Directional immobilization of proteins on gold nanoparticles is essential for their biological activity: leptin as a case study

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1. Materials, general methods, and instruments

All reagents were purchased from Sigma-Aldrich or Acros Organics, unless mentioned otherwise, and used without further purification. Hydrochloric acid for ICP-MS and amino acid analysis from Alfa Aesar (87617, 99.999% metals basis, 36.5% min); nitric acid for ICP-MS from Alfa Aesar (87920, 99.999% metals basis, 65-70%); gold element reference solution for ICP-MS from Romil (E3AU#, 1000 ppm Au). Alkyl-PEG600 thiols ((1-mercaptoundec-11-yl)PEG600)-acetic acid **3** and (1-mercaptoundec-11-yl)-(ω -ethynyl)PEG600 **4** were prepared according to literature.^{1, 2} The synthesis of heterobifunctional linker 3-[2-[2-(2azidoethoxy)ethoxy]ethoxy]propanoic acid and of the THPTA ligand (tris(3hydroxypropyltriazolylmethyl)amine) has been reported previously.² Human recombinant leptin (UniProtKB - P41159) was purchased by Enzo Life Sciences (ALX-201-034) or by R&D Systems (398-LP-01M). Leptins corresponding to these products feature at the N-terminus either an additional alanine or an additional methionine, respectively, according to the manufacturer information, which is confirmed by mass spectrometry (Figure 1 and supporting spectra).

TLC analyses were carried out using silica gel plates Polygram[®] Sil G/UV254 (40×80 mm) from Macherey-Nagel. Flash chromatography was performed on silica gel 40 – 63 μ m (230 – 400 mesh) from Macherey-Nagel.

All glassware employed for nanoparticle preparation was cleaned with *aqua regia* (HCl (37%)/HNO₃ (65%) 3:1). Ultrapure deionized water (Millipore Elix[®] 35 water purification system, 18.2 M Ω cm) was used for the preparation of all aqueous solutions. All solutions used for nanoparticle preparation were filtered through a 0.2 µm membrane filter (cellulose acetate, Whatman[®]). All AuNP samples used for *in vitro* studies were filtered sterile using Costar[®] Spin-X[®] centrifuge tube filters, 0.22 µm pore CA membrane (product #8160 from Corning).

AuNPs were characterized by Dynamic Light Scattering (DLS) (Zetasizer, Malvern Instruments). AuNP core diameter was measured via TEM (JEOL JEM-1011 transmission electron microscope operating at an accelerating voltage of 100 kV). UV/Vis measurements were carried out using a TECAN Infinite M200 Pro plate reader. The concentration of gold was determined via inductively coupled plasma-optical emission spectrometry-ICP-OES² (Agilent 720 ICP-OES) or inductively coupled plasma mass spectrometry-ICP-MS (Thermo Fisher Scientific iCAP Q ICP-MS). The HR-ESI-MS spectra were recorded on a 6540 UHD Q-TOF LC-MS system form Agilent. MALDI mass spectra were acquired on a 4800 Plus MALDI TOF/TOF™ Analyzer from AB Sciex. Sample mineralization and peptide hydrolysis for amino acid analysis were carried out on a pressurized microwave acid digestion system from CEM (Discover SP-D). HPLC-UV analysis were performed on a 1260 Infinity II LC system form Agilent equipped with a diode array detector (DAD) using a Zorbax Eclipse Plus C18 column (100 mm × 4.6 mm id, 3.5 μm). HPLC/ESI-FTMS experiments were carried out on a Dionex UltiMate 3000 HPLC (Thermo Fisher Scientific, CA, USA) system coupled with a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Whaltam, MA, USA) using a Waters - XBridge BEH C18 column (100 mm × 2.1 mm id, 3.5 μm). NMR spectra were recorded on a Bruker AMX-300 spectrometer or on a Bruker Avance 400. ¹H and ¹³C{¹H} NMR spectra were calibrated to TMS on the basis of the relative chemical shift of the solvent as an internal standard. Abbreviations used are as follows: s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, m = multiplet, br = broad signal. Cytofluorimetric analysis were run on a BD Accuri[™] C6 system from BD Biosciences.

Cell cultures

The immortalized human mammary gland adenocarcinoma cell line MCF7 (ECACC catalogue no. 86012803) was obtained from Prof. Cecilia Bucci (Department of Biological and Environmental Sciences and Technologies – University of Salento). Cells were cultivated in Dulbecco's Modified Eagle Medium high glucose (DMEM 4500, Sigma) supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Sigma Aldrich), 2 mM L-Glutamine (Euroclone), 100 U/mL penicillin, and 100 mg/mL Streptomycin (Gibco) at 37 °C under a humidified controlled atmosphere with 5% CO₂. Cells were harvested from subconfluent cultures by incubation with a trypsin-EDTA solution (Sigma Aldrich) and propagated every 3 days. Cultures were periodically monitored for mycoplasma contamination using MycoAlert mycoplasma detection kit (Lonza).

List of used antibodies

Mouse monoclonal anti-LR antibody (MAB867, R&D Systems), mouse monoclonal anti-Actin antibody (A3853, Sigma-Aldrich), rabbit monoclonal anti-pSTAT3 (Tyr705) antibody (D3A7, Cell Signaling Technology), mouse monoclonal anti-STAT3 antibody (124H6, Cell Signaling Technology), goat anti-mouse IgG (H+L)-cross-adsorbed-Alexa Fluor 488 secondary antibody (A-11001, Thermo Fisher Scientific), and goat anti-mouse and anti-rabbit IgG (H+L)-HRP conjugated secondary antibodies (1706515, BioRad).

Statistical analysis

Data relative to nanoparticle uptake quantification and pSTAT3 quantification are presented as scatter plots showing means \pm s.d. (Figures 3 and 4 main text) of 5 independent experiments. Independent experiments were performed on different days using MCF7 cell cultures at passage 20 – 26. Cells were treated with independently prepared AuNP batches. In each experiment, cells were randomized to wells, and wells to treatment. In one experiment, each treatment was carried out in triplicate (technical replicate). All treatments including controls were carried out in each experiment. To compare treatment groups, we used two-way ANOVA (repeated measures ANOVA) followed by the Bonferroni's multiple comparison test. Statistical analysis was carried out using Prism 8 (GraphPad Software, Inc.) software.

List of abbreviations used in organic synthesis: EA=ethyl acetate, Hex=hexane, DCM=dichloromethane, THF=tetrahydrofuran, DMF=*N*,*N*-dimethylformamide, ACN=acetonitrile, EtOH=ethanol, MeOH= methanol, TFA=trifluoroacetic acid, AcOH=acetic acid.

2. Synthesis of azido-terminated alkyl-PEG600 disulfide 5

Note: The reported PEG derivatives are not a single compound but a mixture of homologous compounds with a PEG segment length distribution centered at 13 ethylene glycol units (PEG600). Thiol 5A was prepared according to the procedure developed for the synthesis of **3**.¹

Scheme S1.



Reagents and conditions: a) I₂, MeOH, r.t., quant.; b) 11-azido-3,6,9-trioxaundecan-1-amine (1.8 equiv.), HBTU (2.3 equiv.), DMF, r.t. 19 h, 72%.

Symmetric disulfide (5B): A 59 mM solution of I₂ in methanol was added dropwise and under stirring at r.t. to 3-((1-mercaptoundec-11-yl)PEG600)propionic acid 5A (0.770 g, 0.90 mmol) dissolved in 10 mL methanol until a yellow color persisted. Stirring was continued for additional 10 minutes before the volatiles were removed under reduced pressure. The symmetric disulfide bis-carboxylic acid 5B (quant.) was used without further purification in the next step.

¹H NMR (300 MHz, CDCl₃) δ 3.69 (t, *J* = 6.1 Hz, 1.3 H, -OCH₂-CH₂-COOH), 3.66-3.46 (m, 47.7 H, PEG), 3.35 (t, J = 6.8 Hz, 2.4 H, C₁₀H₂₀-CH₂-O-), 2.59 (t, J = 7.4 Hz, 2H, -S-CH₂-), 2.52 (t, J = 6.1 Hz, 1.2H, -OCH₂-CH₂-COOH), 1.64-1.42 (m, 4.3H, -CH₂-alkyl), 1.35-1.12 (m, 16.1 H, -CH₂- alkyl).

According to NMR analysis, there are ~0.6 propionyl groups per alkylPEG600 chain.

Azido-terminated alkyl-PEG600 disulfide (5). Disulfide bis-carboxylic acid 5B (0.895 g, 0.52 mmol) and 11azido-3,6,9-trioxaundecan-1-amine (411 mg, 1.88 mmol, 1.8 equiv.) were dissolved in 5 mL of DMF (peptide synthesis quality) under Ar. HBTU (464 mg, 1.22 mmol, 1.15 equiv) was added and stirring was continued for 19 h at r.t.. After this period, the reaction mixture was partitioned between 100 mL of EA and 80 mL 0.5 M ag. citric acid solution. The aqueous phase was extracted with an additional portion of EA (50 mL). The combined organic phases were washed with 80 mL 0.5 M citric acid, sat. NaHCO₃ (2×80 mL, and brine (2×80 mL). Drying over Na₂SO₄ and removal of the solvent under reduced pressure afforded the crude product as brownish oil. Purification by flash chromatography (from DCM:EA:EtOH 50/45/5 to DCM:EtOH 80/20) gave product 5 as pale yellow oil (794 mg, 0.376 mmol, 72% yield). Isolated 5 was characterized via ¹H NMR and mass spectrometry (MALDI TOF/TOF™, matrix: α-cyano-4-hydroxycinnamic acid (10 mg/mL) in 50% aqueous ACN containing 0.05% TFA, see supporting spectra).

¹H NMR (300 MHz, CDCl₃) δ 6.57 (bs, 0.49 H, -N*H*-), 3.78-3.52 (m, 55.4 H, PEG+ -OCH₂-CH₂-CONH-), 3.49-3.35 (m, 5.32 H, C₁₀H₂₀-CH₂-O- + -NH-CH₂-CH₂-+ -NH-CH₂-CH₂-+ -CH₂-N₃), 2.67 (t, J = 7.3 Hz, 2H, -S-CH₂-), 2.47 (t, J = 6.0 Hz, 1.2H, -OCH₂-CH₂-CONH-), 1.72-1.51 (m, 4 H, -CH₂-alkyl), 1.42-1.22 (m, 15.6 H, -CH₂alkyl).

According to NMR analysis, all propionyl groups have been functionalized, i.e. there are ~0.6 N₃ groups/ alkyIPEG600 chain.

3. Synthesis of 3-[2-[2-[2-[2-[2-[2-dioxo-3,4-bis(phenylsulfanyl)-2,5-dihydro-1*H*-pyrrol-1-yl]ethoxy]ethoxy]ethoxy]propanoic acid 6

In the literature, the group 2,5-dioxo-3,4-bis(phenylsulfanyl)-2,5-dihydro-1H-pyrrol-1-yl is sometime identified with the synonym 2,3-Bis(phenylthio)maleimide³ or simply dithiophenolmaleimide.⁴⁻⁶

Scheme S2.



Reagents and conditions: *a*) 2,3-dibromomaleimide (5.22 mmol, 0.83 equiv.), PPh₃ (5.22 mmol, 0.83 equiv.), DIAD (5.22 mmol, 0.83 equiv.), THF, 0 °C to r.t., 3 h, 75%; *b*) TFA 13% v/v in DCM (15 equiv.), r.t., 4 h, quant.; *c*) thiophenol (3.70 mmol, 2.2 equiv.), NaOAc (3.53 mmol, 2.1 equiv.), MeOH, r.t., 20 min, 60%. DIAD=diisopropyl azodicarboxylate.

t-Butyl **3-**[2-[2-[2-[2-[2-[2-(2,5-dioxo-3,4-dibromo-2,5-dihydro-1H-pyrrol-1-yl]ethoxy]ethoxy]ethoxy]ethoxy] propanoate (6B). *t*-Butyl 3-[2-[2-[2-(2-hydroxyethoxy)ethoxy]ethoxy]ethoxy]propanoate $6A^7$ (2.02 g, 6.26 mmol) was dried by co-evaporation with toluene (2×10 mL) and dissolved in 5 mL dry THF under N₂, followed by the addition of PPh₃ (1.37 g, 5.22 mmol, 0.83 equiv.). The reaction mixture was stirred at r.t. until a solution was obtained and then cooled to 0 °C. A solution of diisopropyl azodicarboxylate (1.0 mL, 5.22 mmol, 0.83 equiv.) in 5 mL dry THF was then added dropwise and the reaction mixture was stirred for additional 5 min. before 2,3-dibromomaleimide (1.33 g, 5.22 mmol, 0.83 equiv.) was added. The reaction mixture was stirred for 3 h during which it was allowed to slowly reach r.t., and then quenched by the addition of 30 mL of aqueous, sat. NH₄Cl solution and extracted with 100 mL of EA. The organic phase was washed with 30 mL 5% NaHCO₃ in brine and with brine (2×30 mL). Drying over Na₂SO₄ and removal of the solvent under reduced pressure afforded the crude product, which was purified by flash chromatography (from Hex:EA 7:3 to Hex:EA 1:1) to obtain **6B** as pale yellow oil (2.19 g, 3.92 mmol, 75% yield).

¹H NMR (400 MHz, CDCl₃) δ 3.81 (t, *J* = 4.8 Hz, 2H, N-CH₂-), 3.73-3.56 (m, 16H, EG -CH₂-O-), 2.50 (t, *J* = 6.4 Hz, 2H, -CH₂CO-), 1.44 (s, 9H, *t*-Bu).

3-[2-[2-[2-[2-(2,5-dioxo-3,4-dibromo-2,5-dihydro-1H-pyrrol-1-yl)ethoxy]ethoxy]ethoxy]

propanoic acid (6C). Compound 6B (2.06 g, 3.68 mmol) was dissolved in 25 mL DCM under N_2 and 3.8 mL of TFA were added. The reaction mixture was stirred at r.t. for 4 h, after which the starting material was not present anymore according to TLC analysis (Hex:EA 1:1). Removal of the volatiles under reduced

pressure followed by co-evaporation with toluene (3×10 mL) afforded the crude product in quantitative yield. No further purification was necessary.

¹H NMR (400 MHz, DMSO-d₆) δ 3.64 (t, *J* = 5.8 Hz, 2H, N-CH₂-), 3.59 (t, *J* = 6.3 Hz, 2H, EG -CH₂-O-), 3.56-3.44 (m, 14H, EG -CH₂-O-), 2.43 (t, *J* = 6.3 Hz, 2H, -CH₂CO-).

 ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.61, 164.07, 129.20, 69.78, 69.72, 69.65, 69.63, 69.48, 66.76, 66.24, 38.72, 34.75.

HR-ESI-MS (C₁₅H₂₁Br₂NO₈) *m/z*: 501.9674 ([M+H]⁺, calc. 501.9707); 523.9525 ([M+Na]⁺, calc. 523.9526)

¹H NMR (400 MHz, CDCl₃) δ 8.34 (b, 1 H, COO*H*), 7.28-7.16 (m, 10 H, Ph), 3.75 (t, *J* = 6.2 Hz, 2H, N-C*H*₂-), 3.72 (t, *J* = 6.1 Hz, 2H, EG -C*H*₂-O-), 3.66-3.56 (m, 14H, EG -C*H*₂-O-), 2.62 (t, *J* = 6.2 Hz, 2H, -C*H*₂CO-). ¹³C NMR (100 MHz, CDCl₃) δ 175.82, 166.89, 135.71, 131.88, 129.05, 129.04, 128.42, 70.60, 70.58, 70.46, 70.32, 69.85, 67.75, 66.44, 37.97, 34.91.

HR-ESI-MS (C₂₇H₃₁NO₈S₂) *m/z*: 560.1407 ([M-H]⁻, calc. 560.1418); 606.1202 ([M-H+2Na]⁺, calc. 606.1203)

4. Synthesis of "clickable" heterobifunctional linkers 7 and 8 for disulfide rebridging

Scheme S3.



Reagents and conditions: *a*) Propargylamine (0.47 mmol, 1.1 equiv.), EDC·HCl (0.62 mmol, 1.5 equiv.), DCM, 0 °C to r.t., 16 h, 93%; *b*) 11-Azido-3,6,9-trioxaundecan-1-amine (0.67 mmol, 1.1 equiv.), EDC·HCl (0.93 mmol, 1.5 equiv.), DCM, 0 °C to r.t., 16 h, 23 %; EDC·HCl = N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride.

Alkyne-terminated hetero-bifunctional linker 7. Compound **6** (0.235 g, 0.42 mmol) was dissolved in 2 mL dry DCM under N₂ at 0 °C and EDC·HCl (0.120 g, 0.62 mmol, 1.5 equiv.) was added. After 5 min., propargylamine (30μ L, 0.62 mmol, 1.3 equiv.) was added. The reaction mixture was stirred for 16 h during which it was allowed to reach r.t. and then partitioned between 100 mL EA and 35 mL 0.1 M HCl in brine. The aqueous phase was further extracted with EA (2×20 mL). The combined organic phases were washed with 0.1 M HCl in brine (2×35 mL) and dried over Na₂SO₄. Removal of the solvent under reduced pressure afforded the crude product, which was purified by flash chromatography (from Hex:EA 1:1 to EA) to obtain **7** as yellow amorphous solid (0.233 g, 0.39 mmol, 93% yield).

¹H NMR (400 MHz, CDCl₃) δ 7.30-7.16 (m, 10 H, Ph), 4.03 (dd, *J* = 5.3, 2.5 Hz, 2H, -CH₂-C=CH), 3.71 (q, *J* = 5.4 Hz, 4H, N-CH₂- and EG -CH₂-O-), 3.67-3.56 (m, 14H, EG -CH₂-O-), 2.50 (t, *J* = 5.6 Hz, 2H, -CH₂CO-), 2.21 (t, *J* = 2.5 Hz, 1H, -C=CH).

 ^{13}C NMR (100 MHz, CDCl₃) δ 171.69, 166.84, 135.78, 131.95, 129.09, 129.07, 128.47, 71.22, 70.76, 70.66, 70.61, 70.37, 70.33, 70.00, 67.76, 67.06, 38.06, 36.68, 29.06.

HR-ESI-MS (C₃₀H₃₄N₂O₇S₂) *m/z*: 599.1883 ([M+H]⁺, calc. 599.1880); 621.1690 ([M+Na]⁺, calc. 621.1700)

Alzido-terminated hetero-bifunctional linker 8. Compound 6 (0.343 g, 0.61 mmol) was dissolved in 3 mL dry DCM under N₂ at 0 °C and EDC·HCl (0.178 g, 0.93 mmol, 1.5 equiv.) was added. After 5 min., 11-Azido-3,6,9-trioxaundecan-1-amine (148 μ L, 0.67 mmol, 1.1 equiv.) was added. The reaction mixture was stirred for 16 h during which it was allowed to reach r.t. and then partitioned between 100 mL EA and 35 mL 0.1 M HCl in brine. The aqueous phase was further extracted with EA (2×20 mL). The combined organic phases were washed with 0.1 M HCl in brine (2×35 mL) and dried over Na₂SO₄. Removal of the solvent under reduced pressure afforded the crude product, which was purified by flash chromatography (from EA+0.1% EtOH to EA+0.55 EtOH) to obtain 8 as yellow viscous oil (0.108 g, 0.14 mmol, 23% yield).

¹H NMR (400 MHz, CDCl₃) δ 7.31-7.16 (m, 10 H, Ph), 6.79 (bm, 1 H, CON*H*), 3.74 (t, *J* = 6.0 Hz, 2H, N-C*H*₂-), 3.72-3.53 (m, 28H, EG -C*H*₂-O-), 3.45 (q, *J* = 5.3 Hz, 2H, NH-C*H*₂-), 3.40 (q, *J* = 5.1 Hz, 2H, C*H*₂-N₃), 2.50 (t, *J* = 6.0 Hz, 2H, -C*H*₂CO-).

 13 C NMR (100 MHz, CDCl₃) δ 172.02, 166.82, 135.68, 131.88, 129.04, 128.43, 77.48, 77.16, 76.84, 70.78, 70.71, 70.68, 70.67, 70.61, 70.57, 70.43, 70.34, 70.13, 69.92, 69.76, 67.72, 67.27, 50.75, 39.44, 37.99, 36.73.

HR-ESI-MS (C₃₅H₄₇N₅O₁₀S₂) *m/z*: 762.2836 ([M+H]⁺, calc. 762.2837); 784.2663 ([M+Na]⁺, calc. 784.2657)

5. Localization of the functionalized disulfide bridge within the leptin structure.

The software packages used for composing Figure S1 (top) are:

- 1) TURBOMOLE V7.2, 2017, a development of University of Karlsruhe and Forschungszentrum Karlsruhe GmbH, 1989-2007, TURBOMOLE GmbH, since 2007; available from http://www.turbomole.com.
- 2) VMD Visual Molecular Dynamics, molecular graphics software for MacOS X, Unix, and Windows. Software available from https://www.ks.uiuc.edu/Research/vmd/



Figure S1. *Top:* Schematic structural representation displaying the dithiophenolmaleimide linker positioned in proximity of the single disulfide bridge of leptin. The image is composed displaying the structure of human leptin (PDB entry 1ax8⁸) and the dithiophenolmaleimide linker position arbitrarily in

proximity of leptin disulfide bridge. Several sites and orientations of the linker with respect to the single disulfide bridge of leptin were analyzed and the geometry was optimized at a force field level by using the Universal Force Field (UFF) module implemented in Turbomole package. The image was made using the Visual Molecular Dynamics (VMD) molecular graphics software. The inset shows the two sulfur atoms (gold color) next to the maleimide ring. The structure of the linker is truncated at the second oxygen of the ethylene glycol chain (starting from the maleimide-ring) and the two phenyl rings are omitted for clarity; image courtesy of Dr. Stefania D'Agostino, Center for Biomolecular Nanotechnologies, Italian Institute of Technology. *Bottom:* Crystal structure- based model of the interaction between leptin and its cognate receptor. Image from Haglund, E.; Sulkowska, J. I.; Noel, J. K.; Lammert, H.; Onuchic, J. N.; Jennings, P. A. *PLoS Comp. Biol.* 2014, *10*, e1003613. The disulfide bridge controls the 3D-folding of leptin and its biological activity. Its location is ideal for chemical modification and for protein immobilization because no major interaction between leptin and its receptor occur in this region.

6. Hydrolytic stability of dithiophenolmaleimide linker 6

For efficient disulfide rebridging it is mandatory that the 2,3-bis substituted maleimide ring does not open upon hydrolysis.⁶ The rate of hydrolysis of linker **6** was analyzed at different temperatures in phosphate buffer at pH 7.8 (Figure S2). Under these conditions, negligible hydrolysis is observed at +4 °C. Under the same conditions, precursor dibromomaleimide **6C** hydrolyzes much faster (Figure S3, magenta trace). Also a small change in the pH from 7.8 to 8.4 increases substantially the hydrolysis of **6** at +4 °C (Figure S3, grey trace).

The kinetics were followed by HPLC-UV detection on a 1260 Infinity II LC system form Agilent equipped with a diode array detector (DAD) using a Zorbax Eclipse C18 column (100 mm \times 4.6 mm id, 3.5 μ m). For quantitative analysis, chromatograms at 220 nm were integrated.

Eluents: A=water + 0.1% TFA, B= ACN + 0.1% TFA.

Binary gradient for **6**: 0 - 2 min 27.5% B, 2 - 30 min linear from 27.5% to 70% B; flow 0.6 mL/min; t_R=23.3 min.;

Binary gradient for **6C**: 0 - 2 min 10% B, 2 - 25 min linear from 10% to 45% B; flow 1.0 mL/min; t_R=18.0 min.



Figure S2. Kinetics of hydrolysis of dithiophenolmaleimide linker **6** (100 μ M) in 50 mM phosphate buffer at pH 7.8, followed by HPLC-UV detection. Peak areas are normalized to the peak area at t= 0 min.



Figure S3. Kinetics of hydrolysis at + 4 °C for: (black) dithiophenolmaleimide linker **6** (100 μ M) in 50 mM phosphate buffer at pH 7.8; (magenta) **6** in 50 mM NaHCO₃ at pH 8.4; (grey) precursor dibromo maleimide **6C** (100 μ M) in 50 mM phosphate buffer at pH 7.8. Kinetics are followed by HPLC-UV detection. Peak areas are normalized to the peak area at t= 0 min.

7. Preparation of AuNPs 1 and 2

Citrate coated AuNPs with a diameter of 13.5 ± 1.5 nm were synthesized according to a literature procedure.⁹ AuNPs were passivated by formation of self-assembled monolayers of alkyl-PEG600 thiols on their surface.¹ For AuNPs **1** we used mixtures of carboxy-terminated thiol **3** and azide-terminated disulfide **5** while for AuNPs **2** we used mixtures of carboxy- and alkyne-terminated thiols (**3** and **4**). The molar fraction of **4** was 0.1 while it was 0.05 in case of **5** (disulfide). Stock solutions of thiols **3**, **4** in EtOH (5 mM) or disulfide **5** in EtOH (2.5 mM) were freshly prepared and directly used. Final concentrations in the passivation reaction were: 70 - 90 nM AuNPs, 25 mM NaHCO₃, 1 mM thiol equivalents (total) and therefore the reaction contained 20% v/v of EtOH. Stirring at r.t. was continued for 96 h after which AuNPs were purified by ultrafiltration in Amicon Ultra-4 centrifugal filters (regenerated cellulose – 100 kDa from Millipore) washing with 1×4 mL 25 mM NaHCO₃, 1×4 mL 2:8 v/v EtOH/50 mM NaHCO₃ and 2×4 mL 50 mM NaHCO₃ buffer. Purified, passivated AuNPs were taken up in 50 mM NaHCO₃ at a final concentration varying in the interval 200 – 400 nM.

8. Mass spectrometry characterization of functionalized leptins

LC/ESI-FTMS analysis of the site-selective functionalization of leptin via disulfide rebridging.

Analysis were conducted using a Dionex UltiMate 3000 HPLC chromatograph coupled with a Q-Exactive mass spectrometer. This spectrometer allows to record MS spectra with high accuracy (better than 3 ppm) and with high resolving power, set at 70 000 at m/z 200.

HPLC separations were carried out at 40 °C using a XBridge BEH300 C4, 3.5 micron, 2.1 x 100 mm column from Waters. Eluents: water + 0.1% v/v formic acid (eluent A) and acetonitrile + 0.1% v/v of formic acid (eluent B); flow rate 200 μ L/min binary gradient: 0-2 min 35% B, 2-8 min linear from 35% to 45% B; 8-28 min linear from 45% to 55% B (Figure S4).

Several dilution tests were performed to evaluate possible ion suppression at high concentrations; no differences in the concentration ratios among the species of interest were observed. Spectra have been collected from samples containing 10 μ M leptin.

MS full scan acquisitions were performed in positive ion mode, in the *m/z* range 200 - 2500. MS parameters: sheath gas relative flow rate, 20 (arbitrary units); auxiliary gas relative flow rate, 5 (arbitrary units); spray voltage +3.5 kV; capillary temperature 200 °C; S-lens RF level, 100%. Data deconvolution of the high resolution/accurate mass (HR/AM) MS spectra was performed using Protein Deconvolution 4.0 (Thermo Scientific).



Figure S4. Representative extracted ion current chromatograms (XIC) relative to ions at m/z 1644.8682 (green, **lep-6**) and m/z 1610.5552 (magenta, leptin); the elution gradient is shown in black. **lep-6** and leptin mostly co-elute under these conditions.



Figure S5. Deconvoluted MS spectrum obtained for the peak at 13.9 min. Three protein species are identified: m/z 16086.465 (leptin, calc. monoisotopic mass [M+H]⁺=16086.424); m/z 16429.596 (**lep-6**, calc. [M+H]⁺=16429.559); m/z 16447.579 (**lep-6** + H₂O, calc. [M+H]⁺=16447.569), indicative of maleimide hydrolysis.

Speciation of nonselectively functionalized leptin **11** via ESI-TOF MS.

6540 UHD Q-TOF LC-MS system: drying gas temperature 300 °C, drying gas flow rate 10 L/min, capillary voltage 3500V, fragmentor potential 150 V, nebulizer gas pressure 40 psi, and skimmer voltage 45 V.



9. TEM images of leptin-functionalized AuNPs

Figure S6. Representative TEM micrographs of leptin-functionalized AuNPs 14 (a) and 13 (b).

10. Quantification of leptin loading on AuNPs via amino acid analysis

The average number of leptin molecules per nanoparticle for AuNPs 12 - 17 was determined via *amino acid analysis* using the o-phtalaldehyde (OPA) pre-column derivatization method¹⁰⁻¹² as previously described.² The method is very sensitive to sample contaminations, the most important source being dust. *Amino acid standard mix (AA-mix) and L-norvaline internal standard*

AA-mix solution (AAS18 Sigma-Aldrich) in 0.1 M HCl. L-Norvaline: 200 μM in 0.1 M HCl.

Sample microwave-assisted sample hydrolysis:

- 10 mL Pyrex glass vials (CEM Corporation, USA) with a magnetic stirring bar were filled with freshly prepared Piranha solution (3:1 conc. H₂SO₄: 30% w/w H₂O₂) and left stirring for at least 12 h. They were then thoroughly rinsed with water and dried in an oven at 95 °C.
- Typically, 40 µL of AuNPs (≥100 nM) and 10 µL of 200 µM L-norvaline solution in 0.1 M HCl (internal standard) were placed into the cleaned Pyrex glass vials together with 800 µL of 6 M HCl and hydrolyzed for 20' at 175 °C using a pressurized microwave acid digestion system from CEM (Discover SP-D).
- 3. Calibration samples containing known amounts of AA-mix and L-norvaline as an internal standard were treated in the exact same way.

HPLC conditions: Zorbax Extend C18 column, 100 mm × 4.6 mm id, 3.5 μ m, 40 °C, detection at 338 nm. Mobile phase A: (10 mM Na₂HPO₄, 10 mM Na₂B₄O₇) adjusted to pH 8.2 with HCl, mobile phase B was ACN/MeOH/H₂O 45:45:10 (v/v/v). Flow rate: 1.5 mL/min. Gradient: 1 min at 2% B then up to 58% B in 17.5 min, followed by 100% B (total analysis time 24 min). Re-equilibration at 2% B for at least 5 min before starting a new analysis. Quantification was carried out averaging the single values obtained for Val, Arg, Thr, Ile, Leu. For each one of these amino acids, good linearity was observed in the calibration range from 0.5 to 15.0 μ M of AA-mix (R²≥0.97).

11. Leptin Receptor (LR) expression by MCF7 cells

*Flow cytometry experiments were performed to confirm the presence of LRs on the plasma membrane of MCF7 cells.*¹³

MCF7 cells were collected using trypsin-EDTA solution, resuspended in PBS with 2% FBS (cytofluorimetry buffer), and incubated with LR antibody ($2.5 \ \mu g/10^6$ cells) for 20 min at r.t.. After 3 washes with cytofluorimetry buffer (centrifugation 5 min. at $350 \times g$), cells were incubated with Alexa Fluor-488-conjugated secondary antibody ($10 \ \mu g/mL$) for 5 min. on ice in the dark, followed by 3 washes with cytofluorimetry buffer. The samples were analyzed at 488 nm by cytofluorimetry. Cells stained only with the secondary antibody were used as the control. FlowJo software was used to analyze flow cytometry data (Tree Star, Inc., Sac Carlos, CA, USA). For all experiments FSC-A/ SSC-A gates of the starting cell population were used to discriminate between viable cells and cell debris. Singlet and doublet cells were discriminated using FSC-A/ FSC-H gating. Cells stained only with the FITC-conjugated secondary antibody were used as the negative control to distinguish between background staining and specific antibody staining.



Figure S7. Cytofluorimetric assessment of LR expression by MCF7 cell line. a) Based on forward (FSC) and side scatter (SSC) parameters, gating parameters were defined to exclude debris and select live cells for the analysis. b) LR is expressed on MCF7 cells as demonstrated by the shift of the fluorescence intensity distribution to higher intensity values (red histogram). The grey histogram correspond to the fluorescence intensity distribution of cells stained only with secondary antibody (negative control).

12. Leptin-directed cellular internalization of AuNPs

MCF7 cells seeded in 6-well plates were grown in complete medium until they reached sub-confluence. The medium was then changed to DMEM without FBS for to synchronize the cells (21 h). Afterwards, cells were exposed to 5 nM leptin-functionalized AuNPs **13** and **14** or control AuNPs **16** in 0.8 mL DMEM high glucose supplemented with 10% FBS for 3 h. After this period, the medium was removed and cells were

washed with complete medium (2×2 mL) and with PBS (2×2 mL). Cells were detached by treatment with trypsin-EDTA solution (700 μ L for 5 minutes), which was quenched by addition of 1 mL complete medium. The cell suspension was transferred to mineralization glass vials and the wells were rinsed with 1 mL cell culture medium, which was added to the cell suspension. Cells were counted and then pelleted by gentle centrifugation (5 min at 200×g). The supernatant was carefully removed leaving approx. 1 mL above the cell pellet. The vials with the samples were then dried overnight at 90 °C. The dry residues were mineralized in 0.9 mL of a 2:1 v/v mixture of 65% HNO₃ and 36.5% HCl using a pressurized microwave acid digestion system from CEM. Mineralized samples were diluted with H₂O to 9 ml. 1 mL of this solution was further diluted to 5 mL with H₂O containing L-cysteine and Bi which was used as an internal standard. These diluted samples were used for the quantification of gold via ICP-MS and contained 2% v/v acid mixture, 0.1% w/v L-cysteine and 4 μ g/L Bi. The gold concentration was determined by comparison with an external calibration curve (11 concentration levels in the interval 0.2 – 6.2 μ g/L).

13. Internalization assessment of AuNPs via fluorescence confocal microscopy

MCF7 human cells were seeded on glass bottom dishes for confocal microscopy (VWR) and grown overnight in complete medium. The medium was changed to DMEM without FBS to synchronize cells (21 h). The cells were then exposed to 5 nM leptin-functionalized AuNPs **13** and **14** or control AuNPs **16** in 0.8 mL high glucose DMEM supplemented with 10% FBS for 3 h. Cells were washed with complete medium (2×2 mL) and with PBS (2×2 mL) and fixed in 4% paraformaldehyde (freshly prepared in PBS) for 10 min. at r.t.. Nuclei were stained with DAPI dye (4',6-diamidino-2-phenylindole; Life Technologies) for 10 min. at r.t. and actin filaments with phalloidin-Atto 488 (49409 Sigma Aldrich) for 10 min. at r.t.. Finally, cells were washed with PBS (2×2 mL) and examined by confocal laser scanning microscopy (CLSM) using a Leica TCS SP8 microscope (Leica Microsystems) mounting an HC PL APO CS2 63×/1.40 oil objective. AuNPs were imaged in reflection mode at 627 – 637 nm.¹⁴



Figure S8. Confocal microscopy images of MCF7 cells exposed to AuNPs **13** and **14** or negative control AuNPs **16** confirm the internalization of leptin-functionalized AuNPs. Blue: nuclei, green: actin filaments, magenta: AuNP reflection signal.

14. Western blotting based quantification of pSTAT3 in MCF7 cells

Preparation of cell lysates for western blotting

MCF7 cells were plated in six-well plates at about 800000 cells/well in complete DMEM. After overnight incubation to allow cell adhesion, cells were synchronized in serum-free medium (21 hours) and then treated with AuNPs, leptin, or only DMEM for the required time (as detailed in Figures 4, S9, and S10). In all cases, during treatment no FBS was present in the DMEM medium. After treatment, cells were washed two times with ice-cold PBS and whole cell lysates were prepared by scraping cells in 200 μ l of ice-cold radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich) for 15 min on ice. After 10 min centrifugation at 18000×g at 4 °C, the clear supernatants were collected and the protein content was quantified by Micro BCA Protein Assay kit (Thermo Fisher Scientific). The cell lysates were either directly used for western blotting or immediately stored at -80 °C until further use.

Total cell lysates containing 30 μ g of proteins were separated by 7.5% polyacrylamide gels and electroblotted onto poly(vinylidene difluoride) (PVDF) membranes (Bio-Rad Laboratories). The blots were blocked with 8% dry nonfat milk (Sigma-Aldrich) in TBS containing 0.05% Tween 20 for 1 h at r.t., washed 3 times with TBS with 0.1% Tween 20 for 10 min at r.t., and horizontally cut between 75 and 50 KDa. Membranes were incubated overnight at 4 °C with the primary antibodies. The upper part of the

membrane was incubated with pSTAT3 antibody (1:1000) in TBS with 0.1% Tween 20, 5% BSA, and 0.02% NaN₃. Alternatively, incubation was performed with STAT3 antibody (1:1000) in TBS with 0.1% Tween 20, 5% dry nonfat milk, and 0.02% NaN₃. The lower part was incubated with actin antibody (1:2000) in TBS with 0.1% Tween 20, 5% BSA, and 0.02% NaN₃. Membranes were then washed 3 times with TBS with 0.3% Tween 20 and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:2500) in TBS with 0.1% Tween 20 for 1 h at r.t.. Finally, proteins were visualized according to manufacturer's instruction by enhanced chemiluminescence detection systems (Bio-Rad Clarity Max[®] for pSTAT3 and Bio-Rad ECL+[®] for STAT3 and ACTIN analysis) using a Typhoon Trio Variable Mode Imager System (GE Healthcare). Protein band intensity was quantified with ImageQuant TL software (GE Healthcare). The data shown in Figure 4 are ratios of the intensity of the bands of pSTAT3 over STAT3, each normalized by the intensity of the corresponding actin band to account for slightly different lane loading.



Figure S9. MCF7 cells show increased phosphorylation of STAT3 in response to leptin treatment. a) Representative immunoblot analysis of pSTAT3 and actin expression in MCF7 untreated or treated with 100 nM leptin for various intervals of times (from 5 to 90 minutes). b) Quantification of pSTAT3 on actin normalized to the untreated sample. Results are averages of 3 independent measurements using cells at passage number 20 - 26.

We have tested whether linker functionalization was affecting the biological activity of leptin **10** and **11** (**10** modified site-selectively at the disulfide bridge and **11** modified randomly). The activity of leptin **10** and **11** was compared to that of unmodified leptin using the STAT3 phosphorylation assay above reported. The results of the western blot analysis show only minor differences between modified and unmodified leptins, which are well within the experimental variability of these measurements (Figure S10).



Figure S10. STAT3 is phosphorylated at comparable levels by threating MCF7 cells for 20 minutes with either 100 nM leptin or 100 nM linker-modified leptins (**10** and **11**).

15.References

- (1) Maus, L., Dick, O., Bading, H., Spatz, J. P., and Fiammengo, R. (2010) Conjugation of Peptides to the Passivation Shell of Gold Nanoparticles for Targeting of Cell-Surface Receptors. *ACS Nano 4*, 6617-6628.
- Maggi, V., Bianchini, F., Portioli, E., Peppicelli, S., Lulli, M., Bani, D., Del Sole, R., Zanardi, F., Sartori, A., and Fiammengo, R. (2018) Gold nanoparticles functionalized with RGD-semipeptides: a simple yet highly effective targeting system for αVβ3 integrins. *Chem. Eur. J. 24*, 12093-12100.
- (3) Yudhistira, T., Mulay, S. V., Lee, K. J., Kim, Y., Park, H.-S., and Churchill, D. G. (2017) Thiomaleimide Functionalization for Selective Biological Fluorescence Detection of Peroxynitrite as Tested in HeLa and RAW 264.7 Cells. *Chem. Asian J. 12*, 1927-1934.
- Schumacher, F. F., Nobles, M., Ryan, C. P., Smith, M. E. B., Tinker, A., Caddick, S., and Baker, J. R.
 (2011) In Situ Maleimide Bridging of Disulfides and a New Approach to Protein PEGylation. Bioconjugate Chem. 22, 132-136.
- (5) Eissa, A. M., Wilson, P., Chen, C., Collins, J., Walker, M., Haddleton, D. M., and Cameron, N. R.
 (2017) Reversible surface functionalisation of emulsion-templated porous polymers using dithiophenol maleimide functional macromolecules. *Chem. Commun. 53*, 9789-9792.
- (6) Ravasco, J. M. J. M., Faustino, H., Trindade, A., and Gois, P. M. P. (2019) Bioconjugation with Maleimides: A Useful Tool for Chemical Biology. *Chem. Eur. J.* 25, 43-59.
- (7) Warnecke, A., and Kratz, F. (2003) Maleimide-oligo(ethylene glycol) Derivatives of Camptothecin as Albumin-Binding Prodrugs: Synthesis and Antitumor Efficacy. *Bioconjugate Chem.* 14, 377-387.
- (8) Zhang, F., Basinski, M. B., Beals, J. M., Briggs, S. L., Churgay, L. M., Clawson, D. K., DiMarchi, R. D., Furman, T. C., Hale, J. E., Hsiung, H. M., et al. (1997) Crystal structure of the obese protein leptin-E100. *Nature 387*, 206-209.
- (9) Grabar, K. C., Freeman, R. G., Hommer, M. B., and Natan, M. J. (1995) Preparation and Characterization of Au Colloid Monolayers. *Anal. Chem.* 67, 735-43.
- (10) Alkaitis, M. S., Nardone, G., Chertow, J. H., and Ackerman, H. C. (2016) Resolution and quantification of arginine, monomethylarginine, asymmetric dimethylarginine, and symmetric dimethylarginine in plasma using HPLC with internal calibration. *Biomed. Chromatogr. 30*, 294-300.

- (11) Perucho, J., Gonzalo-Gobernado, R., Bazan, E., Casarejos, M. J., Jiménez-Escrig, A., Asensio, M. J., and Herranz, A. S. (2015) Optimal excitation and emission wavelengths to analyze amino acids and optimize neurotransmitters quantification using precolumn OPA-derivatization by HPLC. *Amino Acids 47*, 963-973.
- (12) Molnár-Perl, I. (2011) Advancement in the derivatizations of the amino groups with the ophthaldehyde-thiol and with the 9-fluorenylmethyloxycarbonyl chloride reagents. *J. Chromatogr. B 879*, 1241-1269.
- (13) Dieudonne, M.-N., Machinal-Quelin, F., Serazin-Leroy, V., Leneveu, M.-C., Pecquery, R., and Giudicelli, Y. (2002) Leptin mediates a proliferative response in human MCF7 breast cancer cells. *Biochem. Biophys. Res. Commun. 293*, 622-628.
- (14) Yuan, L., Wei, W., Li, J., Sun, Z., Wang, H., Zhang, X., and Chen, Y. (2009) Facile method for CLSM imaging unfunctionalized Au nanoparticles through fluorescent channels. *J. Nanopart. Res.* 11, 1219-1225.