μL —equivalent to 500 nM NO in assay mixture if all NO was released immediately), light transmission was decreased to 35%. At an increased volume of the diazeniumdiolated heparin spermine conjugate (40 μL , equating to 2 μM total NO), almost 0% transmission was observed, indicating that platelets are not aggregating at all. For such experiments, it is necessary to ensure that the prevention of platelet activation is indeed from the NO released from the conjugate. Therefore, after release of all the NO from the NONOate, the resulting heparin–spermine conjugate was injected into the PRP solution (Figure 4b). As shown, it is clear that the NO is responsible for the inactivation of the platelets. A transmission of nearly 0% is obtained with the NO loaded onto the conjugate, but upon removal, the same dose of the compound increases transmittance to 50% (within 10% of the control's value), demonstrating that the conjugate without NO is not responsible for the inhibition of platelet aggregation. These results provide further evidence that the new heparin–spermine–NONO species exhibits dual-acting anticoagulant properties.

CONCLUSION

The new diazeniumdiolated heparin–spermine conjugate described herein functions as a novel, dual-acting anticoagulant, exhibiting both antithrombin properties due to the heparin backbone, as well as antiplatelet activity from the NO reservoir on the attached spermine moiety. The combination of heparin with NO creates an inhibitor of both the intrinsic and extrinsic coagulation pathways. Further *in vitro* studies such as activated clotting time (ACT), partial thromboplastin time (PTT), and thromboelastography will need to be conducted during the next phase of this project, to fully understand how this new dual-acting agent functions in common blood tests employed to

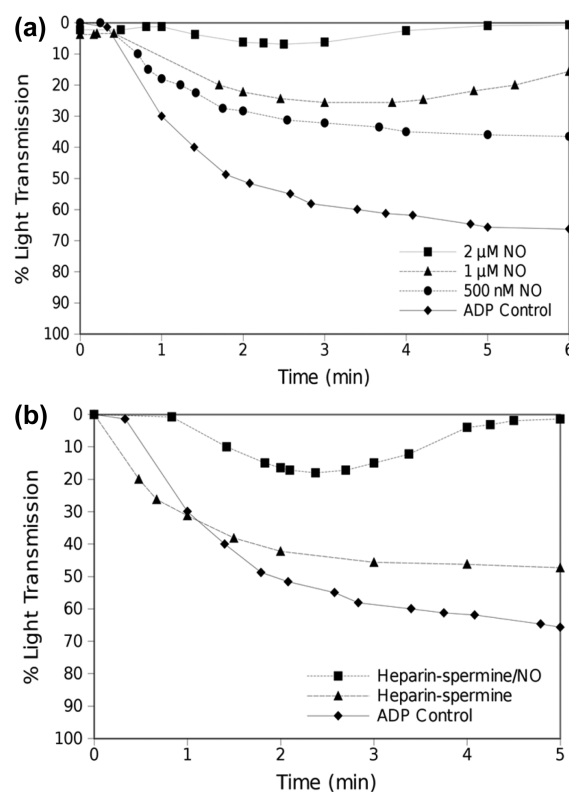


Figure 4. (a) Various volumes of the heparin–spermine NONOate to yield solutions that have total NO release capability of 0.5 μM NO (●), 1 μM NO (▲), and 2 μM NO (■), along with the ADP control (◆). The samples were measured against a PPP blank containing the same amount of heparin–spermine that is in the 2 μM NO aliquot. (b) Aliquots (40 μL) of the heparin–spermine conjugate with NO (■) and without NO (▲) compared to the control containing solely PRP with a 2 μL injection of ADP (◆).

assay the potential thrombosis status of patients. With additional future animal testing for optimal dosing determinations and cytotoxicity assessment, this new antithrombotic agent has potential for clinical application in several different

medical situations, ranging from stroke and myocardial infarction to use as a short-term combined anticoagulant in extracorporeal procedures such as coronary artery bypass surgery.

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Author Contributions

D.J.S. performed the experiments under the guidance of Drs. Handa and Meyerhoff.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank the National Institute of Health (Grants EB-000783 and K25HL11213) for supporting this work.

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