Medicinal Chemistry

Deutsche Ausgabe: DOI: 10.1002/ange.201409639
Internationale Ausgabe: DOI: 10.1002/anie.201409639

Clotting Activity of Polyphosphate-Functionalized Silica Nanoparticles**

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Abstract: *We present a silica nanoparticle (SNP) functionalized with polyphosphate (polyP) that accelerates the natural clotting process of the body. SNPs initiate the contact pathway of the blood-clotting system; short-chain polyP accelerates the common pathway by the rapid formation of thrombin, which enhances the overall blood-clotting system, both by accelerating fibrin generation and by facilitating the regulatory anticoagulation mechanisms essential for hemostasis. Analysis of the clotting properties of bare SNPs, bare polyP, and polyP-functionalized SNPs in plasma demonstrated that the attachment of polyP to SNPs to form polyP-SNPs creates a substantially enhanced synergistic effect that lowers clotting time and increases thrombin production at low concentrations. PolyP-SNP even retains its clotting function at ambient temperature. The polyP-SNP system has the potential to significantly improve trauma-treatment protocols and outcomes in hospital and prehospital settings.*

Controlling hemorrhage is a major focus in the treatment and stabilization of many trauma patients. Uncontrolled blood loss is the leading cause of battlefield deaths, even though less than 5% of soldiers who subsequently reach a hospital die of their wounds.[1] In civilian hospitals, hemorrhage results in 15–25% of trauma deaths.[2] These data suggest that treatment should focus on stopping bleeding prior to hospital arrival. Bleeding management is currently aimed at volume resuscitation and surgical intervention to limit blood loss.[3] However, these measures often do not address the source or mechanism of the bleeding and ultimately can limit the possible options to control it, especially in the prehospital setting.

Currently, there are three major approaches for controlling prehospital hemorrhage. The oldest method employs mechanical devices that compress the wound to minimize the area through which blood can escape the damaged vessel.[4] Agents such as kaolin or chitosan (Figure 1) are useful as field therapeutics for the management of external hemorrhage and are widely utilized by military forces as a first-response treatment.[5,6] However, these compounds cannot be administered systemically and therefore lack utility for internal injuries with an intrinsic noncompressible hemorrhage.

Recombinant human factor VIIa (rFVIIa) is currently licensed for the management of bleeding episodes in patients with hemophilia and certain cases of warfarin overanticoagulation. Off-label use of rFVIIa is also commonly seen in certain other hemorrhagic conditions.[7] Although anecdotal reports of clinical response abound, a significant number of concerns regarding safety remain owing to reported thrombotic complications.[7] Drug-storage requirements and the

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[**] This research was funded by the US Army Medical Research and Material Command under Contract Number WQ81XWH-11-2-0021, by the US Army Medical Research and Materiel Command and the Telemedicine and Advanced Technology Research Center under Contract Number W911NF-10-2-0114, and by NIH grant R01 HL047014. Partial support was provided by the Institute for Collaborative Biotechnologies grant W911NF-09-0001 from the U.S. Army Research Office. The content of the information does not necessarily reflect the position or the policy of the Government, and no official endorsement should be inferred. 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 MINECO for financial support within the framework of project number CTQ2011-29336-C03-02.

Supporting information for this article, including experimental details of the synthesis and characterization of all new compounds as well as clotting assays, is available on the WWW under http://dx.doi.org/10.1002/anie.201409639.

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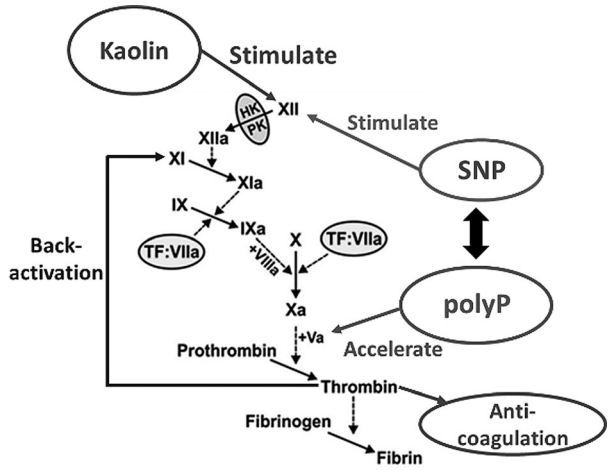

Figure 1. Simplified coagulation cascade. The SNP surface induces the activation of FXII, whereas short-chain polyP enhances the rates of activation of FV and FXI, thus leading to an earlier thrombin burst. Rapid thrombin generation leads to back-activation of FXIa to accelerate the coagulation cascade and facilitate increased activity in the anticoagulation pathway, which is designed to limit the spread of coagulation to uninjured vessels. Kaolin acts only to activate FXII through the intrinsic pathway.

extreme cost of recombinant-protein medication further limit field use.[8]

The ideal agent to control noncompressible hemorrhage in the prehospital setting would have the following properties: 1) long shelf life and stability in weather extremes, 2) reasonable cost, 3) promotion of key functions of the blood-clotting system at the injury site through the common pathway, including coagulation, regulatory anticoagulation, and fibrin formation, and 4) lack of off-target adverse effects. Such an agent could initiate treatment during the transport phase when the patient is at the greatest risk for exsanguination. We propose that targeted short-chain-polyphosphate-laden silica nanoparticles (polyP-SNPs) have the potential to fulfill these requirements.

Because it accelerates through the common pathway the key functions of the blood-clotting system rather than initiating endogenous-coagulation-enzyme production, short-chain polyP is a reasonable candidate for the management of hemorrhage.[9] The delivery of a polyP payload on a nanoparticle carrier could potentially be optimized to target the site of injury while minimizing the impact on the systemic circulation. Effective targeting and control would in theory minimize the risk of thrombotic complications that limit procoagulant therapy. Besides the potential for better biosystem safety, the production cost for polyP is low as compared to that of recombinant proteins. Upon attachment to inorganic oxides, polyP also has the potential for long-term stability under a variety of storage conditions.

In response to an injury, human platelets secrete short-chain polyP of approximately 60–100 monomers. Platelet-secreted polyP has a variety of wound-healing therapeutic effects, including enhanced activation of factors XI and V, which ultimately leads to enhanced factor X activity, and limitation of the activity of tissue factor pathway inhibitor, thus resulting in accelerated thrombin generation.[10] The incorporation of polyP also impacts clot structure and leads to resistance to fibrinolysis.[11] As a result of the approximately 90 min half-life of polyP in plasma, the procoagulant effects are temporally limited.[10–12] This endogenous metabolism of polyP offers much better biocompatibility than currently available agents, such as kaolin, which are not metabolized.

Whereas long-chain polyP is a potent activator of the contact pathway, short-chain polyP released from platelets has a relatively poor capacity to activate factor XII.[9] We employed polyP with a relatively short chain length for these studies to maximize the enhancement of downstream coagulation enzymatic steps (common pathway), while minimizing contact-pathway activation.

As noted above, platelets serve as a polyP-delivery agent, secrete procoagulants and clotting factors that promote blood coagulation, and initiate the formation of a clot-dissolving enzyme that degrades blood clots during the healing process. In our studies following up on the extensive research originally carried out by the Morrissey group,[9–12] we used free polyP as a benchmark. In the research we report herein, delivery-agent bifunctionality is introduced by using SNPs as a procoagulant carrier for the polyP.

Silica is generally considered to be a nontoxic material, and it is often used in drug-delivery studies.[13] However, owing to its negative surface charge, silica is also a contact activator.[5a,14] Consequently, the ideal construction of a polyP-bearing silica nanoparticle would shield the silica from exposure to the systemic circulation, with targeting and exposure of the silica carrier surface and polyP at the site of internal hemorrhage. Current materials used for treating external hemorrhage generally contain particles in the micrometer range, which are too large to readily traverse capillaries and unsuitable for use as intravenous therapeutics.[15] We consequently developed an approach for the synthesis of particles with a diameter of 50–100 nm (Figure 2a, Table 1).[5a,15]

Table 1: ζ potential and surface charge of the particles when dispersed in water or phosphate-buffered saline solution (pH 7.4).

Compound, medium[a]	ζ potential [μV]	Size [nm]
SNPs, H_2O	-24.4 ± 0.3	55.97 ± 2.19
SNPs, PBS	-52.5 ± 2.1	74.00 ± 6.24
PolyP-SNPs, H_2O	-44.3 ± 0.3	53.79 ± 1.75
PolyP-SNPs, PBS	-52.0 ± 3.2	78.18 ± 2.86

To attach the highly anionic polyP to an oxide, we followed the model of Lorenz et al., who used zirconia, which like silica has a negative surface potential, as the scaffold for applications in protein separation and purification.[16] This attachment strategy exploits the Lewis acid properties of an oxide surface to bind polyP, thus overcoming the electrostatic repulsion.[17]

^{31}P NMR spectroscopy and the change in surface charge qualitatively demonstrated polyP attachment. PolyP-SNPs were digested in acid to break down polyP into phosphate monomers. ^{31}P NMR spectroscopic tests on the digested

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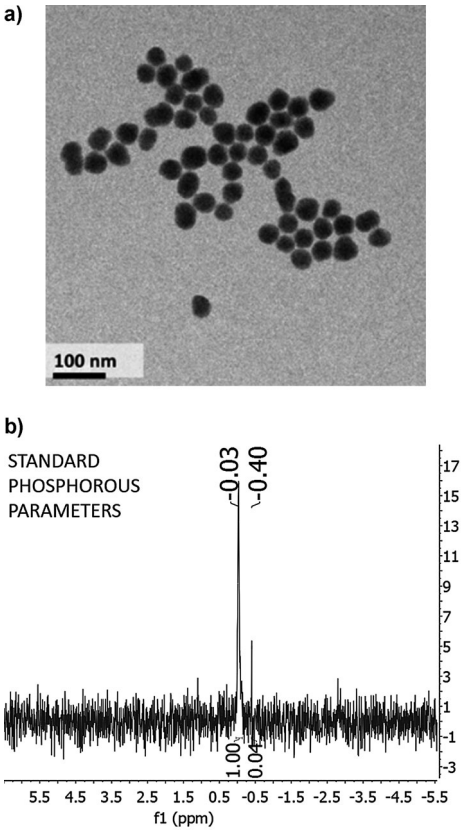

Figure 2. a) TEM images of polyP-SNPs. b) ^{31}P NMR spectrum of digested polyP-SNPs showing evidence of phosphorous.

sample detected the presence of phosphorous. The change in surface charge also suggested the presence of polyP in undigested polyP-SNPs (Table 1; see Figure 2 in the Supporting Information). Under physiological pH conditions in phosphate-buffered saline solution (PBS), both SNPs and polyP displayed a negative surface charge. In deionized water, the SNP surface charge ranged from −15 to −25 mV (Table 1; see Figure 2 in the Supporting Information). In simulated body fluid (SBF) under physiological pH conditions, SNPs had a surface charge between −50 and −60 mV. PolyP is negatively charged at this pH value as a result of a pK_{a1} value between pH 1 and 2 (for all internal phosphates) and a pK_{a2} value between pH 7.2 and 8.2 (for the two terminal phosphates).[18] Upon functionalization of the SNP surface with polyP, the ζ potential of the nanoparticles decreased from −20 to −30 mV to roughly −40 to −50 mV in water, thus confirming the attachment of the polyP. In SBF, both types of particles exhibited a strongly negative charge below −45 mV.

To quantify the polyP loaded on the SNP surface, we examined the digested phosphate solutions with a malachite green assay and by inductively coupled plasma atomic emission spectroscopy (ICP-AES). Malachite green identified PO_3 concentrations of 56, 26, and 23 nmol per milligram of SNPs. ICP-AES indicated that the 26 nmol PO_3 sample had a PO_3 concentration of 29.6 nmol per milligram of SNPs. Assuming each polyP chain has 70 PO_3 monomers, these data suggest 100–200 polyP molecules are attached to each SNP.

By not using tissue factor to initiate clotting, we focused our clotting assays on the intrinsic (common) and the contact activation pathways. Negatively charged particles, such as the aluminosilicate kaolin (QuikClot Combat Gauze) used for external injuries, activate the contact pathway.[4,5a] Because polyP is a poor clot initiator, polyP attachment shields SNPs in the systemic circulation, which is beneficial for intravenous control of haemorrhage. We measured the impact of both bare SNPs and polyP-SNPs on clot time by thromboelastography (TEG) on pooled normal plasma (PNP; Figure 3a; see

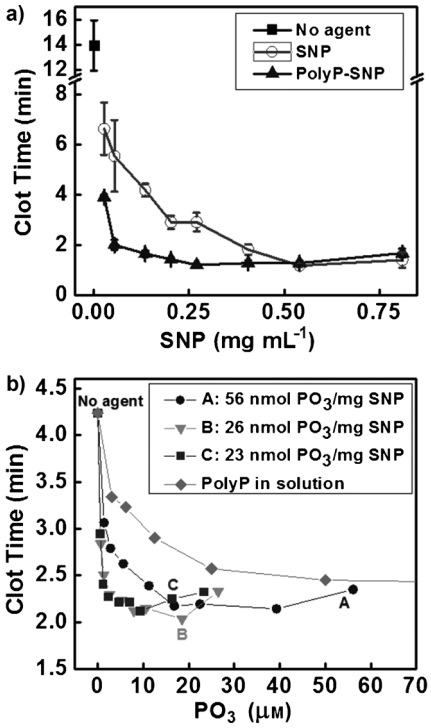

Figure 3. a) Graph showing that polyP-SNPs cut clot time (*R* value by TEG) roughly by half relative to that observed with bare SNPs below 0.3 mg mL^{-1}. b) Graph showing that polyP loaded onto silica lowers clot time relative to that observed with bare polyP, as measured by fibrometry.

Figures 4 and 5 in the Supporting Information). Both particles decreased the time to initial clot formation (*R*) in a concentration-dependent manner; however, polyP-SNP was more potent at concentrations below 0.5 mg mL^{-1}. The clot time reported in Figures 3 and 5 refers to the time to initial clot formation, which is key in illustrating how polyP-SNP successfully accelerates clotting. The agent used did not affect the overall size of the clot formed, only the time required to reach peak clot size. However, polyP has previously been shown to improve overall clot formation, in part by limiting the effect of fibrinolysis in plasma containing tissue plasminogen activator.[11,12]

Next we evaluated the procoagulant activity of polyP-SNPs formed with differing loads of polyP. The ability to promote coagulation was measured by adding polyP-SNP or polyP in solution to PNP. Coagulation was evaluated by measuring the time to clot formation on a fibrometer (Fig-

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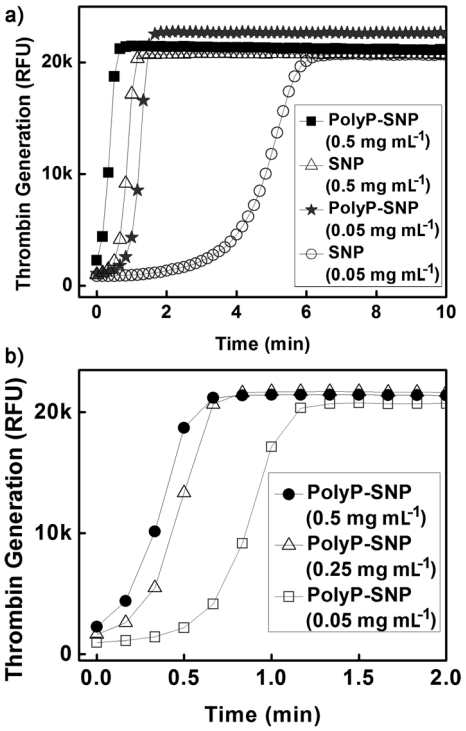

Figure 4. a) Graph showing that thrombin generation is more rapid with polyP-SNPs than with bare SNPs. b) Graph showing that polyP-SNPs are able to generate a rapid thrombin burst even at low concentrations. Thrombin generation was measured by the use of a thrombin-sensitive fluorescent dye.

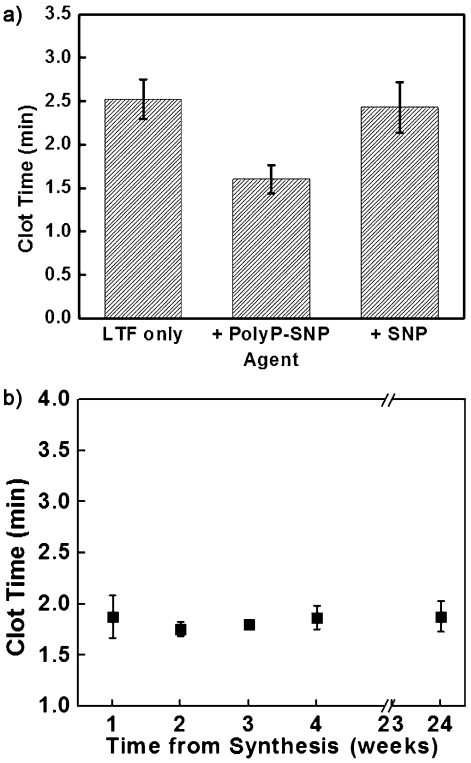

Figure 5. a) Graph showing that the addition of polyP-SNPs to LTF shortens clot time in FXII-deficient plasma relative to that observed with LTF only or LTF + SNP. LTF is required to initiate clotting. b) Graph showing that polyP-SNPs suspended in aqueous solution retained their procoagulant function after weeks of storage under ambient conditions.

ure 3b). In comparing the polyP payload, polyP-SNPs were more potent at activating the contact pathway than polyP in solution.

To further explore the relative activities of our materials, we evaluated the ability of the materials to generate thrombin, the terminal enzyme of the coagulation cascade and the primary determinant of the rate of fibrin formation.[19] PolyP-SNP again substantially outperformed its bare counterpart (Figure 4a).

We next evaluated whether polyP-SNPs were able to enhance the generation of downstream coagulation enzymes (common pathway). We eliminated any potential impact on contact activation by utilizing factor XII deficient plasma and initiating coagulation with a small amount of relipidated tissue factor (LTF, 63 pM). As expected, bare SNPs did not affect TEG clot time in this system (Figure 5a). In contrast, polyP-SNP did shorten the time to physical clot formation. This result indicates that the polyP is accessible for binding to the relevant downstream coagulation proteins. Additionally, this response could also be evaluated in the clinical setting by comparing tests such as prothrombin time (PT) and partial thromboplastin time (PTT).

One of the problems facing emergency medical personnel is that current intravenous treatments have a significantly short half-life at ambient temperature. Even pure polyP nanoparticles remain stable for hours.[9b] In comparison, the attachment of polyP to silica greatly enhanced the stability and procoagulant function of polyP from hours to weeks. After bench-top storage at room temperature as both a powder and an aqueous suspension, polyP-SNP clotting times remained constant for weeks (Figure 5b). The strong negative surface charge of polyP-SNPs also minimized aggregation in aqueous suspensions over the same time period. Injectable drugs with a long shelf life can be used by emergency medical personnel prior to hospital arrival without concern that the particles will degrade without refrigeration. Thus, the polyP-SNP system could become the first prehospital intravenous injection designed to treat internal injuries by accelerating the clotting system at bleeding sites.

In this study, we successfully attached polyP to the surface of small-diameter SNPs and demonstrated that these polyP-SNPs are more potent than bare SNPs at promoting coagulation, probably owing to the ability of polyP to accelerate the common pathway for active clotting processes. PolyP-SNPs, like polyP in solution, are able to enhance downstream coagulation reactions, thus resulting in a shorter time to clot formation. Even after long-term storage at regular room temperature, the polyP-SNP system retained its procoagulant ability. The polyP-SNP construct is consequently promising as a prohemostatic agent. Further exploration of methods to limit contact activation in vivo will be necessary for its use as a systemic agent.

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Keywords: hemorrhage · nanoparticles · polyphosphates · silicates · trauma

Zitierweise: *Angew. Chem. Int. Ed.* **2015**, *54*, 4018–4022
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Received: September 30, 2014
Revised: December 19, 2014
Published online: January 29, 2015

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