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Discrete Bimodal Probes for Thrombus Imaging

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Abstract

Here we report a generalizable solid/solution phase strategy for the synthesis of discrete bimodal fibrin-targeted imaging probes. A fibrin-specific peptide was conjugated with two distinct imaging reporters at the C- and N-terminus. In vitro studies demonstrated retention of fibrin affinity and specificity. Imaging studies showed that these probes could detect fibrin over a wide range of probe concentrations by optical, magnetic resonance, and positron emission tomography imaging.

Keywords

fibrin; gadolinium; copper; positron emission tomography; magnetic resonance imaging; fluorescence

Thrombosis, the formation of a blood clot within an artery or vein, is implicated in many of the leading causes of death worldwide: heart attack, stroke, pulmonary embolism, and deep vein thrombosis.¹ The *in vivo* detection and characterization of thrombus is essential for disease diagnosis, for guiding therapy and for monitoring treatment. Several imaging modalities can be used for noninvasive identification of thrombus in a clinical or preclinical setting, and these include, positron emission tomography (PET), magnetic resonance (MR) imaging, and optical imaging (OI). Each modality has its own strengths and weaknesses; therefore combinations of modalities are increasingly used to provide synergies.^{2–5} Concurrently, multimodal imaging probes are being developed to exploit the gains in hybrid imaging systems for the improved detection of disease.^{6–9} We present here thrombus-targeted imaging probes employing positron-emitters, MR relaxation agents and fluorophores.

For molecular imaging of thrombus-related biomarkers, fibrin offers the potential for high disease specificity and sensitivity. This is because fibrin is present in all thrombi but is only found in clot and not in circulating blood. Discrete peptide-based probes,^{10–17} antibodies,¹⁸ and nanoparticles^{19–24} have been used to image fibrin. For imaging solid thrombi, peptide-based probes may be more advantageous than antibodies or nanoparticles because their small size offers higher potential to penetrate the clot. However, unlike nanoparticles which are insensitive to the incorporation of multiple imaging reporters, it is a challenge to modify a short peptide with two imaging reporters and still maintain target affinity and specificity.⁹ For instance, the incorporation of hydrophobic fluorescent dyes may induce non-specific protein binding.²⁵ We reasoned that conjugation of a hydrophilic metal complex might

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Supporting Information Available: Experimental procedures, probe synthesis and characterization, details of fibrin binding, relaxivity, and imaging measurements are provided in supporting information. This material is available free of charge via the internet at <http://pubs.acs.org>.

offset the hydrophobicity introduced by the organic dye. In order to minimize the impact of the imaging reporters on fibrin binding, we positioned each reporter at the N- and C-terminus of the peptide. To this end, we describe fluorescent/MR and fluorescent/PET labeled peptides that maintain fibrin affinity and specificity after derivatization.

In our previous work, we noted that derivatizing both the C- and N-terminus of fibrin-specific peptides with Gd chelates not only increased detection sensitivity, but also protected those probes from exopeptidase degradation.²⁶ Peptide substitutions at the C- and N-termini were relatively insensitive to fibrin affinity, presumably because of the conformational rigidity imparted by the cyclic peptide core. To introduce different imaging reporters, we reasoned that a mixed solid/solution phase synthetic approach could be used to incorporate a fluorophore (OI) at one terminus and a chelator for Gd (MRI) or ⁶⁴Cu (PET) at the other terminus. Probe synthesis is outlined in Scheme 1 where the peptide and N-terminal modification are synthesized on resin. After resin cleavage, deprotection, and cyclization, the C-terminus is modified in solution. Complexation with Gd³⁺ or ⁶⁴Cu²⁺ is the final synthetic step.

In our probe design we employed the established fibrin-specific cyclic peptide used in the MR thrombus agent EP-2104R, and maintained the modification with *p*-xylenediamine to introduce a primary amine at the C-terminus.¹⁴ The isothio-cyanate of fluorescein (FITC) was used to incorporate a fluorescent reporter at the C-terminus. For attachment of FITC at the N-terminus it was necessary to introduce a linker, since direct thiourea bond formation at the N-terminus is not stable under the peptide deprotection conditions. The tris(*t*-butyl) ester of DOTA was used to couple the chelate as a monoamide to either the C- or N-terminus. After removal of the *t*-butyl protecting groups, either gadolinium(III) or copper(II) was introduced. The probes are denoted FI-^NPep-Gd, etc where FI is fluorescein, Gd is GdDOTA-monoamide, Cu is CuDOTA-monoamide, and ^NPep refers to the peptide; in the example FI-^NPep-Gd, the fluorescein is conjugated to the N-terminus. Full synthetic details are described in the supporting information.

Binding of the probes to human fibrin was assessed in a plate-based assay as previously described.^{10,14} In this assay, a fibrinogen solution is aliquoted into a 96-well plate and then polymerized to fibrin by addition of CaCl₂ and thrombin. The fibrin gel is then dried onto the base of the plate by evaporation at 37 °C. Rehydration with an equivalent volume of liquid results in a known concentration of polymeric fibrin based on fibrinogen monomer. Solutions of Gd-^NPep-FI, Cu-^NPep-FI and FI-^NPep-Gd ranging from 0.1 to 50 μM in either tris buffered saline (TBS) or citrated rat plasma were added to each of the wells of a dried fibrin (7 μM) microtiter plate. After 2 h incubation, the concentration of free probe, [free], was assayed by ICP-MS or by fluorescence-HPLC. The concentration of fibrin-bound species, [bound], in the clot was determined by subtraction ([bound] = [total] – [free]). The binding data were fit to a stoichiometric binding model with stepwise association constants K₁, K₂ and K₃ (Figure S1). The affinities of Gd-^NPep-FI and FI-^NPep-Gd for human fibrin (Table 1) were similar and comparable to EP-2104R. Not surprisingly, replacement of Gd(III) with Cu(II) did not significantly change the fibrin affinity of the probe.

One concern with using organic fluorophores for *in vivo* applications is the potential for non-specific binding of the hydrophobic fluorophore. Non-specific protein binding would adversely impact the distribution of the probe and its specificity for fibrin *in vivo*. In our previous work with this type of peptide, we found that peptide derivatives bind to two equivalent binding sites on fibrin.^{10,14} When fluorescein is incorporated into the molecule, there is a third, weaker fibrin binding event that we attribute to a non-specific interaction. We were concerned that non-specific binding to other proteins could lower the effective affinity of the probe for fibrin, and so we measured fibrin affinity in the presence of blood

plasma proteins. However the apparent fibrin affinity of Gd-^NPep-Fl measured in rat plasma was only 2–3 fold lower than when measured in buffer (Table 1), indicating that this probe maintains high specificity for fibrin.

Relaxivities of the Gd-containing probes as well as the commercial contrast agent [Gd(HP-DO3A)(H₂O)] (Pro-Hance®, gadoteridol) were measured at 1.4T, 37 °C in either pH 7.4 TBS, 30 μM fibrinogen solution in TBS, or 30 μM fibrin gel in TBS (Table 2). The relaxivities in TBS are much higher than for small Gd(III) complexes such as [Gd(HP-DO3A)(H₂O)], and this relaxivity increase is due to the increased molecular weight resulting in slower rotational diffusion. Protein binding further decreases the correlation time and results in increased relaxivity.²⁷ This is reflected by the 50% higher relaxivity of Fl-^NPep-Gd in fibrin gel compared to buffer alone. On the other hand there is little to no increase in relaxivity observed in fibrinogen solution implying that these probes do not bind fibrinogen as was seen previously with this peptide sequence. The lack of fibrinogen binding is important since there is ~7 μM fibrinogen in circulating blood plasma. The specificity of the probes for fibrin over the structurally similar fibrinogen precursor may be expected to enhance target:background *in vivo*. In addition, there was no change in the relaxivity of [Gd(HP-DO3A)(H₂O)] when measured in TBS, fgn, or fibrin gel suggesting no protein binding, and indicating that the mechanical properties of the gel do not affect relaxivity. We note that the relaxivities for the probes in the presence of fibrin are quite high (21.2 mM⁻¹s⁻¹ for Gd-^NPep-Fl and 18.8 Fl-^NPep-Gd) at this clinically relevant field strength and comparable to other high relaxivity compounds such as fibrin-bound EP-2104R and albumin-bound MS-325 at 1.4T.^{14,28} Relaxivity will decrease with increasing field strength but based on the field dependence of the related molecules,²⁹ we expect higher relaxivity than simple Gd(III) chelates at fields up to 9.4T.

The relaxivity results were corroborated by imaging studies on a clinical 1.5T MRI scanner. Here we compare the commercial contrast agent [Gd(HP-DO3A)(H₂O)] with equimolar Gd-^NPep-Fl. Figure 1 shows T1-weighted MR images of 6 tubes containing water, fibrinogen solution, or fibrinogen solution that has been polymerized to fibrin gel and then separated by mechanical agitation. Tubes A and B contain no gadolinium and represent the baseline signal intensity. Tubes C and D contain 25 μM [Gd(HP-DO3A)(H₂O)] in fibrinogen or fibrin gel, respectively. There is a slight enhancement in signal (16.5%) compared to tube B due to the dilute, low relaxivity contrast agent. However the enhancement is uniform in both C and D, indicating that [Gd(HP-DO3A)(H₂O)] cannot distinguish the fibrin clot on the wall of tube D from the unclotted solution. Tube E shows equimolar Gd-^NPep-Fl in fibrinogen solution and the signal enhancement (59% compared to tube B) is much greater than [Gd(HP-DO3A)(H₂O)] due to the higher relaxivity of Gd-^NPep-Fl. Tube F shows that when a fibrin clot is prepared and separated, that Gd-^NPep-Fl localizes in the clot and generates high clot signal intensity (156% increased compared to tube B) and strong contrast between the clot and the supernatant with a very high contrast to noise ratio of 39.

We performed a similar experiment using a IVIS fluorescence spectrum imager. Figure 2 compares 50 nM Gd-^NPep-Fl to equimolar fluorescein in either fibrinogen solution or in a separated fibrin clot. In this study, fibrinogen was clotted (Tubes B and C) and the tubes were centrifuged to separate the clot from the supernatant as apparent from the photograph of the tubes (Figure 2, top). The fluorescence image overlayed on the photograph clearly shows that Gd-^NPep-Fl localizes in the clot and is depleted from the supernatant whereas untargeted fluorescein is evenly distributed between the clot and the supernatant. In Figure 2E we show a fluorescence confocal microscopy image of a fibrin gel in the presence of Gd-^NPep-Fl. The probe highlights the web-like fibrin mesh at the microscopic level.

Finally we compared the Cu-64 versions of both probes to ^{64}Cu DOTA in a similar experimental paradigm, using PET imaging on a clinical PET scanner. The images in Figure 3 demonstrate that the dual probes can visualize fibrin with PET imaging. For the fibrinogen solutions (Figure 3A, 3C, 3E), the ^{64}Cu is distributed equally throughout the samples giving rise to uniform signal intensity. When the fibrinogen is clotted and the tube spun down (tube B), there was no difference in the PET image between tubes A and B indicating that untargeted ^{64}Cu DOTA cannot be used to detect fibrin. The ^{64}Cu DOTA is distributed equally in the clot, at the bottom of tube B, and in the liquid above the clot. The ^{64}Cu DOTA experiment also demonstrates that clot formation does not physically trap the molecule. On the other hand, the fibrin-targeted dual probes both demonstrate an ability to detect fibrin. Tubes D and F indicate that radioactivity is centered in the bottom of each tube, i.e. in the clot, and very little activity remains in the liquid above the clot.

In conclusion, we have described four new fibrin-targeting probes that can be modified to be detectable in multiple modalities and still maintain fibrin affinity and specificity. These bimodal probes offer the opportunity for fibrin imaging over a range of spatial resolutions (OI > MRI > PET) and detection sensitivities (PET > OI > MRI). This proof-of-concept study utilized inexpensive reagents; however our mixed solid/solution phase synthesis is generalizable and amenable to incorporating any bifunctional chelator or fluorophore using standard amine bioconjugation techniques. Combinations of labels may have important applications where high sensitivity, low resolution PET imaging can detect the presence of thrombus throughout the body and then be used to guide where high resolution MR or catheter-based optical imaging should be performed. For instance, one such clinical application could be to detect the presence of coronary artery thrombus and guide stent placement.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Makuc DM, Marcus GM, Marelli A, Matchar DB, Moy CS, Mozaffarian D, Mussolino ME, Nichol G, Paynter NP, Soliman EZ, Sorlie PD, Sotoodehnia N, Turan TN, Virani SS, Wong ND, Woo D, Turner MB. *Circulation*. 2012; 125:188. [PubMed: 22215894]
2. Beyer T, Freudenberg LS, Czernin J, Townsend DW. *Insights Imaging*. 2011; 2:235. [PubMed: 22347950]
3. Catana C, Procissi D, Wu Y, Judenhofer MS, Qi J, Pichler BJ, Jacobs RE, Cherry SR. *Proc Natl Acad Sci U S A*. 2008; 105:3705. [PubMed: 18319342]
4. Schlemmer HP, Pichler BJ, Schmand M, Burbar Z, Michel C, Ladebeck R, Jattke K, Townsend D, Nahmias C, Jacob PK, Heiss WD, Claussen CD. *Radiology*. 2008; 248:1028. [PubMed: 18710991]
5. Townsend DW. *J Nucl Med*. 2008; 49:938. [PubMed: 18483101]
6. Frullano L, Catana C, Benner T, Sherry AD, Caravan P. *Angew Chem Int Ed Engl*. 2010; 49:2382. [PubMed: 20191650]

7. Frullano L, Meade TJ. *J Biol Inorg Chem*. 2007; 12:939. [PubMed: 17659368]
8. Jaffer FA, Libby P, Weissleder R. *Arterioscler Thromb Vasc Biol*. 2009; 29:1017. [PubMed: 19359659]
9. Kuil J, Velders AH, van Leeuwen FW. *Bioconjug Chem*. 2010; 21:1709. [PubMed: 20812730]
10. Kolodziej AF, Nair SA, Graham P, McMurry TJ, Ladner RC, Wescott C, Sexton DJ, Caravan P. *Bioconjug Chem*. 2012; 23:548. [PubMed: 22263840]
11. Makowski MR, Forbes SC, Blume U, Warley A, Jansen CH, Schuster A, Wiethoff AJ, Botnar RM. *Atherosclerosis*. 2012; 222:43. [PubMed: 22284956]
12. Miserus RJ, Herias MV, Prinzen L, Lobbes MB, Van Suylen RJ, Dirksen A, Hackeng TM, Heemskerk JW, van Engelshoven JM, Daemen MJ, van Zandvoort MA, Heeneman S, Kooi ME. *JACC Cardiovasc Imaging*. 2009; 2:987. [PubMed: 19679287]
13. Nair SA, Kolodziej AF, Bhole G, Greenfield MT, McMurry TJ, Caravan P. *Angew Chem Int Ed Engl*. 2008; 47:4918. [PubMed: 18496805]
14. Overoye-Chan K, Koerner S, Looby RJ, Kolodziej AF, Zech SG, Deng Q, Chasse JM, McMurry TJ, Caravan P. *J Am Chem Soc*. 2008; 130:6025. [PubMed: 18393503]
15. Uppal R, Ay I, Dai G, Kim YR, Sorensen AG, Caravan P. *Stroke*. 2010; 41:1271. [PubMed: 20395615]
16. Uppal R, Catana C, Ay I, Benner T, Sorensen AG, Caravan P. *Radiology*. 2011; 258:812. [PubMed: 21177389]
17. Zhang Z, Kolodziej AF, Greenfield MT, Caravan P. *Angew Chem Int Ed Engl*. 2011; 50:2621. [PubMed: 21370351]
18. Morris TA, Gerometta M, Yusen RD, White RH, Douketis JD, Kaatz S, Smart RC, Macfarlane D, Ginsberg JS. *Am J Respir Crit Care Med*. 2011; 184:708. [PubMed: 21680946]
19. Cai K, Kiefer GE, Caruthers SD, Wickline SA, Lanza GM, Winter PM. *NMR Biomed*. 2012; 25:279. [PubMed: 21751273]
20. McCarthy JR, Patel P, Botnaru I, Haghighyeghi P, Weissleder R, Jaffer FA. *Bioconjug Chem*. 2009; 20:1251. [PubMed: 19456115]
21. Pan D, Caruthers SD, Hu G, Senpan A, Scott MJ, Gaffney PJ, Wickline SA, Lanza GM. *J Am Chem Soc*. 2008; 130:9186. [PubMed: 18572935]
22. Pan D, Caruthers SD, Senpan A, Yalaz C, Stacy AJ, Hu G, Marsh JN, Gaffney PJ, Wickline SA, Lanza GM. *J Am Chem Soc*. 2011; 133:9168. [PubMed: 21599030]
23. Pan D, Pramanik M, Senpan A, Yang X, Song KH, Scott MJ, Zhang H, Gaffney PJ, Wickline SA, Wang LV, Lanza GM. *Angew Chem Int Ed Engl*. 2009; 48:4170. [PubMed: 19418503]
24. Pan D, Roessl E, Schlomka JP, Caruthers SD, Senpan A, Scott MJ, Allen JS, Zhang H, Hu G, Gaffney PJ, Choi ET, Rasche V, Wickline SA, Proksa R, Lanza GM. *Angew Chem Int Ed Engl*. 2010; 49:9635. [PubMed: 21077082]
25. Choi HS, Nasr K, Alyabyev S, Feith D, Lee JH, Kim SH, Ashitate Y, Hyun H, Patonay G, Strekowski L, Henary M, Frangioni JV. *Angew Chem Int Ed Engl*. 2011; 50:6258. [PubMed: 21656624]
26. Zhang Z, Kolodziej AF, Qi J, Nair SA, Wang X, Case AW, Greenfield MT, Graham PB, McMurry TJ, Caravan P. *New J Chem*. 2010; 2010:611.
27. Caravan P, Ellison JJ, McMurry TJ, Lauffer RB. *Chem Rev*. 1999; 99:2293. [PubMed: 11749483]
28. Caravan P, Cloutier NJ, Greenfield MT, McDermid SA, Dunham SU, Bulte JW, Amedio JC Jr, Looby RJ, Supkowski RM, Horrocks WD Jr, McMurry TJ, Lauffer RB. *J Am Chem Soc*. 2002; 124:3152. [PubMed: 11902904]
29. Caravan P, Farrar CT, Frullano L, Uppal R. *Contrast Media Mol Imaging*. 2009; 4:89. [PubMed: 19177472]

Figure 1. MR Imaging of Fibrinogen (fgn) and Fibrin Clots

1-mL centrifuge tubes were scanned on a clinical 1.5T MRI at room temperature. Top row shows axial slices corresponding to the point indicated by the line through the coronal images (bottom row). A) pure water; B) 15 μ M fgn; C) 25 μ M [Gd(HP-DO3A)] in 15 μ M fgn; D) 25 μ M [Gd(HP-DO3A)] in 15 μ M fibrin gel; E) 25 μ M Gd-^NPep-FI in 15 μ M fgn; F) 25 μ M Gd-^NPep-FI in 15 μ M fibrin gel.

Figure 2. Fluorescence Images of Fibrinogen (fgn) and Fibrin Clots

Phantoms (A–D) were scanned on an IVIS Spectrum imager. Fluorescence images (bottom) are overlaid on photographs (top) of 1-mL glass tubes containing 1-mL of A) 50 nM Gd-^NPep-Fl in 6 μ M fgn; B) 50 nM Gd-^NPep-Fl in 6 μ M fibrin gel; C) 50 nM fluorescein in 6 μ M fibrin gel D) 50 nM fluorescein in 6 μ M fgn. E) Fluorescence confocal microscopy image of fibrinogen polymerized on a microscopy slide in the presence of Gd-^NPep-Fl.

Figure 3. PET Imaging of Fibrin Clots

Phantoms in 4 mL glass culture tubes were scanned on a clinical PET head scanner. Each phantom contained fibrinogen (fgn) at a final concentration of 6 μM , 2.3 nmol of peptide or DOTA, a total volume of 2 mL, and a total activity of 10 μCi . A) ^{64}Cu DOTA in fgn; B) ^{64}Cu DOTA in fibrin gel; C) Fl- $^{\text{N}}$ Pep- ^{64}Cu in fgn; D) Fl- $^{\text{N}}$ Pep- ^{64}Cu in fibrin gel E) ^{64}Cu - $^{\text{N}}$ Pep-Fl in fgn; F) ^{64}Cu - $^{\text{N}}$ Pep-Fl in fibrin gel.

Scheme 1. Multimodal Probe Synthesis

Cartoon depicting mixed solid/solution phase synthesis of multimodal probes. See Supporting Information for full synthetic details.

Table 1**Fibrin Association Constants**

Stepwise association constants (μM^{-1}) for bimodal probes binding to human fibrin measured in buffer (B) or blood plasma (P). Standard deviations in parentheses. NA= not applicable.

Compound	K_1	K_2	K_3
Fl-^NPep-Gd (B)	0.89 (0.25)	0.23 (0.09)	0.018 (0.006)
Gd-^NPep-Fl (B)	1.00 (0.34)	0.19 (0.11)	0.23 (0.11)
Gd-^NPep-Fl (P)	0.53 (0.08)	0.064 (0.029)	0.05 (0.034)
Cu-^NPep-Fl (B)	0.75 (0.29)	0.34 (0.18)	0.14 (0.06)
EP-2104R	1.01 (0.39)	0.18 (0.06)	NA

Table 2
Relaxivity of Gd-containing Probes

Relaxivities ($\text{mM}^{-1}\text{s}^{-1}$) were measured at 1.4T, 37 °C in either pH 7.4 TBS, 30 μM fibrinogen (fgn) solution in TBS, or 30 μM fibrin gel in TBS. Uncertainties estimated at $\pm 10\%$.

Compound	r_1 (TBS)	r_1 (fgn)	r_1 (fibrin)
Fl- ^N Pep-Gd	12.5	11.4	18.8
Gd- ^N Pep-Fl	16.4	17.6	21.2
[Gd(HP-DO3A)(H ₂ O)]	3.00	3.04	3.04