Electronic Supporting Information

Functionalization of colloidal nanoparticles with a discrete number of ligands based on a "HALO-bioclick" reaction

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Material and methods:

1. Production, purification and characterization of HALO-GFP

Plasmid and bacterial strain

A modified DNA sequence encoding for HALO-GFP was synthesized by Eurofins Genomics (Ebersberg, Germany) to obtain sequences containing NaeI and NatI restriction sites, respectively, at 5' and 3' positions.
HALO-GFP sequence was cloned in a pGEX6P-1 vector between NaeI and NatI restriction sites to obtain HALO-GFP as GST-fusion proteins containing a PreScission protease recognition site.
Plasmidic DNA (pGEX/HALO-GFP) was sequenced and used to transform *E. coli* expression strain BL21 [DE3].

HALO-GFP production in bioreactor

A stirred tank bioreactor (Biostat A Plus, Sartorius) with an operating volume of 2 L was used for HALO-GFP production. A seed culture was prepared by inoculating the plasmid-trasformed *E.coli* cells into a 1,000 mL Erlenmeyer flask with 200 mL of FRS01 medium (triptone 10 g L^{-1}; yeast extract 5 g L^{-1}; 2 mM $MgSO_4 \cdot 7H_2O$, 0.5 g L^{-1}; glycerol 5g L^{-1}), containing ampicillin (100 μg mL^{-1}). The cells were grown at 30 °C for 24 h in an orbital shaker (Innova 40R, New Brunswick Scientific) at 120 rpm. The optical density was measured at 600 nm (OD_{600}). Based on the OD_{600} value, the culture volume to be inoculated in the bioreactor was calculated to get a starting OD_{600} = 0.1.
The medium utilized in the bioreactor cultivation was the same of the shake flask (FRS01) with an increased amount of glycerol (20 g L^{-1}), to sustain a high biomass production. The cells were grown until OD_{600} = 1 was reached. Afterwards, HALO-GFP expression was induced with the addition of 2 g L^{-1} of lactose. The cells were grown overnight in batch mode. During the growth, the pH was not controlled, leaving pH to decrease over time, from 7 to 5.5. Dissolved oxygen

tension (DOT) was kept controlled at 25% of the O_2 saturation to avoid that cells, consuming oxygen during the cultivation, underwent anaerobic metabolism. Control was achieved by cascade changing the agitation speed (between 120 and 1,000 rpm), maintaining an airflow of 2 L min^{-1} and by using a proportional-integral-derivative control strategy. Value of volume of gas inlet × volume of the growth medium × minute (vvm) flushed into the bioreactor during the process was 1. The initial temperature was set at 30 °C and then lowered to 20 °C after lactose addition, while the other parameters were kept unchanged. After 24 h, the culture broth was collected, split into pre-calibrated bottles (about 300 ml for each bottle) and the culture medium was discarded by centrifugation (5,500 ×g for 10 min at 4 °C). The cell pellets were then resuspended in 2 mL g^{-1} of PBS (pH 7.3) containing: 10 μg mL^{-1} DNase, 0.19 mg mL^{-1} phenylmethanesulfonyl fluoride (PMSF) and 0.7 mg mL^{-1} pepstatin. Cells were sonicated in ice (10 cycles of 30 s, 12% amplitude, Branson Digital Sonifier 450) and centrifuged (20.000 ×g for 1 h at 4 °C). The supernatant was collected and HALO-GFP was purified under native conditions using Pierce Glutathione Superflow Agarose (Thermo scientific) columns exploiting the GST tag. After column equilibration, the resin was incubated with HALO-GFP soluble fraction for 2 h at 4 °C. The flow-through was discarded and the resin-bound HALO-GFP was incubated with 1 column volume (CV) of cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 8) containing 40 μL of PreScission Protease (GE Healthcare) for 16 h at 4 °C under weak shaking. After GST cleavage, HALO-GFP was collected in the eluate by washing the column with 20 CV of cleavage buffer and the collected fractions were analyzed through SDS-PAGE gel.

Circular dichroism

For the circular dichroism analysis, the purified HALO-GFP was suspended in phosphate buffer 10 mM (PB) at a final concentration of 10 μM. The CD spectra were acquired with a JASCO J-715 spectropolarimeter using a quartz cuvette with an optical path of 0.5 cm; 15 acquisitions per sample were performed in the wavelength range from 180 nm to 270 nm and the scan speed was set at 100 nm per minute.

HALO-GFP enzymatic activity assay

The catalytic activity of HALO was evaluated through a qualitative colorimetric test based on the use of a chromophore to monitor the decrease in pH that occurs following the dehalogenation reaction.[1] For this experiment, bromothymol blue was used as a pH indicator (BTB) and N,N-Bis (2-hydroxyethyl)-2-aminoethanosulfonic acid (BES) was used as reaction buffer. A sufficient volume of solution containing 1 mM BES and 50 μM BTB, pH 7.8, was first prepared. Right before the test was carried out, part of the solution was mixed with 10 mM dichloropropane (DCP) and left under stirring for 1 h. Then 200 μl of reaction buffer were mixed with 100 μl of cleavage buffer containing HALO-GFP (20 μg) into a 1 cm-path length cuvette. The absorbance of the solution at 620 nm was monitored every 3 min for 21 min (Nanodrop 2000c Thermo Fisher Scientific).

2. Synthesis and characterization of PMDA-AuNPs

DDT-AuNPs synthesis

Thiol-derivatized AuNPs with a core diameter d_c of about 4 nm were synthetized according to the Brust-Schiffrin two phases method with some modifications.[2,3] In brief, in a large separation funnel, a solution containing 2.17 g of tetraoctylammonium bromide in 80 mL of toluene was mixed with a solution containing 0.3 g of tetrachloroauric acid in 25 ml of MilliQ water. The organic phase was separated after several rounds of shaking and was transferred in a large bowl. A solution containing 0.344 g of sodium borohydride ($NaBH_4$) in 25 mL of MilliQ water was added dropwise to the organic phase under vigorous stirring (1,300 rpm), resulting in a color change to intense red that indicates the formation of AuNPs. The solution was left stirring for about 1 h and was transferred into a separator funnel. The resulting NP solution was washed with HCl and NaOH (25 mL, 10 mM) and MilliQ water (25 mL) for three times while removing the aqueous phase after each step of washing. Finally, the remaining organic solution was transferred into a 250 mL round bottom flask and left under stirring overnight (600 rpm) to get thermodynamically stable NPs with narrow size distribution, mediate by Ostwald ripening. After the synthesis of TOAB-AuNPs, a ligand exchange procedure was carried out to obtain dodecanethiol-stabilized AuNPs (DDT-AuNPs). 10 mL of dodecanethiol (DDT) was added and the solution heated at 65 °C for 2 h under stirring. After cooling down to room temperature, the solution was poured into separate glass vials and centrifuged at 3,000 ×g for 5 min to remove big agglomerates. The supernatant containing DDT-AuNPs was collected and the precipitate discarded. DDT-AuNPs were then precipitated by centrifugation at 900 ×g for 5 min by adding methanol as a nonsolvent and finally the pellet containing the AuNPs was resuspended in chloroform.

Phase transfer of DDT-AuNPs to aqueous solution using PMDA polymer

DDT-AuNPs suspended in chloroform were transferred from organic to aqueous solution by wrapping an amphiphilic polymer around their surface, resulting in monodisperse and water-soluble NPs according to previously published protocols.[3] The amphiphilic polymer, poly(isobutylene-alt-maleic anhydride)-graft-dodecyl (PMDA) was synthetized by linking dodecylamine to 75% of the anhydride rings of polyisobutylene-*alt*-maleic anhydride (average molecular weight M_w = 6,000 g mol^{-1}, Sigma). For the coating procedure an aliquot of polymer ($V_{p)}$ at a concentration of C_p = 0.5 M (concentration referring to the monomer units of the polymer, as dissolved in chloroform) was added to AuNPs suspended in chloroform. The amount of polymer per NP scales with the effective surface area A_{eff} of one single NP and with the total number of NPs. Calculation details are shown below:
In the case of spherical NPs, A_{eff} is given as the surface of a sphere:

$$A_{eff} = 4Ï€\left(\frac{d_{eff}}{2}\right)^2 = Ï€\, d_{eff}$$

d_{eff} is the effective diameter, which is the core diameter d_c (as determined by TEM) plus two time the thickness of the DDT shell. Here, d_c was determined to be 3.8 nm, and d_{eff} was estimated as 4 nm. In a solution with volume $V_{NP\ sol}$ and NP concentration C_{NP}, the number of NP moles is:

$$n_{NP} = C_{NP} * V_{NP_{sol}}$$

Therefore, the total number of NPs is:

$$N_{NP} = n_{NP} * N_A \ (N_A = Avogadro's\ number)$$

The total effective surface area of NPs in solution thus can be calculated as follow:

$$A_{total\_eff} = N_{NP} * A_{eff} = C_{NP} * V_{NP_{sol}} * N_A * A_{eff}$$

The number of monomer units that needs to be added per nm^2 of effective surface area ($R_{p/area}$) is experimentally determined (for this experiment $R_{p/area}$= 100 nm^{-2}). The number of polymer monomers N_P that needs to be added to the NP suspension thus is:

$$N_P = R_{p/area} * A_{total\_eff}$$

It follows that, for a polymer stock solution of monomer concentration C_p, the volume V_p to be added to the NP suspension was calculated based on the following equation:

$$V_P = N_P/(NA * Cp)$$

The mixture containing DDT-AuNPs and PMA-g-dodecyl dissolved in chloroform was stirred manually for 5 min at room temperature (RT) and then the chloroform was completely evaporated in a rotary evaporator under heating at 40 °C. Few milliliters of anhydrous chloroform were added to the flask to reconstitute the solid film and again the solvent was removed under reduced pressure. This step was repeated 3 times to obtain a homogeneous coating. After the last step, the remaining solid film in the flask was reconstituted in alkaline sodium borate buffer (SBB 30 mM, pH 12 adjusted with NaOH) under vigorous stirring. Then the water-soluble polymer-coated NPs (PMDA-AuNPs) were concentrated in centrifugal filter (Merk Millipore Amicon, 100 kDa) and the polymer excess removed by means of ultracentrifugation (OptimaXE, Beckman Coulter Life science ultracentrifuge, 100,000 ×g; 1 h at 4 °C). The supernatant was discarded, and the pellet was resuspended in 30 mM SBB, pH 8. This washing procedure was repeated 3 times and then the PMDA-AuNPs were characterized by TEM analysis and the concentration quantified by UV-vis absorption spectroscopy. In particular the absorbance at 450 nm was detected and the PMDA-AuNPs concentration was calculated assuming a molecular extinction coefficient of 3.62×10^6 M^{-1} cm^{-1}.[4]

3. Synthesis of 1- and 2-HALO-AuNPs

PEGylation of PMDA-AuNPs (1PEG- and 2PEG-AuNPs)

For the introduction of a discrete number of functional groups, standard bioconjugation chemistry using 1-ethyl-3-(3–dimethylaminopropyl)carbodiimide hydrochloride (EDC) was used exploiting the carboxylic groups on the surface of polymer-coated AuNPs. The 6 kDa diamine-PEG (NH_2–PEG–NH_2) was selected as an optimal spacer "bridge" for the covalent linkage between the nanoparticles and HALO-GFP, because of its sufficiently high molecular weight which allowed us to clearly distinguish the distribution in discrete bands in the gel electrophoresis. For the functionalization, stock solutions of PMDA-AuNPs with the concentration C_{NP} = 6 μM in 30 mM SBB, pH 8 were prepared. The same buffer was used to dissolve the diamine–PEG and EDC. Equal volumes of the NPs solution (C_{NP} = 6 μM) and the PEG solution (C_{PEG} = 3 mM) were mixed and split into 20 μL samples. The ratio of PEG to NPs molecules was kept constant: C_{PEG}/C_{NP} = 500 (the large excess of PEG was chosen to prevent inter-particle crosslinking via the two PEG-terminal amino groups). Then, 10 μl of an EDC solution of appropriate concentration was added to the samples. The molar ratio EDC/NPs was optimized testing different conditions (C_{EDC}/C_{NP} = 2, 4, 8, 16, 32 and 64) and the ratio 4 was selected as optimal. The samples were mixed, allowed to react for 60 min and analyzed through gel electrophoresis. 2% agarose gels were prepared with 0.5% Tris/borate/EDTA buffer (TBE) and 6 μL of gel-loading buffer containing bromophenol blue and 30% glycerol was added to the samples. After sample loading, the gel was run for 90 min at 100 V cm^{-1}. The bands corresponding to 1PEG-AuNPs and 2PEG-AuNPs were cut out and

immersed separately in 0.5 % TBE in dialysis membranes (molecular weight cutoff of 3,500 Da). Again, an electric field was applied for 40 min at 100 V cm^{-1}. Finally, the buffer containing the extracted NPs was collected, the NPs were centrifuged for 1 h at 2,700 ×g to remove the precipitates and filtered using a 0.22 μm syringe filter (Millipore Corporation, Italy).

Chlorohexyl HALO substrate (linker) conjugation

The conjugation reaction between 1PEG- and 2PEG-AuNPs and the HALO substrate (linker) was carried out in 30 mM SBB, pH 8. The linker was added to the nanoparticle solution (molar ratios to 1PEG- and 2PEG-AuNPs 1.5 and 3 respectively) and the reaction was incubated overnight at 4 °C under weak shaking. Afterwards, the linker excess was removed by centrifugal filtration (Amicon Merk Millipore 50 kDa) at 4,000 ×g for 10 min. The linker removal was monitored by UV-vis absorption spectroscopy analysis and the complete removal of the linker excess was achieved after 15 centrifugal filtration rounds.

HALO-GFP conjugation

The conjugation between HALO-GFP and the linker-functionalized NPs (1L-PEG-AuNPs and 2L-PEG-AuNPs) occurs spontaneously via the enzymatic reaction. HALO-GFP dissolved in 30 mM SBB, pH 8, was added to 1L-PEG-AuNPs and 2L-PEG-AuNPs in an excess of 1.5 and 3 times, respectively. 1PEG-AuNPs and 2PEG-AuNPs were used as controls for the non-specific binding. After 1 h incubation at room temperature, the protein excess was removed using centrifugal filter tubes (Amicon Merk Millipore 15 kDa). To remove most of protein only adsorbed on the AuNPs surface, but not covalently bound, eight rounds of washing were required.

Spectrofluorimetric analysis

Fluorescence spectra were recorded using a Fluoromax-4P spectrofluorimeter from Horiba Scientific (NJ, USA). Samples were excited at 480 nm and spectra were recorded in a wavelength range between 500 and 600 nm. The maximum fluorescence emission of HALO-GFP was detected at 509 nm. For the analysis, samples were diluted in 30 mM SBB, pH 8, to reach a fixed nanoparticles-relative concentration of 50 μg mL^{-1} for all samples.

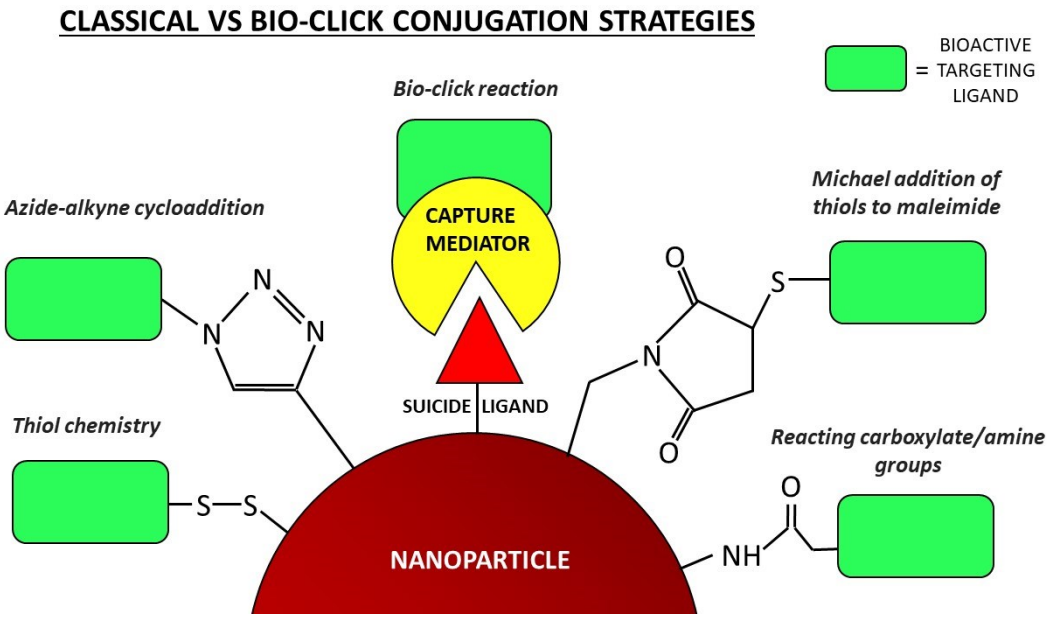

Figure S1. Scheme of bio-click and classical conjugation strategies.

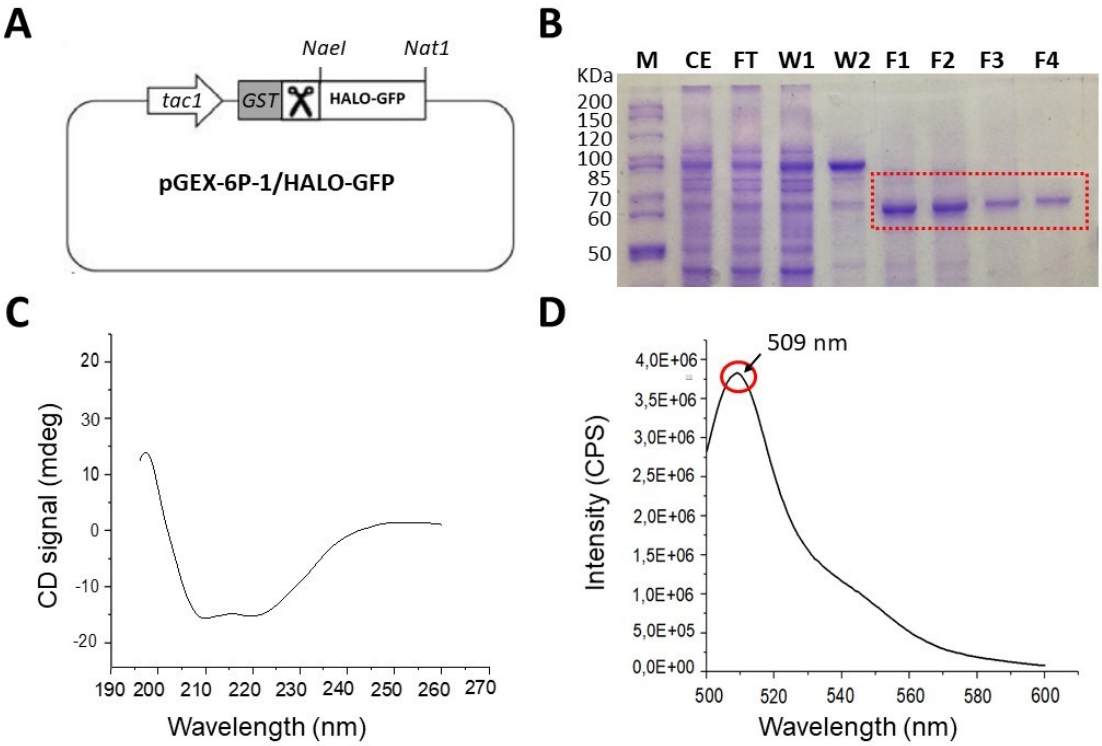

Figure S2. A) HALO-GFP plasmid: the GST tag is expressed in fusion at the N-terminal of the HALO-GFP sequence. B) SDS-PAGE: after the GST cleavage, the HALO-GFP purified fractions (F1-4 framed in red) have a molecular weight of about 60 KDa; M = marker, CE=crude extract, FT = flow-through, W = washing. C) Circular dichroism analysis: the HALO-GFP CD spectrum indicates that the recombinant protein retains a well-defined secondary structure after the purification process. D) Fluorescence analysis: the GFP expressed in fusion with HALO shows an emission spectrum with the peak at 509 nm, comparable to the wild type GFP fluorescence spectrum.

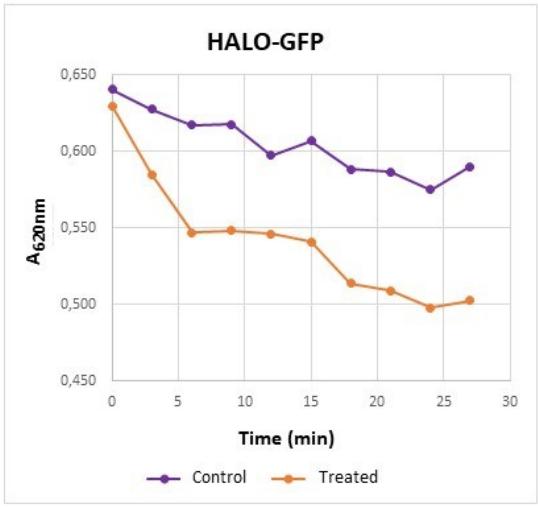

Figure S3: HALO-GFP enzymatic activity assay. The decrease of absorbance at 620 nm ($A_{620\ nm}$) indicates a significant pH change that occurs following the HALO-mediated dehalogenation reaction. The same reaction mixture without DCP substrate (violet line) was used as control.

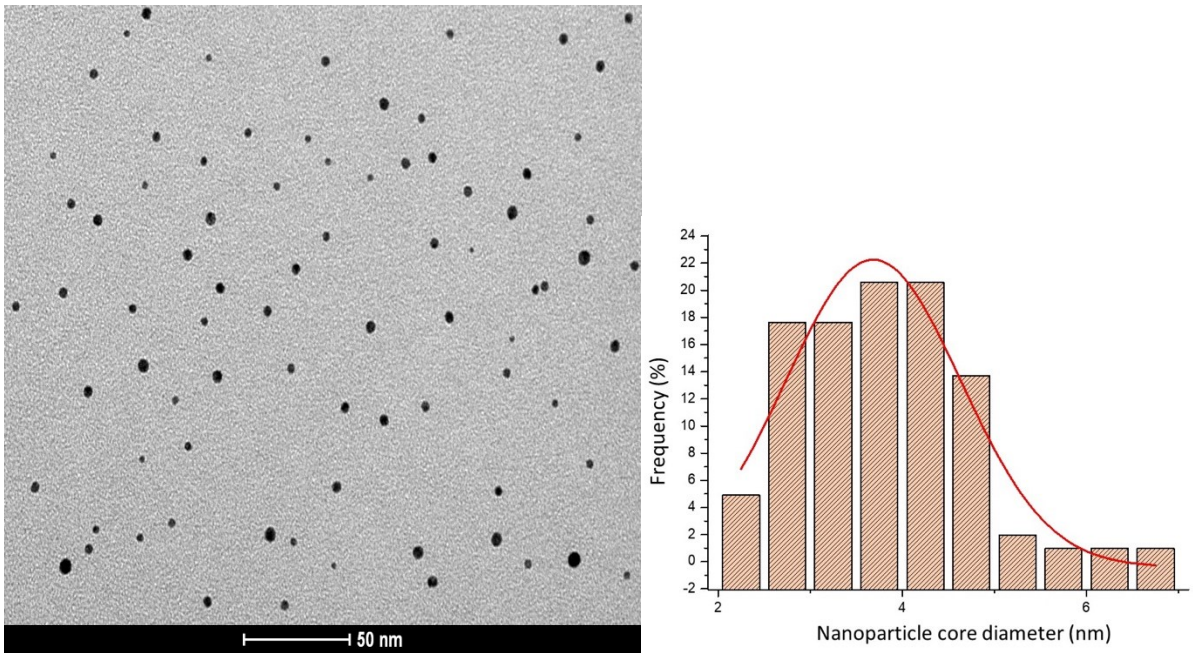

Figure S4: TEM analysis. Image and particle size distribution histogram (n = 100) of PMDA-AuNPs

	C_{NP} (µM)	$V_{NP\ sol}$ (µl)	C_{PEG}/C_{NP}	C_{PEG} (6 kDa) (mM)	$V_{PEG\ sol}$ (µl)	C_{EDC}/C_{NP}	C_{EDC} (µM)	$V_{EDC\ sol}$ (µl)
Control	6	10	-	-	-	-	-	-
A	6	10	500	3	10	2	12	10
B	6	10	500	3	10	4	24	10
C	6	10	500	3	10	8	48	10
D	6	10	500	3	10	16	96	10
E	6	10	500	3	10	32	192	10
F	6	10	500	3	10	64	384	10

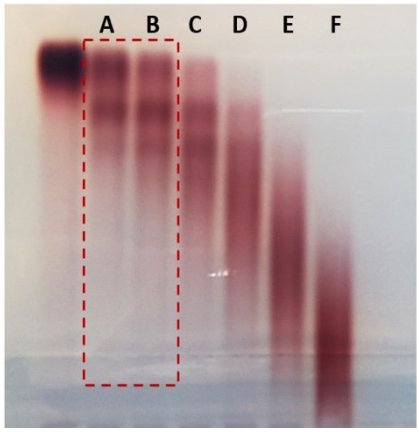

Figure S5: Optimization of EDC/PEG ratio for PEGylation of PMDA-AuNPs.

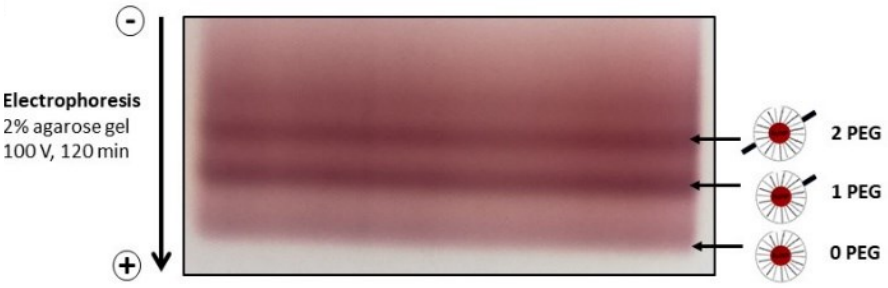

Figure S6: Agarose gel electrophoresis. By applying an electric field, the negatively charged PMDA-AuNPs migrate toward the positive pole in dependence of their size, which depends on the number of PEG molecules conjugated to the NP surface. At the end of the run, the different nanoparticle populations are separated in distinct bands.

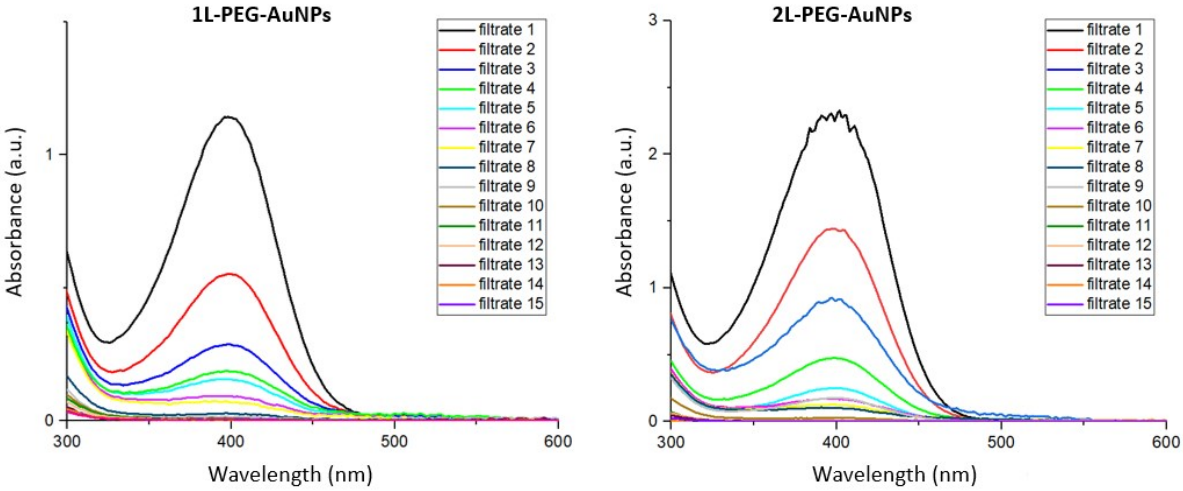

Figure S7: Linker excess removal by centrifugal filtration.

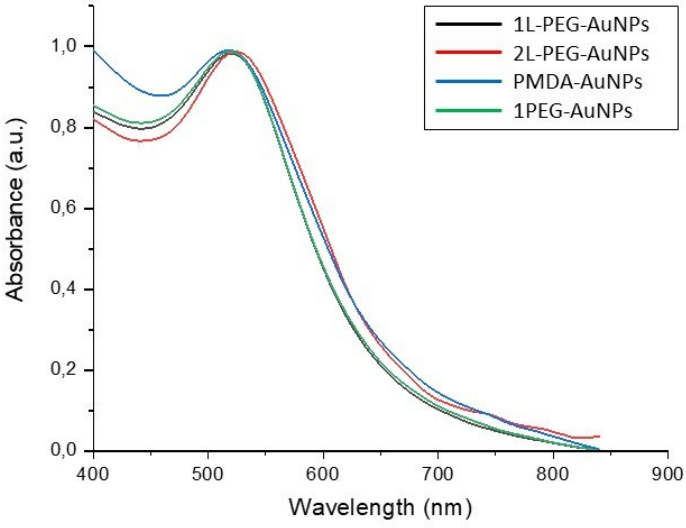

Figure S8: 1L-PEG-AuNPs and 2L-PEG-AuNPs UV-vis absorption spectroscopy characterization.

Table S1: Characterization of nanoparticles populations

	Hydrodynamic diameter (nm)	ζ potential (mV)
PMDA-AuNPs	12.4 ± 0.5	–34.4 ± 5.2
1PEG-AuNPs	14.1 ± 0.2	–30.5 ± 6.4
2PEG-AuNPs	16.9 ± 0.4	–25.92 ± 2.6

References

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