

Vesicular Aptasensor for the Detection of Thrombin

Andreas Müller and Burkhard König*

Institut für Organische Chemie, Universität Regensburg, 93040 Regensburg, Germany

Fax: +49 943 1717; Tel: +49 943 4575; E-mail: burkhard.koenig@chemie.uni-regensburg.de

Electronic Supplementary Information

Table of Contents

1. General Methods and Material.....	S-2
2. Synthesis	S-3
2.1 Synthesis of <i>N</i> -(2-(2-(2-aminoethoxy)ethoxy)ethyl)hexadecanamide (3).....	S-3
2.2 Synthesis of <i>N</i> -(2-(2-(2-maleimidoethoxy)ethoxy)ethyl)hexadecanamide (Mal-C₁₆).....	S-4
2.3 NMR Spectra of Mal-C₁₆	S-5
3. Preparation and Characterization of Vesicles.....	S-6
3.1 Preparation of Functionalizable Luminescent Vesicles.....	S-6
3.2 Aptamer-Functionalization of Vesicle Surfaces.....	S-7
3.3 Dynamic Light Scattering (DLS) of Vesicles.....	S-8
4. Determination of Binding Constants.....	S-9
5. References.....	S-10

1. General Methods and Material

General

Commercially available solvents of standard quality were used. Starting materials were purchased from either Acros or Sigma-Aldrich and used without any further purification. Phospholipids were purchased from Avanti Polar Lipids Inc. Thrombin from bovine plasma (40–300 NIH units / mg protein) and elastase from porcine pancreas (Type IV) were obtained from Sigma-Aldrich. Human α -thrombin was available at Haematologic Technologies Inc. The thiolated aptamers were synthesized by IBA GmbH Göttingen.

Thin layer chromatography (TLC) analyses were performed on pre-coated TLC sheets ALUGRAM Xtra SIL G/UV₂₅₄ with 0.2 mm layer thickness from Macherey-Nagel. The detection was done by staining with KMnO₄. Flash column chromatography was performed on silica gel 60 (70–230 mesh) from Macherey-Nagel.

Melting Points

Melting points were determined on a Stanford Research Systems OptiMelt MPA100 with a heating rate of 1 °C / min.

NMR Spectra

For NMR spectroscopy, a Bruker Avance 300 (¹H: 300.1 MHz, ¹³C: 75.5 MHz, T = 293 K) was used. All chemical shifts are reported in δ [ppm] (multiplicity, coupling constant *J*, number of protons, assignment) relative to the solvent residual peak of CDCl₃ as the internal standard (¹H: δ = 7.26 ppm, ¹³C: δ = 77.16 ppm). The coupling constants are given in Hertz [Hz]. Abbreviations used for signal multiplicity: ¹H-NMR: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad.

Mass Spectra

Mass spectra were measured on an Agilent Q-TOF 6540 UHD mass spectrometer.

Fluorescence Measurements

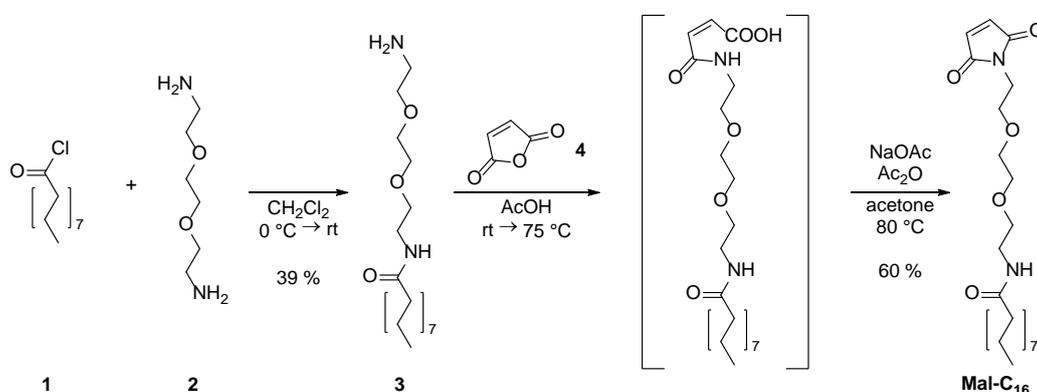
Fluorescence spectroscopy was carried out on a HORIBA FluoroMax-4 fluorescence spectrophotometer with temperature control with 10 × 4 mm Hellma quartz cuvettes at 21 °C. For better comparison of the different measurements, the relative fluorescence changes ($\Delta I_{\max} / I_{0,\max}$) were calculated.

Dynamic Light Scattering

Dynamic light scattering was performed on a Malvern Zetasizer Nano ZS at 25 °C using 1 cm disposable polystyrene cuvettes. Data analysis was performed using the Malvern Zetasizer software.

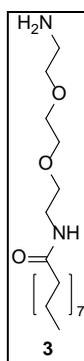
2. Synthesis

Compound **Rho-C₁₈**¹ was prepared according to the literature procedure. The amphiphilic maleimide **Mal-C₁₆** was synthesized by amide formation of palmitoyl chloride (**1**, Scheme S1) and bisamine **2** followed by reaction of amphiphilic amine **3**² with maleic anhydride (**4**).



Scheme S1: Synthesis of amphiphilic maleimide **Mal-C₁₆**.

2.1 Synthesis of *N*-(2-(2-(2-aminoethoxy)ethoxy)ethyl)hexadecanamide (**3**)

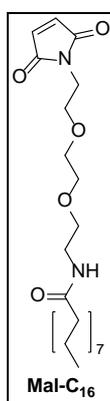


A solution of palmitoyl chloride (**1**, 3.00 g, 10.9 mmol, 1.00 eq) in CH_2Cl_2 (20 mL) was added dropwise over 4 h to a stirred solution of 2,2'-(ethylenedioxy)bis(ethylamine) (**2**, 16.2 g, 109 mmol, 10.0 eq) in CH_2Cl_2 (40 mL) at $0\text{ }^\circ\text{C}$ under nitrogen atmosphere. The reaction

mixture was allowed to warm to room temperature and stirring was continued for 17 h at 25 °C resulting in the formation of a thick white precipitate. Aqueous NaOH (1.0 M, 90 mL) was added to the reaction mixture and the organic solvent was removed by rotatory evaporation under reduced pressure. The aqueous suspension was filtered and the obtained white precipitate was washed with water. After re-suspension of the solid in water (100 mL), the aqueous mixture was extracted with CH₂Cl₂ (3 × 80 mL) and the combined organic phases dried over Na₂SO₄. Evaporation of the solvent provided amide **3**² as a white solid (1.64 g, 4.25 mmol, 39 %), which was used for the next step without any further purification.

$R_f = 0.20$ (MeOH/CH₂Cl₂ 20:80). — ¹H-NMR (CDCl₃, 300 MHz): $\delta = 0.87$ (t, $J = 6.7$ Hz, 3H, Me), 1.24 (m, 24H, (CH₂)₁₂), 1.54–1.67 (m, 2H, CH₂CH₂C=O), 1.82 (br s, 2H, NH₂), 2.16 (t, $J = 7.6$ Hz, 2H, CH₂CH₂C=O), 2.88 (t, $J = 5.2$ Hz, 2H, CH₂NH₂), 3.41–3.49 (m, 2H, CH₂NH), 3.49–3.59 (m, 4H, 2 × CH₂O), 3.59–3.64 (m, 4H, 2 × CH₂O), 6.25 (br s, 1H, NH).

2.2 Synthesis of *N*-(2-(2-(2-maleimidoethoxy)ethoxy)ethyl)hexadecanamide (Mal-C₁₆)



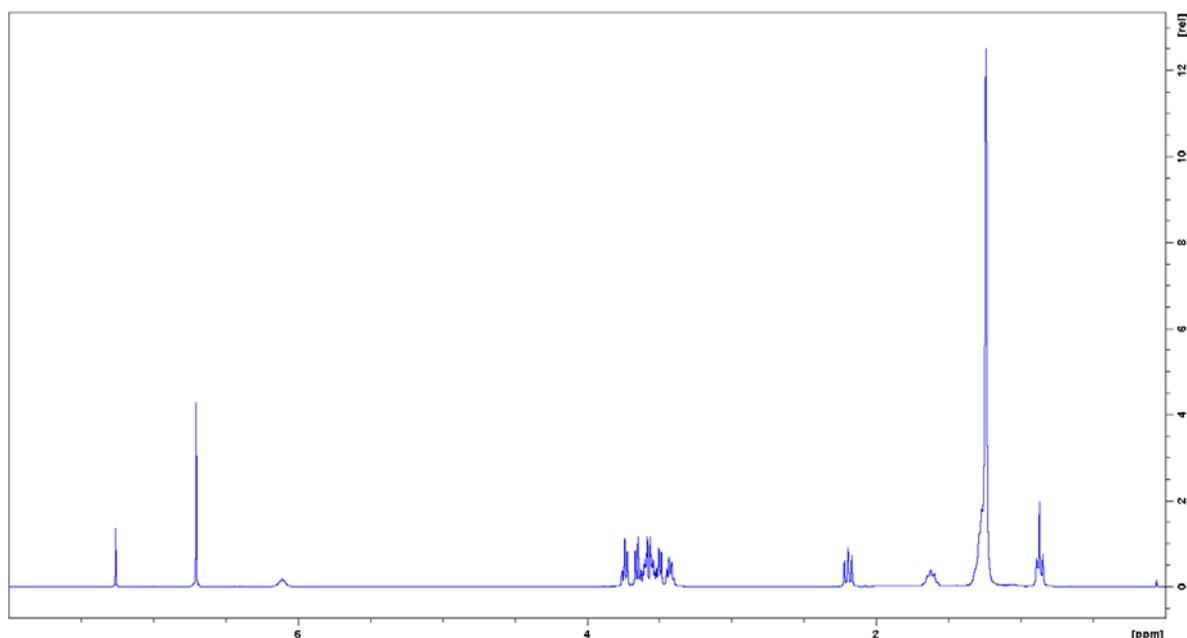
To a solution of amphiphilic amine **3** (1.60 g, 4.14 mmol, 1.00 eq) in AcOH (55 mL), maleic anhydride (**4**, 446 mg, 4.55 mmol, 1.10 eq) was added. The reaction mixture was stirred at 25 °C for 17 h and subsequently at 75 °C for 3 h. Acetic acid was distilled off under reduced pressure followed by dissolution of the obtained white residue in acetone (90 mL). Sodium acetate (1.09 g, 13.2 mmol, 3.20 eq) and acetic anhydride (55 mL) were added and the reaction mixture was stirred at 60 °C for 14 h and at 80 °C for 6 h. After removal of the solvent and excessive Ac₂O *in vacuo*, CH₂Cl₂ (50 mL) and saturated NaHCO₃ solution (50 mL) were added and the aqueous layer was extracted with CH₂Cl₂ (50 mL). The combined organic phases were dried over Na₂SO₄. The crude product was obtained after evaporation of

the solvent as a brownish solid. Column chromatographic purification (SiO₂, EtOAc/PE 4:6 → 7:3) provided pure compound **Mal-C₁₆** as white solid (1.29 g, 2.77 mmol, 67 %).

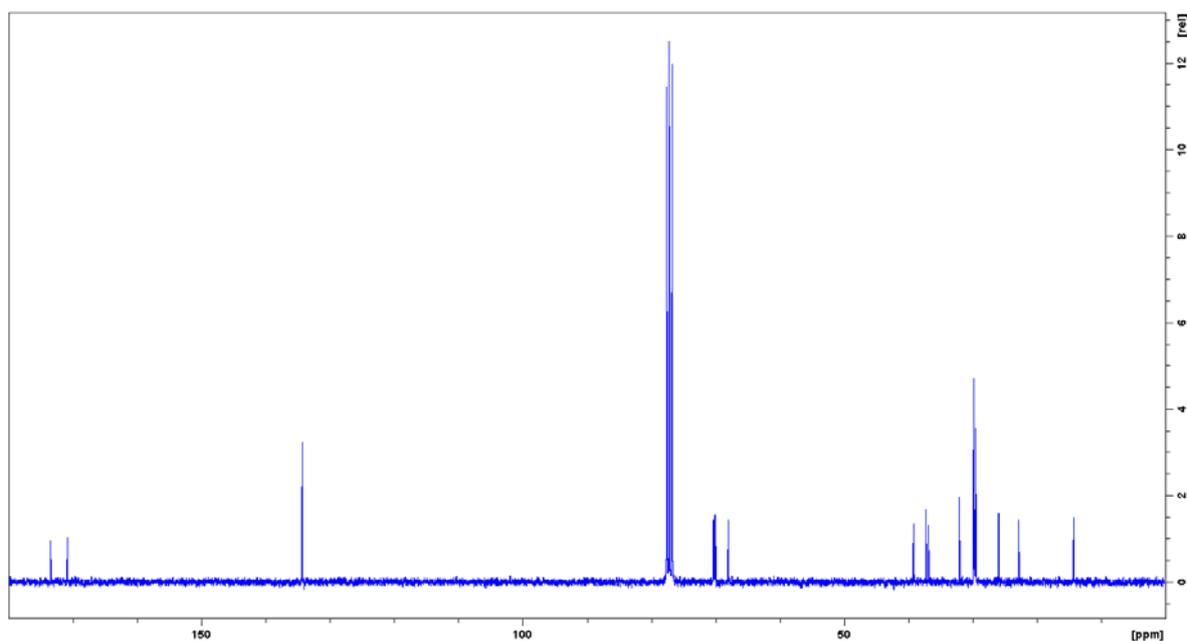
$R_f = 0.58$ (MeOH/CH₂Cl₂ 10:90). — MP: 74–77 °C. — ¹H-NMR (CDCl₃, 300 MHz): $\delta = 0.87$ (t, $J = 6.7$ Hz, 3H, Me), 1.24 (m, 24H, (CH₂)₁₂), 1.56–1.68 (m, 2H, CH₂CH₂C=O), 2.19 (t, $J = 7.7$ Hz, 2H, CH₂CH₂C=O), 3.38–3.46 (m, 2H, CH₂NH), 3.47–3.53 (m, 2H, CH₂N), 3.53–3.62 (m, 4H, 2 × CH₂O), 3.62–3.68 (m, 2H, CH₂O), 3.71–3.77 (m, 2H, CH₂O), 6.11 (br s, 1H, NH), 6.70 (s, 2H, CH=CH). — ¹³C-NMR (CDCl₃, 75 MHz): $\delta = 14.27$ (Me), 22.83 (CH₂), 25.92 (CH₂), 29.49 (CH₂), 29.53 (CH₂), 29.66 (CH₂), 29.79 (CH₂), 29.83 (CH₂), 32.05 (CH₂), 36.88 (CH₂), 37.23 (CH₂), 39.19 (CH₂), 67.99 (CH₂O), 69.98 (CH₂O), 70.13 (CH₂O), 70.33 (CH₂O), 134.32 (CH=CH), 170.83 (CH₂NC=O), 173.49 (NHC=O). — HRMS (ESI): $m/z = [C_{26}H_{46}N_2O_5 + H]^+$ calculated 467.3479; found 467.3484.

2.3 NMR Spectra of Mal-C₁₆

¹H-NMR (CDCl₃, 300 MHz)



^{13}C -NMR (CDCl_3 , 75 MHz)



3. Preparation and Characterization of Vesicles

3.1 Preparation of Functionalizable Luminescent Vesicles

The surface-reactive luminescent vesicles were prepared analogously to formerly established protocols.³

Vesicles V-Pyr

Amphiphilic pyrene **Pyr-C₁₈** was prepared *in situ* according to Scheme S2 by mixing stock solutions of *N*-(1-pyrenyl)maleimide (**5**, 1.00 mM in CHCl_3 , 100 μL) and 1-octadecanethiol (**6**, 1.00 mM in CHCl_3 , 100 μL) in a small glass vessel followed by addition of methanol (500 μL). The reaction mixture was left at room temperature for 30 min and its completion was checked by TLC (EtOAc/PE 3:7). The organic solvent mixture was removed at 75 °C. Stock solutions of DSPC (2.00 mM in CHCl_3 , 900 μL) and **Mal-C₁₆** (1.00 mM in CHCl_3 , 100 μL) were added to the remaining residue to yield a molar ratio of DSPC / **Pyr-C₁₈** / **Mal-C₁₆** = 90 : 5 : 5. The organic solvent was evaporated under a gentle stream of nitrogen and the remaining film of amphiphiles was dried in high vacuum. Aqueous HEPES buffer solution (25.0 mM, pH 7.4, 1.00 mL) was added to obtain a total amphiphile concentration of 2.00 mM. The sample was sonicated for 5 min at room temperature resulting in a slightly turbid

spectra of the vesicle solution were measured after certain time intervals. The surface functionalization reaction was completed after 2–4 h.

3.3 Dynamic Light Scattering (DLS) of Vesicles

Vesicle size distributions were determined by dynamic light scattering. Figure S1 shows the typical size distribution of freshly extruded vesicles. After aptamer-functionalization of the surface and titration of thrombin, no apparent crosslinking of the vesicles was observed (Figure S2).

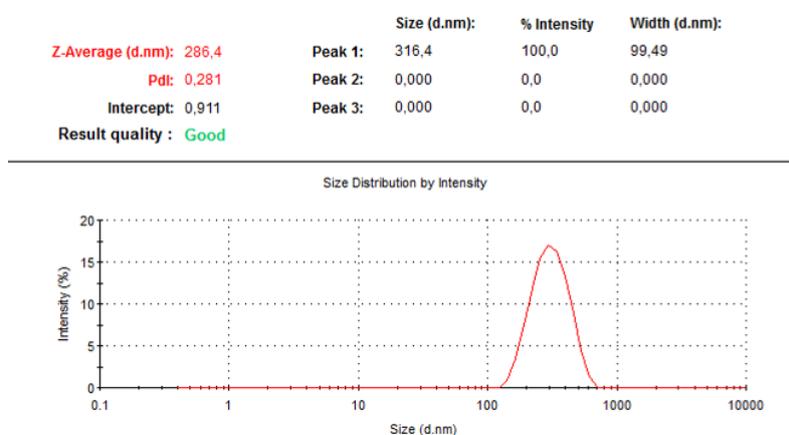


Figure S1: Typical size distribution of freshly prepared vesicles **V-Rho**.

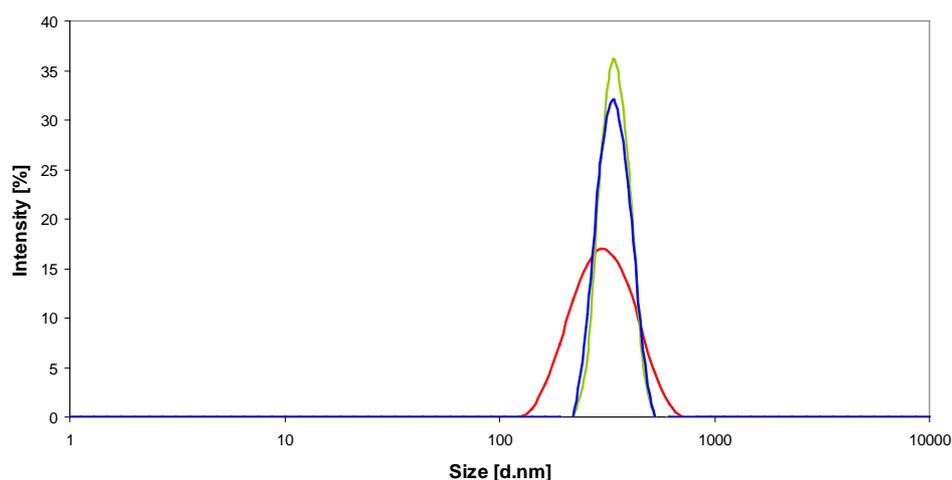


Figure S2: Comparison of vesicle size distribution before aptamer-functionalization (red curve), after functionalization (blue curve) and after addition of thrombin to functionalized liposomes **V-Rho-Thr** (green curve).

4. Determination of Binding Constants

A freshly prepared solution of aptamer-functionalized vesicles was diluted with HEPES buffer up to 2.5 times and filled into a fluorescence cuvette. Aliquots of the analyte solution were added and the fluorescence spectrum was measured after each addition. All fluorescence spectra were corrected for dilution. For titration of thrombin, the vesicle solution contained a 10 mM concentration of KCl. Binding constants were obtained by non-linear curve fitting (ΔI_{\max} versus total concentration of added analyte) using the mathematical algorithm for 1 : 1 binding.⁴

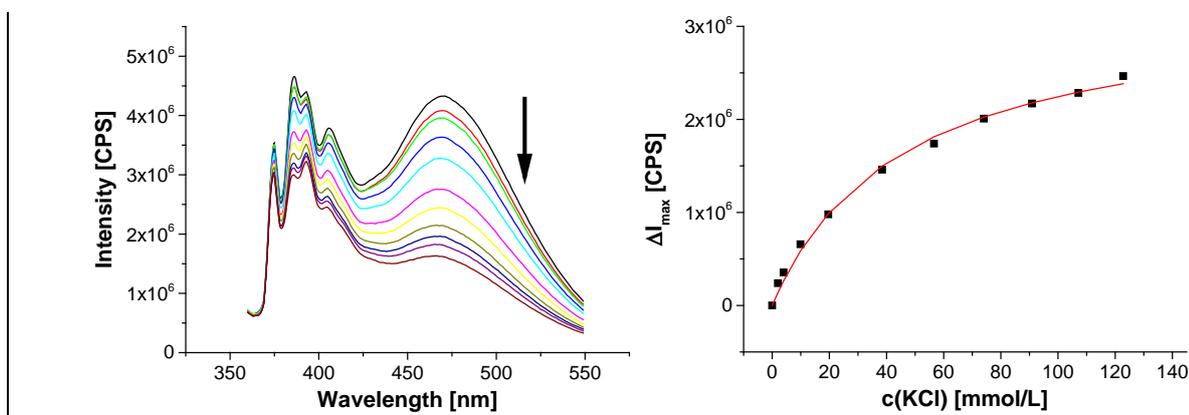


Figure S3: Fluorescence emission change of **V-Pyr-Thr** upon addition of KCl and non-linear curve fitting; $c(\text{Aptamer}) = 2.00 \mu\text{M}$, $\lambda_{\text{ex}} = 345 \text{ nm}$, $\lambda_{\text{em}} = 470 \text{ nm}$.

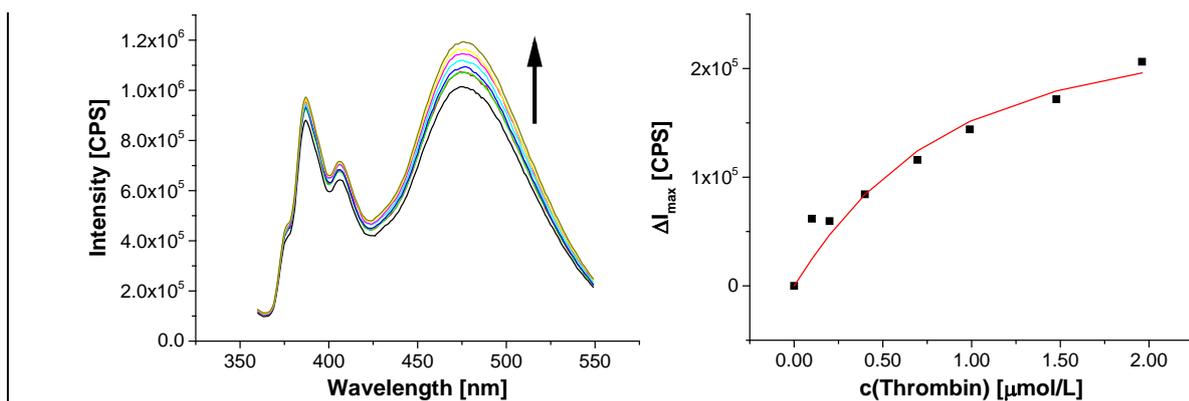


Figure S4: Fluorescence emission change of **V-Pyr-Thr** upon addition of bovine thrombin and non-linear curve fitting; $c(\text{Aptamer}) = 0.50 \mu\text{M}$, $\lambda_{\text{ex}} = 345 \text{ nm}$, $\lambda_{\text{em}} = 470 \text{ nm}$.

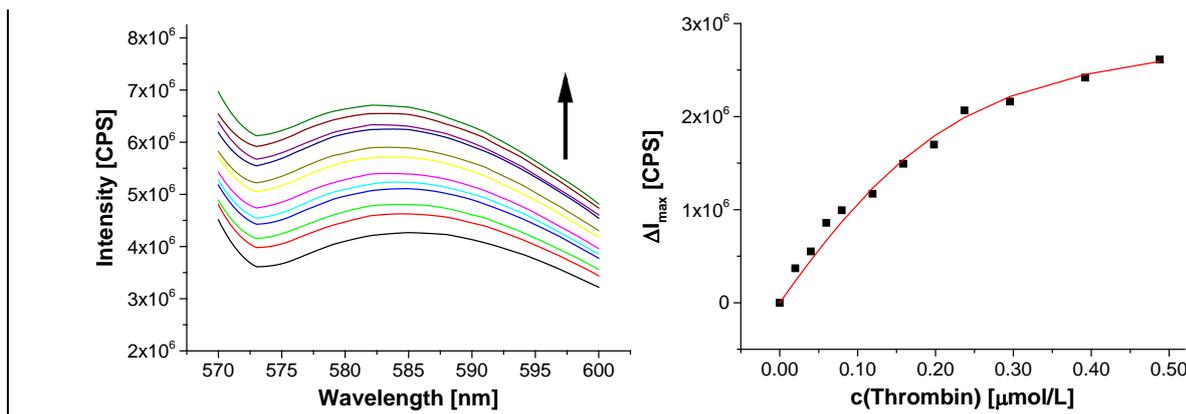


Figure S5: Fluorescence emission change of **V-Rho-Thr** upon addition of bovine thrombin and non-linear curve fitting; $c(\text{Aptamer}) = 0.20 \mu\text{M}$, $\lambda_{\text{ex}} = 557 \text{ nm}$, $\lambda_{\text{em}} = 585 \text{ nm}$.

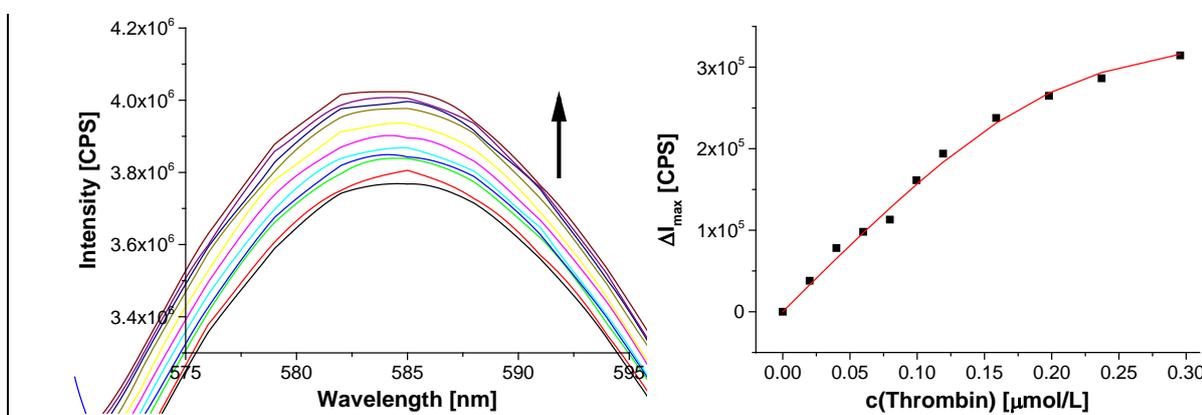


Figure S6: Fluorescence emission change of **V-Rho-Thr** upon addition of human thrombin and non-linear curve fitting; $c(\text{Aptamer}) = 0.20 \mu\text{M}$, $\lambda_{\text{ex}} = 557 \text{ nm}$, $\lambda_{\text{em}} = 585 \text{ nm}$.

5. References

- 1 P. M. Keller, S. Person and W. Snipes, *J. Cell. Sci.*, 1977, **28**, 167–177.
- 2 (a) *US Pat.*, 2 387 201, 1945; (b) *CN Pat.*, 103 739 510, 2014.
- 3 (a) B. Gruber, S. Stadlbauer, A. Späth, S. Weiss, M. Kalinina and B. König, *Angew. Chem. Int. Ed.*, 2010, **49**, 7125–7128; *Angew. Chem.*, 2010, **122**, 7280–7284; (b) S. Banerjee and B. König, *J. Am. Chem. Soc.*, 2013, **135**, 2967–2970.
- 4 A. E. Hargrove, Z. Zhong, J. L. Sessler and E. V. Anslyn, *J. Chem.*, 2010, **34**, 348–354.