Supporting Information

for

Binding and Uptake into Human Hepatocellular Carcinoma Cells of Peptide-Functionalized Gold Nanoparticles

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Table of Contents

1. Experimental Procedures.

General: Solvents were purified by standard methods. All commercially available reagents and substrates were used as received.

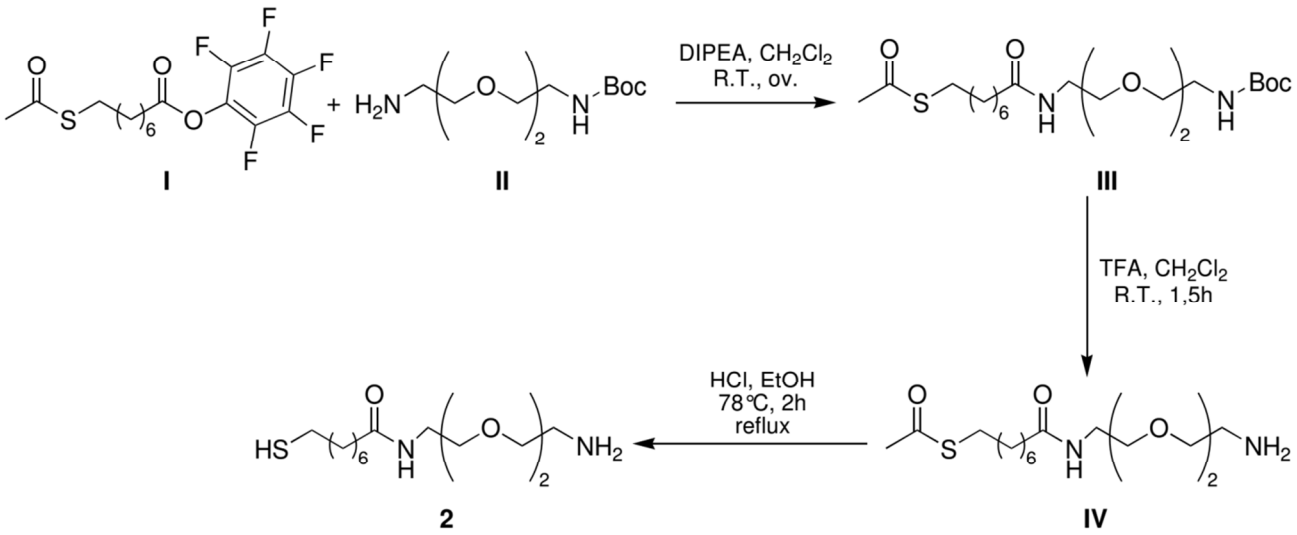
TLC analyses were performed using Merck 60 F_{254} precoated silica gel glass plates. Column chromatography was carried out on Macherey-Nagel silica gel 60 (70-230 mesh).

NMR spectra were recorded using a Bruker AC 250 spectrometer operating at 250.13 MHz for ^{1}H, 62.9 MHz for ^{13}C and Bruker AV300 spectrometer operating at 300 MHz for ^{1}H, 75.5 MHz for ^{13}C and 121.5 MHz for ^{31}P. Chemical shifts are reported relative to internal Me_4Si. Multiplicity is given as follow: s = singlet, d = doublet, t = triplet, q = quartet, qn = quintet, m = multiplet, br = broad peak.

ESI-MS mass spectra were obtained with an Agilent Technologies LC/MSD Trap SL mass spectrometer. TEM images were recorded on a Jeol 300 PX electron microscope. One drop of sample was placed on the sample grid and the solvent was allowed to evaporate. UV-Visible spectra and kinetic traces were recorded on Cary 50 spectrophotometer equipped with thermostatted multiple cell holders. IR spectra were recorded on a Nicolet 5700 FT-IR spectrophotometer and reported in cm^{-1}. AuNPs were dissolved in methanol at the concentration of 10 mg/mL then 20 μL of the solution casted onto a NaCl plate by Eppendorf micropipette and dried using a hotplate.

2. Synthesis of thiol 2

Thiol **2**was prepared according to the following scheme:



2.1 perfluorophenyl 8-(acetylthio)octanoate (I)

8-Bromooctanoic acid (2.00 g, 8.96 mmol) was dissolved in acetone (60 mL) and potassium thioacetate (1.33 g, 11.65 mmol) was added. The mixture was refluxed for 48 hours, the solvent was evaporated and the solid residue dissolved in CH_2Cl_2 (20 mL). The organic solution was extracted with water (5 x 20 mL) and dried with Na_2SO_4. After solvent evaporation 1.70 g (87%) of 8-(thioacetyl)-octanoic acid were obtained as an orange oil. ^{1}H-NMR ($CDCl_3$, 300 MHz), δ: 2.86 (t, 2H, 3 Hz, -S-*CH_2*-CH_2-), 2.35 (t, 2H, 3 Hz, -CH_2-*CH_2*-COOH), 2.32 (s, 3H, *CH_3*-CO-S-), 1.60 (m, 4H, -S-CH_2-*CH_2*-$(CH_2)_3$-*CH_2*-CH_2-COOH), 1.30 (m, 6H, -CH_2-(*CH_2*)$_3$-CH_2-).

8-(Acetylthio)-octanoic acid (1.70 g, 7.79 mmol) and pentafluorophenol (1.86 g, 10.13 mmol) was dissolved in CH_2Cl_2 (30 mL) and *N*-(3-Dimethylaminopropyl)-*N*′-ethyl-carbodiimide hydrochloride (EDC, 1.94 g, 10.13 mmol) was added. The mixture was stirred for 12 hours under nitrogen. The organic solution was extracted with water (3 x 20 mL) and dried with Na_2SO_4. After solvent evaporation, the crude product was purified by flash chromatography (silica gel, eluent: CH_2Cl_2/Petroleum Ether 3:7). 2.45 g (82%) of **1** were obtained as a yellow oil.

^{1}H-NMR ($CDCl_3$, 300 MHz), δ: 2.86 (t, 2H, 3 Hz, CH_3-CO-S-*CH_2*-CH_2-), 2.65 (t, 2H, 3 Hz, -CH_2-*CH_2*-COOH), 2.32 (s, 3H, *CH_3*-CO-S-), 1.77 (qn, 2H, 3 Hz, -S-CH_2-*CH_2*-$(CH_2)_3$-), 1.58 (qn, 2H, 3 Hz, -$(CH_2)_3$-*CH_2*-CH_2-COOH), 1.37 (m, 6H, -CH_2-(*CH_2*)$_3$-CH_2-).

2.2 Synthesis of *tert*-butyl (2-(2-(2-aminoethoxy)ethoxy) ethyl)carbamate (TEG) (II)

2,2′-(ethylenedioxy)diethylamine (5 g) was dissolved in 100 mL of methanol in a three-necked round-bottom flask. To this solution were slowly added, simultaneously, in a period of 2 h, a suspension of K_2CO_3 (10 g) in 50 mL of methanol and a solution of *tert*-butoxycarbonylanhydride (11 g, 0.9 equiv) in 25 mL methanol. The suspension was then kept under stirring overnight and filtered, and the solvent was evaporated. The crude oil was dissolved in CH_2Cl_2 (100 mL) and extracted with a 5% aqueous solution of K_2CO_3 (2 x 50 mL) and then with water (2 x 50 mL). The dried organic layer was evaporated, and the collected material was flash chromatography ($CHCl_3$) to give 7 g (83 % yield) of *tert*-butyl (2-(2-(2-aminoethoxy)ethoxy) ethyl)carbamate as a colorless liquid. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.53 (m, 4H, -CH_2-*CH_2*-O-$(CH_2)_2$-O-*CH_2*-CH_2-); 3.44 (m, 4H, -O-*$(CH_2)_2$*-O-); 3.22 (m, 2H, -CH_2-*CH_2*-NH-CO-); 2.78 (t, 2H, NH_2-*CH_2*-CH_2-); 1.35 (s, 9H, -O-C-*$(CH_3)_3$*).

2.3 Synthesis of S-(2,2-dimethyl-4,15-dioxo-3,8,11-trioxa-5,14-diazadocosan-22-yl) ethanethioate (TEG) (III)

Perfluorophenyl 8-(acetylthio)octanoate (880 mg, 1 equiv) was dissolved in 20 mL of dry dichloromethane and *N*,*N*-diisopropylethylamine (0.56 mL, 1.1 equiv.) was added dropwise. The reaction was stirred in a cold bath and the compound **II** (800 mg, 1.1 equiv) was dissolved in 10 mL of DCM then added to the reaction mixture. The resulting mixture was stirred at room temperature overnight then washed with water (4 x 10 mL) and dried with $MgSO_4$. The dried organic layer was evaporated to give 250 mg (86 % yield) of S-(2,2-dimethyl-4,15-dioxo-3,8,11-trioxa-5,14-diazadocosan-22-yl) ethanethioate. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.60 (m, 4H, -CH_2-*CH_2*-O-$(CH_2)_2$-O-*CH_2*-CH_2-); 3.55 (t, 4H, 2 Hz, -O-*$(CH_2)_2$*-O-); 3.46 (t, 2H, 2 Hz, CO-NH-*CH_2*-CH_2-); 3.30 (q, 2H, 2 Hz, -S-*CH_2*-CH_2-); 2.84 (t, 2H, 3 Hz, -O-CH_2-*CH_2*-NH-CO-); 2.31 (s, 3H, *CH_3*-CO-S-); 2.17 (t, 2H, 3 Hz, -CH_2-*CH_2*-CO-NH-); 1.70 – 1.50 (m, 4H, -S-CH_2-*CH_2*-$(CH_2)_3$-*CH_2*-CH_2-CO-NH-); 1.44 (s, 9H, -O-C-*$(CH_3)_3$*); 1.35 – 1.20 (m, 6H, -S-CH_2-CH_2-*$(CH_2)_3$*-CH_2-CH_2-CO-NH-).

2.4 Synthesis of S-(8-((2-(2-(2-aminoethoxy)ethoxy)ethyl)amino)-8-oxooctyl) ethanethioate (TEG) (IV)

The compound **III** was dissolved in CH_2Cl_2 (5 mL) and 3 mL of trifluoroacetic acid (TFA) were added. The reaction mixture was stirred at R.T. for 1.30h. The solvent was evaporated and the crude of the reaction was obtain as a white salt used in the following step without puritification. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.76 (t, 2H, 2 Hz, -O-*CH_2*-CH_2-NH_2); 3.70 – 3.60 (m, 6H, CH_2-*CH_2*-O-*$(CH_2)_2$*-O-);

3.50 (m, 2H, -O-CH_2-*CH_2*-NH_2); 3.27 (q, 2H, 2 Hz, -S-*CH_2*-CH_2-); 2.85 (t, 2H, 3 Hz, -CO-NH-*CH_2*-CH_2-O-); 2.33 (s, 3H, *CH_3*-CO-S-); 2.30 (t, 2H, 3 Hz, -CH_2-*CH_2*-CO-NH-); 1.65 – 1.45 (m, 4H, -S-CH_2-*CH_2*-$(CH_2)_3$-*CH_2*-CH_2-CO-NH-); 1.40 – 1.20 (m, 6H, -S-CH_2-CH_2-*$(CH_2)_3$*-CH_2-CH_2-CO-NH-).

_N_-(2-(2-aminoethoxy)ethyl)-3-mercaptopropanamide (2)

0.045 g (0.125 mmol) of **IV** were dissolved in ethanol (1.5 mL). A 6 M HCl solution in water (1.5 mL) was added and the mixture was stirred at 78 °C for 3 hours. The reaction mixture was allowed to cool and the solvent evaporated to obtain 0.038 g (95%) of **2** as a yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.62 – 3.56 (m, 4H, -O-*CH_2*-CH_2-NH_2); 3.47 – 3.42 (m, 2H, -CO-NH-*CH_2*-CH_2-O-); 3.28 – 3.20 (m, 4H, CH_2-*CH_2*-O-*$(CH_2)_2$*-O-); 3.06 – 2.97 (m, 2H, -O-CH_2-*CH_2*-NH_2); 2.41 – 2.35 (m, 2H, -S-*CH_2*-CH_2-); 2.09 (t, 2H, 7.4 Hz, -CH_2-*CH_2*-CO-NH-); 1.57 – 1.41 (m, 4H, -S-CH_2-*CH_2*-$(CH_2)_3$-*CH_2*-CH_2-CO-NH-); 1.32 – 1.16 (m, 6H, -S-CH_2-CH_2-*$(CH_2)_3$*-CH_2-CH_2-CO-NH-).

3. Synthesis and characterization of monolayer protected gold nanoparticles (AuNP)

Monolayer protected gold nanoparticles (**AuNP**) were prepared according to a previously reported two-step procedure.[1] All the glassware used in the AuNP preparation was washed with aqua regia and rinsed with distilled water. $HAuCl_4$ is strongly hygroscopic and was weighted within a dry-box.

A solution of $HAuCl_4 \cdot 3H_2O$ (100 mg, 0.254 mmol) in water (4 mL) was extracted with a solution of tetraoctylammonium bromide (5 g, 9.14 mmol) in N_2 purged toluene (250 mL). To the resulting reddish-orange organic solution, dioctylamine (3.36 g, 13.92 mmol) is added (the amount of dioctylamine was calculated[1] in order to obtain 2 nm nanoparticles). The mixture is vigorously stirred under N_2 for 30 min. During this period of time the color of the mixture fades. A solution of $NaBH_4$ (93.0 mg, 2.46 mmol) in H_2O (1 mL) is then rapidly added. The color of the solution turns rapidly to black due to nanoparticles formation. After 2 hours of stirring, the aqueous layer is removed. To the above nanoparticle solution, the desired thiols (0.119 mmol) dissolved in 3 mL of isopropanol were rapidly added (in the case of MPGN coated with a mixture of 1 and 2 thiols, thiol 2 is added first and thiol 1 is added after 5 minutes). The reaction mixture is evaporated and the resulting crude is dissolved in MeOH and purified by gel permeation chromatography with Sephadex LH-60.

3.1. Characterization of 1-AuNP

TEM analysis of the different samples of small nanoparticles (Figure S1) yields an average diameter for the MPGN of 1.8±0.5 nm.

Formula for 1-**AuNP** is $Au_{180}\mathbf{1}_{70}$, as previous determined.[2)]

NMR analysis (Figure S2) indicates monolayer formation (broadening of all signals), as confirmed by diffusion-filtered experiments (not shown).

Characterization of 1-AuNP

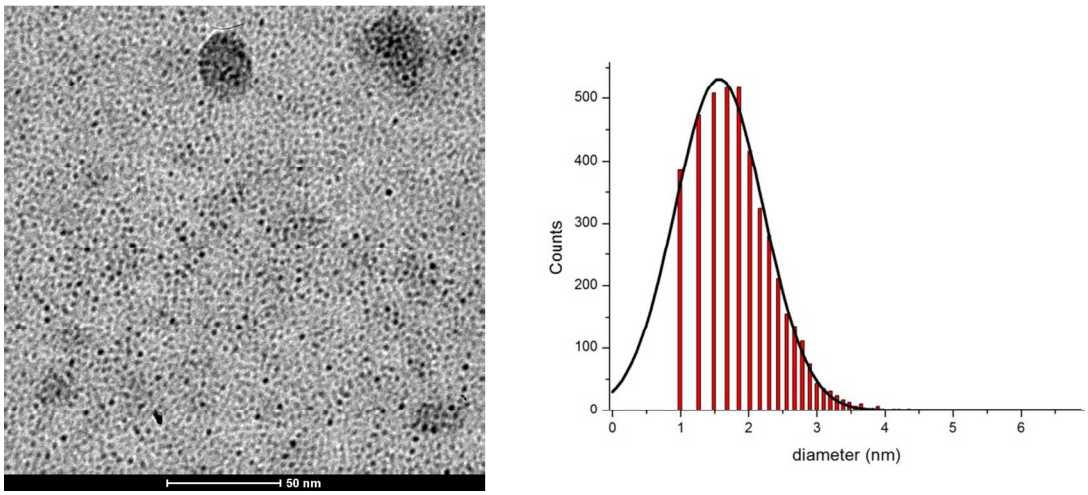


Figure S1: Sample TEM image of 1-**AuNP** and size distribution: average diameter = 1.8 nm (σ= 0.5 nm).

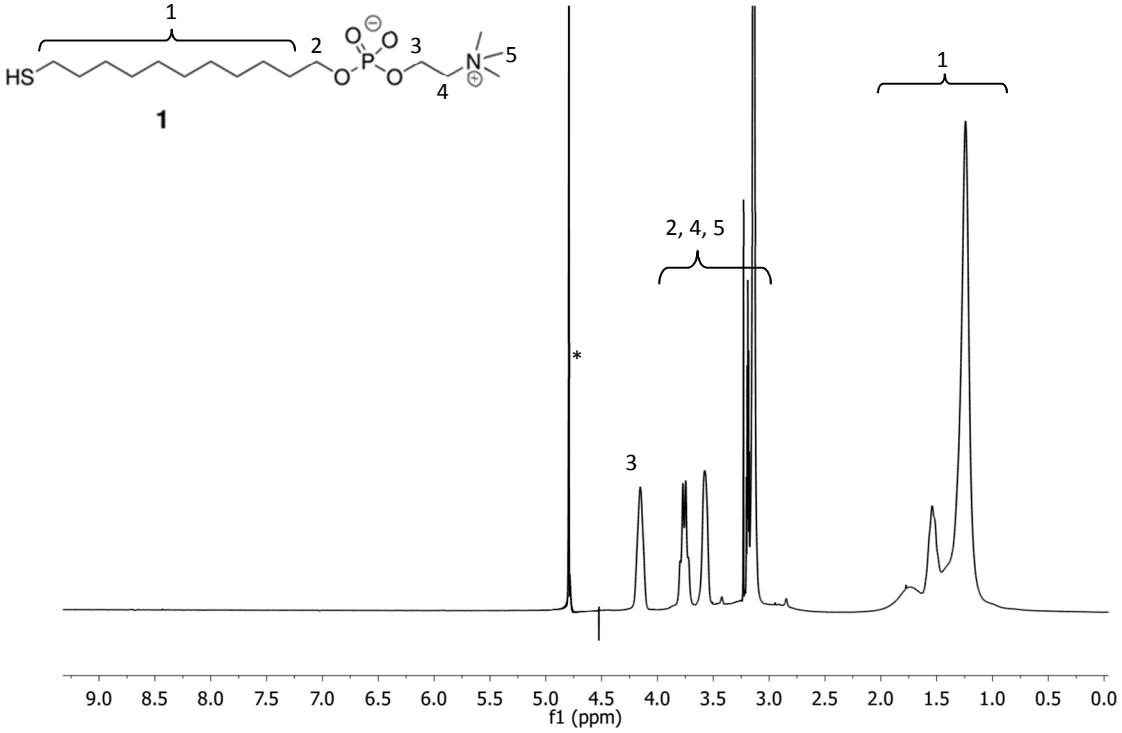


Figure S2: ^{1}H-NMR (250 MHz) spectrum in MeOD of the **1-AuNP** (* indicates the residual solvents and impurities)

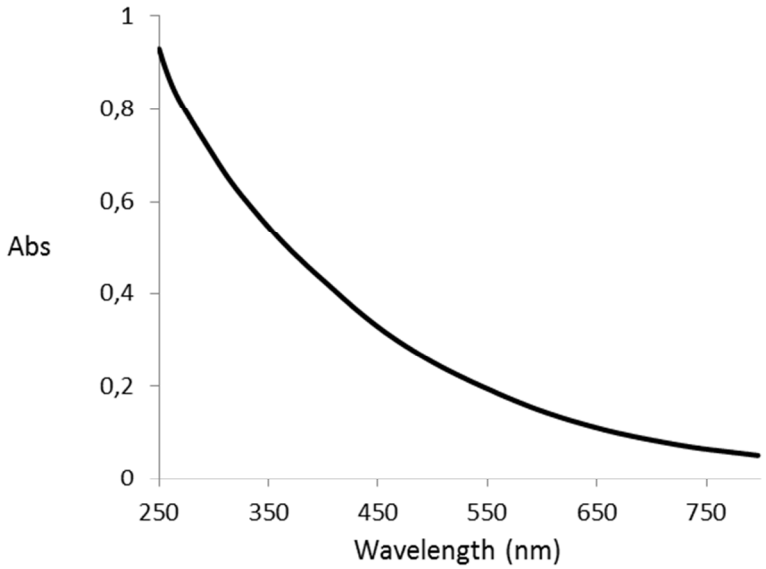


Figure S3: UV spectrum of the **1-AuNP**

3.2. Characterization of 1/2-AuNP

TEM analysis of the different samples of small nanoparticles (Figure S4) yields an average diameter for the MPGN of 1.9±0.7 nm.

Formula for **AuNP1/2** is and $Au_{180}\mathbf{1}_{63}\mathbf{2}_{7}$, as previous determined.[2]

NMR analysis (Figure S5) indicates monolayer formation (broadening of all signals), as confirmed by diffusion-filtered experiments (not shown).

Characterization of 1/2-AuNP

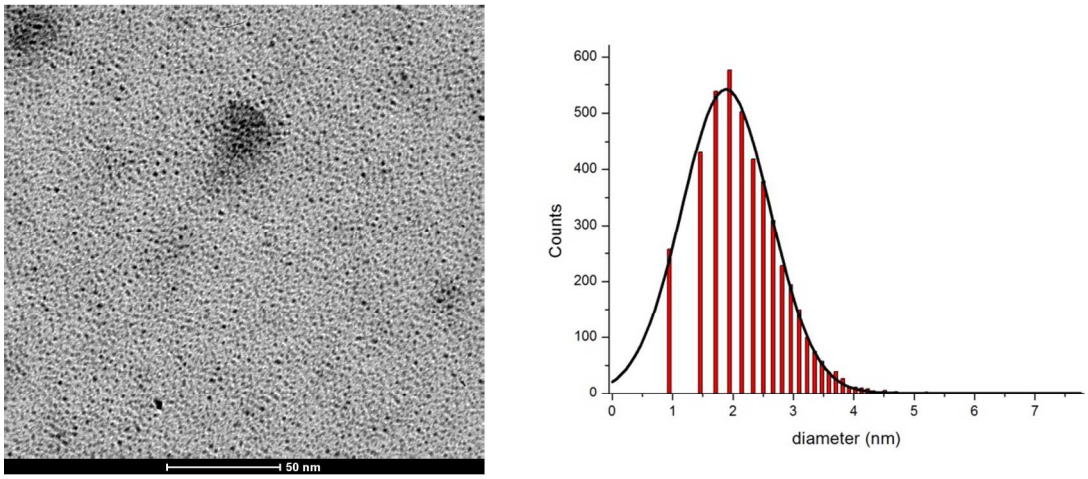


Figure S4: Sample TEM image of **1/2-AuNP** and size distribution: average diameter = 1.9 nm (σ= 0.7 nm).

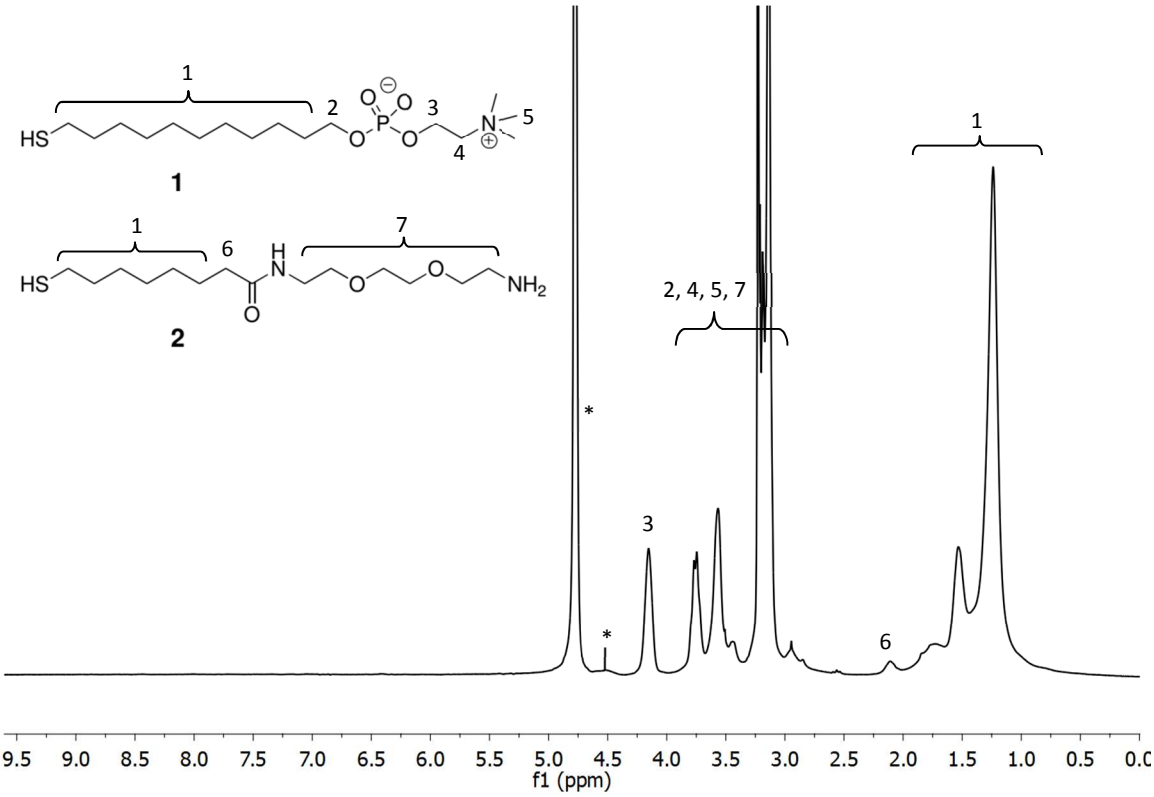


Figure S5: ^{1}H-NMR (250 MHz) spectrum in MeOD of the **1/2-AuNP** (* indicates the residual solvents and impurities)

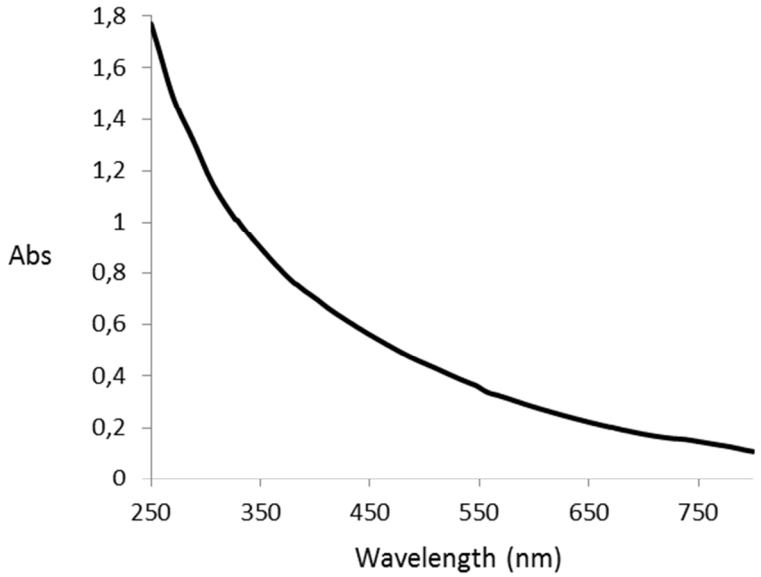


Figure S6: UV spectrum of the **1/2-AuNP**

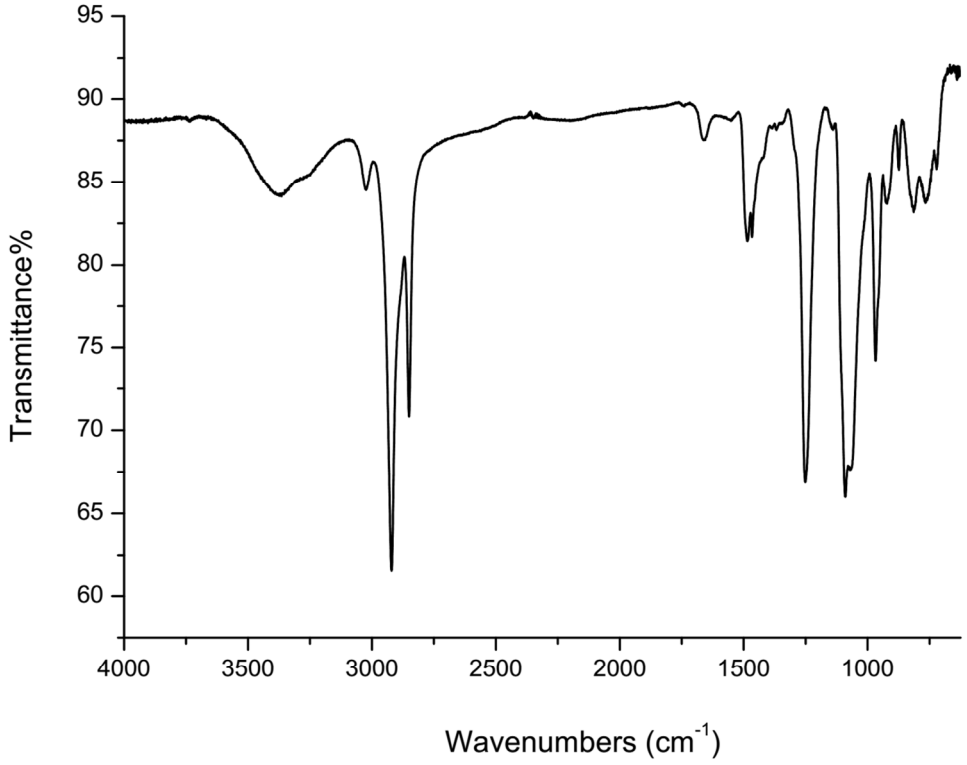


Figure S7: IR spectrum of the **1/2-AuNP**

3.3. Conjugation of 1/2-AuNP with MPBS

1/2-AuNP (34 mg, 1 equiv) was dissolved in 3 mL of methanol and *N*,*N*-diisopropylethylamine (10 uL, 10 equiv.) was added dropwise. 3-(maleimido)propionic acid *N*-hydroxysuccinimide ester (16.5 mg, 10 equiv) was dissolved in 1.4 mL of dry DMF then added to the reaction mixture. The reaction mixture was stirred at R.T. for 16h. The solvent was evaporated and the resulting crude is dissolved in methanol and purified by gel permeation chromatography with Sephadex LH-60.

Characterization of 1/2-AuNP conjugated with MPBS

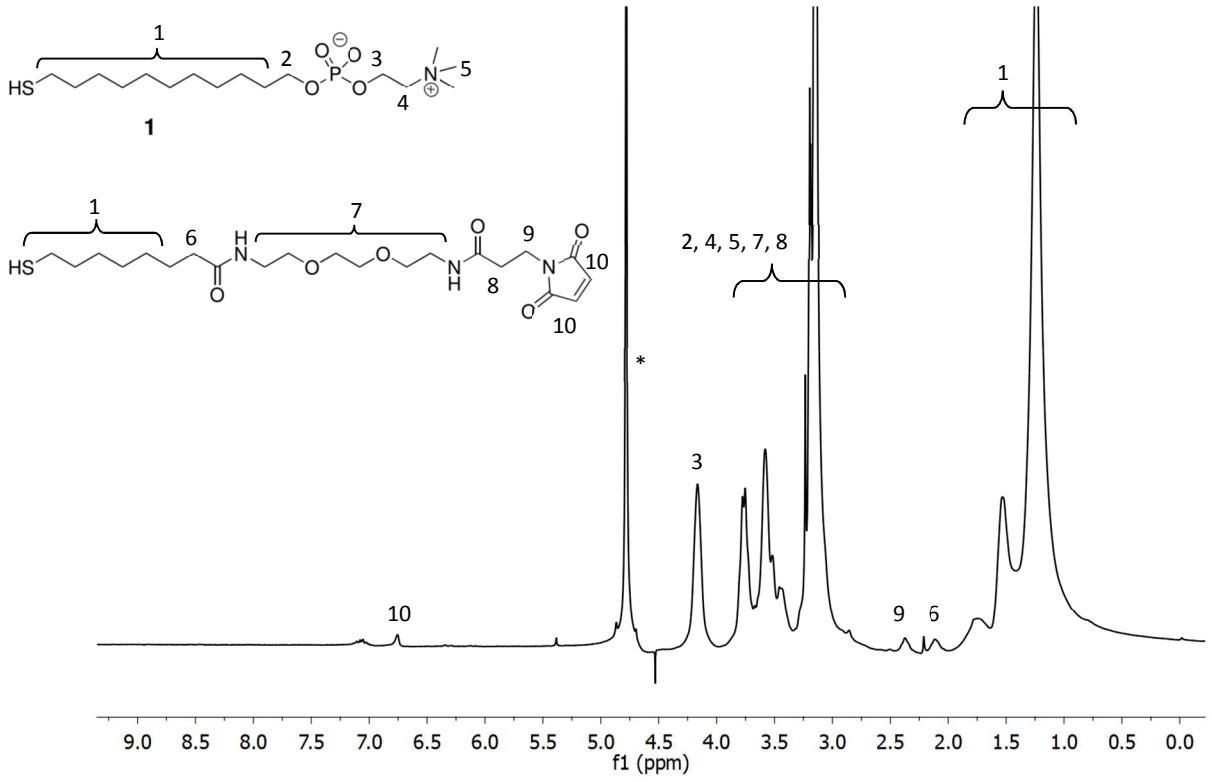


Figure S8: ^{1}H-NMR (250 MHz) spectrum in MeOD of the **1/2-AuNP** after conjugation with MPBS (* indicates the residual solvents and impurities)

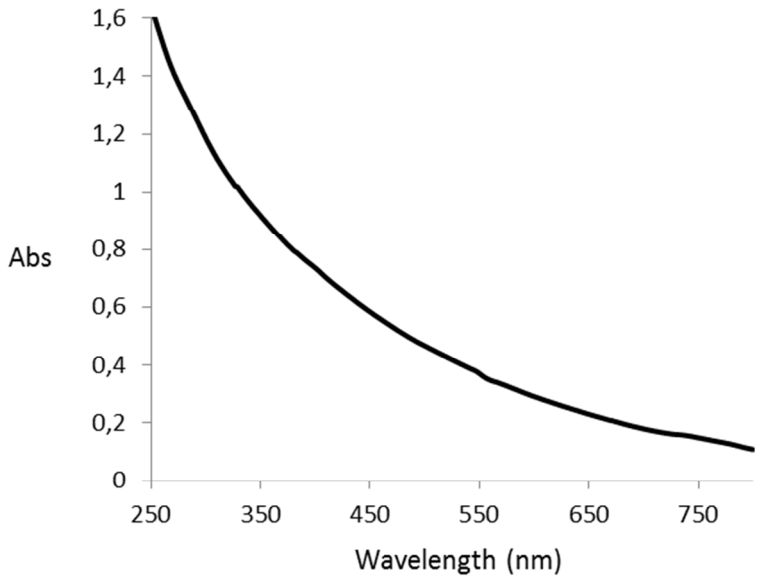


Figure S9: UV spectrum of the **1/2-AuNP** after conjugation with MPBS

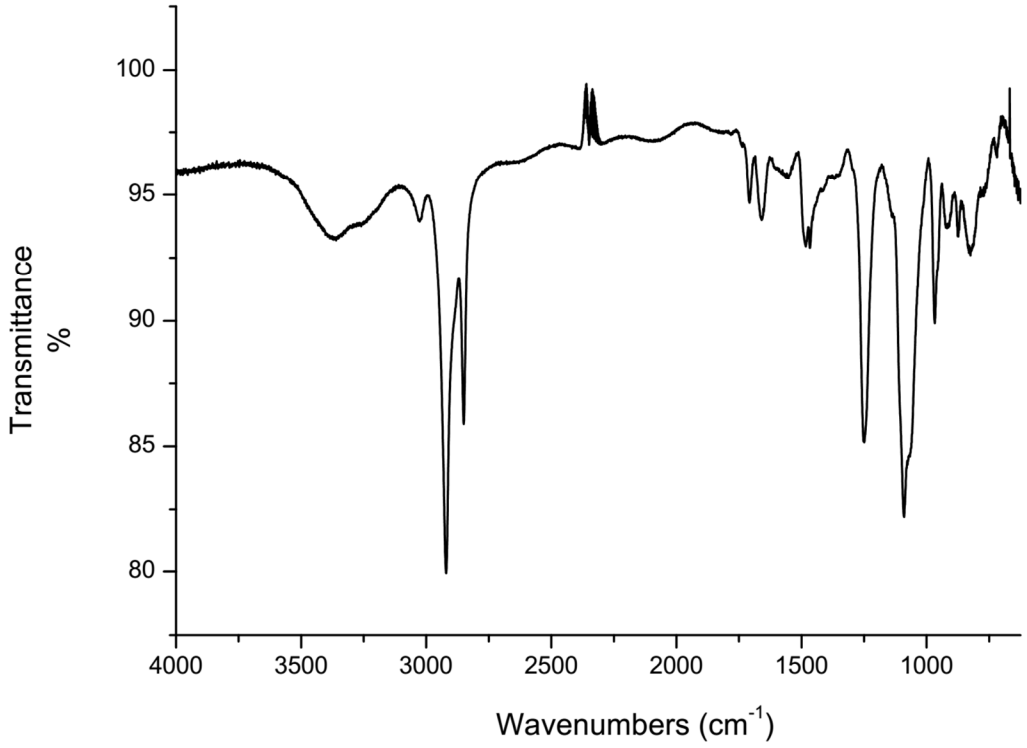


Figure S10: IR spectrum of the **1/2-AuNP** after conjugation with MPBS

3.4. PreS1-AuNP

1/**2**-AuNP conjugated with MPBS (25 mg, 1 equiv.) was dissolved in 4 mL of dry methanol and triethylamine (3.2 µL, 5 equiv.) was added slowly. CysPre-S1(21-47) peptide (14.42 mg, 1 equiv.) is dissolved in 2 mL of dry methanol then added to the reaction mixture. The reaction mixture was stirred at R.T. for 16h in N_2 atmosphere. The solvent was evaporated and the resulting crude is dissolved in methanol and purified by gel permeation chromatography with Sephadex LH-60.

Characterization of PreS1-AuNP

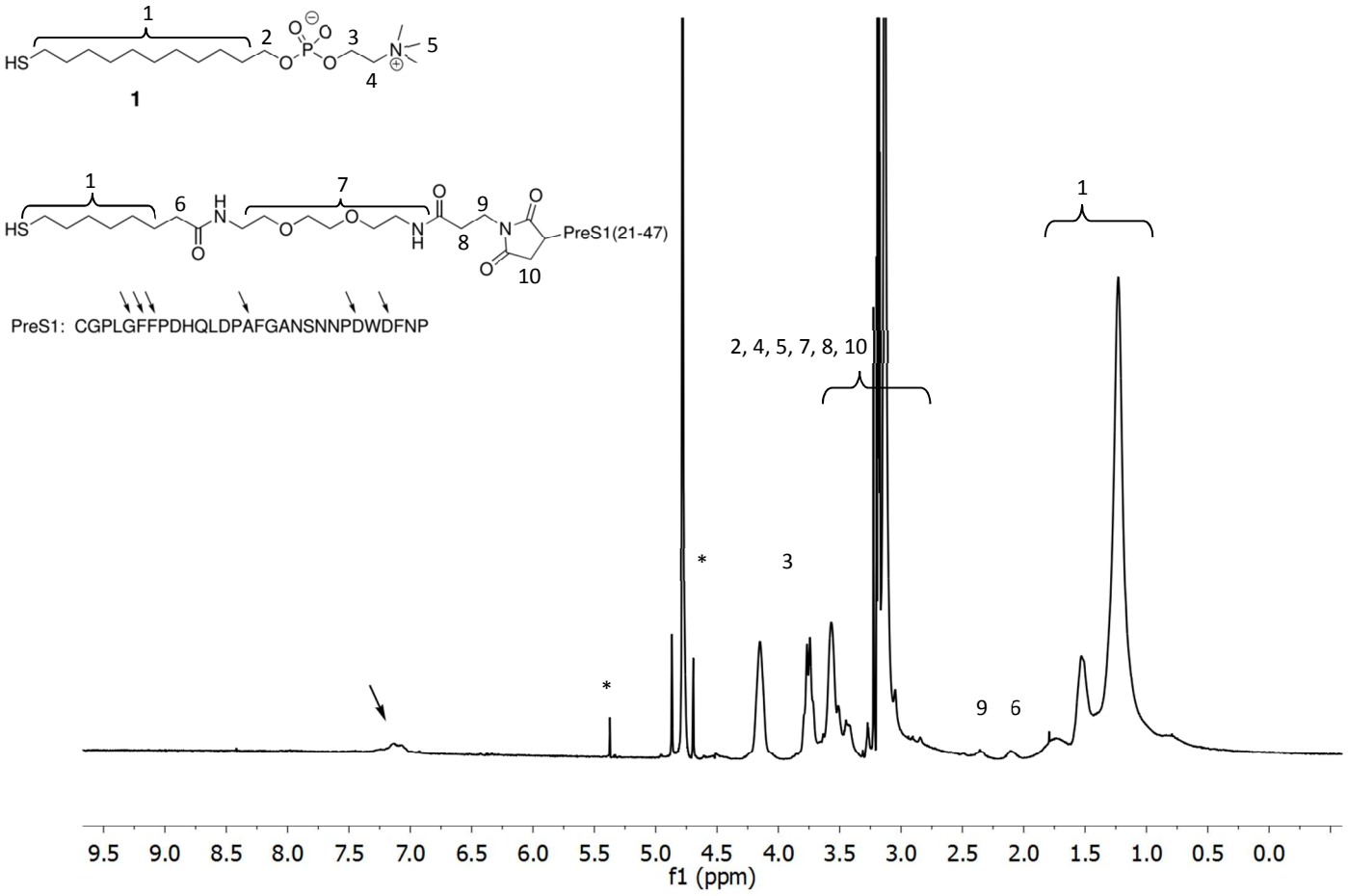


Figure S11: ^{1}H-NMR (250 MHz) spectrum in MeOD of the **PreS1-AuNP** (* indicates the residual solvents and impurities, \ indicates aromatic signals of the peptide)

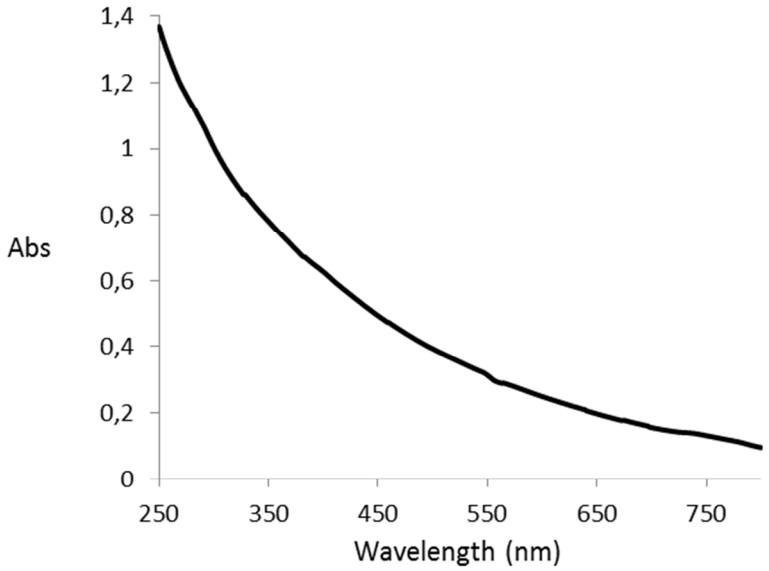


Figure S12: UV spectrum of the **PreS1-AuNP**

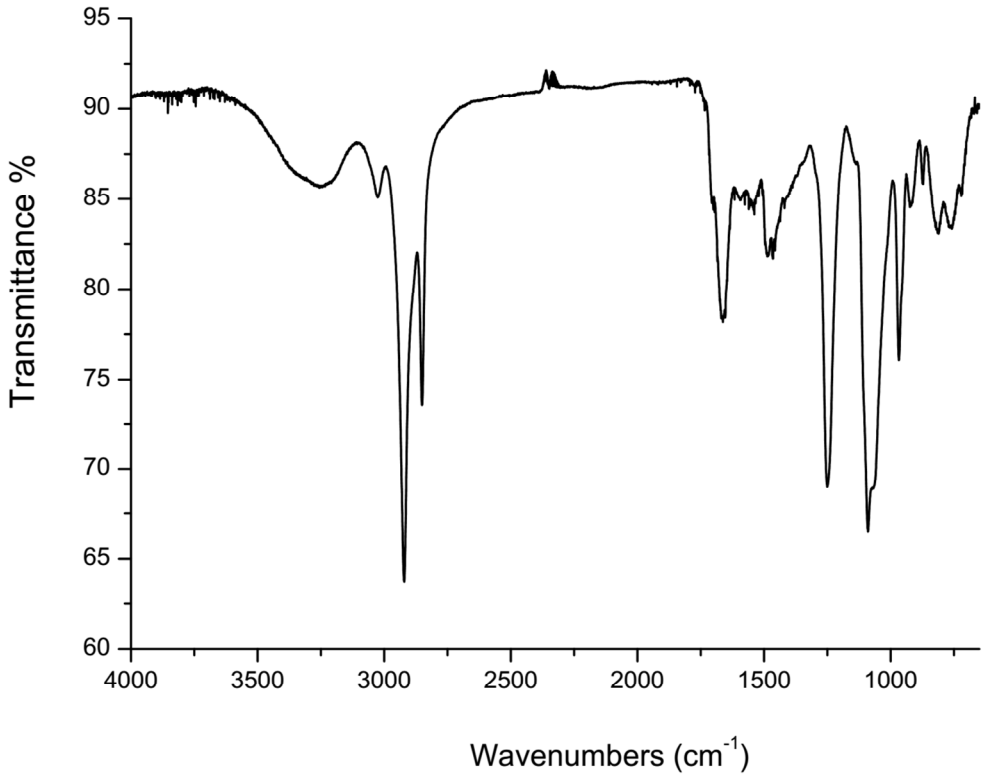


Figure S13: IR spectrum of **PreS1-AuNP**

4. Surface Plasmon Resonance analysis

SPR analysis were performed using a Biacore X100 instrument (GE Healthcare, Uppsala, Sweden). A dextrane-coated gold chip (CM5) was activated by flowing a 1:1 mixture of 0.2 M N-ethyl-N-(3-dimethylaminopropyl) carbodiimide and 0.05 M N-hydroxysuccinimide in water. A continuous flow of HEPES pH 7.4 was maintained. SERPINB3 (50 μg/mL) in 10 mM sodium acetate (pH 5) was immobilized on the activated chip surfaces at a flow rate of 10 μL/min to obtain an immobilization level of Response Bound of 1661 RU and Response Final of 1840 RU. Excess of activated carboxylic groups on the chip was blocked with ethanolamine.

AuNP kinetic assays were performed following the instrument built-in standard protocol using HBS-EP+ buffer at pH 7.4 (0.1 M HEPES, 1.5 M NaCl. 30 mM EDTA, 0.5% v/v P20 surfactant) by subsequent 180 sec injections of AuNP solutions at increasing concentration (0.005-0.157 nM) using NaCl 3 M as regeneration solution.

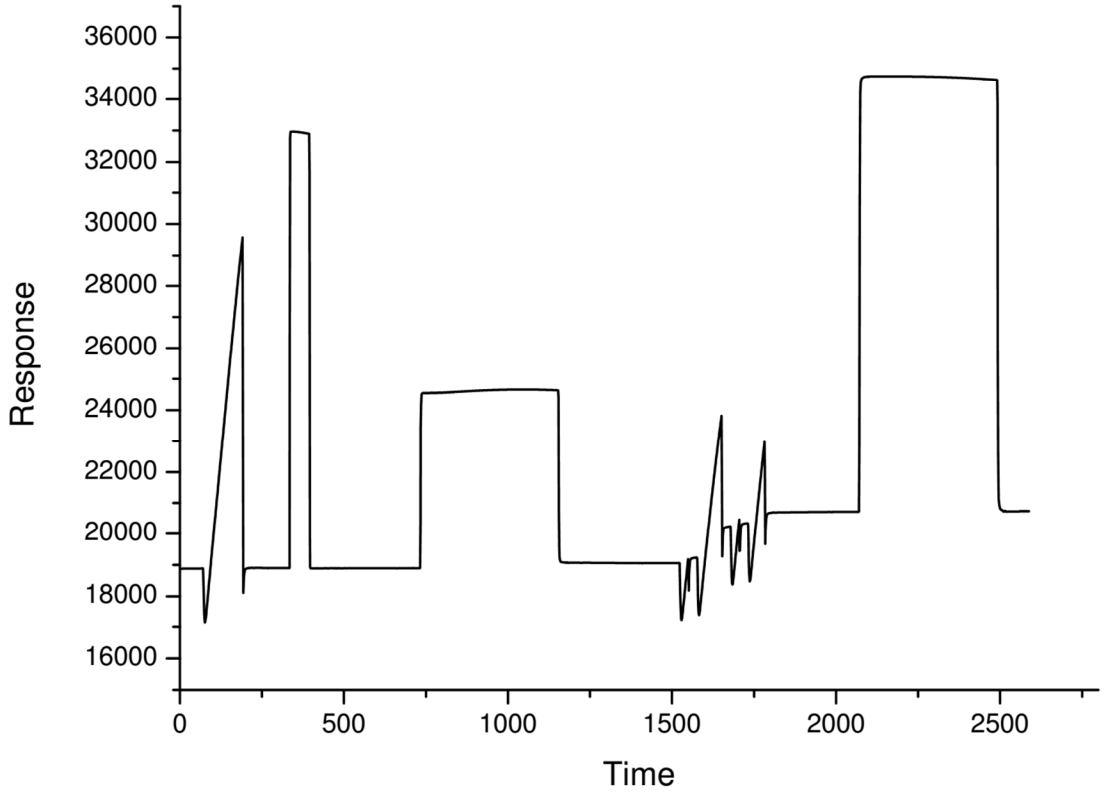


Figure S14: Sensogram of the immobilization SERPINB3 (50 μg/mL) in 10 mM sodium acetate (pH 5)

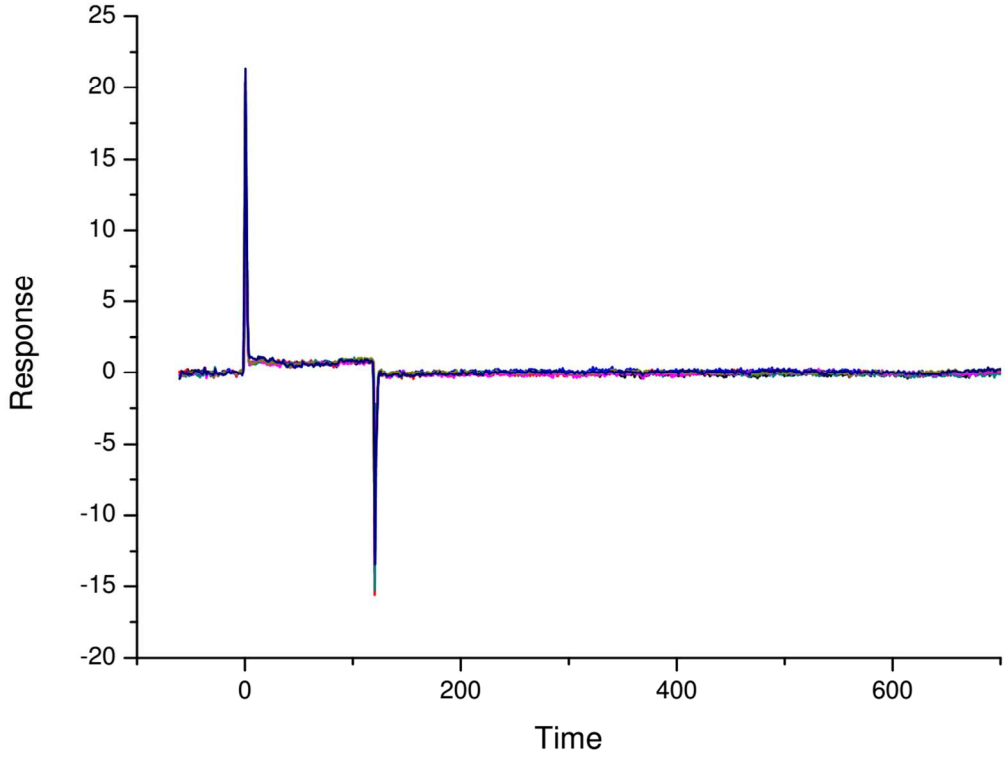


Figure S15: Kinetic assays Multi Cycle of PreS1

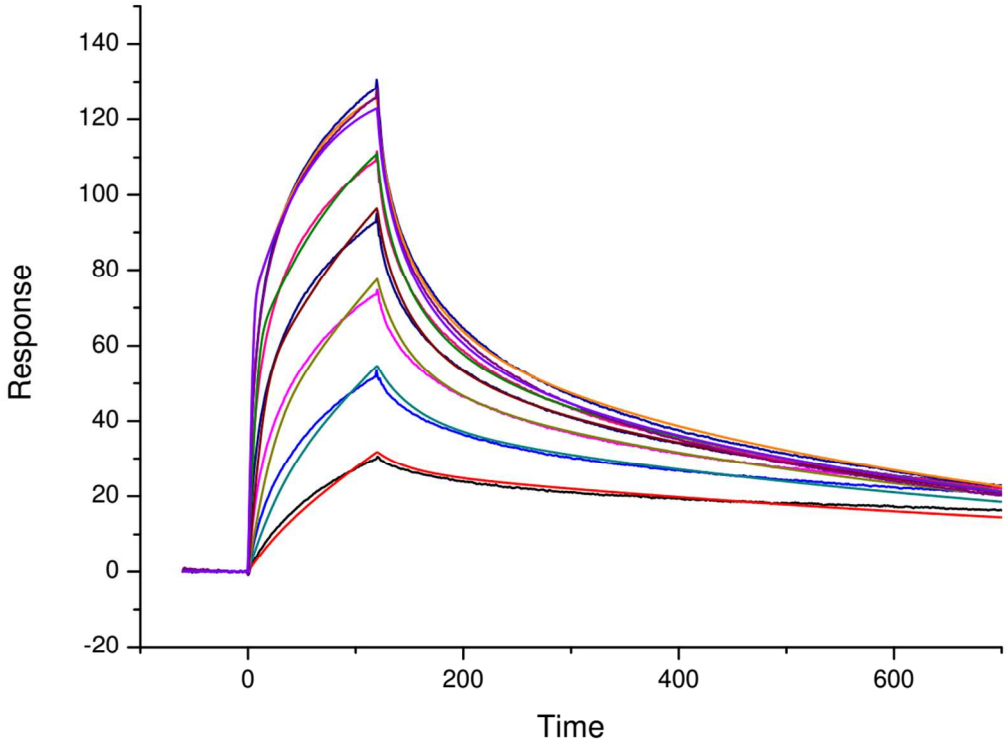


Figure S16: Kinetic assays Multi Cycle of **1-AuNP** and fitting as heterogeneous ligand

Conc (nM)	**ka1 (1/Ms)**	**kd1 (1/s)**	**KD1 (M)**	**ka2 (1/Ms)**	**kd2 (1/s)**	**KD2 (M)**	**Rmax1 (RU)**	**Rmax2 (RU)**
	8.8×10^{9}	0.095	1.1×10^{-11}	1×10^{8}	0.002	1.8×10^{-11}		
0.005							48	761
0.01							66	456
0.02							67	254
0.04							67	146
0.078							70	93
0.157							76	70
0.157							77	65

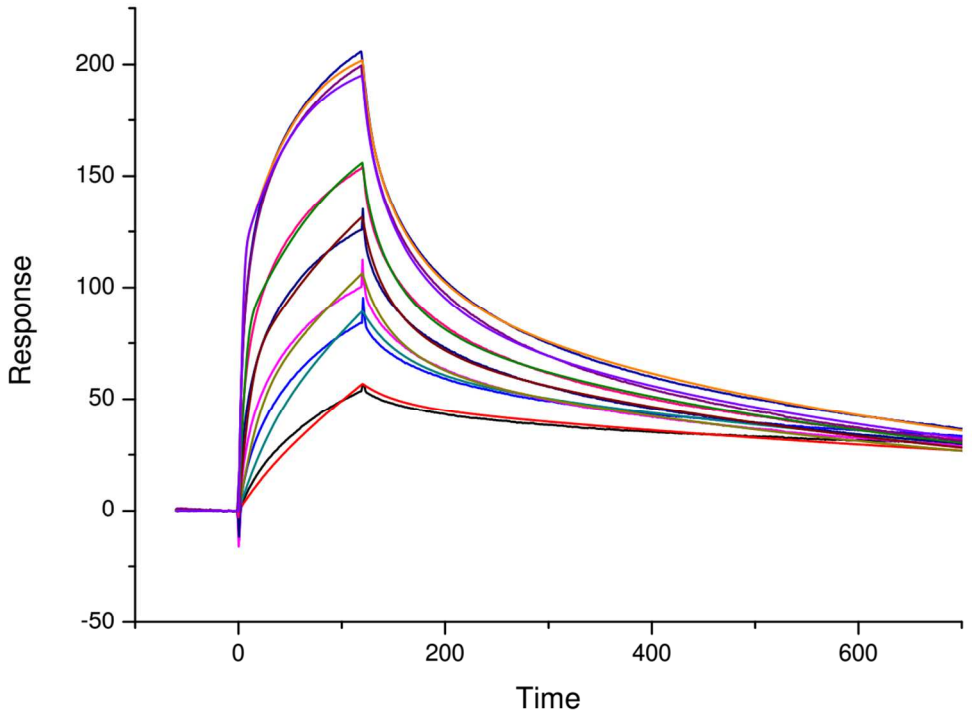


Figure S17: Kinetic assays Multi Cycle of **PreS1-AuNP** and fitting as heterogeneous ligand

Conc (nM)	**ka1 (1/Ms)**	**kd1 (1/s)**	**KD1 (M)**	**ka2 (1/Ms)**	**kd2 (1/s)**	**KD2 (M)**	**Rmax1 (RU)**	**Rmax2 (RU)**
	7.4×10^{9}	0.081	1.1×10^{-11}	1×10^{8}	0.002	1.6×10^{-11}		
0.005							101	1306
0.01							109	670
0.02							87	316
0.04							88	188
0.078							94	129
0.157							122	108
0.157							124	98

4.1 Nanoparticles-SB3 interaction investigated by capture-capture SPR investigations

A dextrane-coated gold chip (CM5) was functionalized with an anti-SB3 antibody using the same procedure described above. Single cycles capture-capture kinetic assays were performed following the instrument built-in the standard protocol using HBS-EP+ buffer at pH 7.4 (0.1 M HEPES, 1.5 M NaCl. 30 mM EDTA, 0.5% v/v P20 surfactant) by injection of SB3 (32 μM, 600 sec) or buffer, and subsequent injections of AuNP solutions at increasing concentration (0.010-0.157 nM).

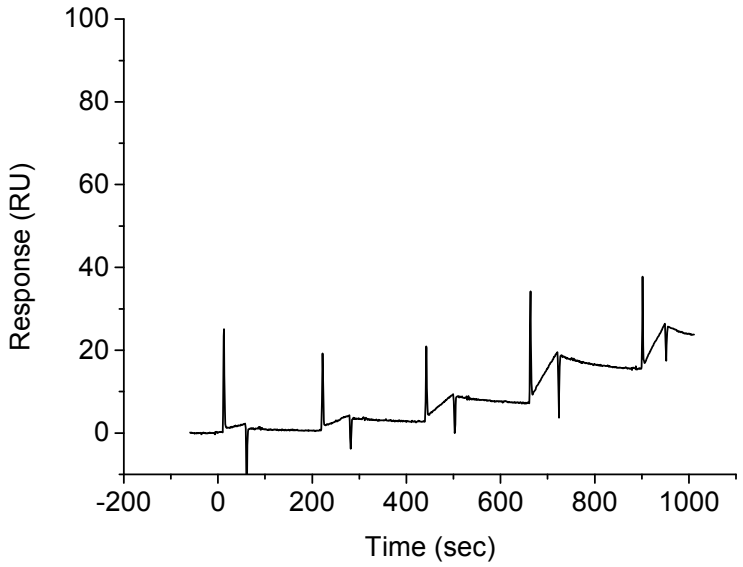


Figure S18. SPR analysis of the interaction between SB3 and nanoparticles: Kinetic assays Single Cycle of **1**-AuNP on an anti-SB3 functionalized chip.

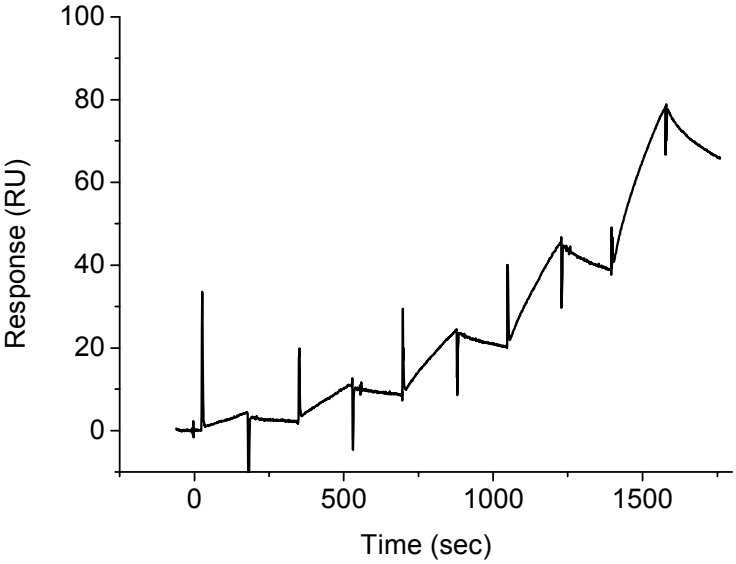


Figure S19. SPR analysis of the interaction between SB3 and nanoparticles: Kinetic assays Single Cycle of **1**-AuNP on an anti-SB3 functionalized chip after injection of SB3 (32 μM, 600 sec).

5. Western blot experiments.

Fifty μg of total protein content from each cellular extract, obtained by cellular protein extraction using RIPA lysis buffer, were loaded on 10% polyacrylamide gel. Antigenic detection was carried out by enhanced chemiluminescence (Amersham, Arlington Height, IL) and densitometric analysis was assessed using VersaDoc Imaging system (Bio-Rad Laboratories, Hercules, CA). The following antibodies were used: monoclonal anti-SERPINB3 antibody (0.5 μg/ml , R&D SYSTEMS Minneapolis, MN), monoclonal anti-β-actin antibody (1:1000, Sigma-Aldrich, St. Luis, MO) and anti-mouse horseradish peroxidase conjugated secondary antibody (1:1000, Amersham Bioscience, Arlington Height, IL).

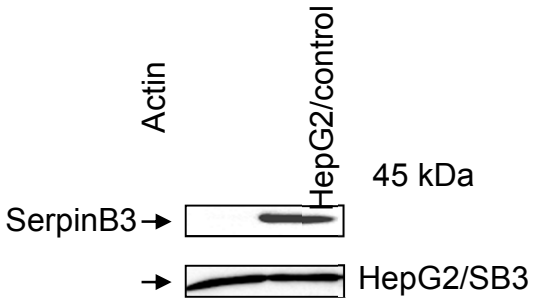


Figure S20. Western blot analysis of SerpinB3 in HepG2 transfected cell lines. The immunoblotting was carried out using 0.5 μg/ml of monoclonal anti-SerpinB3 antibody and monoclonal anti-β actin antibody (1:1000) as housekeeping protein for loading control

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[1)] Manea, F.; Bindoli, C.; Polizzi, S.; Lay, L.; Scrimin, P. (2008) Expeditious synthesis of water-soluble, monolayer-protected gold nanoparticles of controlled size and monolayer composition. *Langmuir, 24*, 4120-4124.

[2)] Guarino, G.; Rastrelli, F.; Scrimin, P.; Mancin, F. (2012) Lanthanide-based NMR: a tool to investigate component distribution in mixed-monolayer-protected nanoparticles. *J. Am. Chem. Soc., 134*, 7200-7203.