

Reversing coagulopathy and internal hemorrhage with functionalized nanoparticles

Damien Kudela^{*}, Anna May-Masnou^{*,**}, Stephanie A. Smith^{***}, Gary Braun^{****}, Alessia Pallaoro^{*}, Tracy Chuong^{*}, James H. Morrissey^{***} and Galen D. Stucky^{*}

^{*}Department of Chemistry and Biochemistry, University of California, Santa Barbara, CA 93106, USA, dkudela@chem.ucsb.edu, stucky@chem.ucsb.edu

^{**}Departament d'Enginyeria Química, Universitat de Barcelona, c/ Martí i Franquès, 1-11, 08028, Barcelona, Catalunya, Spain

^{***}Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, IL, USA, jhmmorris@illinois.edu

^{****}Sanford-Burnham Medical Research Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA

ABSTRACT

Trauma-induced hemorrhage remains a leading cause of preventable death. Silica nanoparticles (SNPs) – long known for their procoagulant properties – have recently been used as a first-response treatment. Functionalizing SNPs with polyphosphate (polyP) – analogous to the polyP produced by activated platelets to induce clotting – to form SNP-polyP leads to decreased clotting times. The decrease in clotting time is even more apparent when studied under traumatic conditions such as hemodilution and hypothermia. In addition to external treatment, the SNP-polyP particles can be protected with polyethylene glycol (PEG) and targeted to treat internal hemorrhage.

Keywords: hemorrhage, polyphosphate, lethal triad, silica nanoparticles, drug delivery,

1 INTRODUCTION

Controlling blood loss is a major focus in treating trauma patients. Uncontrolled hemorrhage accounts for nearly 50 % of all battlefield deaths and 25-30 % of deaths in civilian hospitals [1], [2]. Current treatment calls for surgical intervention and resuscitation to stem blood loss and limit damage. However, little can be done to slow bleeding prior to hospital arrival. Patients suffering trauma-induced hemorrhage are susceptible to coagulopathy – the fundamental breakdown of the coagulation cascade – and death before surgery can stem the bleeding. Roughly 25 % of trauma patients are coagulopathic upon hospital admission [3]. Coagulopathy, hypothermia and acidosis form the “lethal triad.” An effect of coagulopathy, hemodilution, results from the system wide depletion of procoagulant factors as a result of exsanguination, consumption and fluid replacement [4]. Hypothermia, the second member of the lethal triad, occurs when the body temperature drops below 37 °C. The drop in temperature leads to a decreased rate of formation for many coagulation factors.

Morrissey et al. have shown that polyP - linear chains of phosphate anions - induces coagulation depending on chain length [5–7]. In response to vessel injury, *in vivo* activated platelets secrete 60-100 monomer polyP chains that enhance activation of factor V (FV) while inhibiting the anticoagulant tissue factor pathway inhibitor [7]. The polyP binds to thrombin and back-activates factor XI (FXI). This process leads to faster activated FV (FVa) and thrombin production, which is the integral clotting factor [8]. In this paper, we use a synthetic, heterogeneous 70-mer polyP (P70) because of its availability and similarity to platelet-secreted polyP.

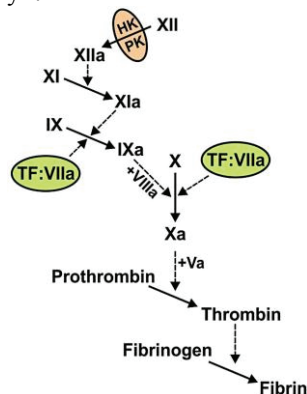


Figure 1: Coagulation cascade. SNP surface produces FXIIa, while P70 leads to faster FVa and thrombin production through an increased formation of FXIa.

Silica nanoparticles (SNPs) have been shown to stimulate coagulation through the contact pathway by activating factor XII (FXII) without immuno-rejection [9]. Lorenz et al. were the first to electrostatically cap polyP to an oxide using a zirconia scaffold for protein separation and purification [10]. Functionalizing an inorganic particle with P70 enhances the negative surface charge and facilitates P70 binding to thrombin cationic sites [11]. Replacing zirconia with silica enables the system to be used in the delivery of coagulation activators [12]. Coating the silica nanoparticle with PEG prevents unwanted coagulation activation, increases the half-life of silica in the blood stream, and limits cellular uptake and protein adsorption to

the underlying active surface [13]. The protected particle can target a hemorrhage site by removing nanoparticle attached PEG molecules in the presence of activated factor X (FXa).

2 EXPERIMENTAL SECTION

2.1 Materials

Polyphosphate was purified from P70 (BK Guilini GmbH, Germany). Frozen citrated pooled normal plasma (PNP) was purchased from George King Biomedical (Overland Park, KS). Phospholipid solutions were purchased in Avanti Polar Lipids: L- α -phosphatidylcholine (PC) and L- α -phosphatidylserine (PS). The other reagents used were from Sigma Aldrich.

2.2 Synthesis of silica nanoparticles and polyphosphate coated silica nanoparticles

SNPs were synthesized following a modified Stöber method [14]. The synthesized silica particles were then dispersed by sonication in Milli-Q water and placed at 30 °C. PolyP was added and the solution was stirred overnight. Successful capping was identified using a dynamic light scattering instrument. PolyP content on the particles was quantified by hydrolysis to monophosphate using calf intestinal alkaline phosphatase (a potent exopolyphosphatase) followed by phosphate analysis using the malachite green microassay [15].

2.3 Determination of the clotting activity

The clotting activity was determined by standard coagulometry on a Stago sT4 coagulometer, and rotational thromboelastometry (TEG) in a thrombelastograph (TEG® 5000, Haemonetics). Coagulometry assays measure clot time. TEG measures initial time for clot formation (R, min), rate of clot formation (α , deg), time until clot reaches 20 mm (K, min) and clot strength or maximum amplitude (MA, mm). The results shown are the average value of typically 4 to 6 replicates. We also monitored the formation of thrombin by fluorescence, using a thrombin-sensitive dye T-butyloxycarbonyl-b-benzyl-L-aspartyl-L-prolyl-L-arginine-4-methyl-coumaryl-7-amide (Boc-Asp(OBzl)-Pro-Arg-MCA)Boc-Asp(OBzl)-Pro-Arg-MCA (Bachem, Torrance, CA), used in previous studies [16], [17].

3 RESULTS AND DISCUSSION

3.1 Clotting activity of silica and polyphosphate coated silica particles

SNPs of ~55 nm were chosen as the core nanoparticle because of their high yield and low clot times. As observed

in Figure 2, 0.5 mg/ml SNP produced the minimum clotting time measured by TEG.

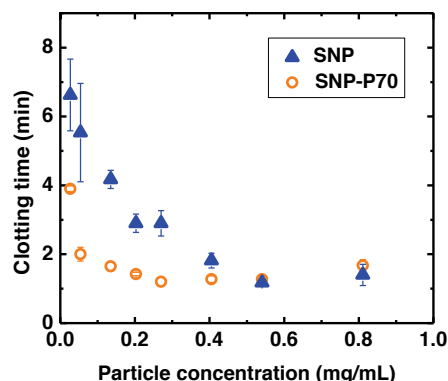


Figure 2: Clot time measured by TEG of bare SNP and SNP-P70 at different concentrations. SNP-P70 lowers clot time versus bare SNP

Clotting activity greatly improved when P70 is attached to the silica particles (Figure 2). SNP-P70 lowers clotting time at much lower concentrations with an optimum concentration around 0.25 mg/mL. Below the optimum concentration, the rise in clot time is not nearly as drastic as that seen for bare SNP.

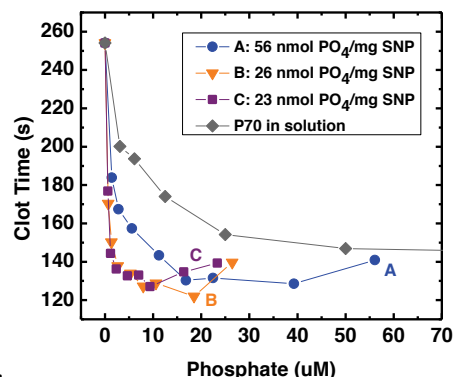


Figure 3: Clot time measured by standard coagulometry of SNP-P70 with different polyP amount versus phosphate content, compared to P70 in solution. SNP-P70 lowers clot time versus P70 in solution.

Compared to P70 in solution, SNP-P70 resulted in lower clotting times for the same amount of polyP, as tested by standard coagulometry (Figure 3).

3.2 Clotting activity under hemodilution and hypothermia

Due to the loss of both procoagulant and anticoagulant factors, dilution significantly impairs clotting when the factor concentration drops below 50 % of the physiological concentration. After reaching the 50 % threshold, it has been reported that the loss of procoagulant materials becomes too much for the cascade system to function [18].

Using TEG and fluorescence to assess clotting activity, a dilution baseline for our system was established using lipidated tissue factor (LTF). The baseline confirmed that clot time increased and thrombin formation decreased below the 50 % threshold. Figure 4 shows the thrombin generation in PNP with LTF using the fluorescence test.

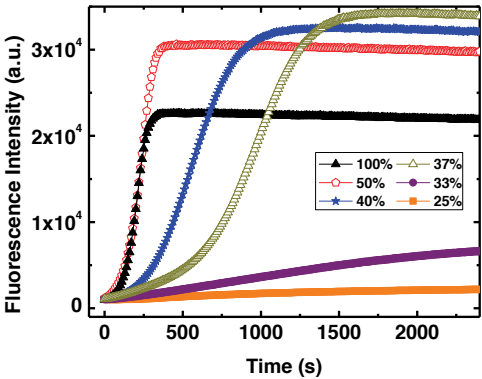


Figure 4: Thrombin generation times from 100 % plasma to 25 % plasma; i.e.: 100 % is 100 % plasma and 0 % dilutant.

Above 50 % PNP, the thrombin generation is rapid. At 40 % PNP (60 % PBS), the thrombin generation slows down and keeps decreasing as PNP dilution increases. Below 33 % PNP, no thrombin is detected. Similar results are obtained using TEG: at 50 % the clotting time increases and keeps increasing when the plasma is more diluted.

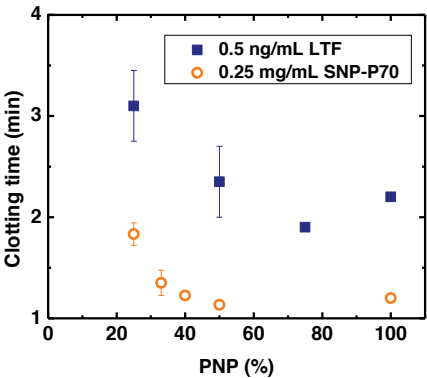


Figure 5: Clot time measured by TEG of diluted plasma with SNP-P70 as clotting agent or LTF. SNP-P70 lowers clotting time in severely diluted samples.

After testing various concentrations of nanoparticles against the LTF baseline, the addition of ~0.25mg/ml SNP improved clot time drastically with PNP dilution. This effect was remarkable, even at drastic hemodilution levels as low as 20 % PNP in the TEG cup (Figure 5). Using the thrombin dye assay at severe hemodilution levels such as 33 % PNP, SNP-P70 generates thrombin quickly whereas LTF cannot.

Hypothermia, the second member of the lethal triad, occurs when the body temperature drops below 37 °C. The drop in temperature leads to a decreased kinetic rate of protein activation [19]. As with the hemodilution assays, a

hypothermia baseline was established using LTF (Figure 7). The addition of SNP-P70 to hypothermic plasma also results in decreased time for thrombin production and clotting time.

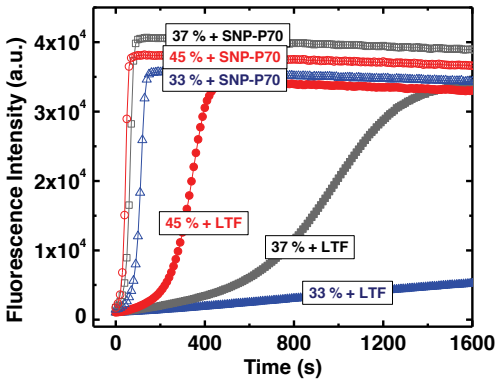


Figure 6: Adding SNP-P70 generates thrombin quickly even under severe plasma dilution.

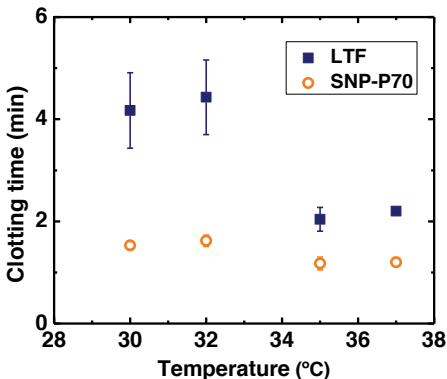


Figure 7: Clot time measured by TEG as a function of temperature of PNP with LTF or SNP-P70. SNP-P70 initiates clots more quickly under hypothermia conditions.

3.3 Clotting activity of protected nanoparticles

Testing the material under adverse, external conditions illustrates only a partial benefit of the SNP-P70 system. The small size of the SNP-P70 nanoparticle also allows it to be injected intravenously to treat internal hemorrhage. In comparison to external hemorrhage, the lack of visible tissue damage greatly complicates treatment. Nanoparticles could be injected into the bloodstream and functionalized to only target bleeding sites. In order to do this, the SNP-P70 particles will likely require a second co-functionalization with polyethylene glycol (PEG) to protect them from initiating clotting in healthy vessels. Deprotection of PEG at the wound site would thereby fully expose the P70 attached to the particle. Found in high concentrations at the wound site, FXa is a candidate enzyme to deprotect the particles if a peptide links the PEG to the particles. We demonstrate here the first steps in the protection of SNP from its activating of FXII (Figure 8).

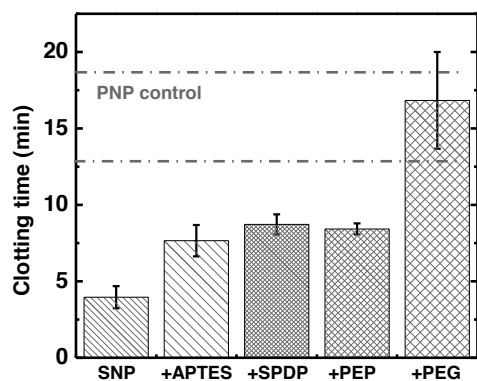


Figure 8: Clotting times of functionalized silica particles. (Pep: peptide, PEG used is 2k).

We attached a low density of amine silane to the SNP, and attached a peptide using SPDP as a heterobifunctional linker. Finally, PEG was attached to the distal end of the peptide. After each functionalization step, the particle was tested to determine its procoagulant activity. Upon addition of PEG, the protected nanoparticle shares a clot range with that of recalcified PNP (12-16 min). This suggests that the PEGylated particles could circulate through blood vessels without initiating spontaneous coagulation.

4 CONCLUSION

Attaching P70 to the surface of SNPs to form SNP-P70 significantly reduces clot time at low concentration using PNP. In all clotting assays, SNP-P70 lowered clot times and thrombin burst times. Most importantly, functionalizing P70 on a silica surface increases its clotting performance when compared to bare silica or soluble P70. Using an APTES bridge to attach PEG to protect the SNP-P70 system allows for the intravenous use of the nanoparticle. The particle can then be tuned to shed the protection PEG layer in the presence of elevated FXa concentrations found solely at wound sites. The SNP-P70 system shows promise as a potential therapeutic agent for both external and internal forms of hemorrhage.

5 ACKNOWLEDGEMENTS

This work was supported by the U. S. Army Medical Research & Materiel Command and the Telemedicine & Advanced Technology Research Center under Contract Number WQ81XWH-11-2-0021 and the Army Research Office under Contract Number W911NF-10-2-0114. The MRL Shared Experimental Facilities are supported by the MRSEC Program of the NSF under Award No. DMR 1121053; a member of the NSF-funded Materials Research Facilities Network. A.M.-M. thanks the Spanish MICINN for the financial support within the framework of the project number CTQ2008-06892-C03-03/PPQ.

Authorship: Conflict-of-interest disclosure: D.K., S. A. S., A. M.-M., G. B., J. H. M. and G. D. S. are

coinventors on pending patent applications covering potential medical uses of SNP-polyP. The remaining authors declare no competing financial interests.

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