

Experimental Section

1. Materials

All chemicals and reagents were purchased from Sigma-Aldrich (Switzerland) and used as received, unless otherwise stated. Hydrochloric acid (37 wt%) was purchased from Honeywell. mPEG-SH, NH₂-PEG-SH and COOH-PEG-SH (5k Da) were purchased from Creative PEGWorks. milliQ water was used throughout all experiments.

2. Synthesis of GNRs

The synthesis route is a slightly modified version of the standard procedure for seeded synthesis of GNRs in a solution of CTAB.^[1] All glassware was cleaned with *aqua regia* and dried in an oven prior to use. A stock solution of HAuCl₄·3H₂O (50 mM) was prepared and kept at 4°C, with the concentration confirmed via ICP-OES and UV-Visible spectroscopy.

Seeds. A CTAB solution (0.1 M, 4.7 mL) was mixed with HAuCl₄·3H₂O (50 mM, 0.025 mL) at 28°C. To this stirred solution, NaBH₄ (10 mM, 0.6mL) was added. The mixture immediately turned light brown indicating the formation of seed particles. N.B. NaBH₄ was weighed out in a glove box, and made up to 10mM in water, this solution was used immediately.

Growth solution. A growth solution was prepared with CTAB (0.1 M, 20 mL), to which HAuCl₄·3H₂O (50 mM, 0.222 mL) was added and mixed by inversion. AgNO₃ was added (10 mM, 0.26 mL), followed by HCl (1 M, 0.384 mL) and mixed by inversion. To this, L-ascorbic acid was added (0.1 M, 0.16mL) and the solution was mixed vigorously until the solution turned colorless. The final addition was the seeds (96 μL), aged for 30 minutes, and followed by brief inversion mixing. The resulting solution was left overnight at 28°C for the GNRs to form.

3. Characterization of GNRs

TEM images of the GNRs were taken using a Phillips CM20 Biotwin operating at 80 kV (Figure S1a). The colloidal suspension was washed twice via centrifugation (1 ml centrifuged at 8,000 x g for 5 minutes) and redispersed in ultra-pure water. This was then drop cast onto a TEM grid and dried in air, before measurements were taken. A minimum of 200 particles were measured to obtain the size distribution and average dimensions.

UV-Visible spectroscopy was done on a Jasco V-670 spectrophotometer, with the pure GNR suspension diluted 5 fold in ultra-pure water (Figure S1b).

Zeta-potential measurements were done on a Brookhaven ZetaPALS, typically in a solution of NaCl (5 mM)(Table S1).

For concentration measurements, the GNR suspensions were washed twice, as described above, before dissolution in *aqua regia*. The treated samples were then measured by ICP-OES using a PerkinElmer Optima 7000 DV (Table S1).

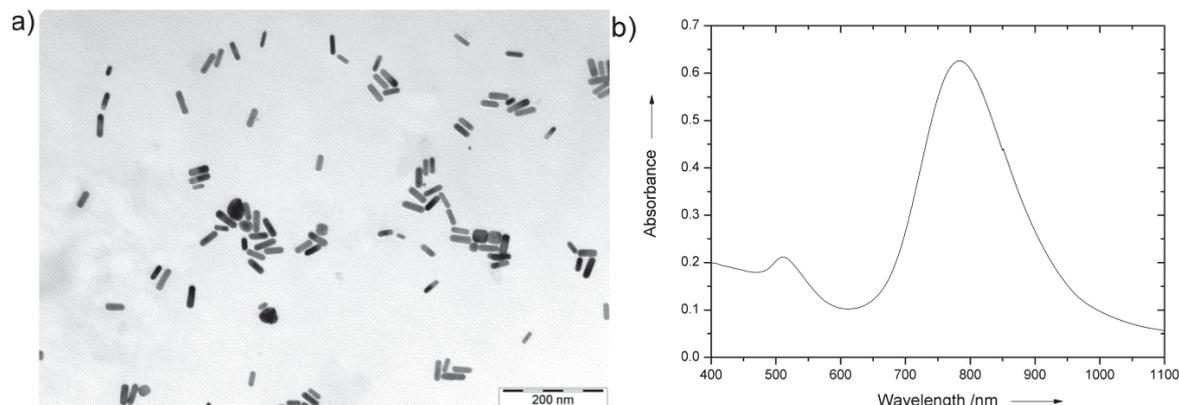


Figure S1. A) TEM image of GNRs as synthesised after 2x purification by centrifugation and b) corresponding UV-Visible spectrum of GNRs with a 5-fold dilution.

Table S1. Characterization of GNR as synthesized with a CTAB coating.

Characteristic	Value
Percentage of initial gold converted into nanoparticles from ICP-OES	62%
Extinction coefficient (@400nm)	$0.01553 \pm 0.0099 \mu\text{g}^{-1} \text{mL cm}^{-1}$
Dimensions (TEM)	$60 \pm 14 \times 19 \pm 3 \text{ nm}$
Aspect Ratio (TEM)	3.2 ± 0.9
Mean surface area per GNR (TEM)	$3580 \pm 972 \text{ nm}^2$
Mean volume per GNR (TEM)	$15200 \pm 4920 \text{ nm}^3$
Zeta-potential	$40.9 \pm 3.9 \text{ mV}$

4. PEGylation of GNRs

The PEGylation of GNRs always occurred over 24 hours at room temperature. Typically, the GNRs were purified twice via centrifugation (10ml centrifuged at $10,000 \times g$ for 20 minutes) with control of redispersion volumes to obtain a final concentration of [CTAB] = 1 mM in water. The thiolated-PEG was then added, maintaining a constant final concentration of [GNR] = 0.6 nM, followed by gentle mixing by swirling overnight.

For the investigations of stability in ethanol, the PEGylated GNRs were centrifuged once, with redispersion of the pellet in a binary mixture of ethanol and water. These GNRs were then characterized periodically with UV-Vis and DLS, over a period of 5 days.

5. Synthesis of GNS-PEG

A citrate reduction procedure was used to produce gold nanospheres (GNS). Following the characterization tools mentioned above, a mean hydrodynamic diameter of 21.4 nm was found by DLS and a core diameter of 18.7 nm by TEM. The core size from the TEM analysis was used to calculate the mean surface area of the sample, and PEGylation proceeded on the unpurified sample as described for the GNRs. The concentration of PEG added was theoretically equivalent to 5 PEG nm⁻². After PEGylation, the hydrodynamic diameter increased to 44.2 nm – corresponding to a layer thickness of roughly 11 nm, indicating a high grafting density where the PEG is in an extended conformation.

6. Preparation of PEG-GNRs by one/two-step methods

One step. The GNRs (10ml), as above, were purified twice by centrifugation leading to a residual [CTAB] = 1 mM. Under vortex mixing, a solution of thiolated-PEG was added (10 mg mL^{-1} , 0.656 mL), equivalent to 100 PEG nm⁻², and mixed by gentle swirling over 24 hours. Unreacted PEG and displaced CTAB were removed by two further rounds of centrifugation.

Two steps. The GNRs (10ml), as above, were purified twice by centrifugation leading to a residual [CTAB] = 1 mM. Under vortex mixing, a solution of thiolated-PEG was added (10 mg mL^{-1} , 65.6 μL), equivalent to 10 PEG nm⁻², and mixed by gentle swirling over 24 hours. The partially PEGylated GNRs were then spun down and resuspended in ethanol (90% v/v ethanol, 10ml). To this, an ethanolic solution of thiolated-PEG (1 mg mL^{-1} in 90% v/v ethanol, 0.656 mL) was added under vortex mixing and gently mixed by swirling over 24 hours. After one final round of centrifugation to remove unreacted PEG and displaced CTAB with resuspension in water, the GNRs were used in the biochemical tests described below.

7. Characterization of GNS, GNR1 and GNR2

The biochemical analysis was undertaken at three different mass concentrations. However, the effective surface area of the nanoparticle suspensions and the number concentrations may both play a role in the biological response upon exposure.^[2] Table S2 shows there is minimal differences in the effective surface area concentration and number concentration between the GNSs and GNRs,

Table S2. The concentrations of NPs expressed in terms of mass, surface area and number concentrations – crucial when comparing different particles in biological systems.

Sample	Mass Concentration	Surface Area Concentration	Number Concentration
GNS	10 $\mu\text{g ml}^{-1}$	$1.5 \times 10^{14} \text{ nm}^2 \text{ ml}^{-1}$	$1.1 \times 10^{11} \text{ GNS ml}^{-1}$
GNR	10 $\mu\text{g ml}^{-1}$	$1.2 \times 10^{14} \text{ nm}^2 \text{ ml}^{-1}$	$3.4 \times 10^{10} \text{ GNR ml}^{-1}$

Table S3. Zeta potential of GNRs after functionalization via a traditional one-step method (GNR1) or a two-step method (GNR2).

Sample	Zeta Potential (mV)
GNR1	-5.4 ± 1.3
GNR2	-4.5 ± 1.1

8. Cell Culture

Primary Macrophages

Monocultures of human-monocyte derived macrophages (MDM) were isolated from human whole blood as previously described.^[3]

9. Biochemical Analysis

Trypan Blue Assay

The ability for CTAB (0.01-1 mM), as well as PEGylated gold nanospheres (GNS), GNRs functionalized by a one-step method with excess PEG (GNR1), and GNRs functionalized by a two-step method with PEG via ethanol washing (GNR2) (0.01, 0.02 and 0.04 mg mL^{-1}) to cause cytotoxicity after 24 hrs suspension exposure was determined via the Trypan Blue assay. Briefly, exposed MDM were cultured and a cell count was performed using a 1:20 dilution of Trypan Blue in cell culture media (Rosewell Park Memorial Institute medium supplemented with 10% fetal calf serum, 1% Penicillin/Streptomycin and 1% L-Glutamine). Using a haemocytometer, a total of 250 cells were counted for each exposure and percentage viability was determined from the positive control (frozen cell cultures at -80°C for 30 mins) (n=3).

Tumor Necrosis Factor-alpha (TNF- α)

The ability for CTAB (0.01-1 mM), PEGylated GNS, GNR1, and GNR2 (0.01, 0.02 and 0.04 mg mL^{-1}) to cause the release of the pro-inflammatory cytokine TNF- α from MDM after 24 hrs suspension exposure was assessed via the use of an enzyme-linked immunosorbent assay (ELISA) diagnostic kit (R&D Systems, Switzerland). As a positive control, lipopolysaccharide (LPS) at a concentration of 0.1 mg mL^{-1} was used (n=3).

Interference with TNF- α protein

The potential for the TNF- α protein to adsorb to the surface of CTAB (0.01-1 mM), PEGylated GNS, GNR1, and GNR2 (0.01, 0.02 and 0.04 mg mL⁻¹) thus eliciting a false negative toxicity, was also assessed. All nano-objects were initially incubated with the TNF- α protein at 10 ng mL⁻¹ (diluted in phosphate buffered saline) for 1 hr at 37°C, 5% CO₂. Samples were then centrifuged at 600 x g to remove all debris prior to being assessed via the ELISA protocol described above (n=3).

Interference of TNF- α adsorbance wavelength

Furthermore, to check for interference of CTAB (0.01-1 mM), as well as PEGylated GNS, GNR1, and GNR2 (0.01, 0.02 and 0.04mg mL⁻¹) at the detection wavelengths for the TNF- α ELISA, all samples were assessed for their absorbance's at these wavelengths individually (n=3).

10. Thermogravimetric analysis (TGA)

The synthesis of GNRs was identical to that as described in section 2; except at a larger scale (200 mL). Of this solution, 120 mL was PEGylated via the two step method, described in section 6. These were extensively purified before freeze drying in preparation for TGA. A Mettler Toledo Star TGA/DSC 1 was used with 40 μ l aluminium pans, where samples were heated to 600 °C at a rate of 10 °C min⁻¹ and a N₂ flow of 30 ml min⁻¹. Characterization by TEM gave dimensions similar to those found in Table S1.

A weight loss of 12.4% was found for the PEGylated GNRs (Figure S2). Using the dimensions from TEM, the ratio of PEG to gold can be calculated thus giving the footprint, or grafting density, of the PEG: 0.89 PEG nm⁻².

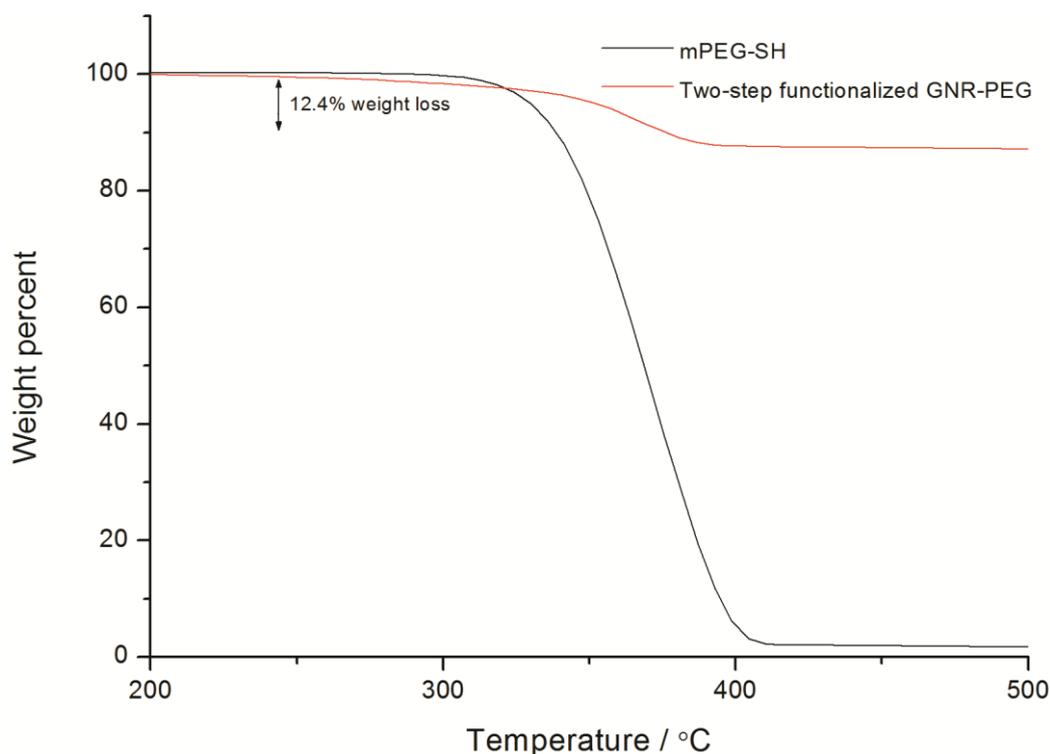


Figure S2. TGA curves of weight loss upon heating the pure polymer (mPEG-SH) or GNRs functionalized by a two-step method in ethanol.

11. ¹H NMR

The GNRs were produced identically as for the TGA experiment. A two-step functionalization procedure with PEG was followed by extensive purification by centrifugation; the GNRs were then concentrated into a final volume of 1 mL (mass of

Au = 28 mg). In order to release the bound molecules, the gold core was dissolved in a combined solution of KI/I₂ and KCN in D₂O over a period of 2 weeks. This is a similar time as found by others, however the addition of KI/I₂ was found as crucial to speed up the dissolution.^[4] A white precipitate was formed, which could be easily dissolved in deuterated methanol and contained the organic material. The NMR spectra of pure CTAB and PEG were analyzed in deuterated methanol on a Bruker 360 MHz (Figure S3) where the triplet at 0.86 ppm was used to identify CTAB, and the peak at 3.67 ppm was used to identify PEG.

The dissolved samples were analyzed on a Bruker 500 MHz, and the spectra are shown in Figure S4. Negligible signals were obtained from the samples in D₂O. In deuterated methanol, both CTAB and PEG could be detected indicating that not all CTAB could be removed.

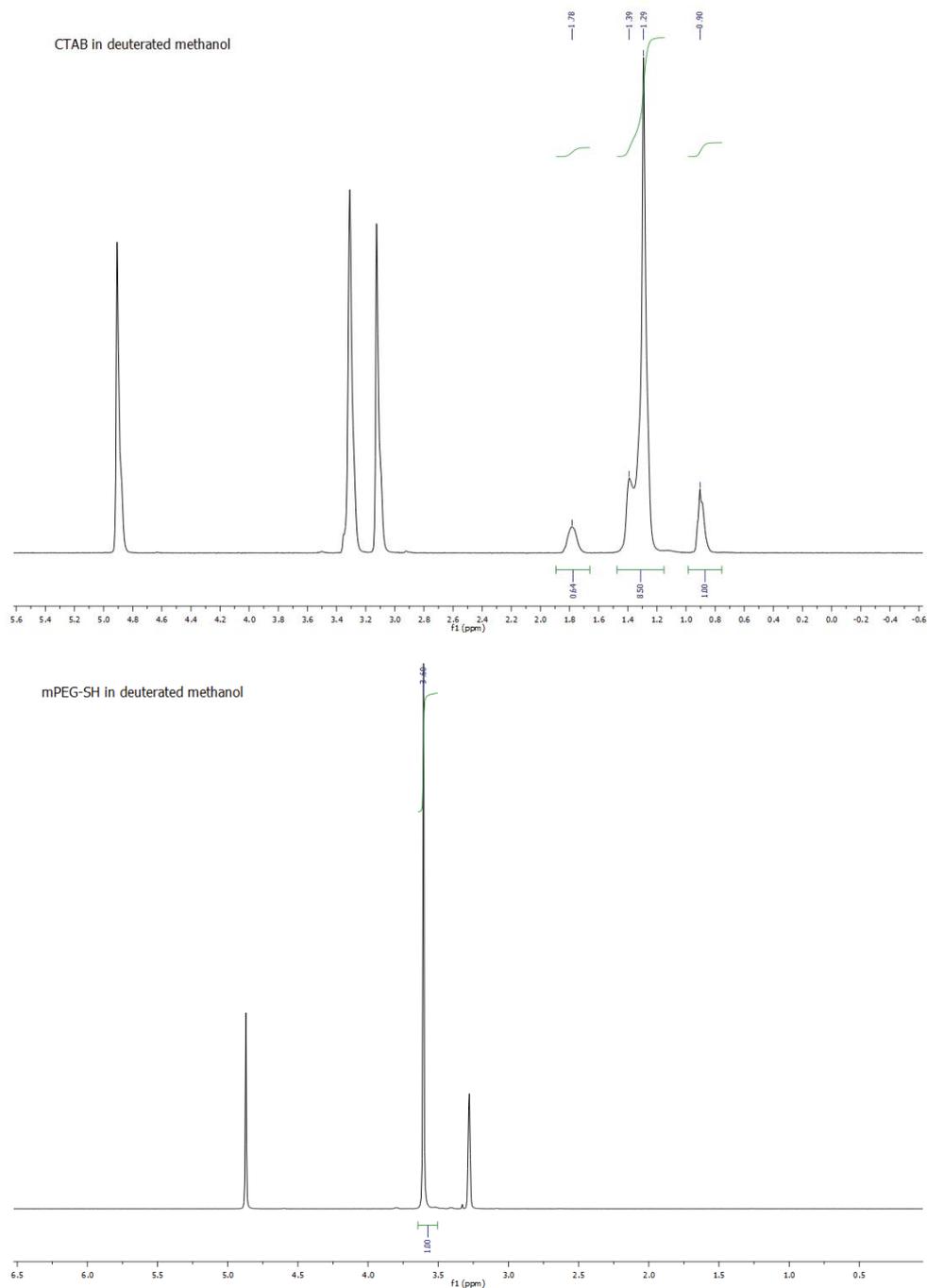


Figure S3. ¹H NMR spectra of CTAB and PEG in deuterated methanol, measured on a Bruker 360 MHz.

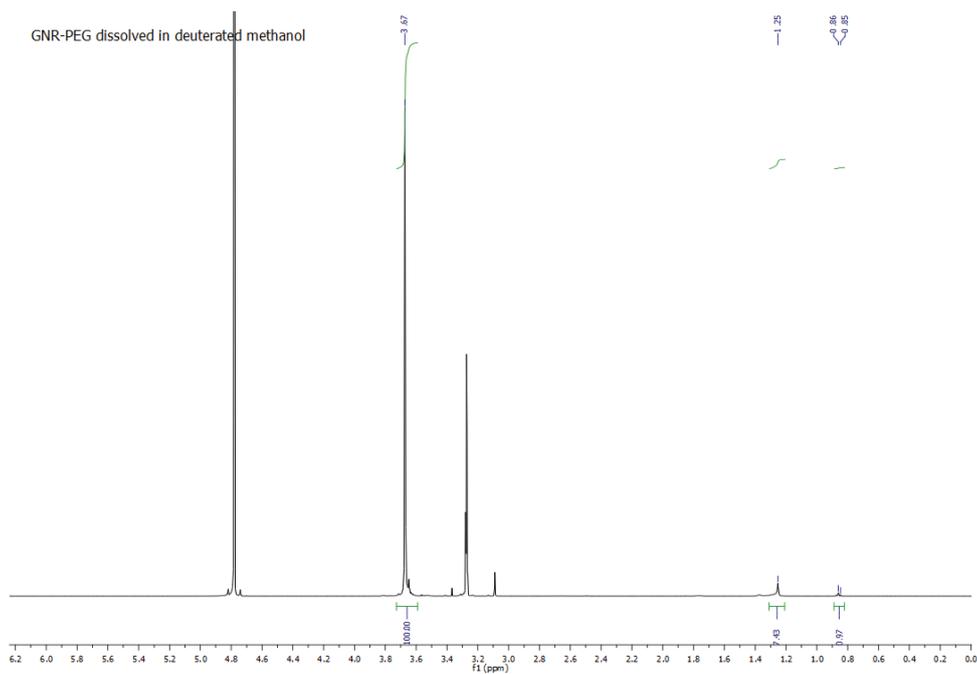


Figure S4. ^1H NMR spectra of GNR-PEG after dissolution and redispersion of the organic precipitate in deuterated methanol, measured on a Bruker 500 MHz.

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- [4] L. Vigderman, P. Manna, E. R. Zubarev, *Angew. Chem. Int. Ed.* **2012**, *51*, 636–641.