Published in final edited form as:

Nanotechnology. 2014 October 3; 25(39): 395101. doi:10.1088/0957-4484/25/39/395101.

Thrombin-Inhibiting Nanoparticles Rapidly Constitute Versatile and Detectable Anticlotting Surfaces

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Abstract

Restoring an antithrombotic surface to suppress ongoing thrombosis is an appealing strategy for treatment of acute cardiovascular disorders such as erosion of atherosclerotic plaque. An antithrombotic surface would present an alternative to systemic anticoagulation with attendant risks of bleeding. We have designed thrombin-targeted nanoparticles that bind to sites of active clotting to extinguish local thrombin activity and inhibit platelet deposition while exhibiting only transient systemic anticoagulant effects. Perfluorocarbon nanoparticles (PFC NP) were functionalized with thrombin inhibitors (either PPACK or bivalirudin) by covalent attachment of more than 15,000 inhibitors to each PFC NP. Fibrinopeptide A ELISA demonstrated that thrombin-inhibiting NPs prevented cleavage of fibrinogen by both free and clot-bound thrombin. Magnetic resonance imaging confirmed that a layer of thrombin-inhibiting NPs prevented growth of clots *in vitro*. Thrombin-inhibiting NPs were administered *in vivo* to C57BL6 mice subjected to laser injury of the carotid artery. NPs significantly delayed thrombotic occlusion of the artery, whereas an equivalent bolus of free inhibitor was ineffective. For thrombin-inhibiting NPs, only a short-lived (~10 minutes) systemic effect on bleeding time was observed, despite prolonged clot inhibition. Imaging and quantification of *in vivo* antithrombotic NP layers was demonstrated by magnetic resonance imaging (MRI) of the PFC NP. ¹⁹F MRI confirmed colocalization of particles with arterial thrombi, and quantitative ¹⁹F spectroscopy demonstrated specific binding and retention of thrombin-inhibiting NPs in injured arteries. The ability to rapidly form and image a new antithrombotic surface in acute vascular syndromes while minimizing risks of bleeding would permit a safer method of passivating active lesions than current systemic anticoagulant regimes.

Keywords

Thrombosis; Nanomedicine; MRI; Nanoparticles

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Disclosures

Samuel Wickline and Gregory Lanza are equity holders and consultants with Kereos, Inc.

Introduction

Direct thrombin inhibition to control acute and/or chronic vascular events is a primary target for pharmaceutical development [1]. In addition to the approved use of dabigatran in chronic atrial fibrillation [2], other next generation anticoagulants are poised for deployment in various scenarios including angioplasty [3] and stent placement [4], unstable angina [5], acute myocardial infarction [6], or prophylaxis against venous thromboembolism [7]. However, potential bleeding side effects from systemic anticoagulant activity, delays in onset, and lack of rapid reversibility still pose significant challenges to widespread clinical adoption [8].

This work aims to show that thrombin-inhibiting nanoparticles provide safer and more effective care by forming an ant clotting surface at sites of acute thrombosis. Our data covers the ant clotting surface formation for two types of nanoparticle, formed with two different types of thrombin inhibitor.

In previous work, we proposed a prototypical antithrombotic nanoparticle (NP) functionalized with the direct thrombin inhibitor, D-phenylalanyl-L-prolyl-L-arginyl-chloromethyl ketone (PPACK) as a potent inhibitor of acute clotting with limited side effects and a capacity for tracking via MRI [9]. Towards demonstrating that this unique therapeutic behavior is relevant to the properties of the base nanoparticle, we have now explored the variation of the active moiety in the thrombin-inhibiting nanoparticle. A part of this study is thus devoted to showing that an inhibitor with properties distinct from PPACK can functionalize thrombin-inhibiting nanoparticles and provide site-specific antithrombotic action via the formation of an antithrombotic surface. While PPACK has a high thrombin affinity and large body of biochemical characterization, the direct thrombin inhibitor bivalirudin is capable of higher specificity for thrombin and has been approved and characterized in multiple clinical scenarios [10,11,12,13]. We thus employed bivalirudin on our nanoparticles as an inhibitor with properties complementary to those of PPACK, testing the hypothesis that thrombin-inhibiting nanoparticles outperform their component inhibitors as anticoagulants for a variety of inhibitory properties [14].

For both types of particle, the research presented here focuses on the phenomenon of thrombin-inhibiting nanoparticles binding to actively forming clots. The thrombin-inhibiting nanoparticle is designed explicitly for acute use, and exhibits unique features of rapid onset, prolonged inhibitory bioactivity at the site of acute clotting (hours), rapid diminution of systemic anticoagulation (within 30 minutes), and a capacity for detecting acute thrombotic events by magnetic resonance imaging of the NPs that are bound to active thrombin at the site of ongoing clotting [9,14,15]. We present data here employing a combination of imaging and biochemistry techniques to support the hypothesis that thrombin-inhibiting nanoparticles act by forming layers that arrest the coagulation cascade at sites of clotting, while permitting the attenuation of effects in the blood pool. Bound to the surface of clots, each of the two types of thrombin-inhibiting NPs show the ability to prevent further clotting induced by a prothrombotic surface. Our results will confirm that the properties of perfluorocarbon nanoparticles permit different clinically utile thrombin inhibitors to be improved in terms of potency and safety by incorporation into an antithrombotic NP layer

capable of sealing sites of active thrombosis. We thus present evidence for the formation of antithrombotic nanoparticle surfaces to explain a new property of thrombin-inhibiting nanoparticles that advances this promising form of nanotechnology towards clinical utility.

Results and Discussion

Formulation of Bivalirudin or PPACK Functionalized PFC Nanoparticles

Carbodiimide coupling enabled the addition of bivalirudin or PPACK at the N-terminus to 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000 in particles prepared as described in the methods (Figure 1).

Stability of the new bivalirudin nanoparticles after addition of bivalirudin was confirmed by dynamic light scattering measurement of particle size and zeta potential. Prior to addition of bivalirudin, the NPs had a mean diameter as measured by dynamic light scattering of 174.2 ± 2.4 nm. After addition of bivalirudin and dialysis to remove uncoupled bivalirudin and excess carbodiimide, the particle diameter was determined as 174.7 ± 1.9 nm, indicating stability of the emulsion during the coupling procedure (Figure 2a). While the particle size was unchanged by bivalirudin addition, the zeta potential shifted following coupling, from -39.64 ± 2.85 mV to -18.66 ± 6.22 mV (Supplementary Figure 1). The shift in zeta potential was indicative of a change in the chemical composition of the particle surface with the replacement of carboxyl termini in 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000 with bivalirudin.

The alteration to the particle surface and the covalent linking of bivalirudin to the lipids was further explored through a quantification assay employing tyrosine fluorescence. Dialysis to remove excess bivalirudin not bound to the surface of the NPs after the coupling procedure was skipped for this procedure. The excess bivalirudin was extracted from the emulsion through Cleanascite centrifugation. A standard curve was acquired to determine the relationship between bivalirudin concentration and tyrosine fluorescence intensity and uncoupled bivalirudin was quantified accordingly. Noting that the quantity of bivalirudin added to each particle was equal to the amount included in coupling minus the excess at the end of coupling, it was determined that approximately 24000 bivalirudin were coupled to each particle (Figure 2b). Both the extent of coupling and the physical properties of the nanoparticles indicated that bivalirudin nanoparticles matched the previously determined stability and inhibitory moiety of PPACK nanoparticles [9].

Newly Formulated Bivalirudin Nanoparticles Efficiently Inhibit Thrombin in Solution

Chromozym TH assay was used to define the activity of thrombin in the presence of various concentrations of free bivalirudin or bivalirudin NPs (Figure 2c). Thrombin digestion of the Chromozym substrate occurred at a linear rate after one-minute incubation of thrombin with bivalirudin or bivalirudin NPs, indicating that the inhibition reaction had reached equilibrium. The model of Stone and Hofsteenge [16], applicable to tight binding reversible inhibitors of serine proteases, was employed to measure the rate of cleavage of the substrate by thrombin after allowing the inhibition reaction to reach equilibrium. According to equation 2, the steady-state rate of substrate digestion v_s was delineated as a function of the thrombin concentration E_t of 12 nmol/L, the variable concentration of the inhibitor I_t , and

the effective inhibition equilibrium constant K_I' . Iterative fits of v_s for equation 2 with constant E_t and variable I_t provided values of K_I' for bivalirudin and bivalirudin NPs.

For free bivalirudin in solution, a K_I' of 121.18 nmol/L was extrapolated. Bivalirudin on NPs exhibited a K_I' of 209.31 nmol/L, illustrating minimal diminution of molecular bivalirudin activity against thrombin when sterically restricted to the particle surface. In contrast, PPACK, used previously as the functional moiety on the first antithrombotic NP, exhibited a K_I' of 12.077 nmol/L according to the tight-binding inhibitor model (not shown). This disparity in affinities agrees with previous literature. The lower affinity of bivalirudin for thrombin is balanced by its higher specificity for thrombin and its ability to displace bound thrombin.

Notably, although the individual bivalirudin molecules on the particle surface exhibited a small diminution of activity, the particle, considered as an inhibiting entity itself, exhibited a much higher affinity for thrombin. Setting I_t to represent the concentration of bivalirudin NPs in equation 2 for the data in Figure 2c, a K_I' of 2.1741 fmol/L was derived from iterative least squares fitting (not shown).

Thrombin-Inhibiting Nanoparticles Inhibit Clot-Bound Thrombin

Fibrinopeptide A (FPA) ELISA experiments further elucidated the versatile and potent means by which bivalirudin or PPACK NPs inhibit thrombin in active clotting processes. The work of Weitz *et al.* used the FPA ELISA to show that bivalirudin and PPACK equally inhibit fibrin generation by thrombin in solution or thrombin bound in clots [17]. Analogous assays demonstrated here that bivalirudin NPs or PPACK NPs also successfully prevented fibrin formation by soluble and fibrin-bound thrombin.

Human alpha thrombin in the fluid phase generated FPA in citrated plasma in a concentration-dependent manner. The addition of bivalirudin, bivalirudin NPs, PPACK, or PPACK NPs to plasma over a range of concentrations resulted in concentration-dependent inhibition of the generation of FPA by thrombin. Inhibition of thrombin in the fluid phase was 6.30%, 14.84%, and 34.55% complete for 5, 10, and 30 nM PPACK on NPs (Figure 3c) and 29.15%, 47.81%, and 69.60% complete for 5, 10, and 20 μ M bivalirudin on NPs (Figure 3a). Thrombin inhibition observed with free inhibitors was similar to that observed with corresponding particles (Supplementary Figure 2a, Supplementary Figure 3a). As with the inhibition of Chromozym TH digestion, this small reduction in bivalirudin effect is overwhelmed by considering that the NP, to each of which approximately 24000 bivalirudin are permanently bound, is an inhibiting entity itself present in much lower concentration than the bivalirudin.

Further FPA ELISAs were employed to examine FPA release by active thrombin bound in fibrin clots. Weitz *et al.* previously determined that bound thrombin is responsible for FPA release from fibrin clots incubated in plasma and that the quantity of FPA release is directly proportional to the clot surface area [17]. For normalization, the surface area of each clot used here was measured by MRI (Supplementary Figure 4). After incubation of the clots with citrated human plasma or plasma containing set concentrations of PPACK, bivalirudin, PPACK NPs, or bivalirudin NPs, FPA concentration in the plasma was determined,

normalized to clot surface area, and compared to FPA release into plasma not containing inhibitors. The extent of thrombin inhibition was measured and compared to the extent of inhibition of fluid phase thrombin by equivalent concentrations of inhibitors. For each inhibitor, the extent of bound thrombin inhibition was within or above error observed in the inhibition of fluid-phase thrombin. Inhibition of FPA release elicited by bound thrombin was 20.03%, 31.59%, and 49.76% complete for 5, 10, and 30 nM PPACK on NPs and 24.06%, 51.66%, and 72.33% complete for 5, 10, and 20 μ M bivalirudin on NPs (Figure 3d, 3b). Similar results were observed for free inhibitors (Supplementary Figure 2b, Supplementary Figure 3b). FPA ELISA data thus indicate that thrombin-inhibiting nanoparticles inhibit thrombin bound in clots and prevented activation of fibrin synthesis due to exposed clots.

Thrombin-Inhibiting Nanoparticles Inhibit Human Plasma Clot Growth In Vitro

After submersion of fibrin clots in plasma, sequential MRIs allowed measurement of changing clot volume. Additional clotting was observed in T2-weighted spin echo MRIs as dark formations extending from the original clot surfaces (Figure 4b). Clot boundaries were drawn at the edge of the new growth in each slice of multislice images, allowing clots to be reconstructed in three dimensional images (Figure 4b, 4c). Demarking the new clot volume as the sum of the volumes of the initial clot and the new growth, clot volume was determined as a function of time after addition of new plasma. Accumulation of clot volume was observed for clots treated with saline, PPACK, or bivalirudin (Figure 4d). At one hour after introduction of plasma clots treated with saline, PPACK, or bivalirudin, clots had grown to $381.17\% \pm 43.48\%$, $355.70\% \pm 44.72\%$, or $314.33\% \pm 14.29\%$ initial volume, respectively. For clots treated with PPACK NPs or bivalirudin NPs, binding of NPs on the clot surface was observable via ^{19}F MRI (Figure 4a). Clots with a protective layer of thrombin-inhibiting NPs (either PPACK or bivalirudin) did not grow when exposed to plasma (Figure 4c, 4d, 4e). Whereas clots without nanoparticle treatment grew sigmoidally to nearly fill the volume of vessel containing the plasma, clots treated with PPACK NPs or bivalirudin NPs did show significant change in volume over one hour (Figure 4d, 4e).

The observation of active clot growth identifies a distinct advantage of nanoparticle-based thrombin inhibition. As evident in Figure 4a, nanoparticles accumulate in quantifiable layers on the surfaces of clots. FPA ELISA data explained above indicate that both nanoparticle and small molecule thrombin inhibitors effectively bind to and inhibit thrombin bound in clots. However, small molecule inhibitors can act only on one thrombin at a time. The thrombin-inhibiting nanoparticles, once bound to the site of active clotting, present thousands of additional thrombin inhibitors. In a cumulative layer, these nanoparticles comprise a thrombin-inhibiting surface that arrests any subsequent progress in coagulation in the surrounding milieu.

Induction, Inhibition, and Imaging Assessment of Acute Thrombosis In Vivo

As described in the methods, diminishing blood flow in the laser-injured carotid was monitored with a Doppler flow probe (Supplementary Figure 5). The progression to zero flow occurred at different times for different treatment groups. In accordance with standard analysis of the rose bengal model, the time to stable occlusion of the carotid delineated the treatment effect for each group [18]. Effective antithrombotics are known to delay the onset

of carotid occlusion. In previous work, PPACK NPs extended thrombotic occlusion time to 145 ± 13 minutes, as compared to a control time of 70 ± 17 minutes [9]. At a single dose of 10 mg/kg free bivalirudin administered 10 minutes prior to induction of injury, the average occlusion time was 83 ± 13 minutes, indicating no significant difference between the saline sham and the bivalirudin treatment as administered. The bivalirudin emulsion, administered at a dose of 1 ml/kg bolus (presenting an equivalent dose of 6.24 mg/kg bivalirudin), delayed the occlusion of the injured carotid to 111 ± 14 minutes (Figure 5a). The bivalirudin nanoparticles significantly extended the stable carotid occlusion time over the control value, but free bivalirudin did not [14].

In previous work, we have reported that the in vivo anticoagulant effect of the nanoparticle is specific to thrombin inhibiting functionality by showing that NPs containing 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000 without a conjugated inhibitor do not extend the time to occlusion (Supplementary Figure 6) [9]. The data here indicate that a superior anticoagulant effect can be demonstrated for nanoparticles with different types of thrombin-inhibiting moieties. The nature of the laser/dye injury is the creation of a procoagulant surface in the artery. As with the in vitro clotting data presented in Figure 3, the nanoparticles had an advantage over single-molecule inhibitors in limiting coagulation induced at a procoagulant surface.

In further in vivo tests, bleeding times, activated partial thromboplastin time (APTT), and prothrombin time (PT) were measured after administration of bivalirudin NPs (Figure 5b, Supplementary Figure 7). For all tests, a time course for the blood pool effect of bivalirudin NPs indicated that the particles exerted a rapidly diminishing systemic anticoagulant effect in the blood pool. Bleeding times were protracted at 10 and 20 minutes after administration of particles, but were indistinguishable from control values at 50 or 100 minutes. Blood samples taken 10 minutes after bivalirudin NP bolus demonstrated extension of APTT and PT over control times, but samples taken at 20 minutes or later manifested no significant effect on coagulation parameters. Similar results were observed for APTT and bleeding times with PPACK nanoparticles in previous work [9]. The thrombin-targeted NPs therefore inhibited occlusion at the site of arterial injury over a prolonged interval, yet fortuitously exhibited a short-lived systemic effect.

Given the problems plaguing interventional and diagnostic strategies for treatment of acute thrombosis [8], these systemic properties of antithrombotic NPs may be ideally suited for use in acute vascular syndromes. NPs outperform bolus PPACK, bivalirudin, and heparin in inhibiting occlusive thrombus formation in a mouse model [9], but induce only a short-lived extension of APTT and PT in the blood pool and allow rapid normalization of bleeding times. Whereas most anticoagulants currently in clinical use increase bleeding times [1,8], this prototypical antithrombotic NP exhibits an anticoagulant effect that is prolonged at the site of acute injury after a short systemic phase.

We posit that this effect is attributable to the demonstrated pharmacokinetics of perfluorocarbon nanoparticles in general [19], combined with binding to clot-incorporated thrombin at sites of injury and coagulation, demonstrated for thrombin-inhibiting nanoparticles in Figures 3 and 4 here. For perfluorocarbon nanoparticles, literature values

assess a rapid blood distribution phase (~10 minutes) and an elimination half-life of ~3 hours. These values essentially reduce the circulating concentration of antithrombotic NPs to a level that no longer exerts anticoagulant activity systemically after 20–50 minutes. Those NPs that do not bind to targeted sites are simply cleared rapidly by the liver and disposed of without toxic side effects [19]. Nevertheless, data in Figures 3 and 4 indicate that the selective buildup of particles at the intended site (of arterial injury) would achieve a sustained thrombin surveillance effect lasting hours.

Detection of Thrombin-Inhibiting Nanoparticles at Sites of Acute Arterial Injury

The perfluorocarbon in the thrombin-inhibiting nanoemulsions generated a signal detectable by magnetic resonance (MR) imaging and spectroscopy [15]. The retention of NPs in injured arteries was visualized with ^{19}F MRI (Figure 6a) [14]. The excised arteries were imaged with proton spin echo sequences in 1 mm slices. Subsequently, ^{19}F images were captured to show the location of NPs relative to the artery. By overlaying a false color fluorine projection image on a proton image slice centered on the injured artery, the retention of thrombin-inhibiting particles in the injured artery was evident. Accordingly, the particles appeared to be sequestered in the clot formed in the injured carotid. Excised arteries obtained after administration of bivalirudin NPs contained NPs in MR-quantifiable amounts. Via quantitative MR spectroscopy, $.34 \pm .02$ fmol of bivalirudin NPs were detected in carotids injured under treatment. In previous work, injured arterial segments contained $.31 \pm .14$ fmol PPACK NPs or $.07 \pm .03$ fmol control NPs [9], indicating specific binding for both types of thrombin-targeted nanoparticle (Figure 6b).

Further exploration of thrombosis-specific binding and detection in larger animals with the use of the thrombin-inhibiting NPs was accomplished by applying PPACK NPs in a cholesterol-fed rabbit model [14]. At 200 minutes after induction of femoral artery injury by introduction of Rose Bengal dye under green laser, a thrombotic mass was detectable by ultrasound echo images. A defect in laminar flow was evident at the site of the injury in ultrasonic Doppler flow imaging (Figure 6c). After PPACK NPs were delivered IV and circulated for two hours, proton imaging with 3T MRI revealed stable thrombus formation that was detectable by black blood, time-of-flight, and phase contrast angiography techniques (Supplementary Figure 8). A subsequent high-resolution ex-vivo image of the injured artery and bound particles was obtained at 11.7T, revealing abundant targeted thrombin-inhibiting NPs at the site of injury coating the thrombus surface (Figure 6d).

The primary implication of the data presented in Figure 6 is the confirmation that thrombin-targeted nanoparticles accumulate at sites of acute prothrombotic arterial injury.

Analogously to the *in vitro* data of Figure 4a, imaging data shows layers of nanoparticles covering thrombus surfaces in the *ex vivo* images of arteries in Figures 6a and 6d. This data thus completes an explanation for the phenomenon of thrombin-inhibiting nanoparticles providing prolonged inhibition of acute thrombus formation while simultaneously providing only a short-lived systemic impact. Layers of nanoparticles are evident in the mouse and rabbit clots and the data presented in Figures 3 and 4 confirm that such layers constitute antithrombotic surfaces.

It is further notable that the perfluorocarbon-core NP is the first known thrombin-inhibiting agent to provide magnetic resonance contrast specific to sites of acute thrombosis. Imaging agents have been described for fibrin [20,21,22], but whether they indicate acute vascular events is uncertain, since fibrin is ubiquitous in many conditions of inflammation [23], wound healing [24], cancer metastasis [25], and atherosclerosis [26,27]. Since traditional treatments for thrombosis feature unpredictable and continued formation of multiple thrombi and microemboli that can go undetected [28], thrombin-inhibiting NPs could provide imaging contrast that permits the detection of otherwise unnoticed thrombotic events, providing a guide for further antithrombotic and thrombolytic intervention.

Ex Vivo Analysis of Clots Formed by Arterial Injury

Following fixation in formalin, occluded mouse arteries were prepared for Carstairs' staining. The clots in the arteries exhibited a distribution of platelets (stained blue) in a fibrin matrix (stained pink) (Supplementary Figure 9). Comparing clots formed in the presence of control NPs (embedded in paraffin from previous work) to clots formed in the presence of bivalirudin NPs, denser and more abundant clusters of platelets were evident in control clots. Color deconvolution and analytical tools in ImageJ were used to evaluate the area of each clot section occupied by platelet staining. Clots formed in the presence of control NPs exhibited platelet staining in $41.31\% \pm 4.09\%$ of the cross-sectional area, as opposed to $27.65\% \pm 2.26\%$ of the cross-sectional area in clots formed in the presence of bivalirudin NPs (Supplementary Figure 10). The result indicates a pervasive effect of thrombin-inhibiting nanoparticles on clot formation. Beyond delaying thrombosis, inhibitor nanoparticles present at the site of persistent injury changed the nature of clots that did form by exerting an impact on platelet deposition. This would presumably entail prevention of platelet activation through inhibition of thrombin's action toward protease activated receptors (PARs) [29].

Conclusions

The onset of thrombosis in acute vascular syndromes coincides with disruption of a normally anticoagulant endothelial barrier either through plaque rupture or focal erosion that exposes an underlying hypercoagulable milieu to circulating clotting factors [2,3,4,5,26]. The conventional management of such events entails the use of combinations of systemic anticoagulants and antiplatelet agents that attenuate clotting ubiquitously until such time as these focal acute events "passivate" and an anticlotting surface is restored [8]. Here, we demonstrate that thrombin-inhibiting NPs immediately form an anticlotting surface localized specifically to sites of arterial injury. The binding of antithrombotic particles at the site of prothrombotic injury, as demonstrated with ^{19}F MRI, establishes a new surface that was demonstrated to be resistant to further thrombin activity. As represented schematically in Figure 7, the elimination of local thrombin activity by a NP anticoagulant surface is expected to mitigate further deposition of fibrin and platelets and provide long-lived surveillance function against further clotting at a disrupted vascular site.

Due to necessity of intravenous administration, the practical utility of thrombin-inhibiting NPs would be focused to acute use, but a number of important indications exist for emergent

application, including acute vascular syndromes, angioplasty [3], microangiopathy [30], acute kidney injury [31], and arteriovenous fistula and/or graft maintenance [32]. Given the success of thrombin inhibiting particles featuring functional moieties with distinctive properties (either PPACK or bivalirudin), the formation of an antithrombotic layer appears to be the critical feature in the success of the nanoparticle strategy in mouse models. Based on this work, we can thus argue for the implementation of an adaptable anticoagulant nanotechnology strategy in the above syndromes. Such a strategy would employ the layer forming property explored here to provide acute antithrombotic care with highly targeted therapeutic effects, imaging contrast specific to sites of thrombosis, and modifiable functional components.

Experimental Section

Formulation of Inhibitor-Functionalized PFC Nanoparticles

PFC emulsions contained 20% (vol/vol) Perfluoro 15-Crown-5 Ether (Exfluor Research Corp.), 2% (wt/vol) of a surfactant mixture, 1.7% (wt/vol) glycerin, and water for the balance. The surfactant contained 99 mole% phosphatidylethanolamine (Avanti Polar Lipids) and 1 mole% 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] (Avanti Polar Lipids) in chloroform:methanol (3:1). The surfactant was dried under vacuum to form a lipid film, which was then combined with the other nanoemulsion components, and emulsified (Microfluidics Inc) at 20000 psi for 4 minutes. Particle sizes were measured following emulsification with the use of a laser light scattering submicron particle analyzer (Brookhaven Instruments) [20].

Amide formation via carbodiimide was employed to functionalize particles containing 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] with bivalirudin or PPACK. After mixing of 1 mL emulsion with 40 mg bivalirudin (Bachem California) or 12.5 mg PPACK (Haematologic Technologies) for one hour, EDCI 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide methiodide (2 mg) was added and the mixture was incubated overnight to achieve coupling. Excess bivalirudin or PPACK and EDCI were removed by overnight dialysis (molecular weight cutoff 3000–5000 g/mol).

Particle size and stability was assessed by dynamic light scattering measurement of hydrodynamic diameter and zeta potential before and after bivalirudin conjugation (Brookhaven Instruments).

The extent of bivalirudin coupling was determined by optical quantification of excess uncoupled bivalirudin after centrifugation of NPs (1000g for 15 minutes) with Cleanascite lipid adsorption reagent (Agilent Technologies). Dialysis was not employed for nanoparticle samples used for determination of bivalirudin coupling. Bivalirudin was detected in the supernatant via tyrosine fluorescence (absorbance 274 nm, emission 303 nm) (Varian) and quantified by comparison to fluorescence from bivalirudin standards. The number of coupled bivalirudin was determined by subtracting the quantified excess from the known amount initially added to the coupling reaction.

Characterization of Thrombin Inhibition by Bivalirudin Nanoparticles

Tosyl-Gly-Pro-Arg-4 nitranilide acetate (Chromozym TH, Roche Applied Science) assay assessed bivalirudin inhibition of thrombin in accordance with previously described methods [33]. 100 μ L of 12 nmol/L thrombin in 1.0M Tris buffer was incubated for one minute at room temperature with selected amounts of bivalirudin or bivalirudin NPs (n=3 for each concentration). 500 μ L (100 μ mol/L) of Chromozym TH thrombin substrate in 1.0 mol/L Tris buffer was added to terminate the bivalirudin-thrombin interaction. Thrombin activity against the substrate was measured via absorbance at 405 nm. The rate of change in absorbance at 405 nm indicated the rate at which thrombin cleaved Chromozym TH. A model appropriate to tight-binding reversible inhibitors like bivalirudin was employed to analyze the Chromozym TH data (equations 1 and 2). Following the methods of Stone and Hofsteenge [16], the model permitted measurement of effective K_I values for bivalirudin and bivalirudin on NPs. For comparison, the model was also applied to published data for PPACK.

$$v_s = (v_0/2E_t) \left[\sqrt{(K_I' + I_t - E_t)^2 + 4K_I' E_t} - (K_I' + I_t - E_t) \right] \quad (2)$$

($K_I' = K_I(1 + S/K_m)$)

In equation (2), the inhibition reaction velocity in the absence of inhibitor is v_0 , the total thrombin concentration is E_t , the steady state (one minute) velocity for inhibitor concentration I_t is v_s , and K_I' is the effective inhibition equilibrium constant for a given Chromozym TH concentration S and substrate-thrombin equilibrium constant K_m .

Characterization of Clot-Bound Thrombin Inhibition

Fibrinopeptide A (FPA) ELISA (American Diagnostica GmbH) was used to measure fibrin generation by thrombin in human plasma or bound in human plasma clots. To assess the PPACK, bivalirudin, PPACK NP [9], or bivalirudin NP efficacy against activity of clot-bound or free thrombin, the methods of Weitz et al. were employed to quantify fibrin generation by thrombin both bound in clots and free in plasma [17].

Human plasma was incubated at 37° C for one hour with selected quantities of thrombin or inhibitor. After incubation, plasma was twice washed with bentonite and centrifuged at 2500g for 20 minutes to remove fibrinogen while leaving FPA produced by thrombin. After addition of 2% Tween 20 (5% vol/vol) to the bentonite-treated plasma, FPA antibody with no demonstrable cross reactivity to fibrinogen or homologous fragments of fibrinogen was introduced for incubation at 37° C for one hour. The antibody-treated plasma was introduced to FPA-coated wells for a one hour incubation at room temperature. After five washes, a secondary antibody complexed with horseradish peroxidase (HRP) was introduced for

incubation at room temperature for one hour. After five additional washes, substrate to the HRP was added to the wells. Digestion of the substrate was concluded after 5 minutes with addition of 0.45 mol/L H_2SO_4 . The absorbance at 450 nm for the substrate/ H_2SO_4 solution was measured with a plate reader (Bio-Rad). The resulting absorbance values were indirectly proportional to the quantity of FPA in the plasma samples, allowing FPA concentration and thus thrombin activity in the plasma samples to be determined by comparison with FPA standards provided by the assay manufacturer.

In additional experiments to test the inhibitory potential of the NP system against clot-bound thrombin, fibrin clots were formed by mixing 563 μL citrated human plasma, 33 μL 500 mM CaCl_2 , and 4 μL 1 U/ μL thrombin, suspended on silk suture in sterile saline, and washed ten times over 24 hours to remove excess fibrinogen and FPA. Plasma then was incubated at 37° C for one hour with selected quantities of inhibitors in the presence of the fibrin clots and FPA generation by clot-bound thrombin was quantified in the plasma as above. The clots were subsequently imaged on a Varian 11.7T magnetic resonance system to quantify the surface area presented by each. The clots were imaged in transverse slices with a T2-weighted spin echo multislice sequence (2.5 s TR, 150 ms TE, 4 signal averages, 128 phase encoding steps, 128 frequency encoding steps, 12mm $\times$ 12mm $\times$ 19.2mm field of view, 16 1.2 mm thick slices). To determine surface area, images were processed and analyzed with custom scripts developed for Matlab.

Inhibition of Human Plasma Clot Growth In Vitro

Further testing of inhibition of bound thrombin was accomplished by evaluating clots suspended on silk suture prepared as above. 50 μL saline (n=3), Bivalirudin (40 mg/mL) (n=3), Bivalirudin NPs (n=3), PPACK (12.5 mg/mL) (n=3), or PPACK NPs (n=3) were added to 2 mL sterile saline surrounding the clots. The clots were incubated with inhibiting agents for 1 hour at 37° C, and then washed three times with sterile saline. The washed clots were immediately imaged with ^1H MRI at 11.7T as above (1.7s TR, 100 ms TE, one signal acquisition, 128 phase encoding steps, 256 frequency encoding steps, 12.7mm $\times$ 12.7mm $\times$ 16mm field of view, 16 1 mm thick slices). Citrated human plasma with 500 mM CaCl_2 was introduced in lieu of saline to surround the clots. The clots were imaged with the same sequence repeatedly for one hour following introduction of plasma. Magnetic resonance images of the clots were manually analyzed in NIH ImageJ to determine changes in clot volume after addition of plasma.

Induction, Inhibition, and Imaging Assessment of Acute Thrombosis In Vivo

All animal experiments were approved by the Washington University Animal Care and Use Committee and are based on National Institute of Health laboratory standards. The in vivo effects of bivalirudin and bivalirudin NPs were determined in mice through application of the rose bengal thrombosis model to the common carotid artery as described previously [33, 18]. 10–12 week old male C57BL/6 mice (weight 25–30 g) were anesthetized with sodium pentobarbital prior to surgical isolation of the right common carotid artery. An ultrasonic Doppler flow probe (Transonic Systems, Ithaca, NY) was applied to the artery to measure relative flow rate in the isolated segment of the carotid for the duration of each experiment. A 1.5 mW 540 nm HeNe laser (Melles Griot, Carlsbad, CA) was focused on the artery.

Bivalirudin NPs (n=6), or free bivalirudin (n=6) were injected as an IV bolus ten minutes prior to inducing thrombotic occlusion of the carotid by injecting rose bengal dye as an IV bolus (50 mg/kg; Fisher Scientific, Fair Lawn, NJ). Occlusion of the carotid artery was noted and experiments were terminated upon the stable (>5 minutes) maintenance of zero flow as measured by the flow probe.

Following the rose bengal experiments, the common carotid arteries were excised, rinsed with saline, and preserved in 10% buffered formalin. Arteries from mice treated with NPs were reserved for analysis with magnetic resonance imaging and spectroscopy prior to histological analysis. Imaging and spectroscopy was conducted with a custom-built single-turn solenoid coil on a Varian 11.7T MR system. ^{19}F spectra capturing signal from the artery and from a perfluorooctylbromide standard were obtained by non-localized spin echo spectroscopy (3 s pulse repetition time (TR), 2 ms echo time (TE), 256 signal averages, 12.88 minute acquisition time). ^{19}F spin echo images (1.1 s TR, 12 ms TE, 512 signal averages, 32 phase encoding steps, 64 frequency encoding steps, 5mm×5mm×1cm field of view) were obtained to depict NP binding in the excised artery. ^1H spin echo images (2 s TR, 14 ms TE, 4 signal averages, 128 phase encoding steps, 256 frequency encoding steps, 5mm×5mm×1mm field of view, 11 slices) allowed coregistration of the fluorine images with an anatomical (proton) image of the artery.

Arteries from each treatment group were preserved in 10% buffered formalin for three days prior to histological analysis with Carstairs' multichromatic staining method. After processing through alcohols and xylenes, the arteries were embedded in paraffin and sectioned at 5-micron thickness. Hydrated sections were treated with 5% ferric alum, Mayer's hematoxylin, picric acid-orange G solution, ponceau-fuchsin solution, 1% phosphotungstic acid, and aniline blue to stain for fibrin, platelets, collagen, muscle, and red blood cells. Images of stained sections were analyzed via color deconvolution in NIH ImageJ.

Bleeding times, activated partial thromboplastin time (APTT), or prothrombin time (PT) were determined in mice at 10, 20, 50, and 100 minutes after injection of thrombin-inhibiting NPs as an IV bolus (n=3 at each time point). For measurement of bleeding times, the tail was transected 0.5 cm from the tip and submerged in warm water. Bleeding time was determined at the cessation of spontaneous flow of blood into the water. For determination of APTT or PT, blood was drawn from the left ventricle into citrate. 80 μL plasma was combined with 80 μL APTT or PT reagent (Beckman-Coulter) for 3 minutes prior to activation with 80 μL calcium chloride (.025 mol/L) followed by determination of coagulation time with a fibrometer.

PPACK NPs were implemented in rabbits to explore the use of thrombin-inhibiting PFC NPs as thrombosis imaging contrast agents in a larger animal model. 12 month cholesterol-fed rabbits were anesthetized via ketamine/xylazine (120 mg/24 mg IM bolus followed by infusion of ketamine and periodic IM bolus of xylazine and acepromazine) in accordance with observed pulse, respiration rate, blood oxygenation, and body temperature. The femoral artery was exposed and illuminated by 1.5-mW 540-nm HeNe laser (Melles Griot, Carlsbad, CA, USA) and 50 mg/kg Rose Bengal was administered as an IV bolus. Laser exposure of

the artery was maintained for 3.5 hours while monitoring flow distal to the site of injury via doppler probe (Transonic Systems, Ithaca, NY, USA). Ultrasound images (Doppler and echo) acquired with a Philips IE33 system every 30 minutes after induction of injury provided a 2 cm by 2 cm longitudinal view of the femoral artery at the site of injury during formation of the arterial thrombus.

At 200 minutes after induction of injury, the site of injury was sutured and the rabbit was subsequently monitored via magnetic resonance angiography on a 3T Philips Achieva MR system to demonstrate non-invasive detection of the injury. PPACK NPs were administered as a 1 ml/kg IV bolus at the beginning of MRI to accumulate at the site of the formed thrombus. Conventional time-of-flight MR angiography, phase-contrast angiography, and dark blood T1-weighted imaging were focused on the injured femoral artery to detect the injury with multiple imaging techniques. After sacrifice, the affected femoral artery was removed and preserved in formalin for ex vivo imaging on an 11.7T Varian MR system. ^{19}F spin echo MRI (1.3 s TR, 13.3 ms TE, 512 signal averages, 32 phase encoding steps, 64 frequency encoding steps, 1.39cm $\times$ 1.15cm $\times$ 1.2mm field of view, 14 slices) identified the retention of NPs in the injured artery. ^1H spin echo MRI (1.5 s TR, 17 ms TE, 4 signal averages, 128 phase encoding steps, 256 frequency encoding steps, 1.39cm $\times$ 1.15cm $\times$ 1.2mm field of view, 14 slices) provided anatomical images for co-registration with ^{19}F MRI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors acknowledge the contributions of Ralph Fuhrhop and Angana Senpan to nanoparticle formulation and preparation, the contributions of Huiying Zhang and Noriko Yanaba to tissue and method preparation for Carstairs staining, the contributions of Cordelia Caradine to animal care during surgical procedures, and the contributions of Joshua Hood to preparation of Figure 7.

Grants

This work was supported by NIH grants R01 HL55520 to Douglas Tollefsen, R01 HL073646 to Samuel Wickline, and U54 HL112303 (Project 5) to Samuel Wickline.

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Fig. 1.
Schematic of bivalirudin and PPACK PFC-core NPs.

Fig. 2.

Analysis of Bivalirudin nanoparticle synthesis. Thrombin cleavage of Chromozym TH indicated thrombin activity after incubation with various inhibitors (a). Increasing concentrations of bivalirudin NPs (purple) or free bivalirudin (black) diminished thrombin activity according to a model for tight-binding reversible inhibition of serine proteases, yielding K_i of 121.18 nM for free bivalirudin (black) and K_i of 209.31 nM for bivalirudin on NPs (purple). Hydrodynamic diameter of NPs was measured before and after conjugation of bivalirudin to the particle surface (b). Before conjugation, the particle diameter was 174.2 ± 2.4 nm. After conjugation, the particle size was measured as 174.7 ± 1.9 nm. Bivalirudin was quantified by tyrosine fluorescence intensity, permitting indirect determination of the quantity of conjugated bivalirudin through measurement of uncoupled excess bivalirudin (c).

Fig. 3.

Fibrinopeptide A (FPA) ELISA determined the quantity of FPA released by thrombin cleavage of fibrinogen. Quantity of FPA release in plasma was dependent on the concentration of thrombin or inhibitor (bivalirudin NPs (**a**) or PPACK NPs (**c**)). Thrombin bound in fibrin clots also generated FPA when clots were incubated in plasma. Addition of inhibitors to the plasma diminished the generation of FPA. The results indicate that bivalirudin (**b**) or PPACK (**c**) NPs perform equally well against bound and free thrombin.

Fig. 4.

^{19}F magnetic resonance signal from the core of NPs was observed on the surface of clots treated with PPACK NPs or bivalirudin NPs (a). After exposure to plasma, clot volume increased for clots treated with saline, PPACK, or bivalirudin (b). No growth was observed for clots treated with PPACK NPs or bivalirudin NPs (c). Representative clot volume growth curves are shown in (d). Final clot volume with saline, PPACK, or bivalirudin treatment increased significantly. Clots treated with PPACK NPs or bivalirudin NPs did not significantly increase in volume (e).

Fig. 5. Time to stable occlusion of the carotid after rose bengal photochemical injury was measured in mice treated with various antithrombotics. Bivalirudin bolus ($n=4$) did not significantly extend time to occlusion, but bivalirudin NP or PPACK NP treatment ($n=5$) did (**a**). In the blood pool, bivalirudin NPs did not exert a significant effect on bleeding time beyond 20 minutes from administration (**b**).

Fig. 6.

^{19}F MRI at 11.7T showed retention of bivalirudin NPs in injured arteries (**a**). ^{19}F MRS identified $.31 \pm .13$ fmol PPACK NPs, 0.34 ± 0.02 fmol bivalirudin NPs, or 0.07 ± 0.03 fmol control NPs bound in acutely injured mouse carotid arteries (**b**). In cholesterol-fed rabbit femoral arteries, development of a thrombus was tracked with Doppler flow ultrasound imaging (**c**). False color ^{19}F MRI at 12T identified the retention of bivalirudin NPs in a three dimensional image of the excised artery (color scale bar represents 15–150 nM concentration) (**d**).

Fig. 7. Schematic representation of clots without (a) and with (b) thrombin-inhibiting NPs. The figures are presented as hypothetical representations of a cross-section of a clot formed in a rabbit femoral artery, as imaged with MRI here as a single slice and in Figure 5d in three dimensions.