A Novel Near-Infrared Fluorescence Sensor for Detection of Thrombin Activation in Blood

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Thrombosis is an important pathophysiologic component of many cardiovascular diseases. Thrombin, a serine protease, plays a central role in thrombosis formation. Detection and imaging of thrombin activity may thus be of considerable biomedical interest. The goal of this study was to design, synthesize, and characterize a novel thrombin-activated near-infrared fluorescence (NIRF) probe. The probe consisted of a thrombin-cleavable peptide spacer (…D-Phe-Pip-Arg…; Pip = pipecolic acid) and contained a terminal fluorescence reporter which was quenched when conjugated to a biocompatible delivery vehicle. A control peptide spacer was synthesized that differed by one amino acid. Following thrombin addition, the probe was activated within minutes. The NIRF signal increased by a factor of 27-fold within 20 minutes, and was inhibited by hirudin, a specific thrombin inhibitor. NIRF optical imaging experiments confirmed rapid activation of the probe in both buffer and human blood. The control probe showed minimal activation in all experiments. In addition to potentially furthering our understanding of thrombin regulation in vivo, the thrombin-activated near-infrared probe may have broad clinical application to the diagnosis of arterial and venous thrombosis.

KEYWORDS:

enzyme activation · fluorescent probes · near-infrared fluorescence · peptides · thrombin

Introduction

Thrombosis is a central pathophysiologic feature of many cardiovascular diseases such as unstable angina and myocardial infarction,[1–3] as well as deep venous thrombosis and pulmonary embolism. Rapid diagnosis of these potentially life-threatening conditions is necessary to minimize their associated morbidity and mortality. Current diagnostic imaging methods are flow-based (X-ray angiography, computed tomography angiography, magnetic resonance angiography, doppler ultrasound) or perfusion-based (nuclear medicine perfusion scans) and suffer from two important limitations. First, these methods do not directly image thrombus and, therefore, cannot reliably distinguish between a thrombotic or nonthrombotic (for example, cholesterol, lipid) obstruction to flow. Second, these methods do not allow assessment of the biological regulation of thrombus formation.

Local hemostatic milieu is critical in vascular thrombus formation; in particular, thrombin, a serine protease, plays a central role in the development of vascular thrombosis. Thrombin cleaves fibrinogen to form fibrin, the scaffolding of thrombosis. In addition, thrombin activates platelets, monocytes, and the vascular endothelium to facilitate thrombus formation.[4] Local detection of thrombin activity might therefore allow direct diagnosis of thrombosis, as well as offering further insight into thrombin regulation.

Molecular imaging is a new paradigm based on imaging specific cellular and molecular targets.[5] Recently, our laboratory has developed a series of near-infrared fluorescence (NIRF) probes for the in vivo detection of specific proteases, including cathepsins and matrix metalloproteinases.[6–9] The probes consist of quenched near-infrared (NIR) fluorochromes conjugated to specific peptide substrates, which themselves are grafted onto an injectable and biocompatible delivery molecule (Scheme 1).[10] The rationale for using NIR fluorochromes rather than visible light fluorochromes is that light at 700 – 900 nm travels though tissue most efficiently as blood does not strongly absorb NIR energy.[11] Upon protease cleavage, the NIR probes fluoresce and can be detected by different imaging systems, for example, fluorescence microscopy, reflectance imaging,[12] or optical tomography.[13]

We hypothesized that thrombin could serve as a relevant optical molecular imaging target in the detection of local thrombus. In this study, we describe the design, synthesis, and

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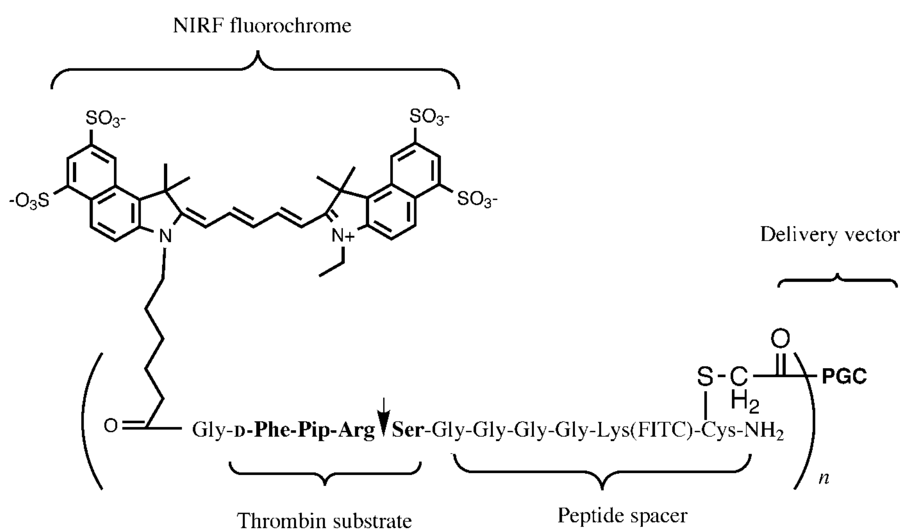

*Scheme 1. Structure of the thrombin-sensitive probe. The thrombin substrate was linked to free amino groups on lysine side chains of the polymeric carrier through a carboxymethyl linkage. PGC indicates a partially pegylated polylysine grafted copolymer.[10] The NIRF fluorochrome, Cy5.5, was attached to the N terminus of the peptide. The bold region represents the thrombin recognition site and the arrow indicates the cleavage site. For synthesis of the control probe, Gly-**D-Phe-Pip-Arg-Pro**-Gly-Gly-Gly-Gly-Lys(FITC)-Cys-NH_2 was used.*

characterization of a first generation thrombin-activatable NIRF imaging probe and report its optical imaging profile compared against a control probe in buffer and in whole human blood.

Results and Discussion

Synthesis

The design of the protease-activatable NIRF probe was based on a long circulating delivery vehicle,[10] the peptide substrate, and a near infrared fluorochrome. The biological fate of the long circulating polymer (a partially pegylated polylysine copolymer) has been extensively studied in animals and humans.[10] The circulation time of the polymer is over 20 hours in humans and is thus ideally suited for vascular imaging applications.[14] We started out by attaching the peptide substrate to unpegylated lysine residues of the polymer.[7] The synthesized 11 residue peptide, Gly-D-Phe-Pip-Arg-Ser-Gly-Gly-Gly-Gly-Lys(FITC)-Cys-NH_2, was designed to contain a thrombin-sensitive substrate, a tetraglycine spacer, a fluorescein tag (FITC = fluorescein isothiocyanate) for quantification, and a cysteine residue for further conjugation. The thrombin-substrate sequence, D-Phe-Pip-Arg, had a D-phenylanaline at the P3 position and an unusual amino acid, pipecolic acid, at the P2 position. The substrate has a reported k_{cat}/K_M of $3.94 \times 10^7 \mathrm{M}^{-1}\mathrm{s}^{-1}$.[15]

We first performed an enzymatic assay to show that the fully designed, C-terminal-extended peptide still served as a substrate for thrombin. By using high-pressure liquid chromatography (HPLC), we found that the peptide was recognized by thrombin and cleaved into two major products (Figure 1 A). In contrast, thrombin proteolysis was eliminated when the serine at the P1′ position was replaced by a proline residue. As shown in Figure 1 B, the control peptide, Gly-D-Phe-Pip-Arg-Pro-Gly-Gly-Gly-Gly-Lys(FITC)-Cys-NH_2, remained intact for two hours following incubation with thrombin.

The peptide was then coupled to the polymer (PGC; protected graft copolymer) using biofunctional iodoacetic anhydride as the connecting linker (Scheme 1). The unpegylated free amino groups on the PGC backbone were capped with iodoacetic anhydride; this converted all amino groups into thiol reactive groups, which were subsequently reacted with peptides. In the final step of the synthesis, monoreactive indocyanine fluorochrome (Cy5.5) was conjugated to the N terminus of each peptide. An average of 23 reporter fluorochromes were attached to each polymeric carrier molecule. With this high number of reporters, fluorescence was efficiently quenched in the inactivated state. Similar conjugation efficiency and optical characteristics were obtained for the control probe.

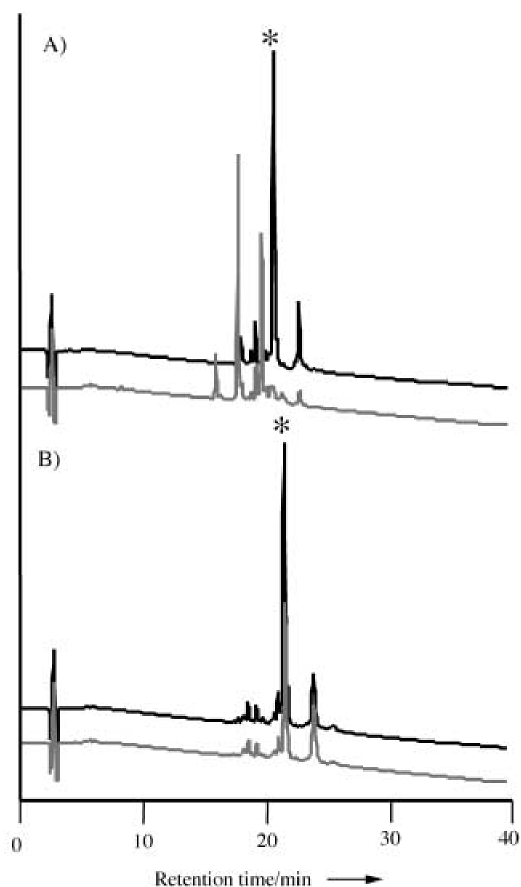

Figure 1. HPLC chromatogram of peptide cleavage by thrombin. A) Thrombin-sensitive peptide (10 μM) was incubated with thrombin (10 Units) in PBS for 1 h. The black and gray chromatograms represent before and after incubation with thrombin, respectively. The peptide substrate was recognized and hydrolyzed. B) As (A), except the control peptide was used. No cleavage was found after incubating with thrombin. Asterisks indicate the intact peptides.

Characterization

The prepared probes were first tested with purified thrombin in phosphate-buffered saline (PBS) buffer as the NIRF signal was recorded over time. Initially both probes showed low NIR fluorescence (150 arbitrary units (AU); Figure 2 A). Following addition of thrombin, the NIRF signal increased from 150 to 4100 AU within 20 minutes (27-fold increase). This was significantly greater activation than with the control probe, where only a 1-fold increase in NIRF signal was observed within the same time frame. There was a clear dose response when the probe was incubated with different amounts of thrombin (Figure 2 B). To further demonstrate the specificity of thrombin activation, we examined probe activation in the presence of hirudin, a direct thrombin inhibitor used in the clinical treatment of vascular thrombosis. When thrombin was added to solutions containing hirudin and the thrombin probe, no NIRF signal increase was detected (Figure 2 C). Furthermore, to show that hirudin did not destroy or alter the optical probe, we added additional thrombin, which overcame hirudin activation and caused a strong NIRF signal (data not shown).

An imaging experiment was subsequently carried out to confirm that thrombin activated the thrombin probe but not other enzyme specific probes. A home-built imaging system which has a bandpass excitation filter at 610 – 650 nm and an emission filter at 680 – 720 nm was used to acquire NIRF images of activation with various probes.[12] Thrombin, control, cathepsin B, and cathepsin D probes were incubated with thrombin, individually. The NIRF and bright-field images were acquired 10 minutes after incubation (Figure 3 A). Without thrombin, there was no detectable fluorescent signal in any of the probes. Within 10 minutes after thrombin addition, however, NIR fluorescence signal was selectively generated by the thrombin probe. The other probes remained silent.

To demonstrate thrombin activation of the probe in human blood, citrated human whole blood was incubated with the thrombin probe and the NIR fluorescence was recorded. There

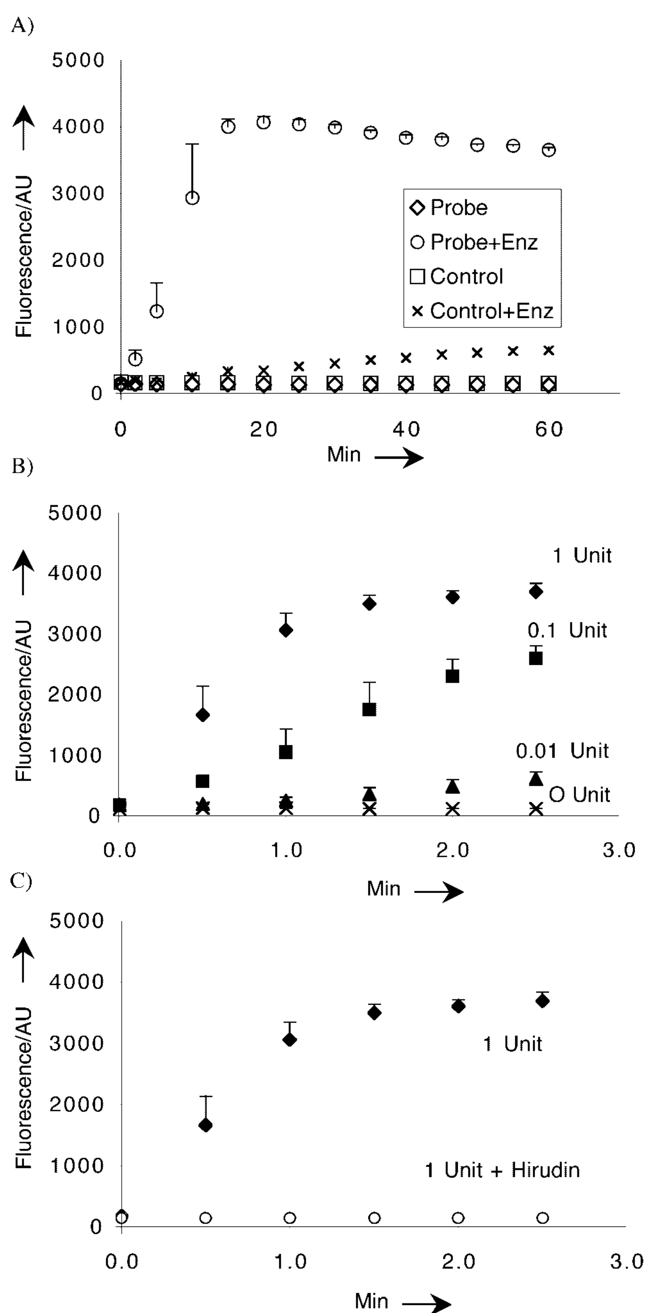

Figure 2. In vitro activation and inhibition of experimental and control NIRF probes. A) The probes (0.5 μM) were incubated with or without thrombin (1 Unit) in PBS solution. B) The thrombin probe was activated with thrombin (1, 0.1, 0.01, or 0 Units). C) Probe activation by thrombin (1 Unit) was completely inhibited by addition of hirudin (5 μg).

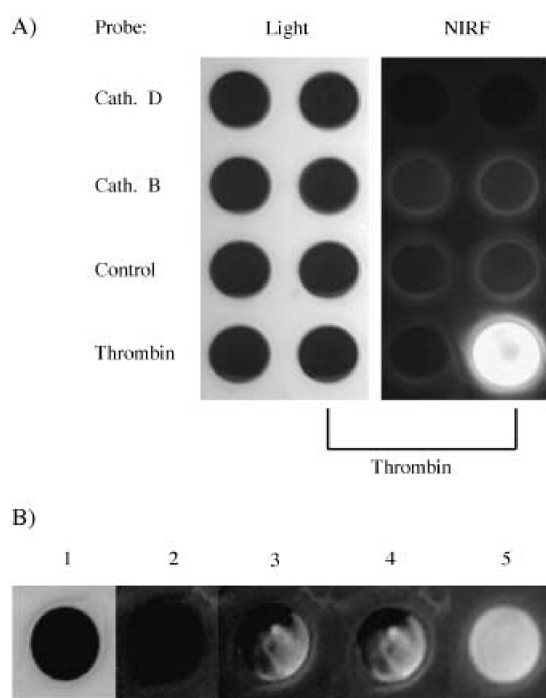

Figure 3. Activation of NIRF probes in buffer and blood. A) Thrombin, control, cathepsin B, and cathepsin D probes (0.5 μM) were incubated with and without thrombin (1 Unit) for 10 minutes, followed by light and NIRF image acquisition. Thrombin activation occurred only with the true thrombin probe. B) Activation of the thrombin probe in whole blood with exogenous thrombin (5 Units). 1) Light and 2) NIRF images before adding thrombin. 3) – 5) NIRF images at 3 minutes, 60 minutes, and 24 hours, respectively.

was no detectable NIRF signal within 30 minutes of incubation of the probe in anticoagulated blood. Following exogenous thrombin addition, the NIRF signal increased within minutes as the blood clotted. Interestingly, as shown in Figure 3 B, the NIRF signal further increased slowly over time. Compared to the probe experiments in buffer, this finding may have been due to restricted mixing of the target probe with thrombin in the semisolid blood clot. Exogenous thrombin was necessary to generate the NIRF signal; this suggests that the anticoagulant effects of sodium citrate inhibited endogenous thrombin generation.

In this study, we have designed a thrombin-sensitive NIR fluorescent probe and demonstrated its ability to detect and image thrombin activity in buffer and human blood. The results indicate that the developed probes have the potential to serve as imaging reporters for thrombus activation in vivo, and biological studies in animal models are currently ongoing. In parallel to this research, we are also developing three-dimensional tomographic imaging systems that would allow quantitative imaging of probe activation in deep tissue in vivo.[13] This targeted optical imaging technology may ultimately contribute to the understanding, diagnosis, and treatment of vascular thrombosis.

Materials and Methods

Thrombin substrate synthesis: Thrombin and a control peptide substrate were synthesized by using an automatic peptide synthesizer (PS3, Rainin, Woburn, MA) with Fmoc (9-fluorenylmethyloxycarbonyl) chemistry. HBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate)/HOBT (N-hydroxybenzotriazole) were used as the activating agents for each coupling cycle. Amino acids, Fmoc-Cys(Trt), Fmoc-Lys(Dde), Fmoc-Gly, Fmoc-Ser, Fmoc-Arg(Pbf), Fmoc-D-Phe, Fmoc-Pro, Boc-Gly, Rink amide MBHA, and coupling reagents for peptide synthesis were purchased from Calbiochem-NovaBiochem (San Diego, CA) (Trt = triphenylmethyl, Dde = (4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl, Pbf = 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl, Boc = benzyloxycarbonyl, MBHA = 4-methylbenzhydrylamine). Fmoc-Pipecolic acid (Pip) was obtained from Advanced Chemtech (Louisville, KY). The sequence of the thrombin substrate was Gly-D-Phe-Pip-Arg-Ser-Gly-Gly-Gly-Gly-Lys(FITC)-Cys-NH_2,[15] and that of the control peptide was Gly-D-Phe-Pip-Arg-Pro-Gly-Gly-Gly-Gly-Lys(FITC)-Cys-NH_2.[16] Peptides were labeled with a fluorescein tag (FITC) at their C terminus for convenient quantification. In the control peptide, proline was exchanged for serine in the P1′ position. Fluorescently labeled peptides were synthesized by using a previously reported method.[7] Following HPLC purification, the molecular mass was determined by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). MS $[M+H]^+$: Thrombin substrate peptide: 1425.5 (calcd), 1425.5 (found); control peptide: 1435.5 (calcd), 1436.2 (found).

Thrombin cleavage assay: Substrate peptide selectivity was tested by an HPLC assay. The thrombin or control peptide (10 μM) was incubated with 10 units of human thrombin (Calbiochem, San Diego, CA) in PBS buffer (200 μL). At different time points, a portion of the reaction solution was removed, and cleavage was quenched with trifluoroacetic acid (TFA; 0.1 %, pH 3.0). The analysis was performed in a Prostar HPLC system (Varian) with a reverse-phase C-18 column (Microsorb-MV, Varian, Walnet Creek, CA) and using 0.1 % TFA in acetonitrile as the elution buffer.

NIRF probe synthesis: The NIRF probes were prepared using a similar approach to that previously described.[7] Briefly, a protected graft copolymer (PGC, $M_W = 500$ kD) consisting of a 35 kD poly-L-lysine backbone and multiple 5 kD methoxypolyethylene glycol side chains served as a delivery vehicle.[10] PGC was reacted with a large excess of iodoacetyl anhydride to convert all remaining amino groups into iodol groups. Specific peptides were then attached through a thiol-specific reaction. Following conjugation, the monoreactive Cy5.5 dye (Amersham-Pharmacia, Piscataway, NJ) was attached to the N terminus of the coupled substrates. The loading of NIRF dye to PGC was quantified by absorption measurements, with an extension coefficient of $250 \times 10^3 \text{M}^{-1}\text{cm}^{-1}$ for Cy 5.5 at 675 nm. On average, each PGC molecule contained 23 NIR fluorochromes.

NIRF probe activation and inhibition in buffer: The thrombin or control probe (0.5 μM, 200 μL in PBS) was incubated with or without thrombin (1 Unit) at room temperature in a 96-well plate (Nalge Nunc, Rochester, NY). The NIRF signal was measured by using a fluorescence microplate reader (SPECTRAmax Gemini, Molecular Devices, Sunnyvale, CA). The excitation and emission wavelengths were set at 675 and 694 nm, respectively.

Thrombin probe activation was detected with various amounts of thrombin with or without recombinant hirudin (lepirudin; Aventis, Strasbourg, France), a thrombin-specific inhibitor. To each well, the thrombin-probe solution (0.5 μM, 200 μL in PBS) was incubated with 1, 0.1, 0.01, or 0 Units of thrombin. In a separate well, premixed thrombin (1 Unit), hirudin (5 μg), and PBS solution (20 μL) were added. The activation of the probe was monitored as previously described.

Thrombin specificity: To determine the specificity of thrombin-probe activation by thrombin, the thrombin, control, cathepsin B,[6] and cathepsin D probes[7, 8] were mixed with exogenous thrombin. Each probe (200 μL, 0.5 μM, in PBS) was incubated with or without 1 Unit of human thrombin for 10 minutes. Light and NIRF images were acquired by using a home-built imaging system.[12] The system contained a bandpass filter at 610 – 650 nm and an emission bandpass filter at 680 – 720 nm (Omega Optical, Brattlebore, VT). Images were detected by a 12-bit monochrome CCD camera equipped with a 12.5 – 75 mm zoom lens (Kodak, Rochester, NY). NIRF image acquisition time was 1/100 s, and the images were analyzed with commercially available software (Kodak Digital Science 1D software, Kodak, Rochester, NY).

NIRF probe activation in human blood: Antecubital venous blood was obtained by phelobotomy from a healthy male volunteer. Blood samples were collected in a test tube with 3.2 % sodium citrate as an anticoagulant. In a clear-bottom 96-well plate (Corning, Corning, NY), a mixture of blood (200 μL) and NIRF probe (0.25 nmole in 2.5 μL water) was pipetted into each well. After 30 minutes incubation of the NIRF probe with blood, 5 Units of exogenous thrombin were added. Light and NIRF images were acquired as described above.

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