Supporting information

Tuning the Surface Chemistry of Gold Nanoparticles to Specifically Image Glioblastoma Cells Using Surface-Enhanced Raman Spectroscopy

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Table S1. Theoretical calculation of molecules required to form a monolayer on 60 nm GNPs. Number of molecules were calculated considering a perpendicular orientation of 1,4-BDT with a footprint of approx. 0.088 nm^2, and a mushroom configuration of PEG with a footprint of 0.35 nm^2. I.e. calculation for 50 and 3% surface coverages of 1,4-BDT and anti-EGFR antibody, respectively.

Gold area for 50% 1,4-BDT surface coverage	5,652 nm^2
N° of 1,4-BDT molecules per GNP	64,000
Residual gold area for total PEG	5,652 nm^2
N° of total PEG molecules per GNP	16,000
Mol of cPEG per GNPs (1.9×10^{10})	2×10^{-10}
N° of cPEG molecules per GNPs (1.9×10^{10})	9.6×10^{13}
N° of cPEG molecules per GNP	5,000
Gold area occupied by cPEG	1,750 nm^2 [1]
Residual gold area for mPEG	3,902 nm^2
N° of mPEG per GNP	11,000
Mol of anti-EGFR antibody per GNPs (1.9×10^{10})	5.3×10^{-12}
N° of anti-EGFR antibody molecules per GNPs (1.9×10^{10})	3.2×10^{12}
N° of anti-EGFR antibody molecules per GNP	164 [2]

[1] Note: this area is considered constant for 1,4-BDT surface coverages lower than 100%.

[2] Note: considering the ratio of 1:1 (ab:cPEG), the maximum number of ab per GNP would be 5,000. Therefore, anti-EGFR antibody surface coverage is 3%.

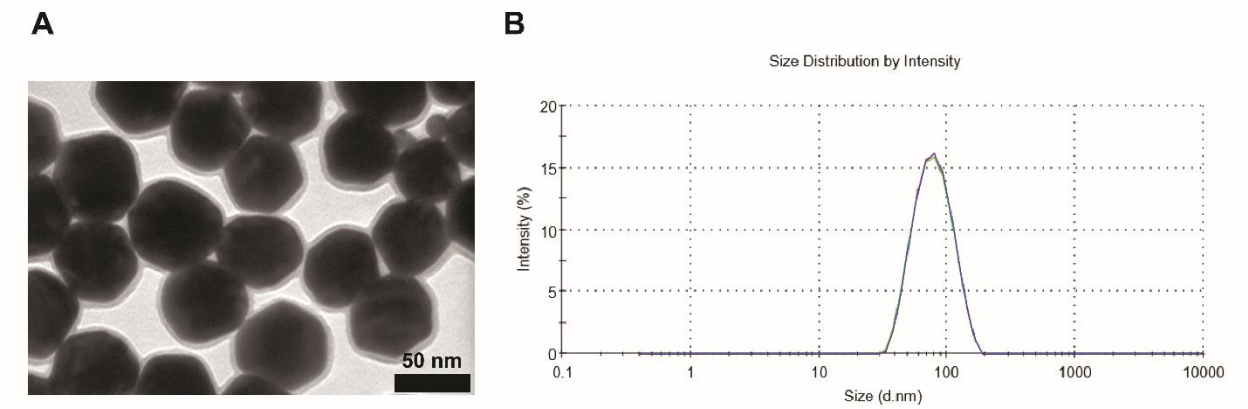

Figure S1. Physical properties of 60 nm bare GNPs in citrate buffer. A) 2 µl of 43 µg/ml GNPs were placed onto standard 3.5 mm copper grids and visualized with EM 900 transmission electron microscope (Zeiss), acceleration voltage 50 kV. B) Homogeneous size distibution is shown by dynamic light scattering measurement. Analysis done with Zetasizer ZS (Malvern Panalytical Ltd, UK).

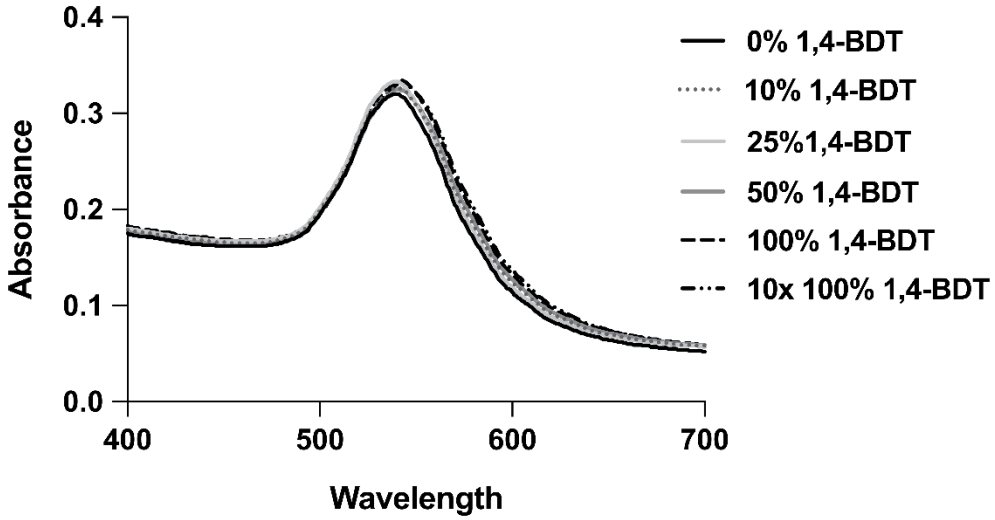

Figure S2. UV Vis spectra of GNPs coated with increasing 1,4-BDT surface coverage. UV-Vis extinction spectra acquired with Sinergy H1 microplate reader (BioTek Instrument, GmbH).

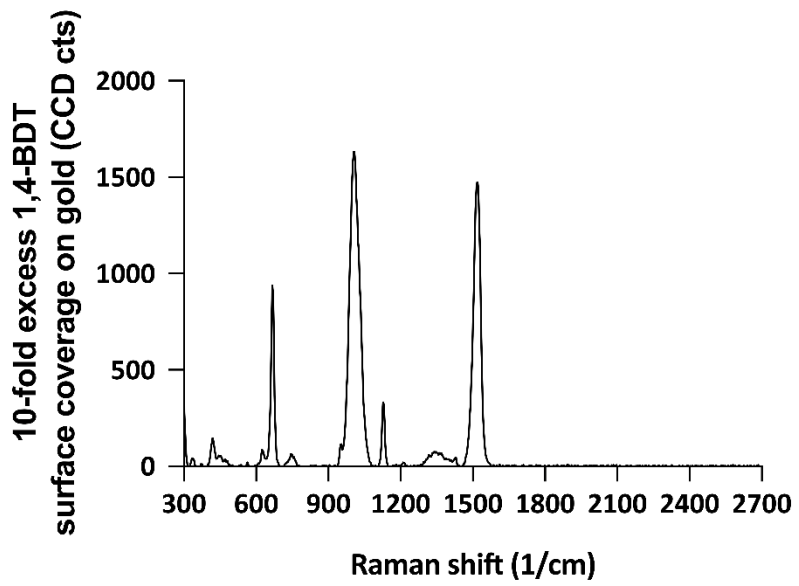

Figure S3. SERS spectrum of 10-fold excess 1,4-BDT absorbed on GNPs surface. Measurements done with confocal Raman microscope (WITec GmbH, Germany), λex 785 nm, laser power 60 mW, integration time 0.5 s, 10 accumulations, 100x objective.

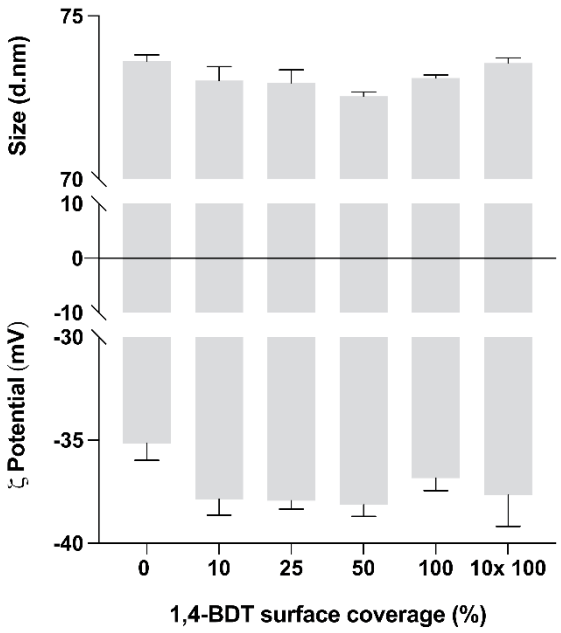

Figure S4. Physical properties of 1,4-BDT GNPs. Particle size is shown on the top graph and negative zeta potential below, both correlated to increasing 1,4-BDT surface coverages. Analysis done with Zetasizer ZS dynamic light scattering instrument (Malvern Panalytical Ltd, UK), T 25°C, pH 7, n=3, mean ± SD.

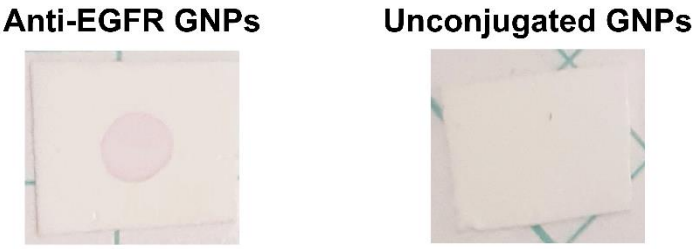

Figure S5. Dot blot of anti-EGFR GNPs bio-functionality. 2 µl of 250 µg/ml human recombinant EGFR (Sino Biological) were spotted onto nitrocellulose membrane. After blocking with 1% (w/v) dry milk for 30 min, membrane was incubated with 70 µl of 40 µg/ml anti-EGFR or unconjugated GNPs for 3 h at RT. Bound GNPs were visually evaluated through the presence of a bright pink spot.

Table S2. Quantified number of antibodies per GNP and relative conjugation efficiency. GNPs with 10% 1,4-BDT surface coverage were conjugated to 3 and 30% anti-EGFR antibody surface coverages. Antibody quantification was performed by incubating GNPs with Alexa Fluor® 680 goat anti-rabbit antibody (Invitrogen) for 1 h at 25°C at 450 rpm. Fluorescence detection was performed by using CLx infrared imaging system (Li-cor Odyssey). The fluorescence of unconjugated GNPs was used as a baseline. The number of antibodies per GNP was determined by using a standard curve and by normalizing the result to total amount of GNPs in the sample as measured by absorbance values.

1,4-BDT surface coverage (%)	Anti-EGFR surface coverage (%)	Quantified anti-EGFR antibody number of molecules per GNP	Conjugation efficiency (%)
10	3	64±4	40
	7.5	67±10	16.5
	30	84±9	5

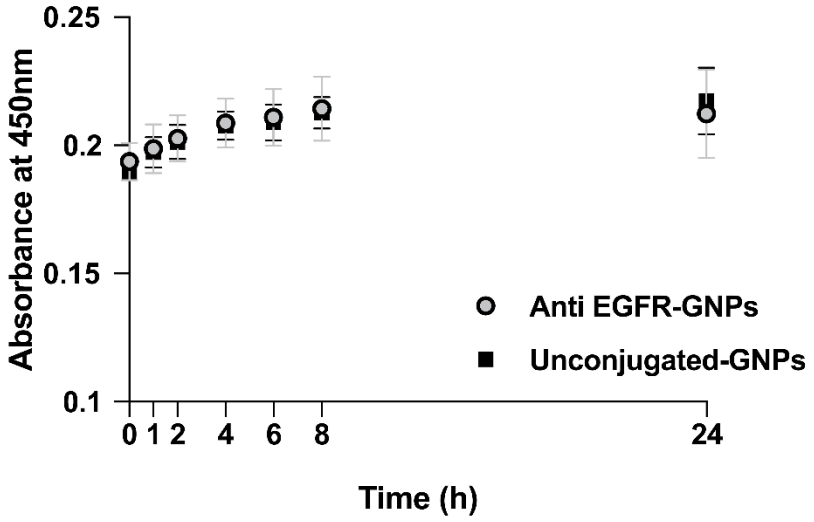

Figure S6. Optical stability of GNPs in cell culture medium. Anti-EGFR and unconjugated GNPs were incubated in DMEM supplemented with 10% FCS and 1% P/S in a ratio of 1:2 and incubated at 37°C at 450 rpm. Absorbance was measured at 450 nm at multiple time points over 24 h, n=3, mean ± SD.

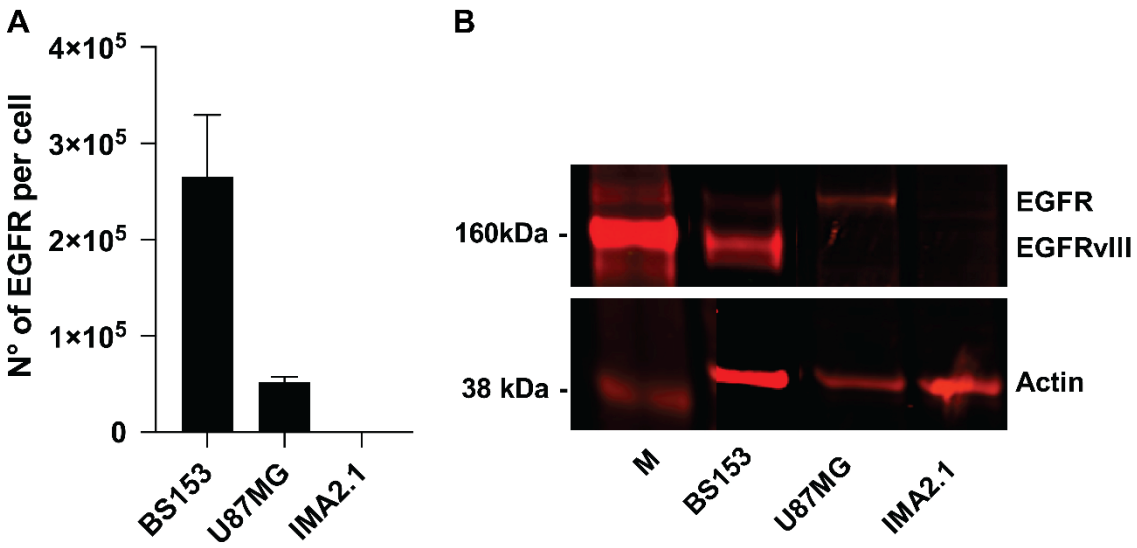

Figure S7. Differential expression of the EGFR protein in GBM cell lines. A) Number of receptors per cell was quantified by mean quantitative flow cytometry. 1,000,000 cells/ml were incubated with a saturating concentration (1 mg/ml) of mouse anti-human EGFR antibody (BioXCell) for 1 h at 4°C in the dark. To prevent EGFR internalization, cells were fixed and incubated with FITC goat anti-mouse antibody. Cells were analyzed with CytoFlex S flow cytometer (Beckman Coulter). Data were subsequently processed with FlowJo 10.4 software, n=3, mean ± SD. B) Western blot detection of EGFR detected at 170 kDa and actin at 42 kDa, EGFRvIII detected at 145 kDa in BS153. 30 µg of protein extracts were separated in a 8% precast polyacrylamide gel (Invitrogen). Proteins were transferred onto polyvinylidene difluoride membrane, blocked and probed overnight at 4°C with rabbit anti-human EGFR and goat anti-human actin antibodies (Abcam), diluted 1:500 and 1:1,000 respectively. Membrane was incubated with IRDye®680 donkey anti goat IgG (Li-cor) and with Alexa Fluor® 680 goat anti-rabbit antibodies, diluted 1:10,000. Blot was then scanned with the CLx infrared imaging system (Li-cor Odyssey).

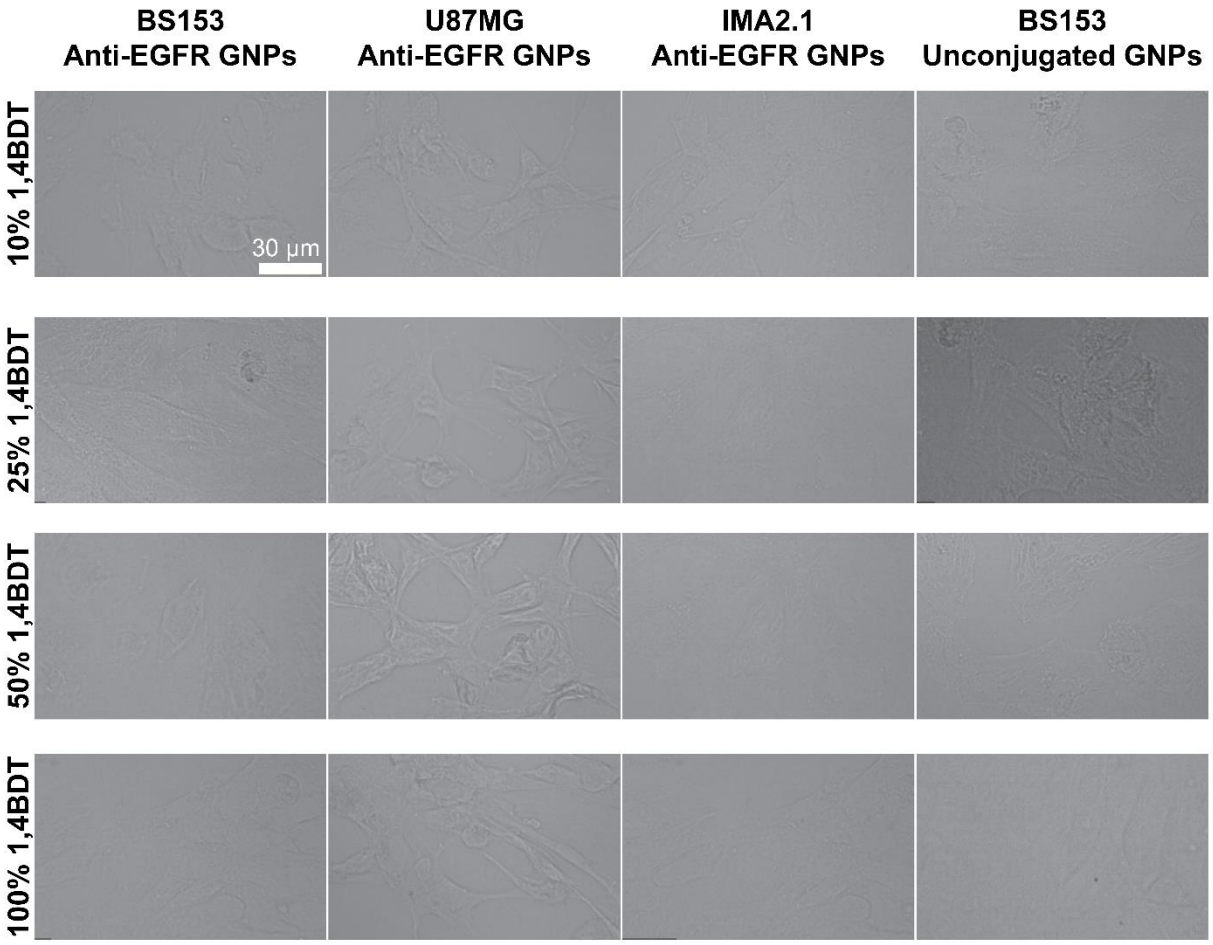

Figure S8. Bright field pictures of GBM (BS153, U87MG) and IMA2.1 cells incubated with anti-EGFR or unconjugated GNPs.

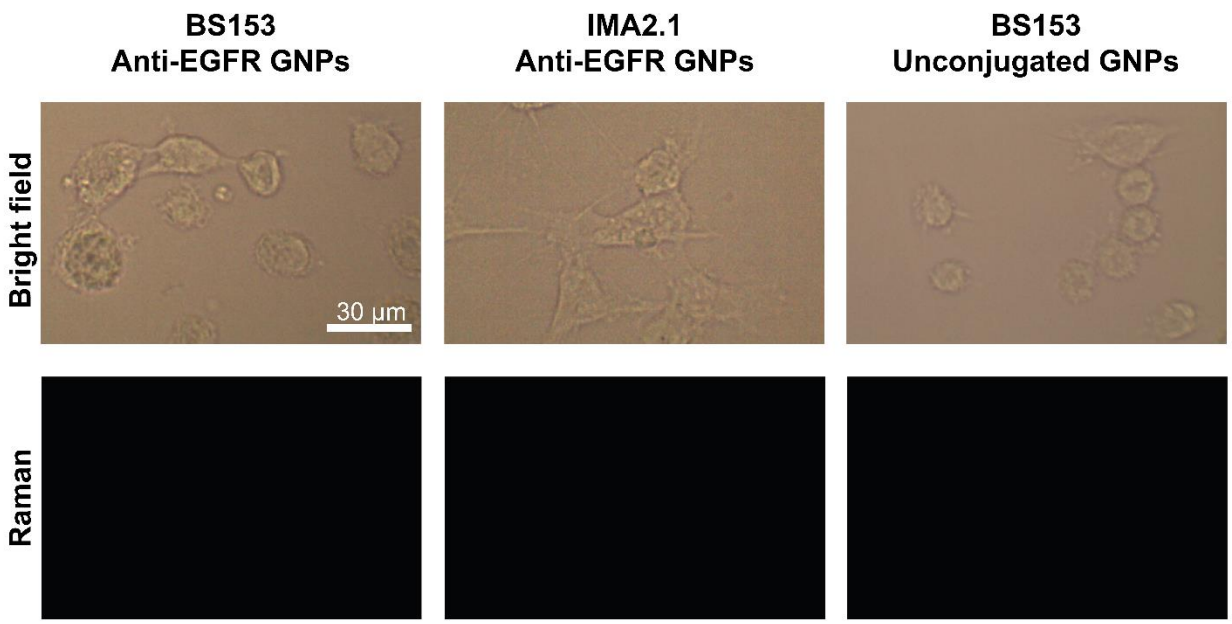

Figure S9. Influence of GNPs surface charge on non-specific binding. After 2 h of antibody conjugation, 1% (v/v) ethanolamine was added to deactivate residual negative charged carboxy-end terminated PEG on GNPs.

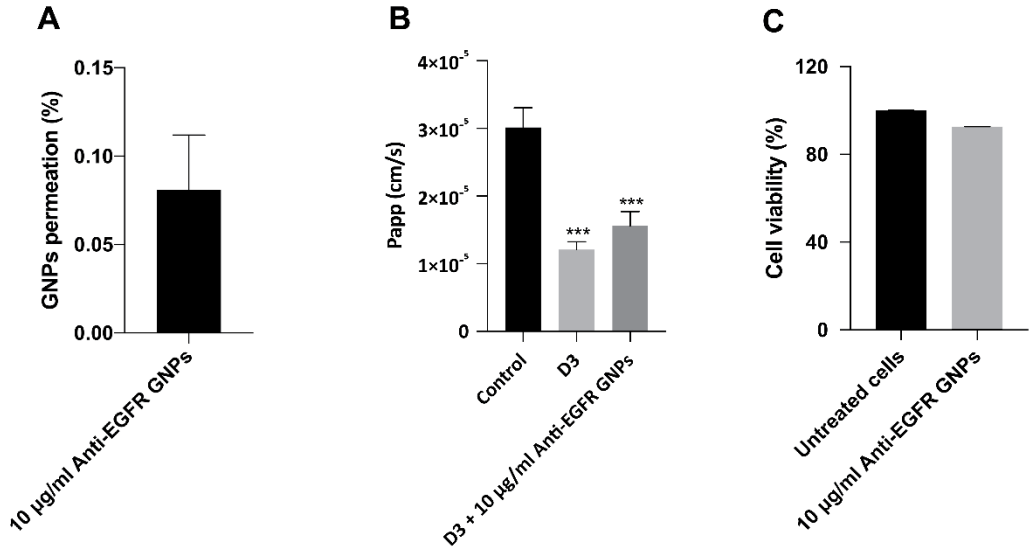

Figure S10. Passage of anti-EGFR GNPs through an *in vitro* blood brain barrier. 20,000 hCMEC/D3 (D3) cells were seeded into 6.5 mm transwell with 0.4 µm pore polyester membrane inserts (Sigma, CLS3470). After 5 days of culture, 150 µl of 10 µg/ml anti-EGFR GNPs were added to the apical chamber and incubated for 24 h. A) About 0.1% of gold was found in the basolateral chamber as measured by ICP-MS, n=3, mean ± SD. B) Cell monolayer integrity remained preserved as evaluated by measuring the paracellular permeability of Lucifer yellow after 1 h of incubation. Fluorescence read at λex 428 nm; λem 540 nm using FlexStation 3.****p≤0.0001 vs control transwells with only collagen, n=3, mean ± SD. Apparent permeability coefficient (Papp) was calculated in cm/s according to the following equation: Papp (cm/s) = VB/(ACAO) x (ΔCB/ΔT), where VB is the volume in the basolateral chamber, A is the surface area of the filter, CAO is the initial concentration in the apical chamber, ΔCB/ΔT is the change of concentration in the basolateral chamber over time. C) Anti-EGFR GNPs were not cytotoxic as determined by measuring the release of formazan. Absorbance read at 450 nm using FlexStation 3. Values expressed as percentage of negative control (untreated cells), n=3, mean ± SD.