Diazeniumdiolated Protamine Sulfate as Bimodal Nitric Oxide Delivery System for Vascular Repair

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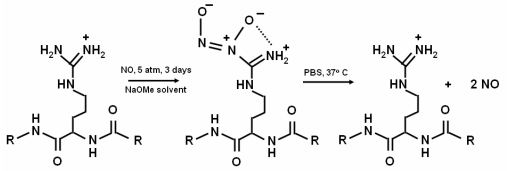
Statement of Purpose: Gold standards for treating occluded arteries due to atherosclerosis remain arterial bypass grafting or angioplasty. However, these procedures often suffer from restenosis secondary to thrombosis and neo-intimal hyperplasia (NIH), linked to impaired local nitric oxide (NO) bioavailability. Localized NO delivery is therefore an attractive solution to alleviate the causes of restenosis. However, current NO delivery strategies are limited in duration of exogenous NO delivery and fail to include mediation of endogenous NO production. We therefore introduce a novel approach that supplements exogenous NO delivery with prolonged induction of endogenous NO. Protamine sulfate (PS) has been reported to induce endogenous NO upregulation[1, 2] and possesses numerous arginine groups that can be used to form diazeniumdiolate groups[3] (sch. 1), which release NO in physiological environments in a controlled manner[4]. Moreover, encapsulation of diazeniumdiolated PS in polymer microparticles may lead to sustained NO delivery by controlled release of both exogenous NO and PS.



Scheme 1 Conversion of protamine to an NO donor by diazeniumdiolation.

Methods: *Diazeniumdiolation of PS:* Protamine sulfate in excess sodium methoxide (NaOMe) solution was exposed to 5 atm pressurized 99.5% NO gas (Matheson, Montgomeryville) for 3 days, rinsed with methanol and diethyl ether and vacuum dried. Diazeniumdiolation was assessed by UV-VIS by dissolving PS/NO in PBS. Absorbance decrease over time was measured to estimate the half-life of the NONO-moieties. PS structure conservation was verified using UV-VIS and MALDI. *In vitro effect of PS:* Effects of PS on porcine smooth muscle and endothelial cell toxicity and growth were assessed by quantifying viability and DNA contents after 24 hour of treatment with PS-supplemented media, using a Live/Green assay and a PicoGreen assay, respectively (Invitrogen, Carlsbad). *Encapsulation and release of PS/NO:* For encapsulation of PS in polymer microparticles, a $W_1/O/W_2$ double emulsion solvent evaporation method was used. PS in deionized water (MQ) was used as W_1, 20 mg/ml PLLA (Mw 300kDa) in dichloromethane (DCM) solvent as the O phase and MQ with a polyvinyl alcohol surfactant as W_2. Particle morphology was assessed using scanning electron microscopy (SEM). Encapsulation efficiency was determined by dissolving microparticles in 0.1 M NaOH, neutralization with 0.1 M HCl and PS content measurement using a microBCA assay (Pierce, Rockford). Nitrite release (as a measure for NO) from particles in PBS, 37°C was measured using standard Griess assay and PS release was measured using microBCA assay.

Results: Diazeniumdiolation was successful, as indicated by a distinct peak at 254 nm in UV absorbance that disappears over time, characteristic for NONO moieties (fig. 1a). The half life of PS/NO was determined to be 129.4 ± 8.3 minutes (n=3) and maximum NO capacity of PS is 201.8 ± 3.7 µmol/g. PS inhibited porcine smooth muscle cell growth at a concentration of 250 µg/ml and stimulated endothelial cell growth (fig. 1b). No toxicity was observed for concentrations of PS lower than 300µg/ml (data not shown). Encapsulation of PS/NO in PLLA microspheres resulted in microspheres with diameters in the 10-200 µm range (fig. 1c), encapsulation efficiency was 65.6 + 4.7 % (n=3) and the maximum load 0.06 mg PS/NO per mg particles. Release experiments showed NO release during just the first days, while NO-inducing PS release continued for app. 2 weeks (fig. 1d).

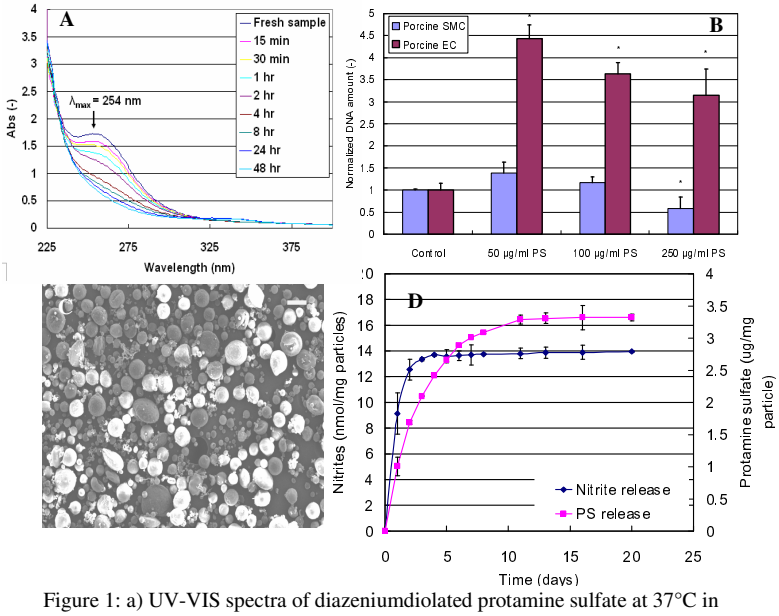


Figure 1: a) UV-VIS spectra of diazeniumdiolated protamine sulfate at 37°C in PBS over time b) Normalized DNA content after 24 hr of treatment relative to the untreated cells c) Morphology of PS-encapsulating PLLA particles. Scale bar 100 µm d) NO and PS release from PS/NO encapsulating PLLA particles Data in b) and d) are mean ± SD (n=3). (*)$p<0.05$ to control.

Conclusions: Currently, we show successful conversion of PS into an NO donor with a half-life of more than 2 hours. Furthermore, PS was successfully encapsulated in PLLA microparticles with high efficiency. Release kinetics show exogenous NO release during the first 3 days, while PS was sustained for 2 weeks, indicating the feasibility of this promising approach. Potentially, this bimodal microparticulate system can be applied perivascularly to prevent restenosis of vein bypass grafts, incorporated into prosthetic grafts or combined with angioplasty treatment to increase patency.

References: [1] Lee Y. et al., Analytical chemistry. 2004;76:545-551. [2] Pevni D. et al. The Annals of thoracic surgery. 2000;70:2050-2053. [3] Southan GJ. et al. Nitric Oxide. 1998; 2: 270-286 [4] Hrabie JA. et al. Chem Rev. 2002; 102(4):1135-54.