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Thrombin-Targeted Liposomes Establish A Sustained Localized Anticlotting Barrier Against Acute Thrombosis

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Abstract

The goal of the present work was to design and test an acute-use nanoparticle-based antithrombotic agent that exhibits sustained local inhibition of thrombin without requiring a systemic anticoagulant effect to function against acute arterial thrombosis. To demonstrate proof of concept, we functionalized the surface of liposomes with multiple copies of the direct thrombin inhibitor, D-phenylalanyl-L-prolyl-L-arginyl-chloromethyl ketone (PPACK), which exhibits high affinity for thrombin as a free agent, but manifests too rapid clearance *in vivo* to be effective alone. The PPACK-Liposomes were formulated as single unilamellar vesicles, with a diameter of 170.78 ± 10.59 nm and a near neutral charge. *In vitro* models confirmed the inhibitory activity of PPACK-Liposomes, demonstrating a K_I of 172.6 nM. In experimental clots *in vitro*, treatment of formed clots completely abrogated any further clotting upon exposure to human plasma. The liposomes were evaluated *in vivo* in a model of photochemical-induced carotid artery injury, resulting in significantly prolonged arterial occlusion time over that of controls (69.06 ± 5.65 min for saline treatment, N=6, 71.33 ± 9.46 min for free PPACK treated 85.75 ± 18.24 min for precursor liposomes; N=4, 139.75 ± 20.46 min for PPACK-Liposomes; $P = 0.0049$, N=6). Systemic anticoagulant profiles revealed a rapid return to control levels within 50 minutes, while still maintaining antithrombin activity at the injury site. The establishment of a potent and long-acting anticoagulant surface over a newly forming clot with the use of thrombin targeted nanoparticles that do not require systemic anticoagulation to be effective offers an alternative site-targeted approach to the management of acute thrombosis.

Keywords

Drug Delivery; Liposomes; Nanoparticles; Anticoagulants; Thrombosis

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Supporting Information Available

Additional supporting figures referenced in the text are presented in the Supplementary Information. This information is available free of charge via the Internet at <http://pubs.acs.org>

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Introduction

The development of new pharmaceutical agents to treat acute arterial thrombosis remains a subject of active investigation to meet the continuing medical need for safe and controllable but powerful anticoagulation.¹⁻³ The deployment of direct thrombin inhibitors such as dabigatran recognizes the central role that thrombin plays in clot formation by proteolytic cleavage of fibrinogen into fibrin, but also its role in activation of platelets and Factor XIIIa, among others.^{4,5} Many of these treatment options display small therapeutic windows, with concerns for deleterious side effects such as bleeding that hamper their clinical adoption.⁴⁻⁷

Previous studies have suggested the potential of nano-scaled drug delivery systems as antithrombotic agents.⁸⁻¹¹ Such treatments would allow for effective, highly localized antithrombotic activity with potentially fewer risks for systemic side effects. Of these candidate nanoscale constructs, liposomes offer many advantages, including their biocompatibility and biodegradability with a history of suitability for regulatory approval.¹²⁻¹⁴ Indeed, liposomes already have achieved clinical translation through the recent labeling of a number of FDA-approved formulations (e.g., Doxil, DaunoXome, DepotDur, Ambisome). Moreover, the potential for encapsulation of drugs within liposomes together with surface conjugation of anticoagulant agents offers a design format that could enable synergistic combination therapy.¹⁵

Herein we describe an antithrombotic agent formed through the conjugation of direct thrombin inhibitors to the surface of unilamellar liposomes. The use of a direct thrombin inhibitor conjugated to the surface of the liposome would allow for high affinity and avidity binding and targeting to thrombin at the site of acute thrombosis. Furthermore, we postulated that the construct would remain active at the site of acute thrombosis for hours by forming an anticlotting surface after binding, while manifesting only a very transient systemic anticoagulant effect, which should mitigate the potential for hemorrhage without the need for an antidote. For proof of concept, the inhibitor used in this study is a short chain peptide, D-phenylalanyl-L-prolyl-L-arginyl-chloromethyl ketone (PPACK), which inhibits thrombin through alkylation of a histidine residue at the active site.¹⁶

Materials and Methods

Liposome Preparation

Unilamellar liposomes were created using standard protocols as previously described.¹⁷ A lipid mixture containing 94 mol% egg phosphatidylcholine, EPC (Lipoid, Newark, NJ), 4 mol% 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, DPPE (Avanti Polar Lipids, Alabaster, AL) was dissolved in chloroform. For fluorescent liposomes, the lipid mixture contained 93.9 mol% EPC, 4 mol% DPPE, 2 mol% DSPE-PEG-COOH, and 0.1 mol% 1,1'-Diocadecyl-3,3',3',3'-Tetramethylindocarbocyanine Perchlorate, DiI (Invitrogen, Carlsbad, CA).¹⁸ The lipid mixture at 10 mg/ml was placed in a heated water bath and dried into a film using rotary evaporation. The resulting lipid film was further dried in a vacuum oven at 40°C overnight to ensure complete evaporation of any residual organic solvent.

After drying, the lipid film was hydrated in phosphate buffered saline (WashU Tissue Culture Lab, St. Louis, MO) and sonicated at level two on a Branson probe sonicator for 2 minutes to resuspend the lipids and generate multilamellar vesicles (MLVs). The MLVs were subjected to extrusion through stacked polycarbonate/polyethylene filters (Whatman Inc, Piscataway, NJ) with a pore size of ~200 nm to generate a suspension of PEGylated, carboxy-terminated unilamellar liposomes.

PPACK-Liposome Formulation

PPACK conjugation was accomplished using amine-carboxyl coupling. Activation of carboxyl groups was accomplished using EDCI, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (Pierce, Rockford, IL), added in excess at 2 mg/ml to the suspension of carboxy-terminated liposomes and mixed for 15 minutes. PPACK (American Peptide Company, Sunnyvale, CA) was added to the activated precursor liposomes at 12.5 mg/ml and allowed to mix overnight. Excess EDCI and uncoupled PPACK was removed from the suspension using dialysis tubing of MWCO 3000-5000 (Spectrum Labs, Rancho Dominguez, CA) and allowed to filter for four hours.

Liposome Characterization

The sizes of the liposomes with or without PPACK incorporated were analyzed by dynamic light scattering (Brookhaven Instruments Corp., Holtsville, NY). Zeta potential values were determined with a PALS Zeta Potential Analyzer (Brookhaven Instruments Corp., Holtsville, NY). Liposomes were diluted in MilliQ Water for zeta potential measurements. Data was acquired in the phase-analysis light scattering mode following solution equilibration at 25°C. For transmission electron microscopy evaluation of liposomes, samples of precursor and PPACK-liposomes were deposited onto formvar-coated Cu grids, followed by application of the vanadium-based NanoVan negative stain (Nanoprobes, Yaphank, NY). The liposomes were visualized using an FEI Spirit TEM at 120 kV.

Conjugation of PPACK to precursor liposomes was confirmed through RP-HPLC quantification of uncoupled PPACK recovered from the supernatant after centrifugation of pre-dialysis PPACK-Liposomes mixed with Cleanascite lipid adsorption reagent (Biotech Support Group, Monmouth, NJ). PPACK elution was accomplished using a C₁₈ reverse phase column (Grace, Deerfield, IL) on a Waters HPLC system. Eluent A was 0.1% Trifluoroacetate (TFA) in water, Eluent B was 0.1% TFA in acetonitrile, and a linear gradient (10-30% B) was established over 20 minutes. PPACK elution was measured at a wavelength of 215 nm. Determination of the liposomal PPACK concentration was accomplished through mass balance of uncoupled PPACK subtracted from total PPACK added during the conjugation step.

The Stewart assay¹⁹ was employed to determine liposome concentration through the measurement of total phospholipid content of the liposome sample. For the phospholipid content measurements, 100 µl of liposomes was diluted up to 2 ml with chloroform, followed by addition of 2 ml 0.1 M ammonium ferrothiocyanate. The mixture was vortexed for 1 min, and the organic phase was removed and measured at 488 nm using a Shimadzu UV-1601 UV-Vis spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). The results were plotted against a standard curve of known lipid concentrations to determine liposomal phospholipid concentration. From this data, given the size of the liposome, liposome concentration was calculated accordingly.

In Vitro Inhibition of Thrombin Activity

A Chromozym TH, Tosyl-Gly-Pro-Arg-4-nitranilide acetate (Roche Applied Science, Indianapolis, IN) assay was utilized to quantify PPACK-mediated inhibition of thrombin in accordance with existing methods.²⁰ Varying amounts of free PPACK or PPACK-Liposomes were incubated for 1 min at room temperature with 100 µl of 12 nM human α-thrombin (Haematologic Technologies, Essex Junction, VT). The thrombin/liposome mixture was then added to 500 µl of 100 µM Chromozym TH in Tris buffer. Thrombin activity against the Chromozym TH substrate was assessed by measuring optical absorbance at 405 nm, indicative of liberation of the 4-nitraniline group of Chromozym TH. To quantify the activity of uninhibited thrombin, the change in absorbance per minute was

recorded. The kinetics of PPACK-thrombin inhibition was fit to a model of thrombin inhibition by hirudin to estimate an apparent inhibition constant K_I' .²¹

Assuming binding to the enzyme causes a significant decrease in inhibitor concentration, the variation of the steady-state velocity can be described in terms of K_I' :

$$v_s = \frac{v_0}{2E_t} \left[\left[\left(K_I' + I_t - E_t \right)^2 + 4K_I' E_t \right]^{\frac{1}{2}} - \left(K_I' + I_t - E_t \right) \right]$$

where the steady state velocity, v_s , is shown as a function of v_0 , the velocity in the absence of an inhibitor; I_t , the total inhibitor concentration; E_t , the total enzyme concentration; and K_I' , the rate constant where the substrate and the inhibitor compete for binding to the active site, as is the case with PPACK inhibition of thrombin.

To test PPACK-Liposome activity against clot-bound thrombin, clots were made by combining 2.25 μ l of 1U/RI bovine thrombin (Sigma-Aldrich, St. Louis, MO), 16.5 μ l of 500 mM CaCl_2 and 281.5 μ l of human plasma, using the cap of a 1.5 ml eppendorf tube as a template. The clots were then transferred to strips of nitrocellulose and aged in saline at 37°C for 2 hours on an orbital shaker. After incubation, 50 μ l of either saline, 12.5 mg/ml free PPACK, Precursor Liposomes or PPACK-Liposomes were added and allowed to incubate with the clots for 1 hour. After liposome incubation, the clots were rinsed with saline and weighed. The clots were then submerged in a mixture of 3.77 ml of human plasma and 220 μ l of 500 mM CaCl_2 and allowed to incubate for intervals of 5, 15, 30, 60 and 180 minutes. At the conclusion of each time point, the clots were rinsed in saline and weighed.

For the visual determination of PPACK-liposome binding to clot-bound thrombin, the previously described protocol was slightly altered. Clots were made as previously described, combining thrombin, CaCl_2 and human plasma in a template. The clots were aged in saline for 2 hours at 37°C on a shaker, followed by the addition of either non-targeted or PPACK-functionalized fluorescent liposomes. The fluorescent liposomes were allowed to incubate for 1 hour at 37°C. After incubation, the clots were rinsed in saline and transferred to 6-well plates with fresh saline for imaging. Competition experiments were accomplished with the addition of 50 μ l of 12.5 mg/ml PPACK followed by 2 hours of incubation and a subsequent saline rinse prior to the addition of fluorescent PPACK-Liposomes. Clots were imaged using a Xenogen *in vivo* imaging system (IVIS) Spectrum Workstation (Caliper Life Sciences, Hopkinton, MA) at excitation and emission wavelengths of 535 and 580 nm, respectively. Radiant efficiency was also measured for the imaged clots to gather a quantitative assessment of PPACK-Liposome binding. Additionally, untreated clots were fixed and embedded in paraffin for histological analysis of fibrin content.

Platelet aggregometry was carried out to assess the inhibition of thrombin-mediated platelet activation by PPACK-Liposomes. Blood collection protocols were approved by the Washington University Institutional Review Board. Human donor whole blood was collected via venipuncture into a syringe containing 3.2% sodium citrate for anticoagulation (1:10 citrate to blood ratio). Platelet-rich plasma (PRP) obtained by centrifuging the anticoagulated whole blood at 180g for 10 minutes at room temperature. The supernatant was carefully collected and the remaining material was further centrifuged at 1200g for 20 minutes at room temperature to obtain platelet-poor plasma (PPP).^{22,23} PPP was used as the standard for 100% light transmission. Platelet aggregometry was conducted on a Model 300 Payton Aggregation System (Payton Scientific, Inc., Buffalo, NY) at 37°C. Briefly, 500 μ l of PRP was mixed with 5 μ l of either water, 150 μ M PPACK, precursor liposomes, or

PPACK-Liposomes and stirred at 1000 RPM in the aggregometer. The mixture was allowed to sit to ensure a stable baseline reading and upon stabilization, 5 μ l of 500 μ M thrombin (5 μ M final concentration) was added to the PRP mixture and allowed to aggregate fully.

In vivo Inhibition of Thrombin Activity

The antithrombotic efficacy of PPACK-Liposome was defined according to their ability to delay the onset of thrombosis in C57BL/6 mice that were subjected to photochemical injury of the carotid artery. The mice were sedated with ketamine (87mg/kg)/xylazine(13mg/kg), followed by isolation of the carotid artery through a midline cervical incision. To follow the kinetics of occlusion after injury initiation, an ultrasonic Doppler probe (Transonic Systems Inc, Ithaca, NY) was placed on the carotid artery to measure the blood flow rate. Saline, free PPACK at 12.5 mg/ml, precursor liposomes (without surface modifications) or PPACK-Liposomes were delivered via tail vein injection as a 1ml/kg bolus 10 minutes before the injection of photosensitive Rose Bengal dye in phosphate-buffered saline to induce arterial injury upon exposure to laser light. A 1.5 mW 540 HeNe laser was focused on the isolated carotid artery. The experiment was terminated upon observation of the flow rate decreasing to < 15% of the original flow and maintaining that level for >5 minutes. The injured carotids were removed and preserved in paraffin for histological analysis of clot composition using the Carstairs stain for fibrin and platelets.²⁴

For observation of liposome localization to the injury site, fluorescent PPACK-Liposomes were administered and the right carotid artery was exposed to photochemical injury for 30 minutes. The mice were euthanized and the injured carotid arteries were excised and embedded in Optimal Cutting Temperature (OCT) medium and sectioned for fluorescence microscopy.

Activated Partial Thromboplastin Time

To measure the effects of PPACK-Liposome treatment on activated partial thromboplastin time (APTT), a series of C57BL/6 mice were injected with a bolus of PPACK Liposomes with terminal left ventricle blood draws at 10, 20, 50, and 100 minutes post-injection. The mice were sedated with ketamine (87mg/kg)/xylazine(13mg/kg) and blood was drawn from the left ventricle into a syringe with 4% sodium citrate for anticoagulation (1:10 citrate to blood ratio). Blood samples were subsequently spun in a microcentrifuge for 15 minutes at 1000g. After centrifugation, the plasma was aspirated and frozen for future use. APTT kits (HemosIL, Lexington, MA) were used to obtain data for each time point. 100 μ l of APTT reagent (colloidal silica with a mixture of synthetic phospholipids, buffer and preservatives) was added to 100 μ l of thawed plasma and allowed to incubate for 3 minutes. 100 μ l of 25 mM calcium chloride was then added to the reagent-plasma mixture and APTT was determined through mechanical agitation of the mixture.

Biodistribution

DiI-labeled PPACK-Liposomes were utilized to assess biodistribution and avenues of liposome clearance after 2 hours of circulation. C57BL/6 mice were given either no injection to serve as a control, or DiI-PPACK-Liposomes (1ml/kg) via tail-vein injection. Mice were euthanized 2 hours post-injection and perfused with saline. The liver, spleen, and kidneys were removed and imaged in a Xenogen IVIS Imaging Workstation as previously described for imaging DiI-labeled liposomes. Total radiant efficiency was measured to give a quantitative measurement of liposome accumulation in the organs.

Statistics

In vivo thrombin inhibition and in vitro clot growth data was analyzed using the Kruskal-Wallis ranked sum test, where $P < 0.05$ demonstrates statistical significance. For all other data, a two-sided Wilcoxon ranked sum test was used, where $P < 0.05$ demonstrates statistical significance. Error bars represent standard error of the mean.

Results

PPACK-Liposome Synthesis and Characterization

After sonication-extrusion treatment of lipid films, liposomes were synthesized with exposed terminal carboxy-PEG ends by incorporation of DSPE-PEG-COOH. The carboxy-terminated ends were activated with EDCI to form amide bonds with the N-terminus of PPACK. (Fig. 1a) The resulting PPACK-Liposome complex, along with the bare liposomes were analyzed with dynamic light scattering to determine the particle size of the resulting formulations. The bare precursor carboxy-terminated liposomes exhibited a mean hydrodynamic diameter of 163.2 ± 3.89 nm, while the PPACK-liposomes exhibited a mean hydrodynamic diameter of 170.78 ± 10.589 nm. (Fig. 1b) TEM images were also obtained to observe liposome morphology. (Fig. S1, Supplementary Information) Zeta potential was used to further characterize the stability of the liposome formulations. The precursor liposomes exhibited a zeta potential of -31.92 ± 11.43 mV, while the PPACK-terminated liposomes exhibited a zeta potential of -13.45 ± 2.00 mV. (Fig. 1b) Because of the positively charged amino acid in the PPACK peptide, this difference in zeta potential confirms successful conjugation of PPACK to the surface of the liposome. In addition, RP-HPLC quantification (Fig. S2A, Supplementary Information) of liposomal PPACK concentration, combined with Stewart assay quantification (Fig. S2B, Supplementary Information) of liposome concentration indicated the coupling of 6254.24 ± 1245.14 PPACK peptides per liposome.

In Vitro Inhibition of Thrombin

The chromogenic substrate Chromozym TH was utilized to measure thrombin activity in vitro. Varying amounts of free PPACK or PPACK-Liposomes were added to a solution of thrombin in order to demonstrate a dose-dependent inhibition of thrombin. After a one-minute incubation time, the thrombin/inhibitor mixtures were added to a solution of Chromozym TH in Tris buffer and the cleavage of Chromozym TH was allowed to occur. The results of the Chromozym TH assay were further used to estimate the apparent K_I for PPACK inhibition of thrombin. The Chromozym TH data was fit to a standard model of tight-binding inhibition of thrombin by hirudin, as previously described.²¹ The results showed a K_I of liposome-conjugated PPACK was 172.6 nM, and that of free PPACK was 12.07 nM. (Fig. 2)

Next, an in vitro assay was developed to study the ability of PPACK-Liposomes to inhibit clot-bound thrombin and prevent further growth of clots after plasma incubation. Standardized clots were produced in vitro by incubating thrombin, plasma and CaCl_2 together in a template for one minute. These clots were then removed from the template and affixed to nitrocellulose paper, followed by exposure to buffer for 2 hours. Then, clots (3-6 clots per group) were exposed to either 5 ml of buffer or 12.5 mg/ml free PPACK, Precursor Liposomes, or PPACK-Liposomes in buffer (50 μl in 5 ml of PBS) for 60 minutes, washed three times, and then incubated in plasma for selected intervals to observe clot growth according to clot weight. After 15 minutes of incubation in plasma, the weight of the saline control and precursor liposomes clots grew to greater than 500% of the initial weight of the pre-incubation clot, totally filling the well of a 6-well plate with new clot. (Fig. 3a) A similar, but more gradual increase in clot size was observed for clots treated with free

PPACK, finally reaching a size approximately 800% of the initial clot weight at the end of the 180-minute plasma exposure time. This significant increase was observed for all subsequent time points and was potentially limited by the restrictions of the size of the well in the plate. (Fig. 3b) Clots treated with PPACK-Liposomes did not grow significantly over 180 minutes. (Fig. 3a, 3b) Accordingly, PPACK-Liposomes completely prevented new clot growth, or “regrowth,” as compared to the control treatments.

To confirm the localization of the PPACK-Liposomes to the clots, liposomes were synthesized with the incorporation of the carbocyanine dye, DiI. Clots (N=6 per group) were grown and incubated with non-targeted liposomes, PPACK-functionalized liposomes, or no treatment. In addition, one group of clots was treated with 12.5 mg/ml free PPACK to block thrombin in the clot, followed by treatment with PPACK-Liposomes to observe any nonspecific interactions of PPACK-Liposomes with the clots. The clots were then imaged with an IVIS Spectrum Workstation after washing away excess, non-bound particles. PPACK-Liposomes exhibited significantly higher amounts of bound particles compared to the control liposomes and the competition group (Fig 4a), as confirmed by a quantitative measurement of total radiant efficiency. (Fig 4b)

PPACK-Liposomes also displayed the ability to effectively inhibit thrombin-mediated activation of platelets through platelet aggregometry measurements. In the presence of water or precursor liposomes, where PPACK is completely absent, platelets rapidly aggregated upon addition of thrombin at a concentration of 5 μ M in the cuvette. In the control group where free PPACK is present in the PRP, platelets exhibited no aggregation throughout the duration of the experiment. PPACK-Liposomes demonstrated a similar ability to ablate platelet aggregation. (Fig. S3, Supplementary Information)

Inhibition of Thrombus Formation in vivo

To delineate the efficacy of the PPACK-Liposomes in vivo, injury of the carotid artery of C57Bl/6 mice was induced by the traditional photochemical/laser method in mice each treated with either saline (N=6), free PPACK (N=4), Precursor Liposomes (N=4) or therapeutic PPACK-liposomes (N=6). The efficacy of liposomes for inhibition of thrombosis was measured by denoted time required to reach complete occlusion. Precursor liposomes with no PPACK functionality served as the liposome control. When injected 10 minutes prior to induction of arterial trauma, the control liposomes yielded an occlusion time of 85.75 ± 18.24 min. This exhibited no significant increase over the saline-treated group of 69.06 ± 5.65 min or the free PPACK group of 71.33 ± 9.46 min. In addition, the free PPACK group showed no significant increase over the saline treated group. In the PPACK-Liposome treated mice, occlusion time was prolonged to 139.75 ± 20.46 min and was significantly increased compared to all other groups. (Fig. 5a) Additionally, the activity of PPACK-Liposomes appeared to exhibit a continuous effect at the injury site as determined by flow rate monitoring throughout the course of the injury. (Fig. S4, Supplementary Information) Site-specificity and fluorescent PPACK-Liposome accumulation in the clot was observed through fluorescence microscopy evaluation of partially formed clots in excised carotid arteries. (Fig. S5, Supplementary Information). The results of this study indicate that the addition of PPACK to the surface of the liposomes provides the needed functionality to serve as a thrombin inhibitor in vivo.

Activated partial thromboplastin time (APTT) testing was carried out in mice treated with PPACK-Liposomes. (Fig. 5b) Liposome treatment resulted in an initial prolongation of APTT up to 65.98 ± 19.59 seconds at 10 minutes after the administration of PPACK-Liposomes. Prolonged APTT was observed 20 minutes after treatment, but followed by a return to the control range within 50 minutes after treatment. The normalization of systemic

APTT values did not affect the prolonged therapeutic action at the site of injury since in vivo inhibition of thrombus formation was observed beyond 2 hours in the carotid artery.

Discussion

The tripeptide agent PPACK is recognized as a potent candidate for antithrombotic applications due to its high affinity for thrombin and low systemic toxicity with an LD₅₀ of >50 mg/kg. However, its fast clearance rate ($T_{1/2} \sim 3$ minutes) in vivo precludes clinical application.¹⁶ To overcome the rapid systemic clearance, we conjugated PPACK to the surface of a liposome, which yields an agent that manifests prolonged anti-thrombin activity at the site of an acute clotting event, yet its systemic anticoagulant activity rapidly wanes after injection. The anticoagulant nanostructure design has the advantage of exposing multiple anticoagulant sites on the surface of the liposome to achieve exceptional thrombin avidity and markedly enhanced local anticoagulation efficacy. Moreover, the aqueous compartment of the liposome offers the potential of encapsulating drugs for possible localized combination anticoagulation or thrombolytic therapy.

PPACK-Liposomes are capable of binding to and inhibiting clot-bound thrombin as shown by the in vitro clot growth assays and fluorescent imaging (Fig. 4). Remarkably, PPACK-Liposomes completely prevented further clot growth in this system, which offers a distinct advantage over heparin that is unable to associate with clot-bound thrombin due to masking of the heparin-binding site on fibrin-bound thrombin.^{25,26} The establishment of a highly focal, long-lasting multivalent anticlotting surface atop a newly forming clot appears to sequester procoagulant materials from serum clotting factors that otherwise might continue to promote coagulation through activation of Factor VIII or other factors.²⁷

In vivo experiments of carotid occlusion time post-injury confirmed the efficacy of the PPACK-Liposomes, demonstrating an ability to prolong the formation rate of occlusive arterial thrombi after the administration of one pre-injury bolus treatment. The use of multiple dosing regimens and treatment of pre-formed thrombi in vivo will be the subject of future investigation to advance towards clinical translation and establish a maximum effective dose.

The prolonged occlusion times in the Rose Bengal mouse model are a consequence not only of the inhibition of thrombin at the site of injury, but also reduced platelet activation and aggregation that depends on thrombin interacting with various platelet receptors²⁸, including GPIIb/IIIa^{29,30}, PAR-1 in humans³¹, and PAR-4 in the mouse.³²⁻³⁴ This activation of platelets by thrombin is completely inhibited in the presence of PPACK-Liposomes as confirmed by platelet aggregometry. (Fig S3, Supplementary Information) This dual functionality likely amplifies the potency of the agent at the site of acute clotting without disrupting alternative mechanisms of platelet activation that would be useful to maintain hemostasis elsewhere. Moreover, despite the prolonged activity of PPACK-Liposomes at the site of injury, the systemic action of PPACK-Liposomes rapidly declines between 20-50 minutes after injection according to activated partial thromboplastin times. This rapid diminution of systemic anticoagulant effect is due to the removal of a sufficient amount of circulating thrombin-targeted liposomes by the liver and spleen (Fig. S6, Supplementary Information) to drop blood pool levels below that required to inhibit further clotting while still providing an enormous focal pool of PPACK at the site of thrombosis to immediately inactivate any subsequently released thrombin.

The limitations of PPACK as an antithrombotic agent also comprise its broader specificity as a protease inhibitor beyond thrombin, although the rapid clearance of the unbound liposomes mitigates this concern. This point might also apply equally to the concern of the

irreversible nature of the interaction with the active site on the thrombin molecule. Nevertheless, it is probable that alternative anticoagulant molecules such as bivalirudin could serve a similar purpose when conjugated to a liposome to enhance specificity in a reversible interaction if required. A pluripotent design with multiple specific factor inhibitors could promote synergism for the formed anticlotting surface without having to completely attenuate any one factor alone.

The use of a liposomal structure may occlude the interaction of some of the PPACK due to potential steric and mobility effects of liposome-bound PPACK, limiting the freedom of PPACK to invade the active site of thrombin. This possibility may explain the increased K_I of the PPACK conjugated liposome versus free PPACK noted in the model-based analyses of affinity, as not all of the included PPACK would be active against thrombin as long as the liposome remained intact. However, due to the high avidity achieved by including copies of PPACK, high local activity should be maintained as shown by the in vivo clot inhibiting experiments.

Previous reports have suggested the potential of nanometer-sized particles to act as effective anticoagulants in vivo, in some cases involving surface modifications to promote anticoagulant activity.^{8,9,11} However, the use of liposomes as anticoagulant structures has the advantage that they are established as a vehicle for biomedical applications with an abundance of FDA-approved liposomal formulations. Furthermore, the opportunity to encapsulate drugs or imaging agents ultimately could facilitate detection and localized treatment of acute thrombi in emergency situations. This benefit is vital—especially when triage to medical versus surgical therapy might necessitate ensuring normalized systemic clotting parameters at the time of decision, which is possible with this agent without the need for an “antidote”.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Schematic of EDCI-mediated PPACK conjugation to carboxyl-terminated liposomes. (B) Size and zeta potential of carboxyl-terminated liposomes and PPACK-Liposomes. Results indicate a hydrodynamic diameter of 163.2 ± 3.89 nm and zeta potential of -31.92 ± 11.43 mV for the carboxyl-terminated liposomes. PPACK liposomes exhibit a diameter of 170.78 ± 10.59 nm and zeta potential of -13.45 ± 2 mV. The rise in zeta potential is indicative of the increased positive surface charge on the liposomes after PPACK conjugation.

Figure 2. Demonstration of inhibition of thrombin activity against the chromogenic substrate Chromozym TH. PPACK-Liposomes exhibited an inhibition constant of 172.6 nM compared to an inhibition constant of 12.077 nM for free PPACK.

Figure 3.

In vitro clot assay demonstrating the inhibition of clot-bound thrombin by PPACK-Liposomes. (A) Measurement of clot growth after incubating saline, free PPACK, Precursor liposomes or PPACK-Liposome-treated clots in plasma for selected time points. Clots treated with saline, free PPACK and Precursor Liposomes all exhibited significant growth after 3 hours of plasma incubation compared to PPACK-Liposome treated clots. (* $P < 0.05$) (B-F) Representative images of clots before (B) and after (C-F) plasma incubation, comparing clots from all treatment groups after 3 hours of plasma incubation.

Figure 4.

(A) Fluorescent images of clots treated with either saline, DiI-labeled carboxyl-terminated liposomes (Non-Targeted) or DiI-labeled PPACK-Liposomes (Targeted). Competition experiments refer to prior inhibition of clot-bound thrombin with free PPACK followed by treatment with DiI-labeled PPACK-Liposomes. (B) Measurement of Total Radiant Efficiency demonstrating that liposome binding to clot was significantly increased with PPACK functionalization. (**P<0.005, N = 6 for non-targeted, PPACK liposomes, and competition groups) Additionally, competition experiments demonstrated specificity through low association of PPACK-Liposomes with clots previously treated with free PPACK.

Figure 5.

(A) Time to occlusion of carotid artery with the Rose Bengal dye injury model in mice treated with Saline, free PPACK, Precursor Liposomes or PPACKliposomes. Occlusion time was significantly increased for the PPACK-Liposome group over all other groups (** $P < 0.005$) demonstrating potent antithrombotic activity in vivo. (B) Activated partial thromboplastin time for PPACK-Liposome treated mice demonstrating a significant increase in APTT (* $P < 0.05$) compared to the normal range 10 and 20 minutes after PPACK-Liposome injection, followed by a return to the control range within 50 minutes after injection.