Synthesis and Calibration of Magnetic-Nanoparticle (Fe/Fe_3O_4) - based Nanoplatforms for Highly Sensitive Fluorescence Detection of Cancer-Related Proteases

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Supplementary Information

1. Fe/Fe_3O_4 Nanoparticle Synthesis

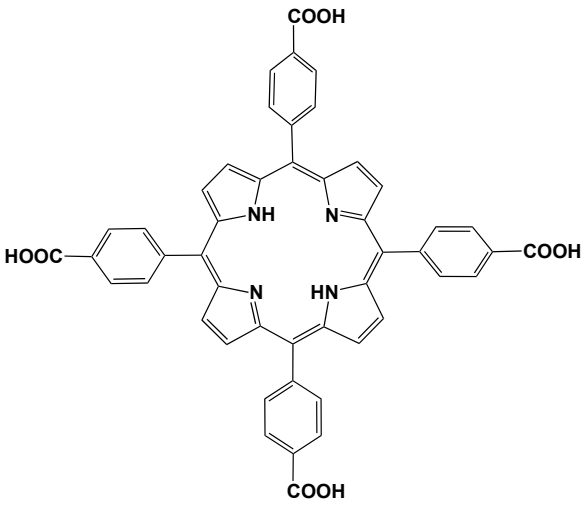
Iron nanoparticles were prepared with slight modification of a literature procedure described by Lacroix et al.[1] A 250 mL, three-necked, round-bottom flask equipped with a magnetic stir bar, one cold water cooled jacket condenser on the middle neck, one septum and one temperature probe on each of the outer necks was charged with 60 mL 1-octadecene (ODE), 0.9 mL oleylamine and 0.831 g hexadecylammonium chloride (HADxHCl). The reaction system was connected to a Schlenk line through the top of the jacket condenser. The reaction mixture was degassed at 120^o C for 30 min with vigorous stirring. After being refilled with argon, the reaction mixture was heated to 180^o C. Three portions of 0.7 mL $Fe(CO)_5$ were injected into the reaction mixture via syringe, every 20 min. The reaction mixture was kept at

180^{o} C for another 20 min after the last injection, and then cooled to room temperature naturally. The supernatant was decanted, and the iron nanoparticles accumulated on the magnetic stir bar were washed with hexane and ethanol. The product was dried in vacuum and stored at room temperature under argon for further use. Based on the iron content of the nanoparticles, which was determined spectrophotometrically after dissolving the nanoparticles in aqueous HCl (1.0 M) and subsequent complexation with ferrozine (sodium 4,4'-(3-(pyridin-2-yl)-1,2,4-triazine-5,6-diyl)dibenzenesulfonate)[2], the yield of the reaction is 95%.

2. Dopamine Coating of the core/shell Fe/Fe_3O_4 Nanoparticles

0.50 g of previously synthesized Fe/Fe_3O_4 nanoparticles was dispersed in 100 mL chloroform via sonication. With vigorous mechanical stirring, a solution of 0.50 g dopamine-hydrochloride in 50 mL chloroform was added drop-wise to the nanoparticle suspension. The reaction mixture was further stirred at room temperature for 24 hours, and then the dopamine coated nanoparticles were collected by centrifugation. After washing with chloroform 5 times, nanoparticles were dried under vacuum. 0.47 g dopamine coated Fe/Fe_3O_4 nanoparticles were collected.

3. Synthesis of (4-carboxyphenyl)porphyrin (TCPP) (1)



The synthetic procedure was a variation of reference 3. 1.50 g 4-carboxybenzaldehyde was dissolved in 80 mL acetic acid. The solution was warmed to 100^{o} C and a solution of 0.67 g pyrrole in 10 mL acetic acid was added dropwise over 20 minutes. Upon completion of addition, the solution was warmed to 120^{o} C slowly and was kept at 120^{o} C for 1 hour. The mixture was cooled to 80^{o} C and 100 mL 95% ethanol was added and then lowered to room temperature, while stirring in 3 hours. Then the mixture was kept in at -15^{o} C for 24 hours. Purple solid was collected by vacuum filtration. The filter cake was washed with cold 50/50 ethanol/acetic acid (3×5mL) and dried under high vacuum (oil pump) overnight. 0.51g of pure product was obtained (25.5% yield). ^{1}H NMR (DMSO-d6) δ: -2.94 (s, 2H); 8.35 (d, 8H); 8.39 (d, 8H); 8.86 (s, 8H); 13.31 (s, 4H). ^{13}C NMR (DMSO-d6) δ: 119.31; 127.90; 130.51; 134.44; 145.42; 167.46. MS-ESI+: m/z 791.2. Molecular weight calculated as 790.2.

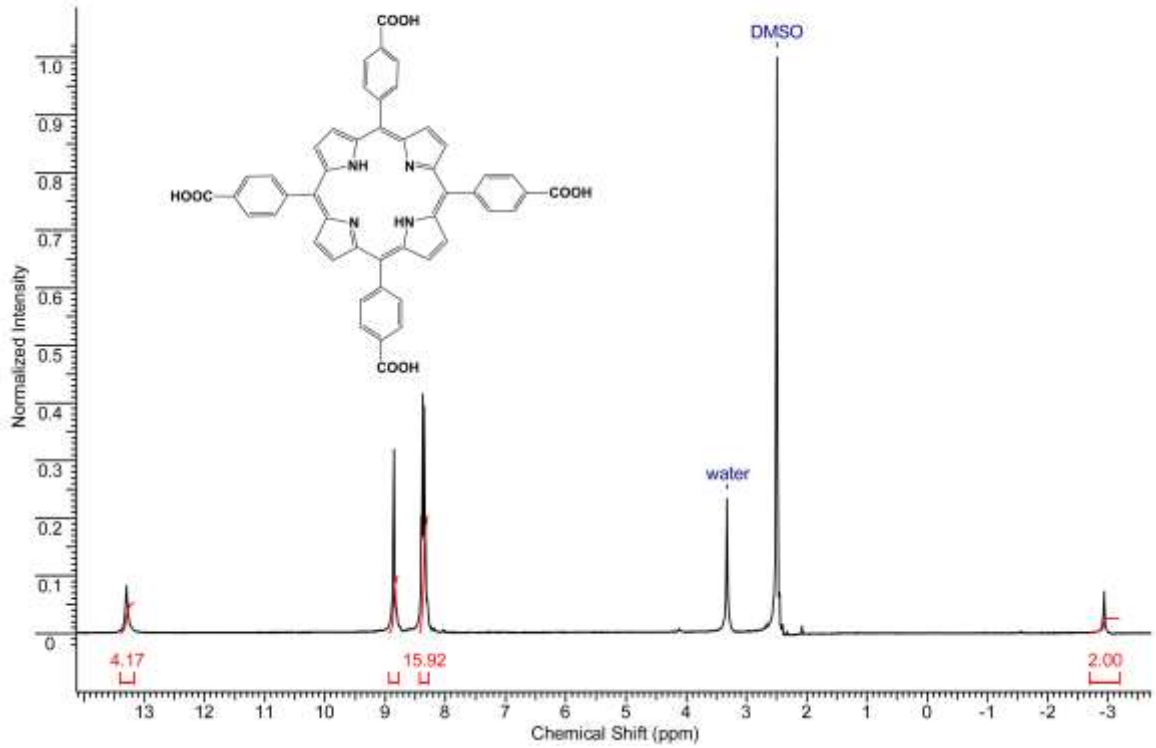


Figure S1: ^{1}H-NMR of (4-carboxyphenyl)porphyrin (TCPP) (Varian, 400 MHz).

4. Cancer-Specific Consensus Peptide Sequence Synthesis

Cancer specific consensus peptide sequences were synthesized via standard Solid Phase Peptide Synthesis (SPPS).[4] Briefly, preloaded trityl-resin was swelled in DCM for 20 min, after washing with DMF, Fmoc-protected amino acids were added sequentially with O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) as coupling agent in a mixture of diisopropylethylamine (DIEA) and DMF. Taking advantage of the solid phase synthesis, the porphyrin TCPP was conjugated to the N-terminal of peptide sequence under standard coupling conditions. Finally, the TCPP-labeled cancer specific consensus peptide sequence was cleaved from the solid phase using 95/2.5/2.5 TFA/TIPS/H_2O solution. The purity of the consensus sequences was examined by using HPLC (RP18 column using $CF_3COOH/H_2O/CH_3CN$ gradients, using a Waters 1525 binary pump HPLC station), as previously described.[5] For all 12 TCPP-labeled consensus sequences, the analytical purity exceeded 95 percent. They were then used for the synthesis of the nanoplatforms without further purification.

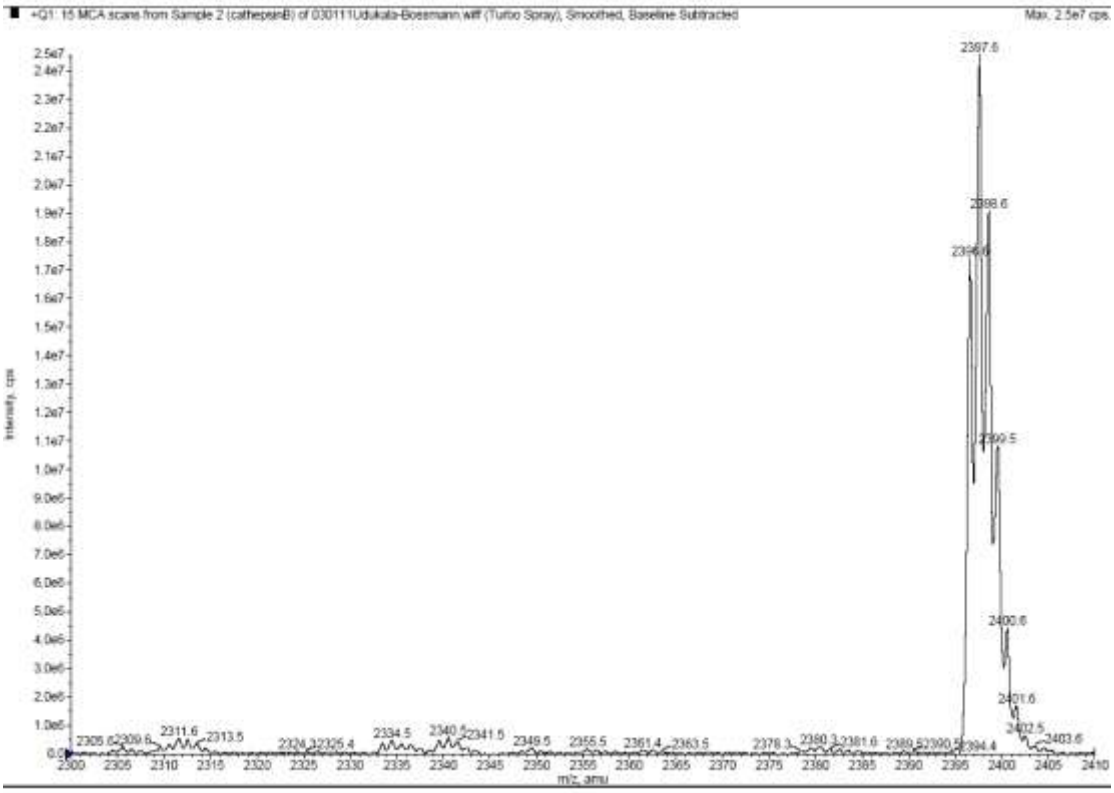
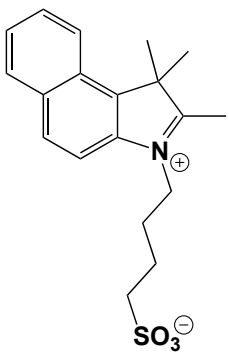


Figure S2: Mass Spectrum (electrospray) of TCPP-labeled consensus sequence for cathepsin B (TCCP-SLLKSRMVPNFN). Calculated mass for $C_{112}H_{135}Cl_3N_{22}Na_4O_{24}S$ = 2,400.84.

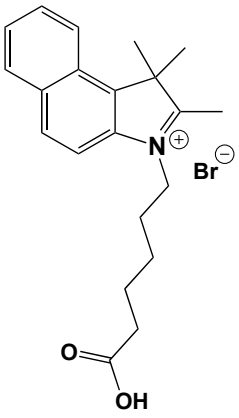
5. Cyanine 5.5 Synthesis

5.1. Synthesis of 4-(1,1,2-trimethyl-1*H*-benzo[*e*]indol-3-ium-3-yl)butane-1-sulfonate (2)



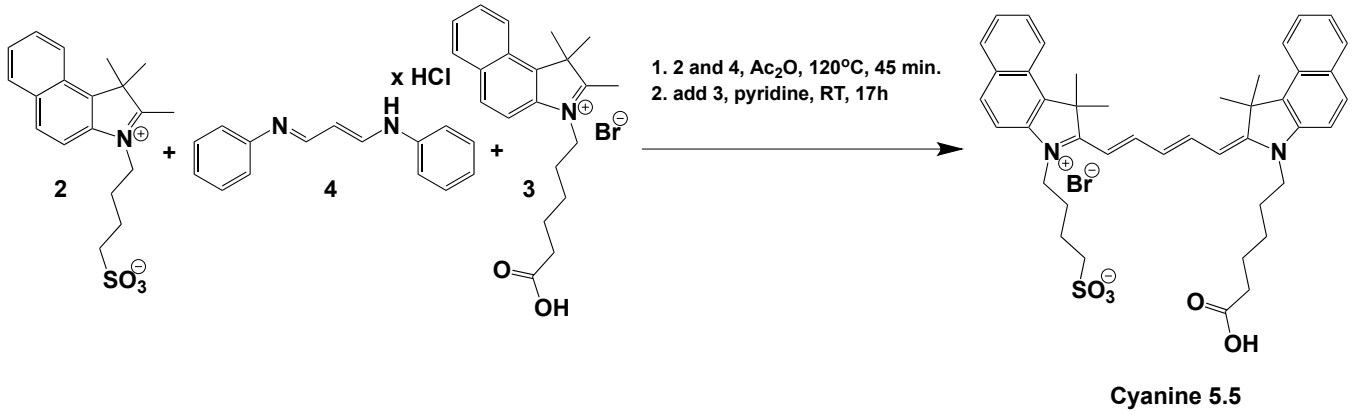
The synthetic procedure was a variation of reference 6. A 50 mL two necked round bottom flask fitted with a magnetic stirrer and a condenser was flame dried. 1,1,2-trimethyl-1H-benzo[e]indole (1.0g, 4.78mmol) was dissolved in dry o-dichlorobenzene (10 mL). 1,4-butanesultone (0.58mL, 5.73mmol) was added drop-wise under a continuous flow of argon. The reaction mixture was, then, allowed to heat up to 130^{o} C for 24h. The reaction mixture was allowed to cool down to room temperature to obtain a blue colored precipitate. The precipitate was triturated with ice-cold diethyl ether (40mL) for 15min. The precipitate was filtered through frit glass, washed with cold diethyl ether (3 x 5mL), dried in vacuum yielding 0.70g of compound 1. Percentage yield: 42%. ^{1}H NMR (DMSO-d6, 400 MHz) δ (ppm): 8.36 (d, 1H), 8.27 (d, 1H), 8.22 (s, 1H), 8.20(d, 1H), 7.76(dt, 2H), 4.60(t, 2H), 3.31(t, 2H), 2.0(qi, 2H), 1.77(m, 2H), 1.75(s, 6H)

5.2. Synthesis of 3-(5-carboxypentyl)-1,1,2-trimethyl-1*H*-benzo[*e*]indol-3-ium (3)



The synthetic procedure was a variation of reference 6. A two-necked 50 mL round bottom flask fitted with reflux condenser and stirring bar was charged with 1,1,2-trimethyl-1H-benzo[e]indole, (1.0g, 4.78mmol) which was flushed with argon three times. After dissolving in dry o-dichlorobenzene (15 mL), (0.93g, 4.78mmol) of 6-bromohexanoic acid was added. The reaction mixture was stirred for 36h at 120^{o} C by using an oil bath. This resulted in a dark blue solution. The reaction mixture was allowed to cool down to room temperature. This cooled solution was triturated with 1:1 diethyl ether and hexane mixture (total volume 90 mL) for an hour. The blue precipitate was filtered off, washed with diethyl ether (3 x 20mL) and dried in vacuum yielding 1.1g of the compound 3. Percentage yield: 56%. ^{1}H NMR (DMSO-d6, 400 MHz) δ (ppm): 12 (br, s, 1H), 8.37 (d, J=8.4Hz, 1H), 8.29 (d, J=9Hz, 1H), 8.21 (d, J=7.8Hz, 1H), 8.14 (d, J=8.8Hz, 1H), 7.79 (t, J=7.0Hz, 7.2Hz, 1H), 7.73 (t, J=7.2Hz, 1H), 4.57 (t, J=7.6Hz, 2H), 2.93 (s, 3H), 2.23 (t, J=7.0Hz, 2H), 1.9 (qi, 2H), 1.76 (s, 6H), 1.56 (m, 2H), 1.45 (m, 2H)

5.3 Synthesis of Cyanine 5.5 (5) (4-(2-((1*E*,3*E*,5*E*)-5-(3-(5-carboxypentyl)-1,1-dimethyl-1*H*-benzo[*e*]indol-2(3*H*)-ylidene)penta-1,3-dien-1-yl)-1,1-dimethyl-1*H*-benzo[*e*]indol-3-ium-3-yl) butane-1-sulfonate, bromide salt)



Cyanine 5.5

The synthetic procedure was a variation of reference 6. A 50mL two-necked oven dry round bottom flask fitted with a magnetic stir bar was flushed with argon. Indolium salt, 1 (0.1g, 0.29 mmol) and malondialdehyde bis(phenylimine) monohydrochloride 2 (0.09g, 0.35mmol) were charged into the 50mL round bottom flask. Acetic anhydride (10mL) was added to this mixture and slowly heated to 120^{o} C in an oil bath and the reaction was monitored through TLC. The reaction was allowed to remain at 120^{o} C for half an hour. The reaction was then cooled to room temperature. An indolium salt, 3 (0.175g, 0.433mmol) in dry pyridine (5mL) was added to the above reaction dropwise. The reaction was allowed to stir at room temperature for 16h. The mixture was, then, concentrated in a rotavap and the residue was dissolved in CH_2Cl_2 (3mL) and loaded on silica column chromatography with CH_2Cl_2:MeOH

solvent system (total volume = 2L). A gradient of 100% to 25% of solvent CH_2Cl_2 was used to obtain 0.21g of a deep blue colored dye. Percentage yield of cyanine 5.5: 92%. ^{1}H NMR (DMSO-d6, 400 MHz) δ (ppm): 8.48 (t, J=12Hz, 2H), 8.24(d, J=8.2Hz, 2H), 8.06(m, 2H), 7.78(m, 1H), 7.67(m, 1H), 7.51(m, 2H), 6.65(dd, J=8Hz, 1H), 6.44(d, J=12Hz, 1H), 6.33(d, J=12Hz, 1H), 4.23(m, 4H), 3.0(m, 2H), 2.08(m, 2H), 1.96(m, 2H), 1.78(s, 16H), 1.57(m, 2H), 1.42(m, 2H)

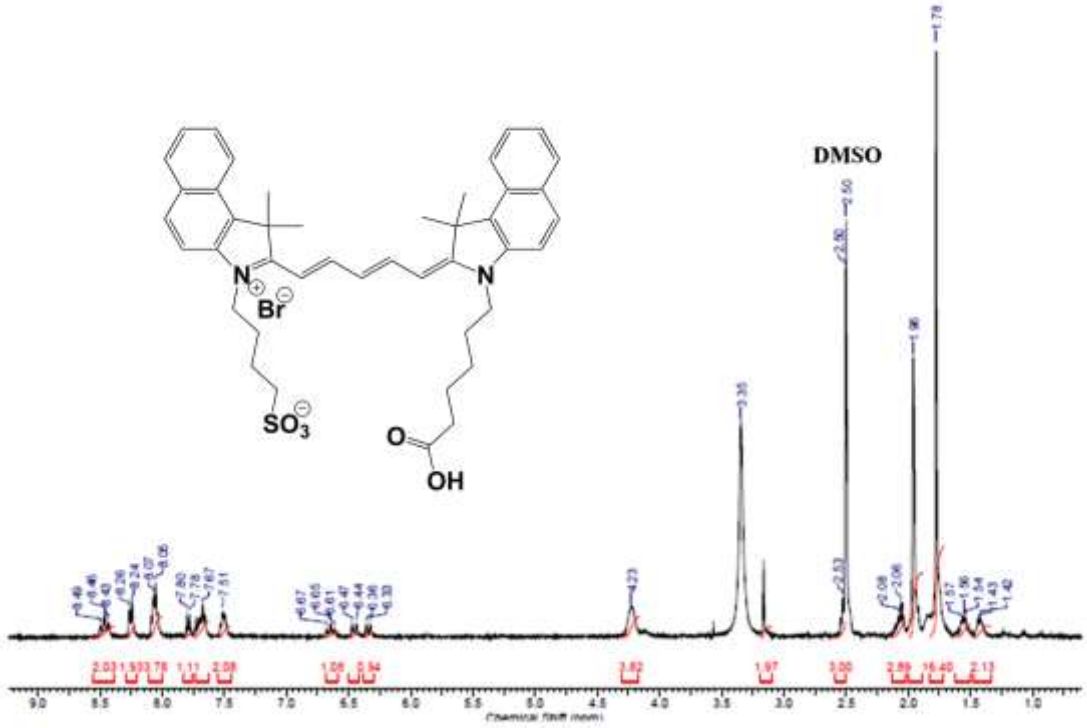


Figure S3: ^{1}H-NMR spectrum of Cyanine 5.5 (Varian, 400 Mhz)

6. Final Assembly of the Nanoplatforms for Protease Detection

200 mg of dopamine coated Fe/Fe_3O_4 nanoparticles were dispersed in 5 mL of DMF. A solution of 3 mmol of Cy5.5, 3.3 mmol of EDC, 1 mmol of DMAP in 1 mL of DMF was added to this dispersion. After sonicating for 1 h, the nanoparticles were precipitated by a magnet, and thoroughly washed with DMF (1 mL × 10). The recovered nanoparticles were redispersed in 5 mL of DMF, and to this dispersion, 2 mmol of TCPP linked peptide sequence, 2.2 mmol of EDC, 1 mmol of DMAP in 2 mL of DMF were added. After sonicating for 1 h, the nanoparticles were precipitated by a magnet (0.55T), and thoroughly washed with DMF (1 mL × 10). After drying in high vacuum, 170-185 mg of nanoplatform can be obtained. The composition of the nanoplatform was analyzed by means of UV-Vis spectroscopy (Agilent HP 8543A). After combining all washing fractions and subsequent solvent removal in high vacuum, TCPP (log ε (420nm) = 5.65) and cyanine 5.5 (log ε (682nm) = 5.17) were taken up

in 1.0 ml methanol and quantitatively measured taking advantage of their high absorption coefficients. The nanoplatforms were dispersed in PBS, and TCPP and cyanine 5.5 were measured by UV/Vis-spectroscopy as well, using dopamine coated Fe/Fe_3O_4-nanoparticles in PBS as the reference. The iron-content of the nanoplatforms was independently determined using the ferrozine assay.[2] This resulting data, together with the size of the Fe/Fe_3O_4-nanoparticles, enables the calculation of the average ratio of TCPP and cyanine 5.5 per nanoparticle.

7.0 Validation of the Nanoplatforms

7.1. TCPP Quenching in Dopamine-coated Fe/Fe_3O_4 Nanoparticles in the Absence of Cyanine 5.5

Figure S4 shows the maximal fluorescence increase observed after incubating a nanoplatform consisting of dopamine-protected Fe/Fe_3O_4 nanoparticles bound to TCPP, by means of the consensus sequence for MMP2 (IPVS-LRSG) in PBS at 25°C for 24h. The observed increase in fluorescence intensity is 65%.

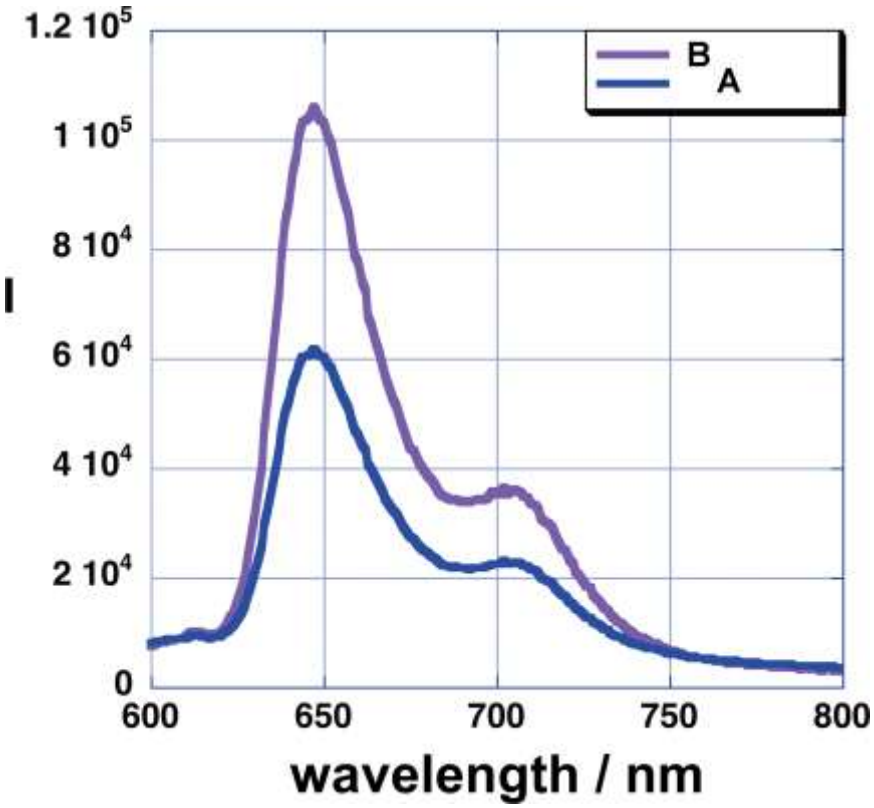


Figure S4: Fluorescence spectra of TCPP when bound to Fe/Fe_3O_4 nanoparticles (32+/-4 TCCP molecules per nanoplatform) (A) and after 24h of incubation with 1 x 10^{-10} mol l^{-1} MMP2 in PBS at 25°C (B). The observed fluorescence increase is 65%.

7.2. The Nanoplatform Designed for MMP13 is Not Activated by MMP9 or When Using a Scrambled Peptide Sequence Instead of the Consensus Sequence

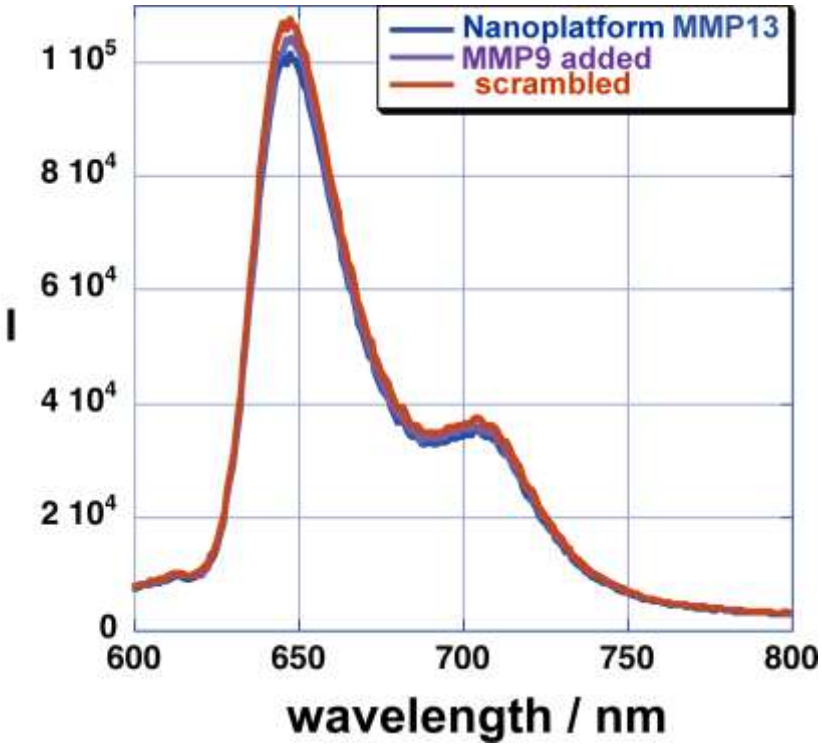


Figure S5: Fluorescence spectra of the nanoplatform for MMP13 in the absence of the protease, as well as in the presence of 1 x 10^{-12} mol l^{-1} of MMP9. As described in the text, the "wrong" protease failed to activate the nanoplatform. Shown is also the fluorescence occurring from a nanoplatform using a scrambled version of the consensus sequence (GRPGAGQVQLGI instead of GPQGLAGQRGIV) in the presence of 1 x 10^{-12} mol l^{-1} of MMP13. Both nanoplatforms were incubated under standard conditions.

8. Control Experiments and Additional Photophysical Information

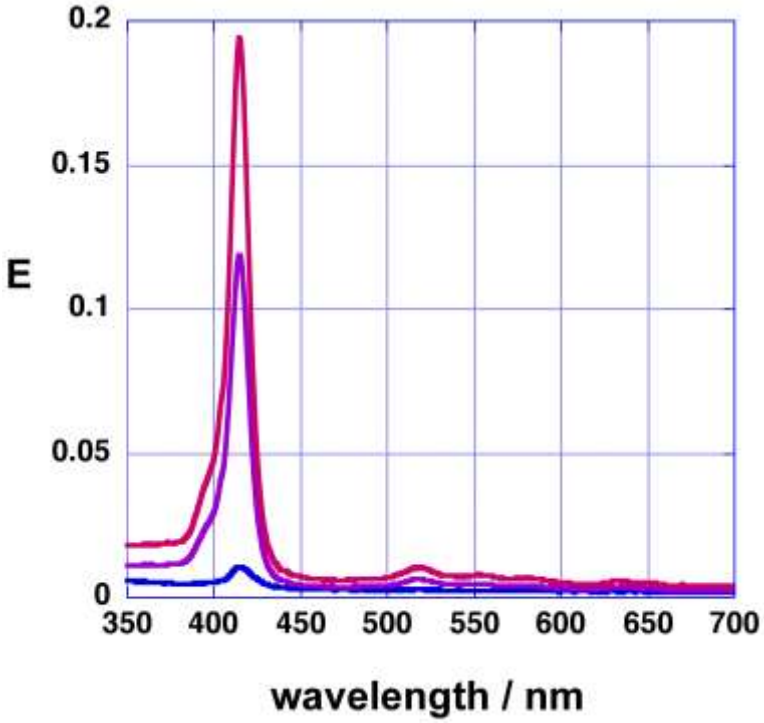


Figure S6: UV/Vis-absorption spectra of TCPP in PBS (5.05 x 10^{-8} M, 3.25 x 10^{-7} M, 5.05 x 10^{-7} M). The maxima of the Soret bands are at 416 +/- 1 nm, indicating that TCPP does not aggregate at pH = 7.4 at the chosen concentrations.

Table S1: Octanol/water Partitioning Coefficients, Calculated by Using Chemdraw Ultra (version 12.0.3.1216)

Protease	**Consensus Sequence**	**Log P when tethered to TCPP at pH 7.4**
H_2TCPP (pH=2)		9.6
H_2TCPP (pH=7)		7.6
MMP 1	VPMS-	4.1
MMP 2	IPVS-	4.6
MMP 3	RPFS-	3.7
MMP 7	VPLS-	4.5
MMP 9	VPLS-	4.5
MMP 11	GGAAN-	3.2
MMP 13	GPQGLA-	2.9
uPA	SGR-	4.7
CTS B	SLLKSR-	1.7
CTS D	SLLIFR-	3.4
CTS K	GPR-	4.5
CTS L	SGVVIA-	3.5

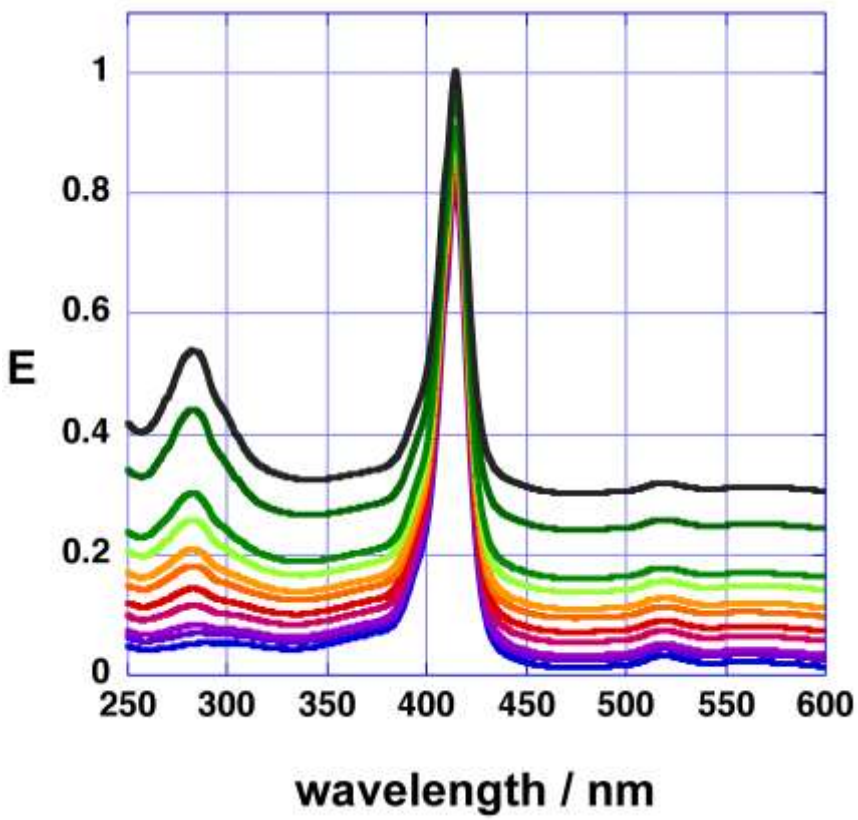


Figure S7: UV/Vis-absorption spectra of TCPP in PBS in the presence of increasing concentrations of dopamine-coated Fe/Fe_3O_4 nanoparticles. These nanoparticles are virtually identical with these employed in the nanoplatforms for protease detection. Nanoparticle concentrations: 0.00167 mg ml^{-1}, 0.005 mg ml^{-1}, 0.0083 mg ml^{-1}, 0.0117 mg ml^{-1}, 0.015 mg ml^{-1}, 0.02 mg ml^{-1}, 0.0267 mg ml^{-1}, 0.033 mg ml^{-1}, 0.067 mg ml^{-1}, 0.10 mg ml^{-1}, 0.167 mg ml^{-1}.

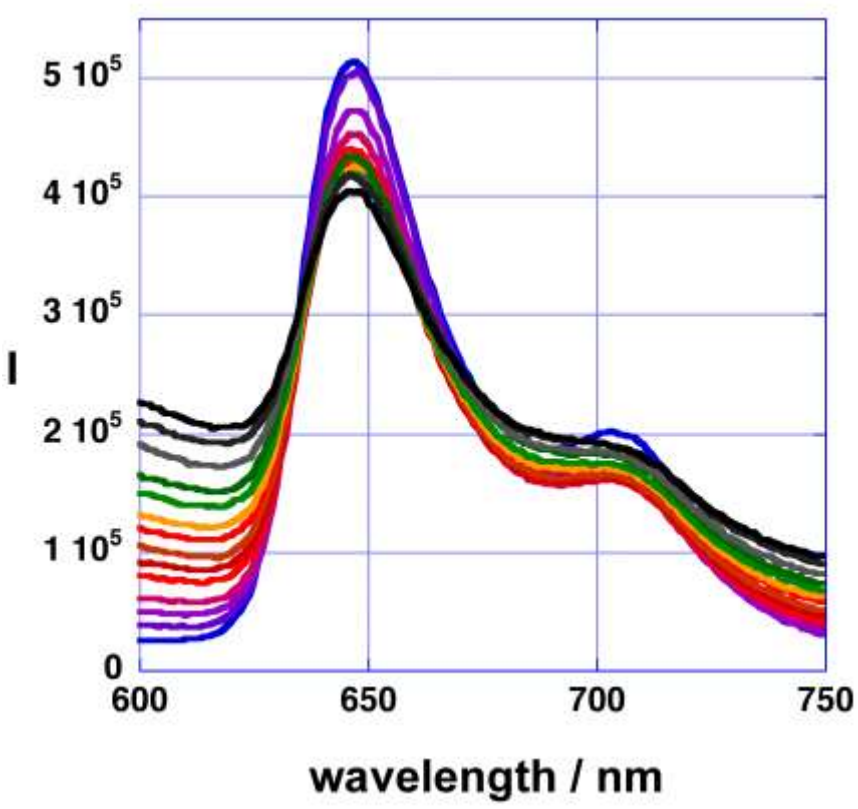


Figure S8: Fluorescence spectra of TCPP in PBS in the presence of increasing concentrations of dopamine-coated Fe/Fe_3O_4 nanoparticles. These nanoparticles are virtually identical with these employed in the nanoplatforms for protease detection. Nanoparticle concentrations: 0.00167 mg ml^{-1}, 0.005 mg ml^{-1}, 0.0083 mg ml^{-1}, 0.0117 mg ml^{-1}, 0.015 mg ml^{-1}, 0.02 mg ml^{-1}, 0.0267 mg ml^{-1}, 0.033 mg ml^{-1}, 0.067 mg ml^{-1}, 0.10 mg ml^{-1}, 0.167 mg ml^{-1}.

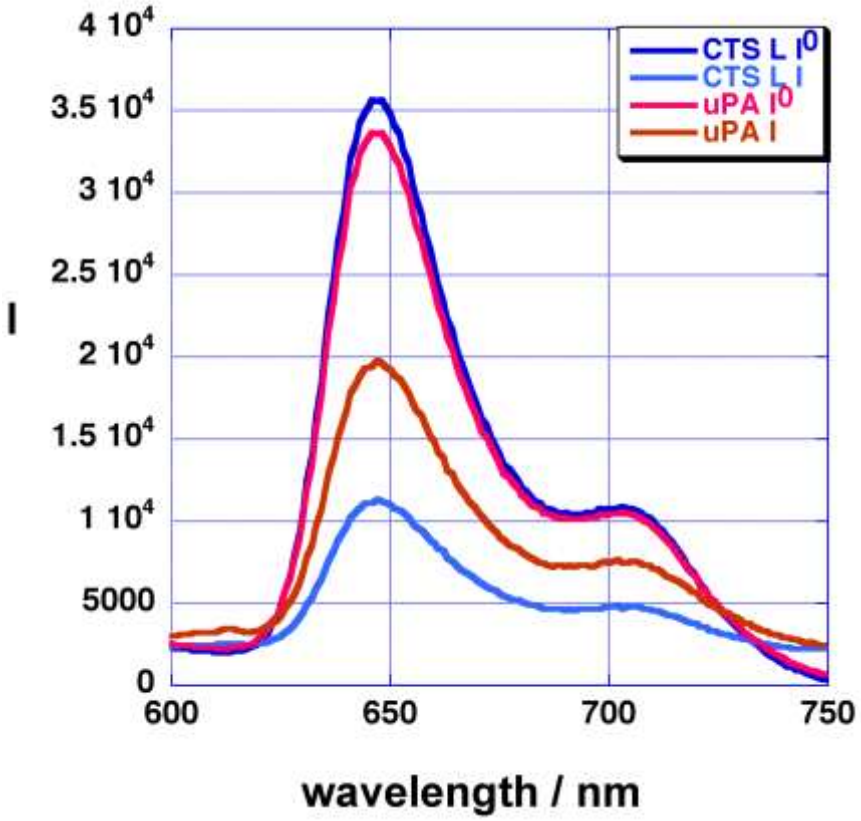


Figure S9: Fluorescence spectra of the nanoplatforms for CTS L and uPA in PBS, before (CTS I^0 and uPA I^0) and after (CTS I and uPA I) attaching cyanine 5.5. We have attached 35 +/- 2 TCPP units and 50 +/- 4 cyanine 5.5 molecules per Fe/Fe_3O_4 nanoparticle.

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