Chapter A4

Overcoming the Limitations in Thrombosis Treatment:

A Bifunctional Chelator as Positron Emission Tomography-Imaging Probe for Detecting Blood Clots

Sarah Spreckelmeyer,\textsuperscript{a,b} Frank M. Lee,\textsuperscript{c} Ed Pryzdial\textsuperscript{c} and Chris Orvig\textsuperscript{a}

\textsuperscript{a} Medicinal Inorganic Chemistry Group, Department of Chemistry, University of British Columbia, 2036 Main Mall, Vancouver, British Columbia, V6T 1Z1, Canada

\textsuperscript{b} Department of Pharmacokinetics, Toxicology and Targeting, Groningen Research Institute of Pharmacy, University of Groningen, Antonius Deusinglaan 1, Groningen 9713 AV, The Netherlands

\textsuperscript{c} Centre for Blood Research, Life Science Centre, 2350 Health Sciences Mall, Vancouver, BC, V6T 1Z3, Canada
1. Abstract

Nuclear imaging techniques such as Positron Emission Tomography (PET) are useful tools for the non-invasive detection of low concentrations of radiotracers in the body. We aimed to design a bifunctional chelator that contains the well-studied $^{68}$Ga chelator H$_2$dedpa and a thiol reactive group for conjugation to the coagulation factor FXa in order to detect and localize blood clots to facilitate surgical removal. In total, four different approaches to obtain the bifunctional chelator were investigated, three approaches using maleimide derivatives for thiol-coupling and one approach using an acrylate derivate. The maleimide functional group obtained in 2 reactions was found to be unstable under basic conditions. Moreover when we tried to synthesize a cyclic maleimide group, ring closure of the maleimide did not occur presumably due to steric hindrance of the carboxylic acids from the picolinic acid moieties. Reaction 4 yielded a promising acrylate analogue of H$_2$dedpa, which showed good reactivity with the thiol group of glutathione. Further experiments have to be conducted to confirm this reactivity and subsequently Ga$^{3+}$ chelation, radiolabeling experiments with $^{68}$Ga$^{3+}$, stability experiments in human serum as well as phospholipid binding experiments need to be performed to obtain a successful diagnostic for blood clots.
2. Introduction

Blood clots are a serious health risk due to their ability to block blood flow and cause heart attacks and stroke. These debilitating and life threatening events are the largest healthcare burden on the globe. Several pharmaceuticals for the treatment of blood clots, based on the physiological clot-dissolving protein tissue plasmin activator (tPA) have been developed; however, these FDA-approved drugs exceed the physiological concentration of tPA by many orders of magnitude, which leads to unfavorable systemic effects and haemorrhagic risk. Despite the development of several potential new drugs, which were tested in major clinical trials, advances have been disappointing and the trend has been toward surgical extraction of the clot. Thrombectomy also has limitations, such as bleeding and difficulties in identification of clot location.¹

The hemostasis of blood has a complex mechanism for maintaining blood fluidity and conversion to insoluble gel in sites of vascular injury. Blood coagulation (Figure 1) and fibrinolysis are usually in equilibrium, constantly repairing trivial lesions in the body. Upon endothelium injury, loss of the endothelial layer activates platelets to change shape and adhesion properties to form the primary hemostatic plug. Platelets also release key clotting proteins, such as Factor V, von Willebrand Factor (vWF) and fibrinogen to promote stronger adhesion of platelets to the site of injury.² An additional flip-flop reaction that exposes negatively charged phospholipids to the outer membrane of platelets provides a surface for the generation of thrombin and fibrin. This mechanism is called secondary hemostasis, which is a carefully controlled proteolytic cascade that forms a clot. The coagulation cascade consists of an intrinsic and extrinsic pathway (not discussed in detail here, but shown for completeness in Figure 1) that both lead to activation of a key serine protease FXa. FXa cleaves prothrombin zymogen in two places, yielding its active form thrombin. Importantly, this process is facilitated by the prothrombinase complex that consists of FXa and FVa assembled on negatively charged phospholipid membranes in the presence of calcium ions. Thrombin then converts
fibrinogen to fibrin, the building block of a haemostatic plug. In addition, thrombin activates more platelets with FXIII and consequently more FXa is synthesized locally at the side of injury (Figure 1). When the concentration of FXa surpasses the threshold of physiological anticoagulants, fibrin can generate a clot. Due to its key role in coagulation, FXa is a prominent drug target for therapeutic anticoagulants and its mechanism of action is well studied.

Fibrinolysis is the counterpart of blood coagulation that breaks down the cross-linked fibrin by plasmin. Plasminogen is the zymogen of plasmin and is produced in the liver. It has an affinity for clots and is incorporated into them. Plasminogen is activated to plasmin by tissue plasminogen activator (tPA) or urokinase. tPA is released slowly into the blood stream by damaged endothelium. Additionally, plasmin stimulates the production of tPA and urokinase via a positive feedback mechanism.

![Figure 1. Pathways of the blood coagulation cascade (reproduced with permission from Haematology, 2nd edition (C.J.Pallister and M.S. Watson), © Scion Publishing Ltd.)](image)

Early detection of blood clots, and particularly at low concentrations, is challenging with the imaging techniques available
Magnetic resonance imaging (MRI), ultrasound (US) and computed tomography (CT). These techniques only look at one part of the body at a time, thereby delaying subsequent treatment and increasing the risk for complications. Nuclear imaging techniques (SPECT, PET) have a high sensitivity for the detection of low concentrations of a radiotracer and single whole-body scans can be obtained. Conjugation of radiotracers to biomolecules, which target to the object to be imaged, is a widely established method.\textsuperscript{5} Applying this approach to the detection of blood clots will help to develop new thrombolytic agents and to pinpoint clots for thrombectomy, thus improving the safety and efficacy of the treatment. Investigation of radiolabeled proteins that bind to blood clotting proteins for tracking blood clots is therefore a worthy endeavour with a great deal of potential for life-saving developments.

Thus far, different tracers have been studied for the diagnosis of blood clots. Most prominent, peptides targeting fibrin or platelet receptors were investigated with $^{99m}\text{Tc}$ (e.g. $^{99m}\text{Tc}$-HYNIC-CGPRPPC in Figure 2)\textsuperscript{6} or $^{111}\text{In}$ for SPECT imaging. In the past 10 years, tracers for PET imaging such as $^{18}\text{F}$-Fluorodeoxyglucose ($^{18}\text{FDG}$) and most recently $^{64}\text{Cu}$-fibrin-binding probe 8 ($^{64}\text{Cu}$-FBP8) coupled to 1,4,7-triazacyclononanetriacetic acid (NOTA)\textsuperscript{7,8} were investigated to detect arterial thrombosis (Figure 2). In the search for a highly localized target other than fibrin, FXa is a promising target, since FXa highly accumulates at the site of clots at the side of endothelial injury due to anionic phospholipid-binding.
Figure 2. Radiotracers discussed in this work.

To our knowledge, there is no literature published on the synthesis of an imaging tracer linked to FXa. Our group has developed numerous ligands with the ability to chelate various radiometals for different purposes. H$_2$dedpa-p-Bn-NH$_2$ (Figure 2) is well studied as a $^{67}$Ga chelator for SPECT imaging; Ga$^{3+}$ has another radioisotope, positron emitter $^{68}$Ga, which makes it suitable for PET imaging.

The aim of this study was to design a bifunctional chelator consisting of the $^{68}$Ga chelator H$_2$dedpa with a thiol reactive group, in order to conjugate it to the coagulation factor FXa via a thiol functional group inserted on FXa as published by Pryzdial et al.\textsuperscript{9} for the detection and localization of blood clots to facilitate surgical removal. Thus, we present here the synthesis approaches of bifunctional H$_2$dedpa that bears a thiol reactive functional group. Different reactions were considered, as summarized in Table 1. We selected to use reaction #5 and #8 from Table 1, since they are well known reactions in biochemistry as they can occur under physiological conditions, whereas the other reactions are only used in basic chemistry under non-physiological conditions. Furthermore the maleimide-linkers have been applied frequently and they are commercially available.\textsuperscript{10,11} The
The thiol reaction with the electron-withdrawing group (EWG) looks promising in the literature as well and is discussed below in more detail.

Table 1. Summarized thiol-reaction with $R_1$-SH as thiol$^{12}$ (EWG = electron withdrawing group, $X$ = halide)

<table>
<thead>
<tr>
<th>#</th>
<th>Functional group</th>
<th>Product</th>
<th>#</th>
<th>Functional group</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$R \equiv X$</td>
<td>$R \equiv S \equiv R_1$</td>
<td>6</td>
<td>$R \equiv$</td>
<td>$R \equiv S \equiv R_1$</td>
</tr>
<tr>
<td>2</td>
<td>$R \equiv X$</td>
<td>$R \equiv S \equiv R_1$</td>
<td>7</td>
<td>$R \equiv S \equiv O \equiv$</td>
<td>$R \equiv S \equiv S \equiv R_1$</td>
</tr>
<tr>
<td>3</td>
<td>$R \equiv X$</td>
<td>$R \equiv S \equiv S \equiv R_1$</td>
<td>8</td>
<td>$R \equiv S \equiv N \equiv$</td>
<td>$R \equiv S \equiv S \equiv R_1$</td>
</tr>
<tr>
<td>4</td>
<td>$R \equiv$</td>
<td>$R \equiv S \equiv R_1$</td>
<td>9</td>
<td>$R \equiv S \equiv S \equiv R_1$</td>
<td>$R \equiv S \equiv S \equiv R_1$</td>
</tr>
<tr>
<td>5</td>
<td>EWG</td>
<td>EWG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

These reactions illustrate the potential of thiol reactions with electron-withdrawing groups in synthetic chemistry.
3. Results and Discussion

3.1 Synthesis

Four different approaches to synthesize a thiol-reactive H$_2$dedpa analogue were attempted, summarized in Figure 3. In the following, the rationale of the different approaches is described and the results are presented.

Approach 1 was aimed to conjugate the linker smPEG4 that contains a maleimide functional group to H$_2$dedpa-p-Bn-NH$_2$. The linker bears an activated N-hydroxysuccinimide (NHS) ester that can easily

![Figure 3. Approaches for the linkage of H$_2$dedpa to a thiol reactive functional group.](image-url)
react with a primary amine to form an amide bond. The product was synthesized, the obtained structure was confirmed by ESI-MS and $^1$H NMR spectroscopy (Figure S1) and further tested for maleimide reactivity (see DTNB assay below).

In approach 2 smTEGK, containing a maleimide group, was conjugated to H$_2$dedpa-p-Bn-NCS. However this approach failed. No product formation was observed. The reason for that might have been lack of product formation, or loss of the product during purification processes that included extraction and HPLC. However, we could not further investigate this due to a limited amount of starting material.

For approach 3, H$_2$dedpa-p-Bn-NH$_2$ was used to synthesize the maleimide functional group directly on the primary aromatic amine without a linker (Scheme 1). H$_2$dedpa-p-Bn-NH$_2$ was synthesized using an established protocol$^{13}$ (Scheme S1). First a model reaction was used to probe the reaction conditions, using glycine as starting material (Scheme 2). Given successful product formation of the model reaction, the same conditions were used for the reaction between H$_2$dedpa-p-Bn-NH$_2$ and maleic anhydride. The intermediate formation was successful, as indicated by ESI-MS and NMR spectroscopies (Figure S2, Figure S3 and Figure S4), but the ring closure to obtain a maleimide functional group did not occur, presumably because of steric hindrance of the carboxylic acids from the picolinic acid moieties. Two approaches were carried out to solve this problem, refluxing the reaction mixture in H$_2$O, and adding concentrated H$_2$SO$_4$ to protonate the carbonyl group as a catalyst, but neither approaches resulted in ring closure (Scheme 1).
Scheme 1. Synthesis of H₂dedpa-p-Bn-maleimide #3

Scheme 2. Model reaction for ring closure for maleimide synthesis.

With approach 4 we succeeded to get promising results. In Scheme 3, the synthesis route of H₂dedpa-p-Bn-acrylate is illustrated. This approach was based on the synthesis of intermediate f (Scheme S1) as published by Boros et al. who evaluated the differences on ⁶⁷Ga radiochemical yield as well as apo-transferrin binding stability between H₂RGD-1 and H₂RGD-2 and found high radiochemical yields (RCYs) with ⁶⁷Ga with both ligands. These ligands bear the cyclic RGD peptide either on the ethylenediamine backbone or on the secondary nitrogens, respectively. It was found, that for both ligands, quantitative labeling could be achieved, with [⁶⁷Ga(RGD-1)]⁺ being more stable after 2h (92 %) than [⁶⁷Ga(RGD-2)]⁺ (72%) assessed in transferrin stability experiments.
Here, the nitro functional group of intermediate f was reduced with Pd/C 20% loading and hydrogen for 2 h to yield compound 1 (Scheme 2). The N-benzyl groups stayed intact due to the short reaction time and less Pd/C loading compared to the established protocol. The N-benzyl protection groups were kept intact in order to prevent side-reactions with acryloyl chloride on the secondary amines. After that, the amine functional group was functionalized with acryloyl chloride to an acrylate 2. Deprotection of the methyl esters of the picolinic acids yielded product 3.

Scheme 3. Synthesis of H$_2$dedpa-N,N’-dibenzyl acrylate #4

The product 3 was analyzed by $^1$H NMR (Figure 4), $^{13}$C NMR (Figure S5), 2D-HSQC (Figure S6) spectroscopy and HR ESI-MS. Concerning the $^1$H NMR spectrum, double bond protons usually show chemical shifts between 4-7 ppm; here, we observed three peaks in that area (6.44-6.39 ppm, 6.23 ppm and 5.80 ppm), each integrating for one
proton (Figure 4). These peaks represent the acrylate double bond. In the $^{13}$C NMR spectrum (Figure S5), alkenes usually have a chemical shift between 115-140 ppm. Here, 127.1 ppm, 128.7 ppm and 129.1 ppm are the chemical shifts for the acrylate alkene protons. This is due to the negative inductive effect of the carbonyl group. The other peaks are difficult to assign, but the integrations of the hydrogens correspond with the theoretical number of hydrogens.

**Figure 4.** $^1$H NMR spectrum of compound 3 (400MHz, MeOD, 25°C).

### 3.1.1. Thiol bioconjugation of compound 3

In the next step, a model reaction was designed to test the thiol bioconjugation reaction ability to the acrylate functional group of compound 3. Glutathione a tripeptide of glutamate, cysteine and
glycine was chosen as a thiol-containing protein analogue, due to its structural similarity to proteins. The reaction scheme is given in Figure 5. As starting material, the tri-acrylate functionalized $H_2$dedpa 4 was used that was synthesized from a by-product during the synthesis of intermediate 2. The product 5 precipitated due to its insolubility in hexylamine. Furthermore, during purification with HPLC, the picolinic esters appeared to be cleaved by trifluoroacetic acid (TFA), as suggested by ESI-MS. The product was analyzed by ESI-MS and $^1$H NMR (Figure 6, where differences between the starting material and the final product are indicated by arrows), $^{13}$C NMR and 2D HSQC spectroscopy. The $^1$H NMR spectrum clearly showed the disappearance of the alkene signals and appearance of additional peaks in the alkyl region for the glutathione protons. The integrations fit to the theoretical values of the number of protons.

To test the reactivity of the acrylate group of compound 3, the compound was incubated with bovine serum albumin (BSA), a thiol-containing protein, and the remaining thiol groups were analysed using DTNB using the same protocol for assessing the reactivity of attempt 1 (see below). Unfortunately, the BSA precipitated in the solvent of the model reaction, making the measurement of the absorbance of DTNB impossible. In the future, the addition of a water/methanol mixture may help to dissolve the BSA. Alternative methods should be developed to validate the reactivity of the acrylate groups with thiol functional groups.
**Figure 5.** Model reaction for approach 4.
**Figure 6.** $^1$H NMR spectra of model reaction of glutathione with #4; starting material (top), product (bottom) (400 MHz, MeOD, 25°C); arrows indicate the differences between the two compounds changes.

### 3.2 DTNB Assay

The product of attempt 1 was further characterized by determining the maleimide reactivity of H$_2$dedpa-smPEG4 compared to the positive control smTEGK (Figure 7). BSA was used as a thiol-containing probe to conjugate to the maleimide functional group of H$_2$dedpa-smPEG4. Unreacted thiol groups of BSA then reacted with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to form a colored compound with absorbance measurable at 412 nm. Thus, a high absorbance indicates a high concentration of unreacted thiols. As seen in Figure 8A, the absorbance decreases with increasing the concentration of the positive control smTEGK until about 75 µM smTEGK, where the concentration of smTEGK is equal to the concentration of the free thiol groups on BSA. When iodoacetamide (IAA), which also reacts with free thiol groups, was added at the highest concentration of smTEGK, no further decrease was observed indicating that all reactive thiols of BSA have been coupled to smTEGK. Figure 8B shows that the absorbance did not decrease with increasing concentration of H$_2$dedpa-smPEG4. This result suggests that the maleimide functional group on H$_2$dedpa-smPEG4 is not functional. This could be due to the presence of impurities or instability of the maleimide functional group. From these results we conclude, that either the synthesis was not successful or the product is not stable under the applied conditions.
**Figure 7.** Structure of positive control smTEGK used in the DTNB assay.

**Figure 8.** DTNB results of approach 1 (A: positive control, B: H₂dedpa-smPEG4).
4 Conclusions

Several approaches were conducted to synthesize a H$_2$dedpa derivative that contains a thiol reactive functional group for functionalization with FXa for detecting blood clots in small concentrations. Unfortunately, the use of the well-studied thiol-reactive maleimide functional group was not successful. However, the synthesis of a H$_2$dedpa-acrylate derivative was achieved and characterized by $^1$H NMR, $^{13}$C NMR and 2D NMR spectroscopies. In addition, a model reaction with the thiol containing glutathione showed promising results concerning thiol-reactivity. In the near future, functionalization with FXa needs to be performed as well as in vitro phospholipid binding studies.

5. Experimental

Materials and Methods

$^1$H and $^{13}$C nuclear magnetic resonance (NMR) spectra were recorded on Bruker AV400, instrument at ambient temperature; the NMR spectrawere expressed on the $\delta$ scale and referenced to residual solvent peaks. Electrospray ionization mass spectrometry (ESI-MS) spectra were recorded on a Micromass LCT instrument also at the Department of Chemistry, University of British Columbia. High-performance liquid chromatography (HPLC) analysis of cold compounds was done on a Phenomenex Synergi 4-µm Hydro-RP 80A column (250 mm x 21.2 mm) on a Waters WE 600 HPLC system equipped with a 2478 dual-wavelength absorbance UV detector run using the Empower software package.

Common starting materials such as 6-bromomethylpyridine-2-carboxylic acid methyl ester$^{14}$, H$_2$dedpa-p-Bn-NH$_2$ and H$_2$dedpa-p-Bn-NCS were synthesized according to the literature.$^{13}$
Approach 1: H$_2$dedpa-\(\text{p}-\text{Bn-smPEG4}\)

H$_2$dedpa-\(\text{p}-\text{Bn-NH}_2\)\textsuperscript{13} (0.008 g, 18.9 mmol, 1.9 eq.) was dissolved in dry DMSO (1 mL) and smPEG4 (0.005 g, 9.9 mmol), \(N,N\)-diisopropylethylamine (DIPEA) (0.005 mL, 29.9 mmol, 3 eq) and 4-dimethylaminopyridine (DMAP) (0.003, 29.9 mmol, 3 eq) were added. After 2 h stirring the reaction mixture at room temperature, the crude was purified via semi-prep reverse-phase HPLC (10mL/ min, gradient A: 0.1% TFA in deionized water, B: acetonitrile, A: 95% to B: 100% for 25 min) to obtain the product as a yellow oil (0.005 g, 74.6 %). $^1$H NMR (D$_2$O, 400MHz) = 8.00-7.98 (d, 2H), 7.93-7.82 (m, 2H), 7.71-7.70 (d, 1H), 7.41-7.39 (d, 2H), 6.96-6.94 (d, 1H), 6.87-6.86 (d, 1H), 6.72-6.71 (d, 1H), 4.22-4.06 (m, 4H), 3.66-6.59 (m, 9H), 3.19 (s, 4H), 3.02 (s, 4H), 2.90 (s, 2H), 2.86 (s, 2H), 2.65 (s, 2H), 2.55 (s, 2H). ESI-MS calcd. for [C$_{41}$H$_{51}$N$_7$O$_{12}$+H]$^+$: 834.3674; found 834.5 [M+H]$^+$

Approach 2: H$_2$dedpa-\(\text{p}-\text{Bn-smTEGK}\)

H$_2$dedpa-\(\text{p}-\text{Bn-NCS}\)\textsuperscript{13} (0.003 g, 7.2 mmol, 1.2 eq) was dissolved in dry DMSO (1 mL). mTEGLys (0.003 g, 6.0 mmol) was added to the solution and the reaction mixture was stirred for 16 h. The crude was purified via semi-preparative reverse-phase HPLC (10mL/ min, gradient A: 0.1% TFA in deionized water, B: acetonitrile, A: 95% to B: 100% for 25 min). No product formation was observed.

Approach 3: H$_2$dedpa-\(\text{p}-\text{Bn-maleimide}\)

Model reaction\textsuperscript{15}

Glycine (0.10 g, 1.33 mmol) was dissolved in glacial acetic acid (10 mL) and maleic anhydride (0.14 g, 1.47 mmol, 1.1 eq) was added under argon atmosphere. After confirmation of the intermediate by ESI-MS, the solvent was removed \textit{in vacuo} and dist. water (5 mL) was added to the crude solid. After heating the reaction mixture to 60°C for 10 min, the suspension became clear and was refluxed overnight. The product
was isolated as a white precipitate after cooling the solution down (0.19 g, 81.5 %).

\[^{1}\text{H}\text{ NMR (DMSO-d}_6\text{, 400MHz)} = 6.06\ (s, 2H), 3.68\ (s, 2H). \text{ }^{13}\text{C NMR (DMSO-d}_6\text{, 101MHz)} = 169.1, 167.2, 135.4\text{ and 39.9. ESI-MS calcd. for }\text{[C}_6\text{H}_5\text{NO}_4\text{+H]}^+: 156.0297; \text{ found 156.1 }[\text{M+H}]^+

\text{H}_2\text{dedpa-p-Bn-maleimide}

\text{H}_2\text{dedpa-p-Bn-NH}_2\ (0.007 \text{ g, 0.02 mmol}) was dissolved in glacial acetic acid (2 mL) and maleic anhydride (0.002 g, 0.02 mmol, 1.1 eq), was added to the reaction mixture. After 16 h stirring of the reaction mixture at room temperature, the intermediate was confirmed via ESI-MS and NMR spectroscopy. The maleimide ring closure reaction (in 2 mL water, refluxing overnight) was unsuccessful.

\[^{1}\text{H}\text{ NMR (MeOD, 400MHz)} = 8.18-8.13\ (m, 4H), 7.84-8.83\ (d, 1H), 7.79-7.77\ (d, 1H), 7.62-7.61\ (d, 2H), 7.32-7.30\ (d, 2H), 6.58-6.55\ (d, 1H), 6.34-6.31\ (d, 1H), 4.76-4.09\ (dd, 4H), 3.79-3.69\ (m, 2H), 3.59-3.48\ (m, 3H), 2.98-2.97\ (m, 1H). \text{ }^{13}\text{C NMR (MeOD, 101MHz)} = 167.0, 164.6, 146.0, 139.6, 133.1, 130.9, 129.7, 126.7, 125.1, 120.9, 58.2, 56.2, 52.5\text{ and 34.3. ESI-MS calcd. for }\text{[C}_27\text{H}_27\text{N}_5\text{O}_7\text{+H]}^+: 534.1989; \text{ found 534.2 }[\text{M+H}]^+

\text{Approach 4: H}_2\text{dedpa-p-Bn-acrylate}

\text{Dimethyl }6,6'-(((3-(4-aminophenyl)propane-1,2-diyl)bis(benzylazanediyl))bis(methylene))dipicolinate, 1

Compound f (Scheme S1) (0.182 g, 0.27 mmol) was dissolved in methanol (10 mL). After adding Pd/C (0.05 g, 0.41 mmol, 1.5 eq) to the solution, the three-neck flask was charged with a H\textsubscript{2}-balloon and the reaction mixture was stirred for 2h until ESI-MS showed the reduction product 1. After that, the crude was purified via semi-preparative reverse-phase HPLC (10mL/ min, gradient A: 0.1% TFA in deionized water, B: acetonitrile, A: 95% to B: 100% for 25 min) to yield the product as an off-white solid (0.054 g, 31 %).
\(^1\)H NMR (MeOD, 400MHz) = 8.04 (d, 2H), 8.00-7.92 (m, 16H), 7.00 (d, 2H), 2.98 (s, 6H), 3.94 (s, 4H), 3.65-3.39 (m, 4H), 3.20-2.96 (m, 3H), 2.60-2.55 (m, 2H). ESI-MS calcd. for \([C_{39}H_{41}N_5O_4+H]^+\): 644.3237; found 644.3 [M+H]^+

**Dimethyl 6,6’-(((3-(4-acrylamidophenyl)propane-1,2-diyldiyl)bis(benzylazanediyl))bis(methylene))dipicolinate, 2**

This synthesis was modified from the literature.\(^{16}\) Potassium carbonate (0.019 g, 0.13 mmol, 1.2 eq) was placed in a three-neck flask, which was evacuated and flushed with argon. Water (1 mL) and acetone (4 mL) were added to the reaction flask and cooled to 0°C. A solution of compound 1 (0.072 g, 0.11 mmol) in 1 mL acetone was added to the suspension, followed by a slow addition of acryloyl chloride (10.8 µL, 0.13 mmol, 1.2 eq). The reaction mixture was stirred for 2h to yield a yellow oil (0.077 g, 0.11 mmol, 99 %). The solvents were removed \textit{in vacuo} and the product 2 was used without further purification since the dominant peak in the ESI-MS was the product peak and HPLC purification was used after the final step.

ESI-MS calcd. for \([C_{42}H_{43}N_5O_5+Na]^+\) 698.3; found: 698.4 [M+H]^+

**H\textsubscript{2}dedpa-p-Bn-acrylate, 3**

To a solution of compound 2 (0.08 g, 0.11 mmol) in a mixture of THF (7.5 mL) and water (2.5 mL) (3:1), lithium hydroxide (0.026 g, 1.1 mmol, 10 eq.) was added and the reaction mixture was stirred for 6 h at room temperature. The solution was neutralized with 0.1 M HCl and the solvents were removed \textit{in vacuo}. Compound 3 was obtained as a yellow oil (0.006 g, 0.11 mmol, 8 %) after purification with semi-prep reverse-phase HPLC (10mL/ min, gradient A: 0.1% TFA in deionized water, B: acetonitrile, A: 95% to B: 100% for 25 min) (t\(_R\) = 18.8 min).

\(^1\)H NMR (MeOD, 400MHz) = 8.41-6.97 (m, 20H), 6.44-6.39 (m, 1H), 6.23 (d, 1H), 5.80 (d, 1H), 4.72-4.45 (m, 4H), 4.01-3.58 (m, 4H), 3.24-2.70 (m, 5H). \(^{13}\)C NMR (MeOD, 101MHz) = 166.1, 144.6, 144.1, 123.2, 131.1,
129.6, 128.0, 127.9, 122.0, 63.6, 58.3, 53.8, 53.2 and 34.8. HR-ESI-MS calcd. for \([C_{40}H_{39}N_5O_5+H]^+\): 670.3029; found 670.3022 [M+H]^+

**Model reaction (glutathione)**

**Dimethyl 6,6'-(6-(4-acrylamidophenyl)propane-1,2-diyl)bis(acryloylazanediyl))bis(methylene))dipicolinate**

Potassium carbonate (0.046 g, 0.33 mmol, 1.2 eq) was placed into a flask, which was evacuated and flushed with argon. Water (1 mL) and acetone (4 mL) were added to the reaction flask and the mixture was cooled to 0°C. A solution of intermediate \(f\) (0.129 g, 0.28 mmol) in acetone (1 mL) was added to the suspension, followed by a slow addition of acryloyl chloride (27.1 µL, 0.33 mmol, 1.2 eq). The reaction mixture was stirred for 2h to yield a yellow oil (0.077 g, 0.11 mmol, 99 %). The solvents were removed *in vacuo* and the product was purified via semi-prep reverse-phase HPLC (10mL/ min, gradient A: 0.1% TFA in deionized water, B: acetonitrile, A: 95% to B: 100% for 25 min., \(t_R = 19.12\) min) to yield the product as a white solid (0.07 g, 42%).

\(^1\)H NMR (MeOD, 400MHz) = 8.04-7.88 (m, 3H), 7.77-7.70 (m, 1H), 7.48-7.39 (m, 3H), 7.29-7.20 (dd, 1H), 7.07-7.00 (m, 2H), 6.81-6.02 (m, 6H), 5.78-5.59 (m, 3H), 4.80-4.57 (m, 4H), 3.98-3.90 (m, 6H), 3.68-3.64 (d, 1H), 2.98-2.92 (m, 2H), 2.66-2.60 (s, 2H). \(^13\)C NMR (MeOD, 100MHz) = 165.3, 164.6, 139.3, 139.3, 130.8, 128.1, 122.8, 119.9, 53.7, 51.5, 47.9, 39.0, 35.1. ESI-MS calcd. for \([C_{34}H_{35}N_5O_7+Na]^+\): 648.2434; found 648.3 [M+H]^+

**H\(_2\)dedpa-triglutathione**

A modified protocol was used.\(^{17,18}\) The starting material \(4\) (0.07 g, 0.11 mmol) was dissolved in 1 mL hexylamine and 200 µL of methanol were added until complete dissolution. Glutathione (0.11 g, 0.37 mmol, 3.3 eq.) was added to the solution and methanol was added again until complete dissolution. After 2h stirring of the reaction mixture, a precipitate was formed. The precipitate was filtered off, dried and purified via semi-prep reverse-phase HPLC (10mL/ min, gradient A:
0.1% TFA in deionized water, B: acetonitrile, A: 95% to B: 100% for 25 min., tR =16.86 min) to yield the product 5 as a white solid (0.02 g, 11 %).

$^1$H NMR (MeOD, 400MHz) = 8.03-7.82 (m, 4H), 7.48-7.33 (4H), 7.10-6.89 (m, 2H), 4.73-4.45 (m, 4H), 4.00-3.92 (m, 5H), 3.46-3.37 (m, 5H), 3.29-3.22 (m, 3H), 3.13-2.59 (m, 35H) $^{13}$C NMR (MeOD, 100MHz) = 169.2, 138.5, 127.8, 120.2, 120.7, 51.3, 40.9, 38.4, 31.3, 30.8, 29.2. ESI-MS calcd. for [C$_{59}$H$_{77}$N$_{14}$O$_{25}$S$_{3}$+H]$^+$: 1479.5260; found 1479.8 [M+H]$^+$

**DTNB assay**

The reactivity of the H$_2$dedpa functionalized compound with the free thiol on bovine serum albumin (BSA) was assessed. BSA (50 µM) was incubated for 15 minutes with different concentrations of the synthesized compound (0, 25, 50, 75, 100 and 150 µM) using a 1 mM stock solution in DMSO of the compound in Tris buffer in a total volume of 100 µL. As a positive control, Lys-PEG4-maleimide was used under the same conditions. Lys-PEG4-maleimide bears a thiol reactive maleimide functional group. Remaining free thiol on BSA was quantified through the addition of 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (Ellmman’s reagent, DTNB) which reacts with free sulfhydryl groups to yield a mixed disulfide and 2-nitro-5-thiobenzoic acid (NTB), a measurable yellow-colored product detectable at 412 nm. Iodoacetamid (IAA) was used as a negative control that alkylates free thiols. As confirmation that BSA is maleimide reactive, commercial rhodamine-maleimide was used and irreversible incorporation into BSA was evaluated by denaturing electrophoresis.

6. References

Supporting Information

Chapter A4

Overcoming the Limitations in Thrombosis Treatment:

A Bifunctional Chelator as Positron Emission Tomography-Imaging Probe for Detecting Blood Clots

Sarah Spreckelmeyer, a,b Frank M. Lee; c Ed Pryzdialc and Chris Orvig a

a Medicinal Inorganic Chemistry Group, Department of Chemistry, University of British Columbia, 2036 Main Mall, Vancouver, British Columbia, V6T 1Z1, Canada

b Department of Pharmacokinetics, Toxicology and Targeting, Groningen Research Institute of Pharmacy, University of Groningen, Antonius Deusinglaan 1, Groningen 9713 AV, The Netherlands

c Centre for Blood Research, Life Science Centre, 2350 Health Sciences Mall, Vancouver, BC, V6T 1Z3, Canada
Figure S1. $^1$H NMR spectra of #1 (400 MHz, $D_2O$ (top) MeOD (bottom), 25°C).
Scheme S 1. Synthesis of H$_2$dedpa-p-Bn-NH$_2$.

Figure S 2. $^1$H NMR spectrum of #3 (400 MHz, MeOD, 25°C).
**Figure S 3.** $^{13}$C NMR spectrum of #3 (101 MHz, MeOD, 25°C).

**Figure S 4.** 2D-HSQC spectrum of #3.
Figure S 5. $^{13}$C NMR spectrum of compound 3 (101MHz, MeOD, 25°C).

Figure S 6. 2D-NMR HSQC spectrum of compound 3.
Part B