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Supporting Information

Regimes of Biomolecular Ultrasmall Nanoparticle Interactions

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Supporting Information

Contents

1 Materials

All chemicals were of highest grade available and used as received. Gold(III) chloride trihydrate (520918), N-(2-Mercaptopropionyl)glycine (280968), O-(2-Carboxyethyl)-O′-(2-mercaptoethyl) heptaethylene glycol (SH-PEG-COOH, 672688), PBS Tablets (P4411), Trizma® base (T1503), Glycine (G8898), Ammonium persulfate (A3678), Ethylenediaminetetraacetic acid disodium salt dehydrate EDTA (252352), Sodium dodecyl sulfate SDS (L3771), N,N,N′,N′-Tetramethylethylenediamine TEMED (T9281), Sucrose (m117), Dodecane (D22110), Acrylamide/bis-acrylamide 40% solution (A7802), DTT-Dithiothreitol (D5545), Ethanol (32294-2), Methanol (24229-2), Trisodium citrate dihydrate (S1804), Tannic acid (16201) and potassium carbonate (P4379) were purchased from Sigma-Aldrich.

In addition: PVC calibration standard 483 nm (PVC000476) were purchased from Analytik Ltd., Color Plus Pre-stained Protein Ladder, Broad Range (10-230 kDa) (P7711S) and Blue Loading Buffer for SDS-PAGE were ordered from New England Bio-Labs (cat. no. B7703S), Micro BCA Protein Assay Kit and Pierce ECL (cat. no. 23235) was purchased from Thermo Scientific., 2D Silver Stain Kit II [Daiichi] (167997) was purchased from Insight biotechnology, SYPRO® orange was purchased from ThermoFisher Scientific, MetaPhor® Agarose (50180) was purchased by LONZA.
Human Plasma was obtained from the Irish Blood Transfusion Service. Total protein content was estimated to be ca. 80 mg/ml by micro bicinchoninic acid assay (µ-BCA). Plasma was defrosted and centrifuged at 16200 RCF for 3 min at prior to use.

2 nm GNP-Glutathione, GNP-aGalPEGSuc and GNP-aGalPEGAmino have been supplied by MidatechPharma®.

2 Experimental Section

2.1 Synthesis of Nanoparticles

2.1.1 Preparation of the ultrasmall gold nanoparticles

2.1.1.1 Synthesis of 2 nm GNP-PEG-COOH

2 nm GNP-PEG-COOH have been synthesized by using a modification of the method reported in literature by Templeton and co-workers.[1] In a 50 mL round bottom flask $HAuCl_4$ (0.078 g, 0.20 mmol) and O-(2-Carboxyethyl)-O′-(2-mercaptoethyl) heptaethylene glycol (SH-PEG-COOH (0.55 g, 1.2 mmol) were dissolved in 9 mL of 6:1 methanol/acetic acid. The solution was stirred for 5 minutes at RT. While the solution was vigorously stirring $NaBH_4$ (0.15 g, 4 mmol) in 3.8 mL of MilliQ water was added drop by drop. The solution become immediately of dark-brown color characteristic of the ultrasmall gold nanoparticles. The reaction was finally stirred for further 30 minutes. The MeOH was removed by rotary evaporation. 5-10 mL of MilliQ water were added to the concentrate water solution containing the nanoparticles. This diluted solution was filtered through a 0.2 µm Millipore® syringe filter and then purified by using dialysis technique (membrane MWCO = 3000) in

order to eliminate the unreacted ligands and the inorganic salts formed during the reaction. The solution was finally concentrated using a centrifugation filter (10,000 MWCO) to a volume of 5-10 mL in order to have a concentration of 8-10 mg/mL.

2.1.1.2 Synthesis of 2 nm GNP-Tiopronin

2 nm GNP-Tiopronin has been synthesized as reported by Templeton and co-workers. [1] In a 50 mL round bottom flask $HAuCl_4$ (0.078 g, 0.20 mmol) and N-(2-mercaptopropionyl)glycine (0.19 g, 1.2 mmol) were dissolved in 9 mL of 6:1 methanol/acetic acid. The solution was stirred for 5 minutes at RT. While the solution was vigorously stirring $NaBH_4$ (0.15 g, 4 mmol) in 3.8 mL of MilliQ water was added drop by drop. The solution become immediately of dark-brown color characteristic of the ultrasmall gold nanoparticles. The reaction was finally stirred for further 30 minutes. The MeOH was removed by rotary evaporation. 5-10 mL of MilliQ water were added to the concentrate water solution containing the nanoparticles. This diluted solution was filtered through a 0.2 µm Millipore® syringe filter and then purified by using dialysis technique (membrane MWCO = 3000) in order to eliminate the unreacted ligands and the inorganic salts formed during the reaction. The solution was finally concentrated using a centrifugation filter (10,000 MWCO) to a volume of 5-10 mL in order to have a concentration of 8-10 mg/mL.

2.1.2 Synthesis of 3 nm GNP-PEG-COOH

3 nm GNP-PEG-COOH have been synthesized by using a modification of the method reported in literature.[2] In a 100 mL round bottom flask, 10 mL of aqueous reducing solution containing trisodium citrate (25 mg, 0.085 mmol), tannic acid (25 mg, 0.015 mmol) and potassium carbonate (5 mg, 0.036 mmol) was prepared and pre-heated to 60 °C. Separately, $HAuCl_4$ (5 mg, 0.013 mmol) was dissolved in 40 mL of water and heated to 60 °C. The gold precursor solution was rapidly added to the reducing solution under vigorous stirring, then the mixture was heated under reflux to the boiling point for 2 min and finally cooled to RT by using an ice-bath. Afterwards, the pH of the colloidal dispersion was adjusted to 8.5 by using aqueous NaOH (500 mM). An orange-brown dispersion was obtained. O-(2-Carboxyethyl)-O′-(2-mercaptoethyl) heptaethylene glycol ligand (2.5 mg**,** 0.0055 mmol) was dissolved in 1 mL of MilliQ water and added to the NP suspension at RT under stirring.[3] After 12 hours, the carboxylated gold nanoparticles were washed 3 times and concentrated with centrifugal filter units (10,000 MWCO).The solution was filtered through a 0.2 µm Millipore® syringe filter and then purified as reported for 2 nm GNP-PEG-COOH.

2.1.3 Synthesis of 5 nm GNP-PEG-COOH

5 nm GNP-PEG-COOH have been synthesized by using a modification of the method reported in literature.[2] In a 100 mL round bottom flask, $HAuCl_4$ (5 mg, 0.013 mmol) was dissolved in 40 mL of water and heated to 60 °C. Separately, 10 mL of aqueous reducing solution containing trisodium citrate (20 mg, 0.068 mmol), tannic acid (5mg, 0.003 mmol) and potassium carbonate (1.75 mg, 0.013 mmol) was prepared and also pre-heated to 60 °C. Subsequently, the reducing solution was rapidly added to the gold precursor solution under vigorous stirring, then the mixture was heated under reflux to the boiling point for 2 min and finally cooled to RT by using an ice-bath. Afterwards, the pH of the colloidal dispersion was adjusted to 8.5 by using aqueous NaOH (500 mM). An orange-red dispersion is obtained. O-

(2-Carboxyethyl)-O′-(2-mercaptoethyl)heptaethylene glycol ligand (2.5 mg, 0.0055 mmol) was dissolved in 1 mL of MilliQ water and added to the NP suspension at RT under stirring.[3] After 12 hours, the carboxylated gold nanoparticles were washed 3 times and concentrated with centrifugal filter units (10,000 MWCO).The solution was filtered through a 0.2 µm Millipore® syringe filter and then purified as reported for 2 nm GNP-PEG-COOH.

Gold particles were characterized by DCS, UV-Vis spectroscopy, DLS and TEM.

2.2 Material Characterization

2.2.1 UV-visible spectroscopy

UV-visible spectroscopy was performed on a Cary 600i UV-visible spectrophotometer using a 1 cm path length Hellma quartz cells, measuring in the 200-800 nm range.The absence of the surface Plasmon resonance band suggest that the dimension of the gold core of the nanoparticles is $\leq$ 2 nm. The concentration of the NPs has been established by using the absorption spectra as described in literature by Haiss et al.[4]

2.2.2 Differential Centrifugal Sedimentation

Differential Centrifugal Sedimentation (DCS) analysis of gold nanoparticles was performed using a CPS Disk Centrifuge DC2400. The measurements were carried out using an 8-24% sucrose density gradient in MilliQ water, with a disc speed set to 24000 rpm while monitoring the 1-500 nm range. Each particle size measurement was calibrated using a PVC standard of nominal diameter 483 nm. 100 µL of standard was injected before each measurement to calibrate the instrument. 100 µL of particles were injected and analysed by DCS.

2.2.3 Dynamic Light Scattering

ζ potential of the NPs suspension was measured by Zetasizer Nano ZS by using a disposable capillary zeta cell. ζ potential measurements reported are an average of three independent measurements, with each measurement consisting of an accumulation of 11 runs.

2.2.4 Transmission electron Microscopy

Samples for HR-TEM and STEM imaging were prepared by evaporating ca. 10 µL of the nanoparticle suspension onto a NetMeshTM Lacey Carbon 300 mesh copper grid or holy carbon grid. FEI Titan TEM and FEI Tecnai G2 20 Twin TEM operating at accelerating voltage of 200 kV were utilized for imaging.

2.2.5 Nuclear Magnetic Resonance

The samples were freeze dried overnight and re-suspended in D_2O. ^{1}H-NMR spectra were recorded at 298 K on Varian Inova 300MHz Spectrometer or Varian NMR System 400MHz Spectrometer.

2.3 Gel Electrophoresis

Gel-electrophoresis was applied to investigate size distribution of the GNPs. Gel-shift assays were performed by using MetaPhor® Agarose and Native gels prepared at different %

following the procedure indicated by the supplier. In addition, denaturalized gel electrophoresis was applied to investigate the protein profile.

2.3.1 Agarose electrophoresis.

Different percentages of agarose gel were prepared by using MetaPhor® Agarose from Lonza in 1 x native buffer (25mM Tris-HCl and 25mM Glycine, pH 8.3). The 1x native running buffer solution was filtrated and degassed before to be used to make the gels. The samples were diluted by using a native running buffer containing 70% of glycerol, loaded in the gel and run using different voltage as difference of potential, using a Mini-Sub Cell GT Cell electrophoresis system from Bio-Rad.

2.3.2 Polyacrylamide gel electrophoresis (PAGE)

In order to investigate the protein adsorption of 3 nm GNP-PEG-COOH in HP and 5 nm GNP-PEG-COOH in HP, 1% agarose gel electrophoresis was performed. The bands were cut, melted and treated using Agarase enzyme (EO0461 from Thermo Fisher Scientific). 1U of Agarose was added to 100 ul of melted agarose and incubated at 42º C for 30 min. The protein content was then denatured by boiling the samples for 5 minutes in loading buffer (62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 0.01% (w/v) bromophenol blue, 40 mM DTT). So prepared samples, containing denatured proteins coated with SDS surfactant (which gives them negative net charge) were separated by size in the moiety of porous 10% polyacrylamide gel (1D SDS-PAGE), in electric field using a Mini-PROTEAN Tetra electrophoresis system from Bio-Rad.

SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Electrophoresis). For two running gels: 10 % SDS – PAGE gel was precast fresh before each experiment as 5.4 mL of MiliQ water, 2.5 mL of 1.5 M Tris – HCl buffer pH 8.9 and 0.1 mL of 10 % SDS are mixed well and 1.8 mL of 40 % acrylamide and 5 µL of TEMED were added and the solution was mixed again. As initiator, 50uL of 10 % ammonium persulfate were added. Gel solution is poured in the frame to polymerize. Stacking gels (4%) are made up with 0.5 mL acrylamide, 1.26 mL 0.5 M Tris-HCL buffer pH 6.8, 50 µL 10% SDS, 3.18 mL MiliQ water, 25 µL APS 10% and 5 µL TEMED and added on top of running gels.

The electrophoresis was run under constant voltage of 130 V for about 45 minutes. The gels were stained with 2D-SILVER STAIN-II reagents (Cosmobio Co.,Ltd) and scanned under white light using a G:Box Chemi XT4 (Syngene).

2.3.3 Mass spectrometry

For the LC-MS/MS analysis, the proteins of the corona complexes were first separated of the protein-NP complexes, by 10% Tris-Glycine SDS-PAGE gel. After running the electrophoresis under constant voltage of 140V for 10 minutes, the gel was stained with Commassie blue and the proteins bands taken from each lane prior to trypsin digestion and mass spectrometry. The gel section containing the proteins was removed using a sterile scalpel and transferred to a clean 0.5 mL sample tube which had been pre-rinsed with acetonitrile. The gel sections were trypsin digested in gel. The samples were resupended in 0.1% w/w formic acid prior to analysis by electrospray liquid chromatography (LC-MS/MS).

All samples were run on a Thermo Scientific LTQ ORBITRAP XL mass spectrometer connected to an Exigent NANO LC.1DPLUS chromatography system incorporating an auto-sampler**.** The raw mass spectral data have been searched against human protein database and analysed using MaxQuant® 1.4.1.2 software. A semi-quantitative assessment of the proteins amount was performed by the method of spectral counting (SpC), which represents the total number of the MS/MS spectra for all peptides attributed to a matched protein. The SpC of each protein identity was normalized to the protein mass and expressed as the relative protein quantity by applying the following equation (Eq.1):

$$NpSpC_k = \left(\frac{(SpC/M_w)k}{\sum_{i=1}^{n}(SpC/M_w)i}\right)x100 \qquad \text{[Eq.1]}$$

Where $NpSpC_k$ is the percentage normalized spectral count for protein k, SpC is the spectral count identified, and M_w is the molecular weight in kDa for protein k. The obtained results have been compared with the method of the label-free quantification (LFQ) performed by MaxQuant.[5] This method, able to accurately and robustly quantify small fold changes on a proteome scale, has the prerequisite that a majority population of proteins exists that is not changing between the samples therefore it has been applied to the samples incubated with the same serum at different percentages.

3 Supplementary Figures

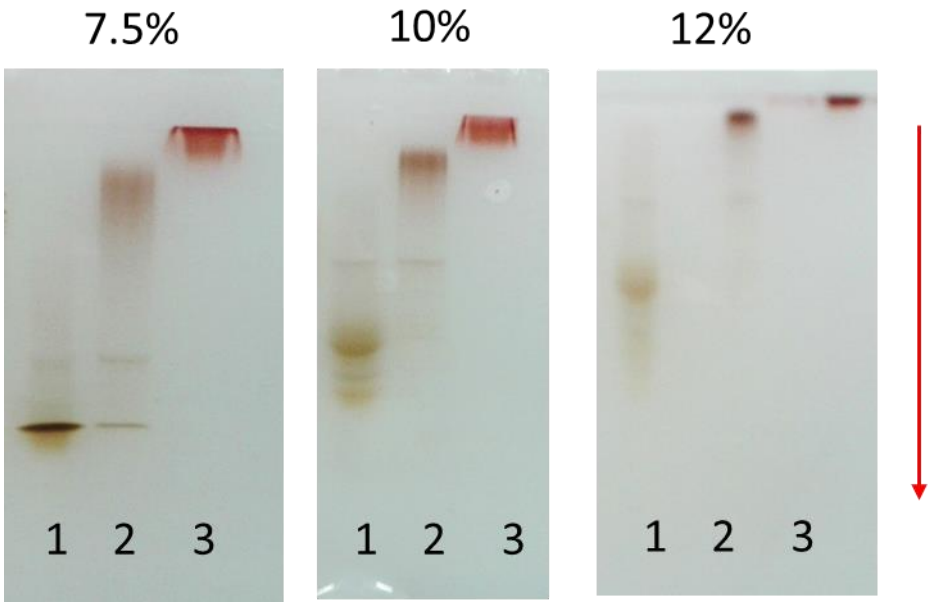


Figure S1. Size distribution investigation of GNP-PEG-COOH by polyacrylamide gel elecrophoresis: (1) 2 nm GNP-PEG-COOH, (2) 3 nm GNP-PEG-COOH, (3) 5 nm GNP-PEG-COOH.

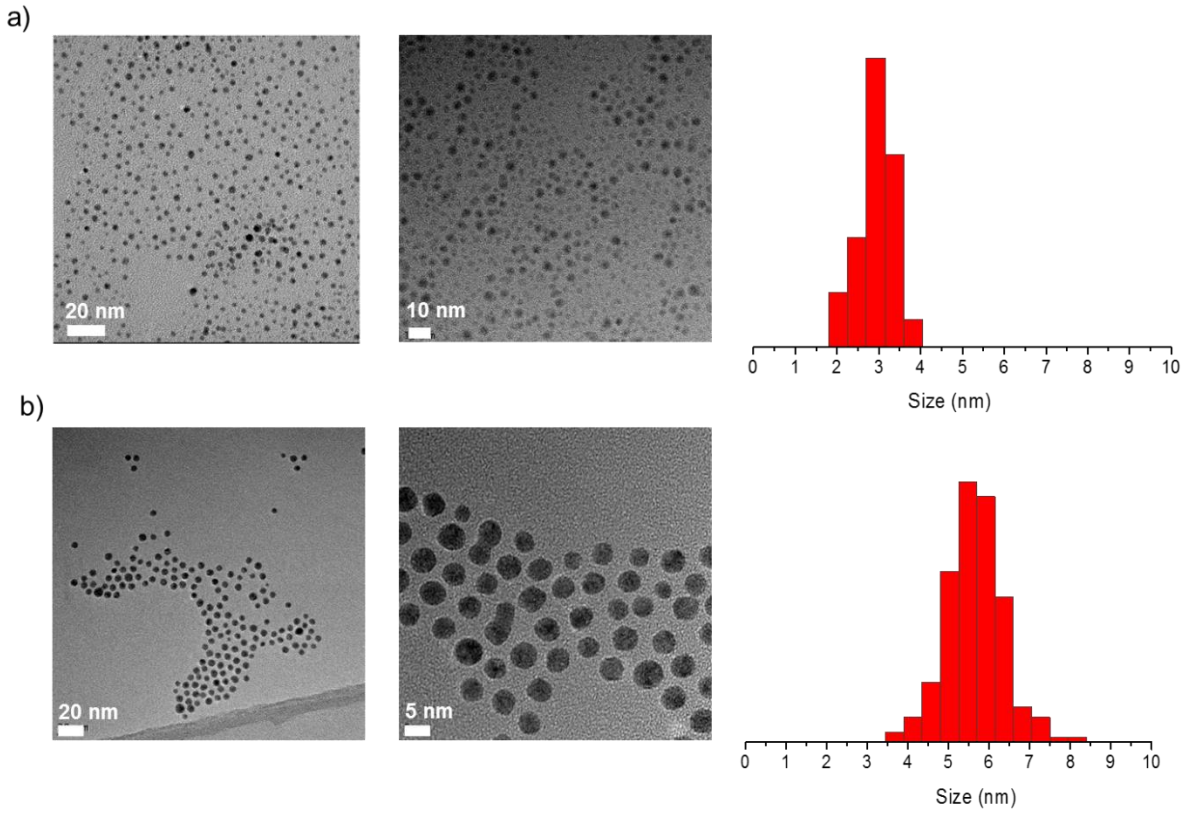


Figure S2. TEM micrographs and size distribution of 3 and 5 nm GNP-PEG-COOH.

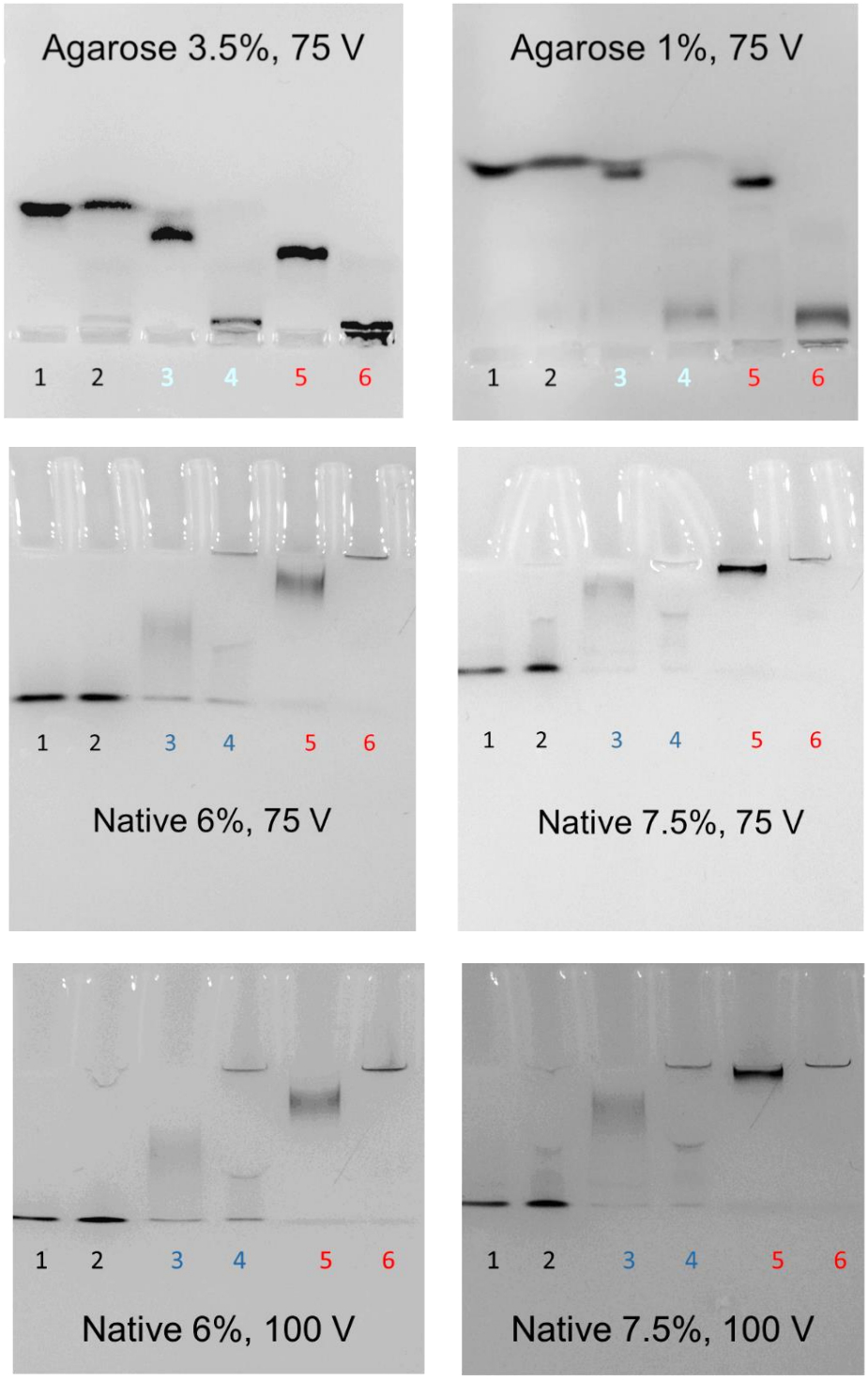


Figure S3. Different pore sizes of agarose and native polyacrylamide gel-assays in 1% native buffer. The numbered lanes refer 5 nm GNP-PEG-COOH in PBS (1) and in HP (2), 3 nm GNP-PEG-COOH in PBS (3) and in HP (4), 2 nm GNP-PEG-COOH in PBS (5) and in HP (6).

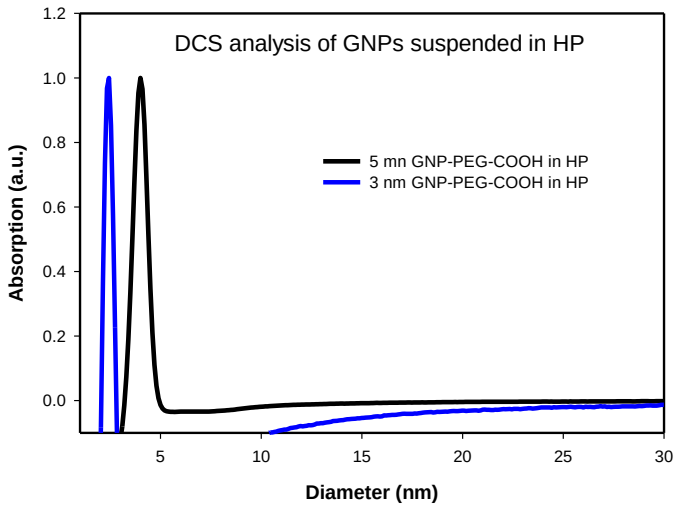
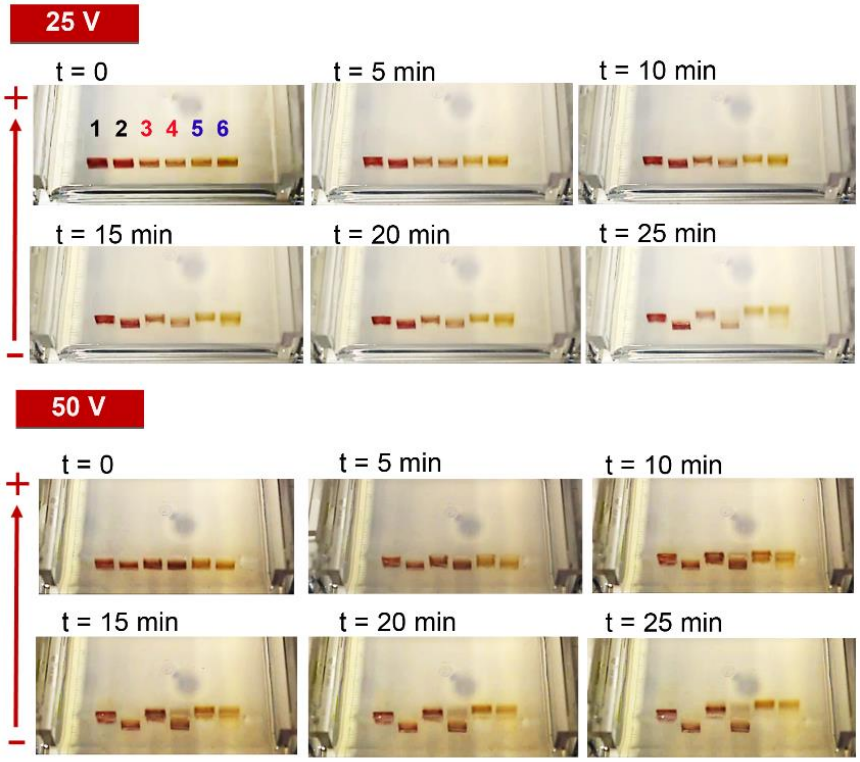


Figure S4. Stability test in human plasma by DCS. 5 nm and 3 nm GNP-PEG-COOH were suspended in HP and analysed by DCS. The sucrose gradient for this experiment was a PBS



solution.

Figure S5. Endpoint images from the high resolution video of a 3.5 % agarose gel-assay experiment showing samples: 1) 5 nm GNP-PEG-COOH in PBS, 2) 5 nm GNP-PEG-COOH in HP, 3) 3 nm GNP-PEG-COOH in PBS, 4) 3 nm GNP-PEG-COOH in HP, 5) 2 nm GNP-PEG-COOH in PBS, 6) 2 nm GNP-PEG-COOH in HP. Running conditions: 25 V (above) and 50 V (below). The endpoint images demonstrate that the electrophoretic mobility of the NPs changes over time. In particular, 5 nm (lane 2) and 3 nm (lane 4) GNP-PEG-COOH in HP are significantly delayed in comparison to the NP control in PBS (lane 1 and 3

respectively). In the first 15 minute of running it is possible to observe a small difference in the electrophoretic mobility also for the 2 nm GNP-PEG-COOH in PBS and HP (lane 5 and 6).

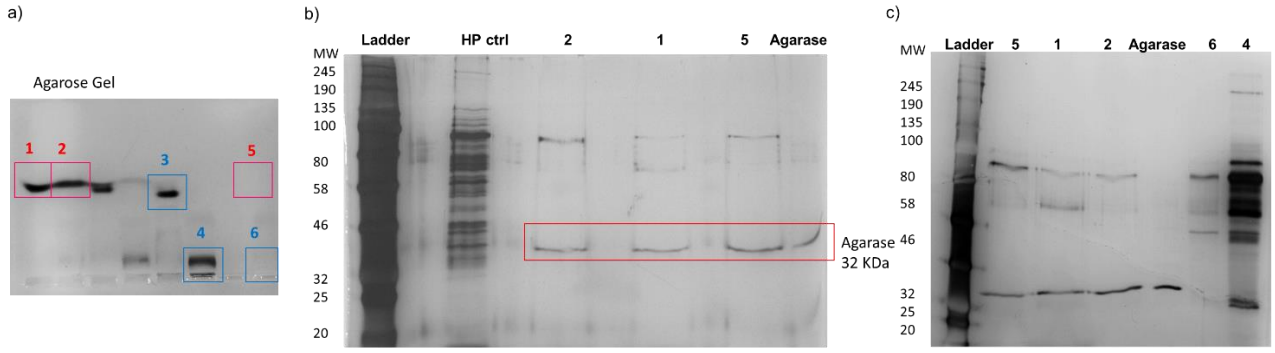


Figure S6. SDS-PAGE analysis of the different fraction recovered from the agarose gel electrophoresis. a) 1 % agarose gel-assay in a native buffer of 2 nm, 3 nm, and 5 nm GNP-PEG-COOH in PBS and in HP. Bands corresponding to 2 nm GNP-PEG-COOH in PBS (1) and in HP (2), 5 nm GNP-PEG-COOH in PBS (3) and in HP (4), and HP (5 and 6). b) and c) SDS-PAGE analysis of the nanoparticles and proteins recovered from the agarose gel.

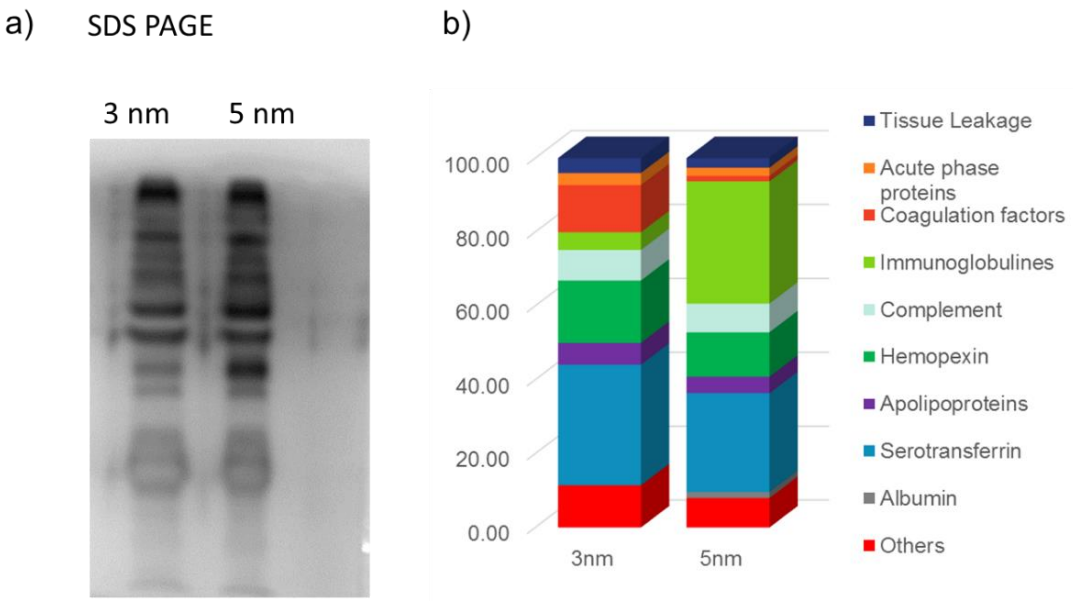


Figure S7. SDS-PAGE (a) and Mass spectrometry analysis (b) of protein corona of 3 nm and 5 nm GNP-COOH isolated by agarose gel electrophoresis.

Protein Name	NSpC (3nm)	Protein name	NSpC (5 nm)
Serotransferrin	10.13	Serotransferrin	6.01
Hemopexin	5.24	Hemopexin	2.70
Ig alpha-1 chain C region	1.29	Ig gamma-2 chain C region	1.49
Histidine-rich glycoprotein	1.00	Ig alpha-1 chain C region	1.05
Insulin-like growth factor-binding protein complex acid labile subunit	0.87	Retinol-binding protein 4	0.96
Retinol-binding protein 4	0.73	Ig kappa chain V-II region RPMI 6410	0.90
Serum amyloid P-component	0.69	Ig kappa chain V-I region AG	0.85
Complement factor B	0.67	Ig kappa chain V-II region TEW	0.75
Lumican	0.63	Beta-2-glycoprotein 1	0.74
Tetranectin	0.63	Ig heavy chain V-III region BUT	0.61
Kininogen-1	0.62	Ig lambda-3 chain C regions	0.61
Apolipoprotein A-IV	0.61	Ig lambda chain V-III region LOI	0.57
Complement factor I	0.60	Complement C1q subcomponent subunit B	0.56
Apolipoprotein C-III	0.58	Complement factor H	0.56
Coagulation factor XIII A chain	0.56	Complement factor B	0.55
WAP four-disulfide core domain protein 3	0.56	Serum amyloid P-component	0.50
N-acetylmuramoyl-L-alanine amidase	0.54	CD5 antigen-like	0.45
Apolipoprotein E	0.53	Ig lambda chain V-IV region Hil	0.44
Antithrombin-III	0.49	Insulin-like growth factor-binding protein complex acid labile subunit	0.44
Complement C3	0.48	Serum albumin	0.36
Kallistatin	0.46	Coagulation factor XIII B chain	0.33
Fibronectin	0.44	Apolipoprotein E	0.24
Carboxypeptidase B2	0.42	Pigment epithelium-derived factor	0.19
Complement factor H	0.40	Vitamin K-dependent protein S	0.17
Complement C5	0.38	Gelsolin	0.13
Alpha-1-antitrypsin	0.32	Ig lambda chain V-III region SH	0.13

Table S1. Mass spectrometry analysis of 3 nm and 5 nm GNP-COOH incubated with human plasma and isolated by agarose gel electrophoresis.

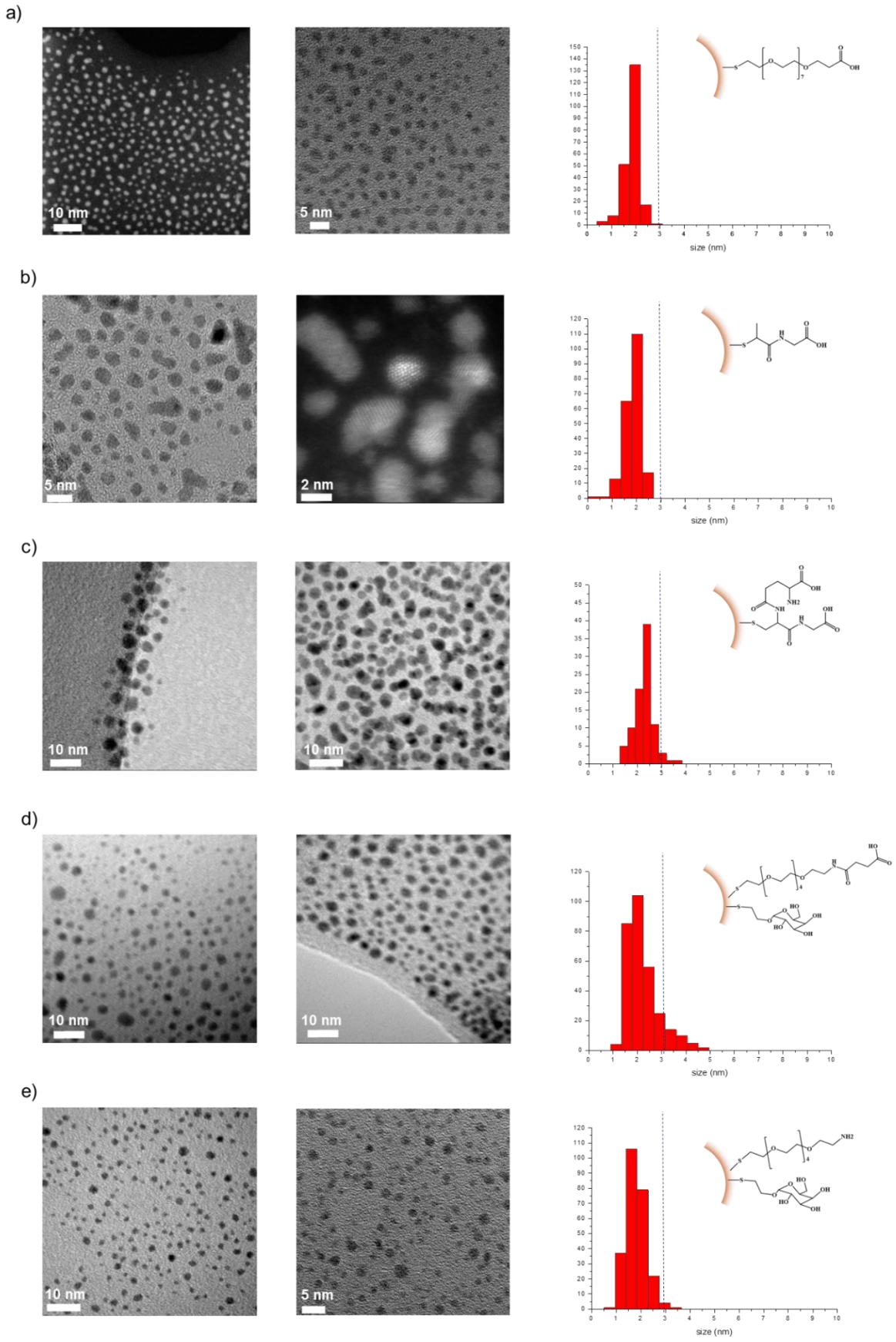


Figure S8. TEM micrographs and size distribution of 2 nm GNPs: a) GNP-PEG-COOH, b) GNP-tiopronin; the micrograph on the right shows individual gold atoms, c) GNP-glutathione

d) GNP-αGalPEGSuc, e) GNP- αGalPEGAmino. The dotted line shows that the nanoparticles distributions are centered around 2 nm and below 3 nm.

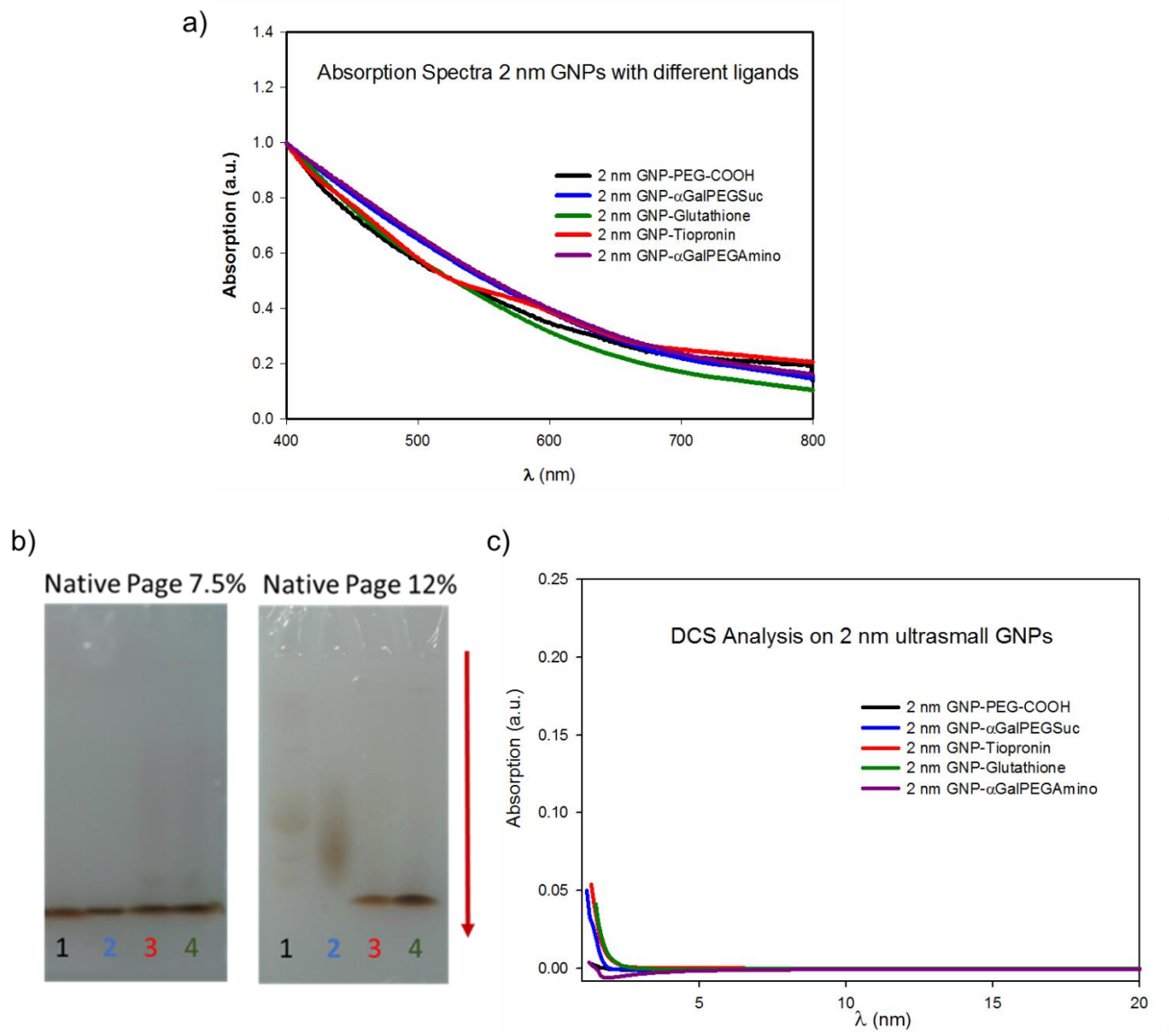
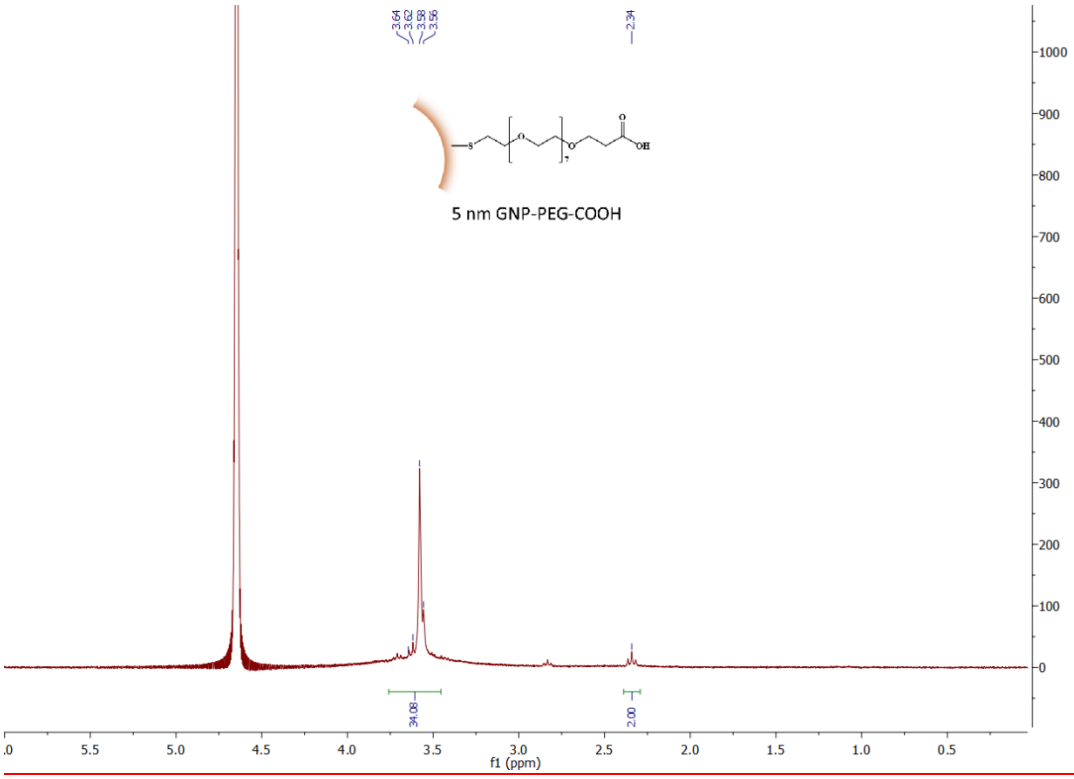
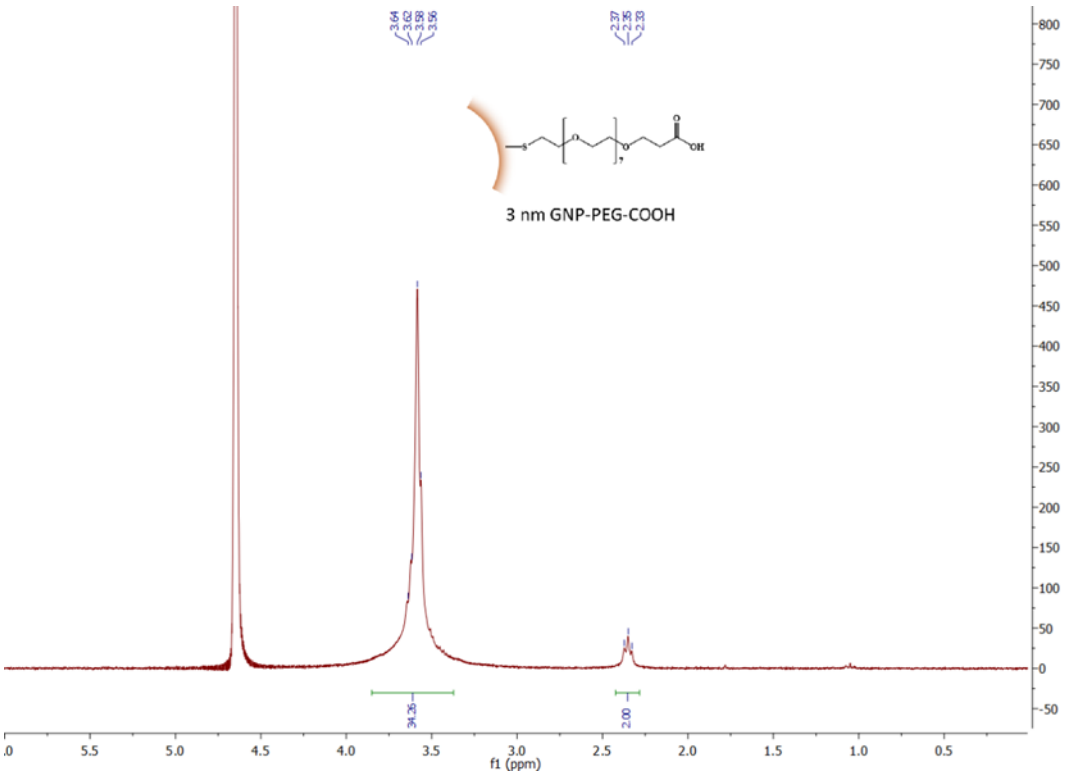


Figure S9. Characterization of 2 nm GNPs: a) DCS, b) Polyacrylamide gel electrophoresis: (1) GNP-PEG-COOH, (2) GNP-αGalPEGSuc, (3) GNP-tiopronin, (4) GNP-glutathione, c) UV-Vis spectroscopy.

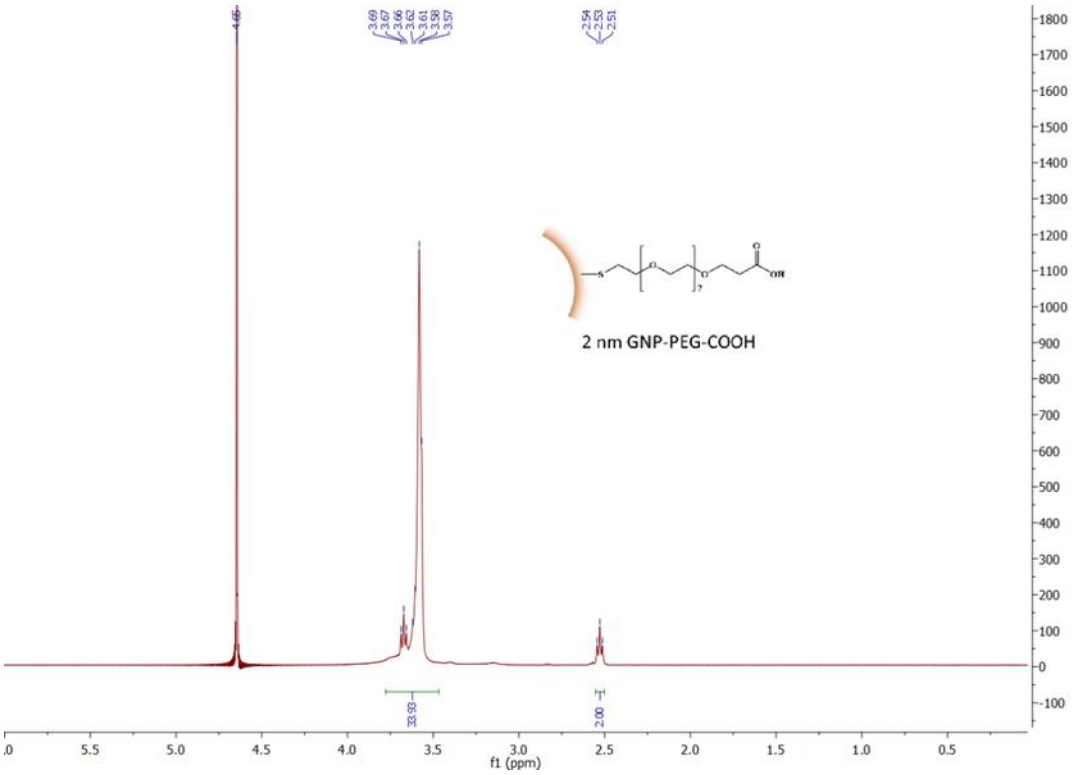
1H-NMR of 5 nm GNP-PEG-COOH



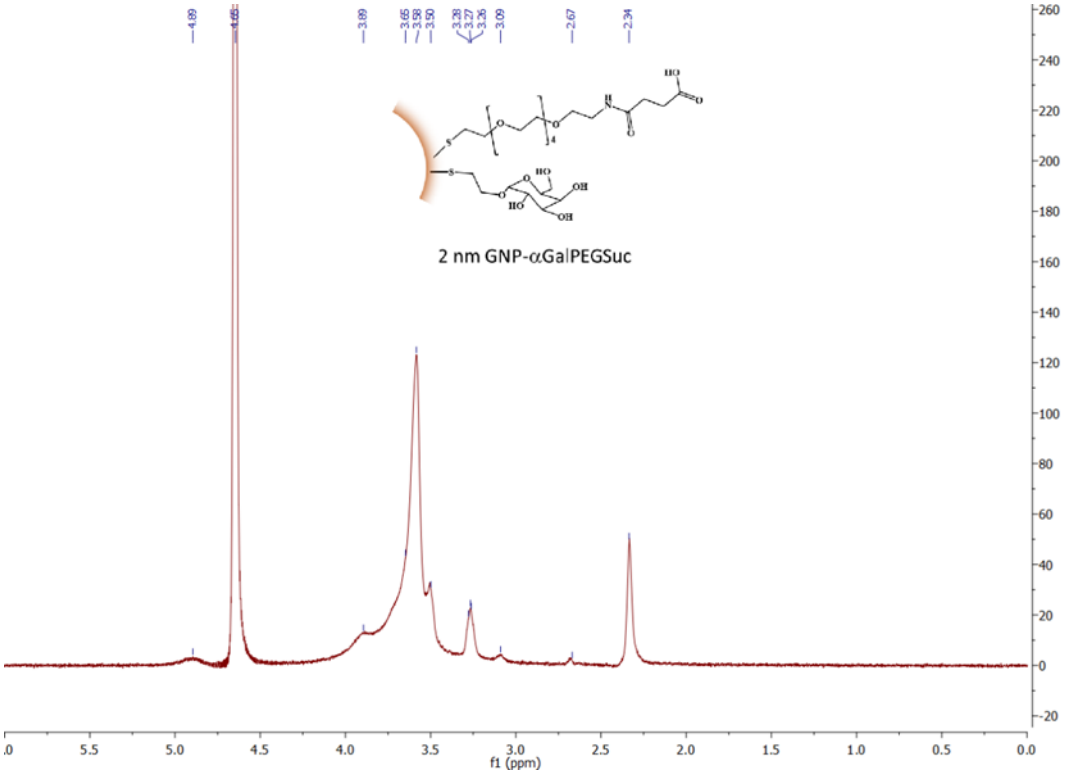
1H-NMR of 3 nm GNP-PEG-COOH



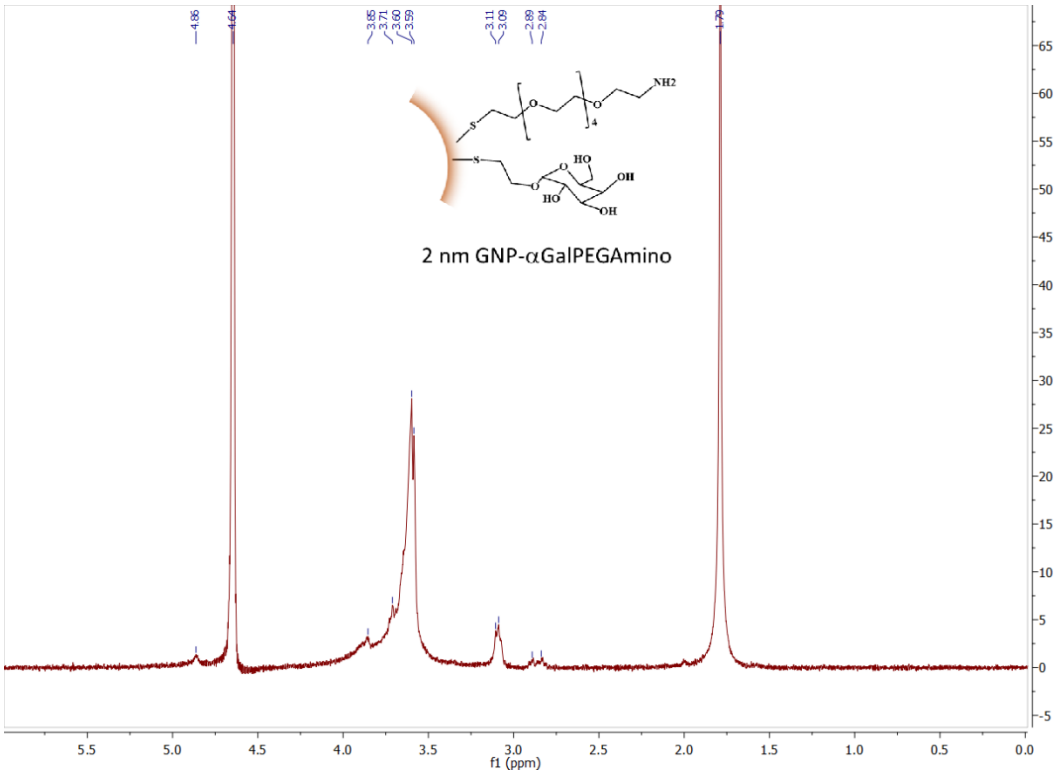
1H-NMR of 2 nm GNP-PEG-COOH



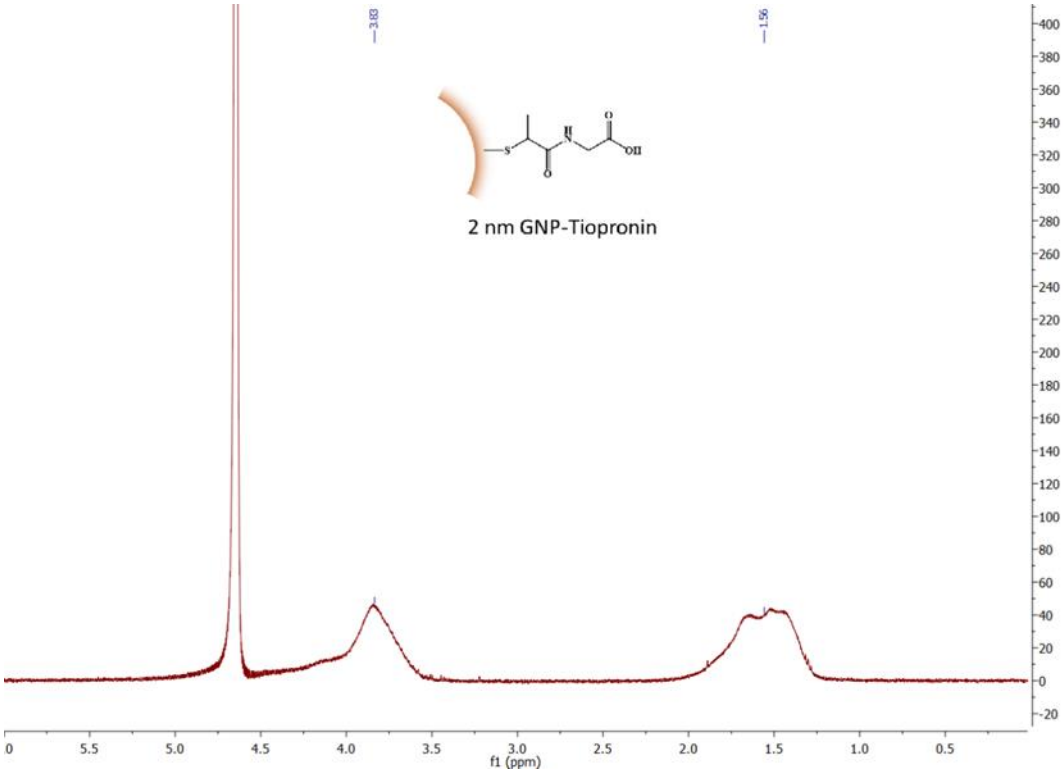
1H-NMR of 2 nm GNP-αGalPEGSuc



1H-NMR of 2 nm GNP-aGalPEGAmino



1H-NMR of 2 nm GNP-Tiopronin



1H-NMR of 2 nm GNP-Glutathione

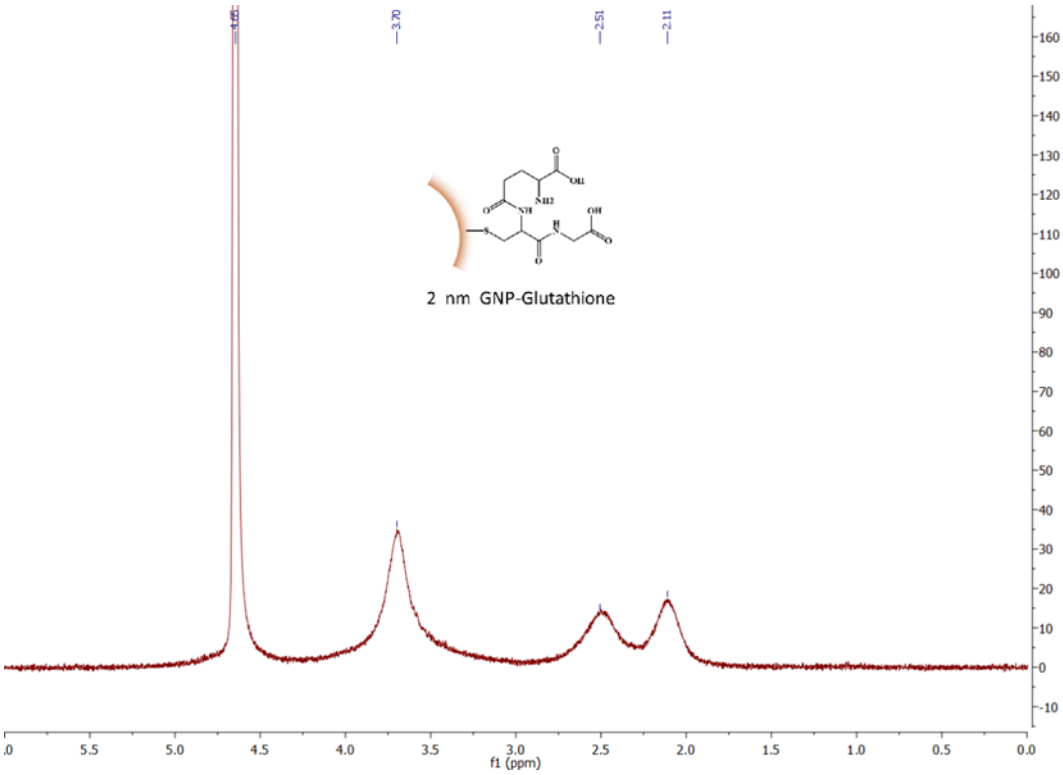


Figure S10. H-NMR spectra of different GNPs. ^{1}H-NMR spectra were recorded at 298 K in D_2O as solvent.[1,6,7]

Sample	TEM (nm)	Number particles	DCS (nm)	Z pot (mV)
5 nm GNP-PEG-COOH	5.2 ± 0.6	100	4.5 (RW)	-18 mV
3 nm GNP-PEG-COOH	3.0 ± 0.5	100	2.5 (RW)	-16 mV
2 nm GNP-PEG-COOH	1.9 ± 0.3	200	1 (RW)	-16 mV
2 nm GNP-Tiopronin	1.8 ± 0.3	200	1 (RW)	-27 mV
2nm GNP-Glutathione	2.3 ± 0.4	100	1 (RW)	-28 mV
2 nm GNP- αGalPEGSuc	2.0 ± 0.7	300	1 (RW)	-15 mV
2 nm GNP-αGalPEGAmino	1.8 ± 0.4	250	1 (RW)	+ 42 mV

Table S2. Summary of the GNPs characterization.

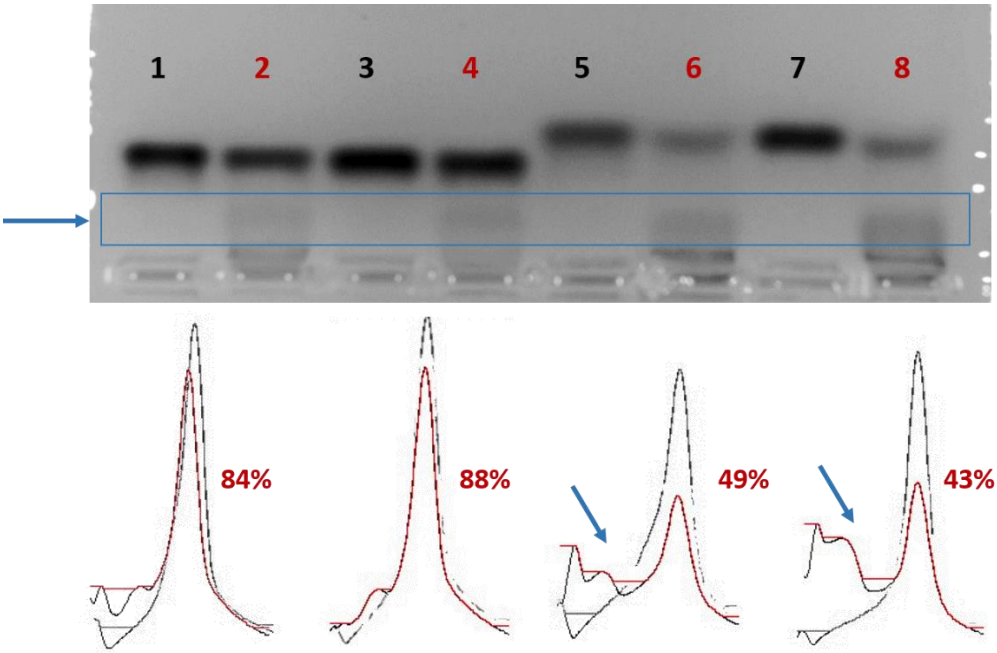


Figure S11. Densitometry of the agarose gel reported in Figure 4 (main text) showing 2 nm GNP-PEG-COOH in PBS (1) and in HP (2), 2 nm GNP- αGalPEGSuc in PBS (3) and in HP (4), 2 nm GNP-tiopronin in PBS (5) and in HP (6), 2 nm GNP-glutathione in PBS (7) and in HP (8). The densitometry analysis highlights a clear bimodal distribution for lane 6 and 8, due to partial interaction of the GNP-tiopronin and GNP-glutathione with the plasma proteins.

4 References

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