Novel Factor XIII Probes for Blood Coagulation Imaging

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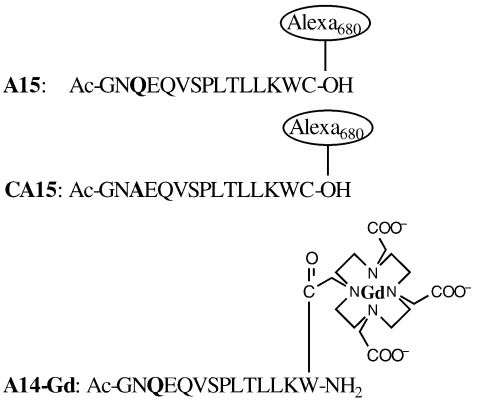
KEYWORDS:

blood coagulation · factor 13 · fluorescent probes · imaging agents · transglutaminase

Thrombosis plays a central role in most cardiovascular diseases, which include myocardial infarction, pulmonary embolism, and deep venous thrombosis.[1] A better ability to diagnose these potentially life-threatening conditions is necessary to minimize the associated morbidity and mortality.[2–4] Thrombosis starts with the activation of thrombin, which converts soluble fibrinogen to fibrin.[5] Thrombin initiates fibrin assembly and the polymerized fibrin is further crosslinked by activated blood coagulation factor XIII (FX13), a transglutaminase.[6, 7] FX13 is a key player in the final stages of blood coagulation; it provides the clot with sufficient stiffness and the necessary resistance against thrombolytic enzymes by incorporating plasmin inhibitors into the crosslinked clot.

In the study described herein, we designed new thrombosis-specific diagnostic probes based on the FX13 transglutaminase activity and previously identified peptide substrates. The specific peptide sequence, N_{13}QEQVSPLTLLK_{24}, extracted from α2-antiplasmin (α2AP), the primary inhibitor of plasmin-mediated fibrinolysis,[8, 9] was used as the primary template for imaging probe synthesis. It has been shown previously that this peptide crosslinks with fibrin in a clot in a specific manner.[8] FX13 catalyzes the formation of a covalent bond between the glutamine residue at position 14 in one fibrin chain and the ε-amino group of a lysine residue in a different chain. The glutamine residue at position 16 is significantly less reactive than glutamine 14. We hypothesized that an effective imaging probe could be covalently attached to blood clots. In particular, two types of imaging probes were synthesized for imaging of blood coagulation: one is a near-infrared (NIR) fluorescence probe and the other is a gadolinium (Gd)-chelating magnetic resonance (MR) probe.

To prepare the NIR fluorescence probe (A15), an acetyl glycine group was added to the N terminus of the α2AP fragment selected for capping. In addition, a tryptophan residue and a cysteine residue were added to the C terminus for quantitation and conjugation purposes, respectively. The peptide was synthesized by solid-phase peptide synthesis, characterized by mass spectrometry (calcd: 1701.9; found: $[M+1]^+$ 1701.6), and then reacted with a sulfhydryl reactive fluorochrome (Alexa Fluor 680C2 maleimide; Molecular Probes, Eugene, OR) through the C-terminal cysteine sidechain, in sodium acetate buffer, pH 6.5 (Scheme 1). The HPLC-purified final product, A15, had excitation and emission wavelengths of 679 and 702 nm, respectively. A control probe (CA15) only modified by replacing glutamine residue at position 14 with an alanine residue was also synthesized.



Scheme 1. *Sequences and structures of FX13 probes.*

The incorporation of an NIR fluorescence probe into a blood clot was studied with the use of mouse blood. After incubating the A15 or CA15 probe (1 μM) with the freshly withdrawn mouse blood for 2 h, the clot was homogenized and washed extensively with phosphate buffered saline (PBS). The clots were then imaged by using a laboratory-built imaging system with 630 + 15 nm excitation and 700 + 20 nm emission.[10] An intense fluorescence signal was observed for the clot incubated with the A15 probe, 11-fold higher than the background emission of the blood (average result from three clots; Figure 1). In comparison, the clot incubated with the CA15 probe showed a significantly weaker fluorescent signal and less than 3-fold signal enhancement could be observed. This result suggests that the glutamine residue at position 14 is critical for the FX13 crosslinking reaction. Once the amide group on the glutamine sidechain was eliminated, the amount of probe attached to the fibrin dropped significantly. The weak fluorescence observed with the CA15 probe may come from less efficient crosslinking through the second glutamine residue at position 16 and/or traced trapped probes.

One potential problem of using light in blood imaging is background absorbance, which is also a problem in other in vivo optical imaging.[11] Hemoglobin and deoxyhemoglobin, the major components of blood, absorb strongly below 650 nm, so the commonly used fluorochromes in the visible range (< 650 nm) are not ideal for blood imaging. Thus, the NIR fluorochrome, which emits between 700 and 900 nm, was used to overcome this limitation, as reported herein and in prior studies.[12–15]

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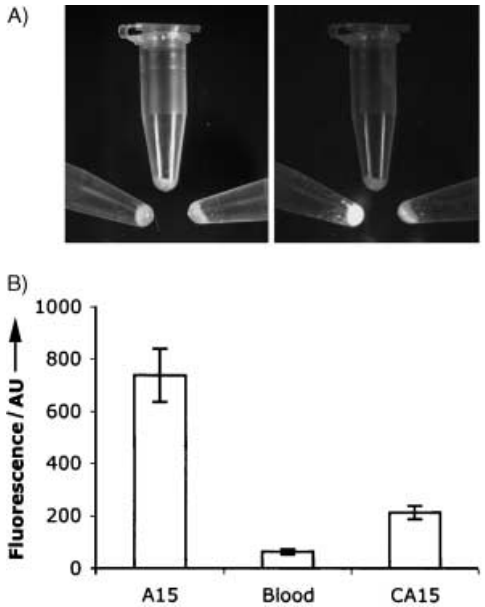
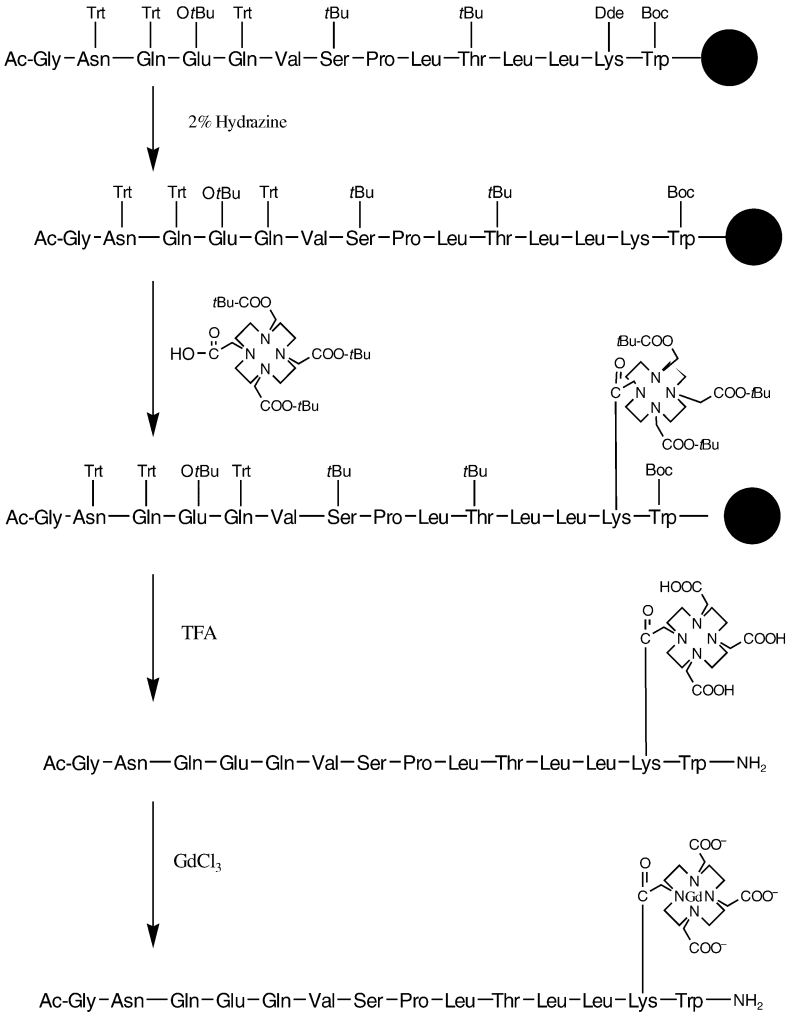


Figure 1. A) Light (left) and NIRF (right) images of a blood clot with A15, no probe, and CA15 (clockwise). B) Fluorescence intensity of clots (number of clots tested = 3).



Scheme 2. Synthetic scheme for A14 – Gd.

In addition to the optical probe, a paramagnetic Gd-containing MR probe was synthesized by anchoring a macrocyclic chelator, 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*′′,*N*′′′-tetraacetic acid (DOTA), to the α2AP peptide (Scheme 2). The peptide was synthesized by using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry with a Lys(Dde) residue and other standard amino acids.[16] After automatic synthesis on the synthesizer, the lysine side chain protecting group Dde was selectively removed by treatment with 2% hydrazine. A protected DOTA analogue, 1,4,7,10-tetraazacyclododecane-1,4,7-tris(acetic acid t-butyl ester)-10-acetic acid (DOTA-3tBu; Macrocyclics, Richardson, TX), was activated by addition of *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) and coupled to the peptide on the solid support. The A14 peptide was then cleaved, purified, and characterized by mass spectrometry (calcd: 2041.6; found: $[M+1]^+$ 2041.8). Gadolinium chelation was carried out by treating $GdCl_3$ with A14 peptide in glycine hydrochloride solution, pH 3.4 at 80 °C for 1.5 hrs. The final product, A14-Gd, was further purified by HPLC and characterized by mass spectrometry (calcd: 2198.8; found: $[M+1]^+$ 2198.5).

As in the optical probe studies, the A14 – Gd probe was tested against mouse blood and it was then imaged by magnetic resonance imaging (MRI). A clinically used Gd complex with diethylenetriamine pentaacetic acid (Gd – DTPA; Magnevist, Berlex, Wayne, NJ) was also included as a control. The blood clots were prepared with A14 – Gd or Gd – DTPA as previously described. After incubation with the compounds, the clots were homogenized and extensively washed with PBS to remove unbound compounds. MR imaging was performed on a 1.5 T clinical imaging system (System 5X; General Medical Systems, Milwaukee, WI) with a 5-inch surface coil. Images were obtained by using a T1-weighted spin echo sequence with the following parameters: repetition time (TR), 400 msec; echo time (TE), 12 msec; field of view, 8 × 6 cm; a matrix of 256 × 256; slice thickness, 1.5 mm. As with the NIR fluorescence results, a significant difference was observed by MRI between the signals with and without the test compounds. The signal intensities were 230.5, 286.8, 299.5, and 483.1 for the blood clot alone and blood clots incubated with 50 μM Gd – DTPA, 30 mM Gd – DTPA, and with 50 μM A14 – Gd, respectively (Figure 2). These results suggest that A14 – Gd crosslinked with fibrin and the retained Gd

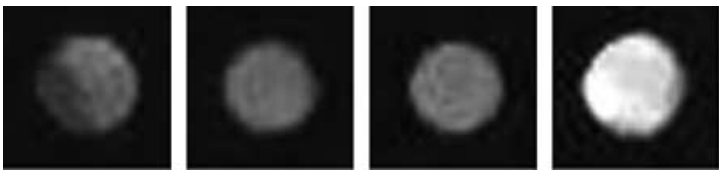


Figure 2. MR images of blood clots. After incubation with no probe, 50 μM Gd – DTPA, 30 mM Gd – DTPA, or 50 μM A14 – Gd (from left to right), the clots were homogenized and extensively washed with PBS to remove unbound compounds. A 1.5-T clinical MR imaging system was used with T1 values of 230.5, 286.8, 299.5, and 483.1, respectively.

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contributed to the dramatic T1 relaxivity changes. Gd–DTPA alone did not provide any meaningful enhancement of the signal, even when a 600-fold excess was used in the experiment.

In conclusion, we have developed new types of imaging probes that have the potential to be used for in vivo imaging of blood coagulation. The specific peptide derived from α2AP labeled with various reporter groups can be covalently attached to fibrin by blood coagulation factor FX13 through transglutamination. Theoretically, the same approach could be applied to other transglutaminases, which are widely found in generic tissue stabilization and also contribute to a variety of diseases, such as cancer, neurodegenerative diseases, and celiac disease.[17, 18]

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[1] R. Corti, M. E. Farkouh, J. J. Badimon, *Am. J. Med.* **2002**, *113*, 668–680.
[2] M. S. Johnson, *J. Vasc. Interv. Radiol.* **2002**, *13*, 13–23.
[3] S. Falati, P. Gross, G. Merrill-Skoloff, B. C. Furie, B. Furie, *Nature Med.* **2002**, *8*, 1175–1181.
[4] R. Taillefer, *Semin. Nucl. Med.* **2001**, *31*, 102–123.
[5] M. W. Mosesson, K. R. Siebenlist, D. A. Meh, *Ann. NY Acad. Sci.* **2001**, *936*, 11–30.
[6] L. Lorand, *Ann. NY Acad. Sci.* **2001**, *936*, 291–311.
[7] L. Muszbek, V. C. Yee, Z. Hevessy, *Thromb. Res.* **1999**, *94*, 271–305.
[8] B. R. Robinson, A. K. Houng, G. L. Reed, *Circulation* **2000**, *102*, 1151–1157.
[9] K. N. Lee, C. S. Lee, W. C. Tae, K. W. Jackson, V. J. Christiansen, P. A. McKee, *Ann. NY Acad. Sci.* **2001**, *936*, 335–339.
[10] U. Mahmood, C. H. Tung, A. Bogdanov, Jr., R. Weissleder, *Radiology* **1999**, *213*, 866–870.
[11] V. Ntziachristos, j. Ripoll, R. Weissleder, *Opt. Lett.* **2002**, *27*, 333–335.
[12] C. H. Tung, R. E. Gerszten, F. A. Jaffer, R. Weissleder, *ChemBioChem* **2002**, *3*, 207–211.
[13] F. A. Jaffer, C. H. Tung, R. E. Gerszten, R. Weissleder, *Arterioscler. Thromb. Vasc. Biol.* **2002**, *22*, 1929–1935.
[14] V. Ntziachristos, C. H. Tung, C. Bremer, R. Weissleder, *Nature Med.* **2002**, *8*, 757–760.
[15] C. H. Tung, Y. Lin, W. K. Moon, R. Weissleder, *ChemBioChem* **2002**, *3*, 784–786.
[16] R. Bhorade, R. Weissleder, T. Nakakoshi, A. Moore, C. H. Tung, *Bioconjugate Chem.* **2000**, *11*, 301–305.
[17] S. Y. Kim, T. M. Jeitner, P. M. Steinert, *Neurochem. Int.* **2002**, *40*, 85–103.
[18] A. J. Cooper, T. M. Jeitner, J. P. Blass, *Neurochem. Int.* **2002**, *40*, 1–5.

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A New Furoxan NO-Donor Rabeprazole Derivative and Related Compounds

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The design of hybrid molecules by combining appropriate pharmacophoric groups with NO-releasing moieties is a promising approach to the production of new drugs with interesting potential for treating a variety of diseases.[1–3] Our research group has been active in this field and we have designed several such products.[1] These compounds include NO-donor nonsteroidal antiinflammatory drugs (NSAIDs)[4, 5] and NO-donor H_2-receptor antagonists.[6] We pursued this line because nitric oxide ($NO^{\bullet}$) has protective effects on the gastric mucosa through a number of mechanisms, such as promotion of mucus secretion, increased mucosal blood flow and decreased adherence of neutrophils to the gastric vascular endothelium.[7] In confirmation of this idea, we herein describe the new hybrid **12**, obtained by joining the 4-alkoxy-3-phenylsulfonylfuroxan substructure to rabeprazole (**1**; Scheme 1). It is known that furoxans are able to release NO at physiological pH, in the presence of thiol cofactors. The mechanism of this release appears to be complex and may

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