Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information

Conserved Effects and Altered Trafficking of Cetuximab Antibodies Conjugated to Gold Nanoparticles with Precise Control of their Number and Orientation.

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I N D E X

1. Materials and Methods.

Materials: sodium citrate, hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$), sodium (meta)periodate ($NaIO_4$, ≥ 99%), Lucifer Yellow CH dipotassium salt (LYCH, 90%), O-(2-mercaptoethyl)-O′-methyl-hexa(ethylene glycol) ($C_{15}H_{32}O_7S$, ≥ 95%) and PD10 desalting columns were purchased from Sigma-Aldrich. Cetuximab IgG1 monoclonal Ab (Erbitux, C225) was purchased from Merck Serono, Spain. Cetuximab mAbs were purified by PD10 desalting columns and transferred into a phosphate buffer (PB) solution 0.1 M, pH = 7.0, before use. Linker 22-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15,18,21-heptaoxadocosanic hydrazide (1) was purchased from SensoPath Technologies, Bozeman, MT, USA. Ultrafiltration spin columns (2 mL, MWCO = 50 kDa, polyethersulfone) were obtained from Sartorius Stedim Biotech GmbH, and dialysis cellulose ester membranes (MWCO = 300 kDa, 10 mL) were obtained from Spectrum Labs. A431 cells (ATCC No. CRL-1555™) were purchased from LGC Standards, Teddington, UK. Alexa Fluor 555-EGF complex (E-35350) was purchased from Life Technologies Ltd., Paisley, UK. M-PER Mammalian Protein Extraction Reagent, Halt Protease & Phosphatase Inhibitor Cocktail (100X) and 0.5 M EDTA solution (100X) were obtained from Fisher Scientific, Madrid, Spain. Rabbit anti-phospho-EGFR (Tyr1173) polyclonal Ab (sc-101668) and mouse anti-EGFR monoclonal Ab conjugated with fluorescein isothiocyanate (sc-120) were obtained from Santa Cruz Biotechnology, Inc. Mouse anti-α-Tubulin monoclonal Ab (T6199), was purchased from Sigma-Aldrich.

Synthesis of AuNPs *(~ 17 nm) in aqueous solution:* AuNPs of 17.2 ± 1.8 nm with a concentration of ~ 10^{12} NP/mL were synthesized by modifications of the classical method of citrate reduction of $HAuCl_4$.[1, 2] Briefly, 65.7 mg of $HAuCl_4 \cdot 3\ H_2O$ were

diluted in 500 ml of milliQ water and the solution was heated up to boiling under vigorous stirring. 15 mL of a sodium citrate 1% (w/v) aqueous solution were added and the solution turned immediately from yellow into dark, and then into red in a few minutes, indicating AuNPs formation. The solution was left to react for 30 minutes and then cooled down under continuous stirring. Before conjugation, AuNPs were centrifuged at 18000 g for 10 minutes and redispersed in borate buffer 2 mM at pH = 8.5 in a 10 times concentrated volume. Final AuNPs concentration for conjugation was ~ 10^{13} NPs/mL (~ 18 nM AuNPs).

Characterization of AuNPs: AuNPs were visualized under TEM, either JEOL 1010 or Tecnai Spirit, at an accelerating voltage of 80 kV. The sample (10 μL) was drop-casted onto ultrathin Formvar-coated 200-mesh copper grids (Ted Pella, Inc.) and left to dry in air. For each sample, the size of a minimum of 200 particles was measured to obtain the average and the size distribution. Digital images were analyzed with Image J software and a custom macro performing smoothing (3 x 3 or 5 x 5 median filter), manual global threshold, and automatic particle analysis provided by ImageJ. UV-Vis absorption spectra from AuNPs were recorded with a Shimadzu UV-2401PC spectrophotometer at room temperature. Purification by dialysis was performed using cellulose ester membranes (MWCO = 300 kDa, 10 mL) against PBS (5000 mL). Fluorescence measurements of the NPs were performed using a SpectraMax M2e microplate reader operating in the fluorescence mode at excitation/emission wavelengths of 494/519 nm. Au content of the cell samples was determined by an Agilent inductively coupled plasma mass spectrometry (ICP-MS) instrument (model: 7500cx) with a detection limit of 0.02386 ppb. Both the supernatant and the pellet samples were digested in aqua regia at 100 ºC before ICP-MS analysis. Ga was used as the internal standard, and the integration time/point and time/mass were 0.1 and 0.3 seconds, respectively, with a 3

times repetition. NP concentration was obtained by analyzing the size distribution by TEM and the precise amount of mass by ICPMS. In detail, one takes the size distribution and count how many NPs of each size there are and calculate their total weight in the distribution, obtaining that for all sizes the number of NPs per unit mass. From here, the total number of NPs is obtained. Confocal images were acquired using a Leica TCS SP5 epifluorescence/reflectance laser scanning confocal microscope with a 63X oil immersion objective. The excitation was provided by an Argon multiline laser. Reflectance images were obtained with 488 nm laser excitation. Images were treated using Leica Microsystems LAS AF lite analysis software. Fluorescence optical microscope images were acquired using an inverted Nikon Ti-E microscope with a 60X objective. Versadoc (Biorad) imaging system was used for chemiluminescent protein detection for Western blot, and bands were quantified using Quantity One software (BioRad). Cell absorbance measurements were performed with Victor 3 microplate reader from PerkinElmer at 450 nm and 620 nm (reference wavelength). Hydrodynamic diameter (DLS) and surface charge (Z potential) were measured in a Malvern ZetaSizer Nano ZS (Malvern Instruments Ltd)..

Preparation of linker-modified Cetuximab mAbs: Cetuximab mAbs were diluted to a concentration of 1 mg/mL in PB solution 0.1 M, pH = 7.0. Then, 10 µL of an aqueous solution of $NaIO_4$ 0.1 M were added to 100 µL of the mAb solution. The mixture was placed in an aliquot wrapped with aluminum foil and left to react for 30 minutes, at RT under gentle shaking. The oxidation rate was slowed down by dilution in 500 µL of PB 10 mM, pH 7.0. Then, 2 µL of linker **1** in a 90% ethanol solution (50 mg/ml) were immediately added to the Abs solution, and it was left to react for 1 hour at RT under gentle shaking. Unreacted linker molecules were removed using ultrafiltration spin

columns, by dilution of the solution in HEPES 40 mM (1 mL, pH = 8.5) and centrifugation at 2000 g for 2-3 min. The purification was repeated 3 times, to obtain a concentrated volume of ~ 300 µL. Cetuximab-**1** pairs concentration was determined spectrophotometrically at 280 nm on the basis of a molar extinction coefficient, ε, of 210.000 $M^{-1}cm^{-1}$. The estimated concentration of Cetuximab-**1** pairs after purification was in the range of 200-400 µg/mL in all cases.

Quantitative analysis of Cetuximab' polysaccharides oxidation: Cetuximab mAbs were diluted to a concentration of 1 mg/mL in PB solution 0.1 M, pH = 7.0. Then, 10 µL of an aqueous solution of $NaIO_4$ 0.1 M were added to 100 µL of the Ab solution. The mixture was placed in an aliquot wrapped with aluminum foil and left to react for 30 minutes, at room temperature (RT) under gentle shaking. The oxidation rate was slowed down by dilution in 500 µL of PB 10 mM, pH 7.0. The sample was immediately dialyzed with a cellulose membrane (MW 3.5 kDa) at RT for 2 hours against 5 L of PB 10 mM, pH = 7. It was followed by two additional dialysis cycles in the same conditions. A 250-fold excess of LYCH (20 µL, 10 mM) was added to the Ab solution and it was allowed to react for 1 hour in the dark at RT under slow agitation. The reaction mixture was dialyzed in the same previously described conditions, by performing three dialysis cycles for a minimum of 2 hours. Fluorescence measurements from the samples were performed in a 96-well plate, using a LYCH calibrate in PB 10 mM, pH = 7, at concentrations ranging from 0 to 10 µM. From these measurements, the concentration of LYCH per well was estimated. The concentration of Cetuximab mAbs in each well was determined by measuring the absorbance at 280 nm with a Shimadzu UV-2401PC spectrophotometer, on the basis of a molar extinction coefficient, ε, of 210.000 $M^{-1}cm^{-1}$. To correct for any LyCH that was nonspecifically adsorbed to the Abs, all the results were compared with nonoxidized Abs that had been incubated with a

250-fold excess of LyCH. Under the purification conditions used, the level of nonspecifically bound LyCH was approximately 0.01-0.02 LyCH molecules/mAb.

Ab Surface functionalization of AuNPs: First, after modification, antibodies were purified by affinity columns, such as Activated Thiol Sepharose 4B medium (dipyridyl disulphide and glutathione as ligands for thiol containing proteins, coupled to the beaded agarose, *sepharose*^{TM}, matrix) to confirm that the derived antibodies are still functional and then incubate them to the NPs." Addition of Cetuximab-**1** pairs to AuNPs (10 mL, ~ 18 nM) was calculated to obtain ratios of 6, 20 and 50 mAb/NP (in solution), which correspond to 3, 6 and 8 mAb/NP (according to quantification with Alexa dye). The mixtures were left to react for 20 minutes at 4ºC under slow agitation to reach equilibrium. Then, a short thiolated PEG (MW 356.48) was added at 3-fold molar excess over the estimated complete NPs coverage (calculated by the number of Au atoms at the surface of the NP and assuming that each thiolated molecule occupies 21.4 Å^2 of the AuNP surface)[3] to cap the remaining bare surface of the NPs. After 5 minutes reaction, dialysis was performed in 2 cycles of 2 hours and a third cycle overnight at 4ºC. A control Ab (goat anti-rabbit polyclonal IgG) was likewise purified to confirm the successful release of free Abs across the dialysis membrane. After the purification step, the samples were preserved in PBS 0.1% (g/mL) BSA.

Antibody Functionality Testing: Small 8 nm AuNPs were employed as labels to observe directly the presence of functional Abs on the 17 nm AuNP surface To confirm the presence of functional Abs on the AuNPs, the formation of dimers and satellite shape NP assemblies was performed and observed by TEM. In first experiment, the formation of dimers was built up from connecting 8 ± 1.5 nm AuNPs and the 17.2 ± 1.8 nm AuNPs. For the dimers superstructures, rabbit anti-BSA Abs and goat anti-rabbit

Abs were coupled to the 8 nm and 17.2 nm Au NPs respectively, with an Ab/NP ratio of 1 for both bioconjugates. A short SH-PEG (MW = 356.48) was added at 3-fold excess over the estimated complete NPs coverage to passivate the total AuNP surface and avoid unspecific interactions as explained in the manuscript. Then, an equimolar stoichiometric amount of both bioconjugates were mixed under slow agitation at 4°C overnight. Afterwards, a gentle centrifugation step (5000 g, 5 minutes) was employed to separate the formed bioconjugates dimers from individual (unbound) bioconugates. The collected pellet was visualized under TEM, revealing the successful formation of the desired NP1-NP2 dimers (see Figure 3a, b). The correct formation of these dimers confirmed the specific recognition between the goat anti-rabbit IgG Fab and its epitope present in the rabbit anti-BSA IgG Fc region, proving that the synthesized bioconjugates retain their biological activity. A much fewer number of dimers and trimers were also observed, probably due to unspecific interactions between the same types of Abs. In a second experiment, satellite-shaped NP superstructures were designed (Figure 3c, d) saturating the surface of the 17 nm AuNPs with the goat anti rabbit Abs (Ab/NP ratio ≈8) and mixing both bioconjugates in an excess (100x) of the 8 nm AuNPs-rabbit antiBSA Abs. These satellite-shaped assemblies were used for the characterization with 3D tomography TEM, which illustrates their three-dimensionality (Figure S4). Finally, in a third experiment (Figure 3e, f), more complex ternary NP superstructures, involving the control of two different Ab-antigen interactions, were prepared. These trimeric superstructures were designed first creating 8 nm AuNP-BSA bioconjugates, and adding them at equimolar stoichiometric amounts to 17 nm AuNPs coupled to rabbit anti BSA Ab. The mixture was left to react 2 hours at room temperature under gentle agitation. Next, the resulting dimer structures were mixed with the 17 nm AuNP coupled to the goat anti-rabbit IgG, which recognize the rabbit anti-

BSA Ab component of the dimers, thus forming the trimeric superstructures. This new mixture was gently stirred overnight at 4°C, and a soft centrifugation step (5000 g, 5 minutes) was then performed to purifiy them from unbound bioconjugates and the collected pellet was visualized by TEM.

Cell culture and NP preparation: epidermoid carcinoma cells (A431) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) under a humidified 10% CO_2 atmosphere at 37 ºC. Previously to the incubation with cells, the bioconjugates at a concentration of ~ 15 nM NPs were irradiated with UV light for at least 3 hours. This is the standard procedure when dealing with growth factor studies[4] since fetal serum, which is intended to keep the cells growing strongly interfere with study units.

AuNP bioconjugates binding studies: plasma membrane sheets from A431 cells were prepared, fixed, and incubated with Cetuximab-**1**-AuNP bioconjugates by optimizing the method from Prior *et al.*[5, 6] In brief, A431 cells were seeded onto sterile glass coverslips in 6-well plates to be 50-60% confluent and sparse when processing begun. After 48 hours, the medium was changed to serum-free medium for 5 additional hours. Cells were then incubated either with Cetuximab-**1**-AuNP (NPC3, NPC6 and NPC8) or unspecific (NPaR) bioconjugates at a concentration of ~ 8 nM NPs for 30 minutes and at 4ºC to avoid receptor-mediated internalization.[7] The incubation was performed in an upright configuration; for instance, a coverslip was placed onto a drop of 100 μL of NP bioconjugates. Coverslips were washed 5 times in PBS after treatment and placed against Formvar and poly-L-lysine coated grids to transfer the cell membranes by exerting a slight pressure on top of them. Grids were immediately placed onto a drop of fixative paraformaldehyde (PFA, 4%), and then quenched in glycine 25 mM, followed

by 5 times PBS and H_2O washes. Finally, the membranes were stained with UA (0.3 %) / methylcellulose (2%) to give them electron contrast and grids were left to air dry in wire loops. Plasma membrane sheets were visualized under a Tecnai Spirit TEM operating at 80 kV.

EGFR blocking studies: cells were seeded on 24-well plates containing sterile coverslips to be 50-60% confluent the day of the experiment and placed in an incubator under a humidified 10% CO_2 atmosphere at 37 ºC. Prior to treatment, cells were starved for 18 hours in serum-free medium (DMEM). Cells were then incubated for 2 hours in DMEM containing Cetuximab alone or conjugated to NPs, and NPaR so as to exclude non-specific interactions. The concentration used was 8 nM of free Cetuximab and NP bioconjugates, The incubation was followed by 2 times PBS washes and staining with 20 ng/mL of Alexa Fluor 555-EGF complex at RT. Cells were immediately washed with cold PBS and fixed with PFA 4% at RT during 15 minutes. Coverslips were washed 3 times with PBS and dried onto filter paper before they were mounted on 40 µL of Moviol with DAPI. Cells were imaged by using an inverted Nikon Ti-E fluorescence optical microscope with a 60X objective.

Quantification of AuNP bioconjugates uptake by ICP-MS: for uptake experiments, cells were seeded at a density of 1.0 x 10^6 cells per well in six-well plates and grown for 48 hours. A431 cells were serum-starved overnight and then were incubated for 2 hours at 37 ºC with 15 nM Cetuximab-**1**-AuNP bioconjugates and NPaR bioconjugates as a negative control. After incubation, cells were washed three times with PBS and the cellular fraction lysed with 1 ml of a M-PER mammalian protein extraction reagent supplemented with a protease and phosphatase inhibitor cocktail (Pierce Chemical Co). The insoluble fraction was centrifuged at 16000 g for 30 minutes. The concentration of AuNPs in the total cellular fraction (before centrifugation) was analyzed by UV-vis

spectroscopy by measuring the absorbance at λ=522 nm. For ICP-MS analysis, plates were prepared as described above, and then incubated for 2 hours at 37 ºC with three different concentrations of 4.5, 9 and 13.5 nM NPs, and NPaR bioconjugates. After the incubation time, the medium of the cells was collected for ICP-MS analysis, and the cells were washed 3 times with PBS (1 mL), trypsinized (Trypsin-EDTA, 1 mL) and centrifuged (400 rcf, 10 minutes). The resulting pellets were also collected for ICP-MS analysis. The percentage of cell-associated NPs was calculated relative to the total number of NPs used in the incubation medium and compared well with the decrease of NPs observed in the incubation medium after cell exposure in all the cases.

Confocal Laser Scanning Microscopy (CLSM) imaging: to perform fluorescence imaging of AuNP bioconjugates uptake, A431 cells were seeded in a 35 mm culture dishes with glass coverslips (Nunc, Villebon sur Yvette, France) at a density of $2.5x10^5$ cells per well, grown for 48 hours and then starved for additional 24 hours in serum-free medium. Starved cells were treated with 15 nM NPC8 and NPaR bioconjugates, for 2 hours. Immediately before imaging, cells were washed three times with cold PBS and the total extracellular EGFR and nuclei stained with an anti-EGFR528 antibody conjugated with fluorescein isothiocyanate (FITC) (Santa Cruz Biotechnology, Inc) and with Hoechst, respectively, for 30 min at 4 ºC (to prevent EGFR internalization). Then, cells were washed gently with cold PBS and fixed with 100% methanol for 10 min at -20 ºC. After fixation, cells were rehydrated with PBS and mounted with ProLong Gold (Life Technologies, Saint Aubin, France). Fluorescence imaging was performed on a Leica TCS SP5 confocal fluorescence microscope. The image processing and analysis was performed using the Bitplane Imaris software

Transmission Electron Microscopy (TEM) analysis: for TEM analysis of AuNP bioconjugates uptake, cells were seeded at a density of 10^6 cells per well in six-well

plates. Serum-starved cells were then incubated either with Cetuximab-**1**-AuNP bioconjugates (NPC3, NPC6 and NPC8) or a unspecific bioconjugate (NPaR) at a concentration of ~ 9 nM of NPs for 2 hours and 24 hours and at 37 ºC. After incubation, the cells were immediately processed for TEM analysis. In brief, cells were thoroughly washed (3 times) with PBS buffer and fixed in a 0.1 M PB solution containing 2.5% glutaraldehyde at 4 ºC for 1 hour. The cells were then rinsed 3 times with PBS and carefully collected in a tube with a cell scraper. The cellular pellet was postfixed in 1% osmium tetroxide solution for 1 hour, rinsed with PBS and distilled H_2O, and dehydrated in a graded series of ethanol (30, 60, 70, 80, and 100%). Warm epoxy resin was added for infiltration and embedding, and it was left to polymerize at 60 ºC for 48 hours. Ultrathin sections (~ 50 nm) were cut with a diamond knive in a Leica Ultracut UCT ultramicrotome, and stained with 5 % uranyl acetate and 2% lead citrate for visualization under TEM.

EGFR downregulation studies: for immunofluorescence analysis of EGFR, A431 cells were seeded in a 35 mm culture dishes with glass coverslips (Nunc, Villebon sur Yvette, France) at a density of $2.0x10^5$ cells per well and after 48 hours, the medium was changed to serum-free medium for additional 24 hours. Starved cells were then treated with 15 nM NPC8 Cetuximab-**1**-AuNP bioconjugates, NPaR bioconjugates and with 100 μg/ml of free cetuximab, for 2 hours or at longer incubation time of 24 hours of treatment + 24 hours of complete fresh medium incubation at 37 ºC. After incubation, cells were washed with PBS and the total extracellular EGFR stained with an anti-EGFR antibody conjugated with fluorescein isothiocyanate (FITC) (Santa Cruz Biotechnology, Inc). Cell nuclei were stained with Hoechst. Cells were rinsed twice with PBS-T and twice with PBS and then fixed with cold methanol for 10 minutes at -20 ºC. After fixation, cells were rehydrated and mounted with ProLong Gold (Life

Technologies, saint Aubin, France). Immunofluorescence images were collected on a Leica TCS SP5 confocal fluorescence microscope. The image processing and analysis was performed using the Bitplane Imaris software.

Western blotting (WB) analysis: adherent A431 cells at a confluency of 60%-70% were starved in serum-free DMEM medium for 24 hours before the NP treatment. Then, cells were treated with 100 μg/ml free Cetuximab or with Cetuximab-**1**-AuNP (NPC3, NPC6 and NPC8) and NPaR bioconjugates at a concentration of 15 nM NPs for 2 hours at 37 ºC. After incubation, cells were washed twice with PBS and stimulated with 10 ng/mL of EGF for 15 minutes. Cells were immediately washed three times with PBS at 4 ºC and and lysed in a mammalian protein extraction reagent (MPER) supplemented with a protease and phosphatase inhibitor cocktail (Pierce Chemical Co). Cell lysates were centrifuged for 15 minutes at 16000 g at 4ºC and the soluble fractions processed for SDS-PAGE and immunoblotting onto PVDF membranes was performed using standard protocols. Proteins were detected using two different primary antibodies; anti-phospho-EGFR (Tyr 1173) produced in rabbit (sc-101668) at a dilution of 1:1000 and a monoclonal anti-α-tubulin produced in mouse (T6199) at a dilution of 1:3000 and visualized with horseradish peroxidase–labeled secondary antibodies, followed by chemiluminescence using a premixed ECL reagent (Luminata™ Forte Western HRP Substrate, Millipore). Versadoc (Bio-rad) imaging system was used for WB imaging and the detected bands quantified using Quantity One software (Bio-Rad). Phosphorylation assays and subsequent Western blot analysis were done as previously descnibed[4]. Briefly, A431 cells were grown to 90% confluency in complete medium and then starved in RPMI and 0.5% BSA for 24 h prior to experimentation. Cells were stimulated with 10 ng/ml EGF in the presence of 10 ug/ml either 225 or C225 for 15 mm at room temperature. Following stimulation, monolayers were washed with ice-cold

PBS containing I msi sodium orthovanadate. Cells were lysed and subjected to SDS-PAGE followed by Western blot analysis. The phosphorylation paterns were determined by probing the blots with a mAb to phosphotyrosin (Upstate Biotechnology, Inc., Lake Placid, NY) followed by detection by the enhanced chemiluminescence method (Amersham, Arlington Heights, IL)." The Elisa experiments have been done in serum supplemented cell culture media.

Flow Cytometry analysis: to confirm the down-regulation of the EGFR recycling after treatment with AuNPs bioconjugates, we performed flow cytometry studies. To perform the experiments, A431 cells were seeded in 6-well plates at a density of 6.0 x 10^5 cells per well and after 48 hours, the medium was changed to serum-free medium for additional 24 hours. Starved cells were treated with 15 nM NPC8 and NPaR bioconjugates or with 100 μg/ml of free Cetuximab, for 2 hours or at longer incubation time of 24 hours of treatment + 24 hours of complete fresh medium incubation at 37 ºC. After incubation, cells were rinsed with cold PBS and the total extracellular EGFR stained during 30 minutes at 4ºC with the anti-EGFR528 antibody conjugated with fluorescein isothiocyanate (FITC) (Santa Cruz Biotechnology, Inc). The cells were then rinsed, trypsinized and resuspended in 1 ml of PBS. Flow cytometry analysis was performed with a BD FACSCalibur flow cytometer by measuring the emission at λ=488 nm.

Cell viability*:* the cytotoxicity of AuNPs bioconjugates was tested in a cell culture system using A431 cells. Cells were seeded onto 96-well plates at a cell density of 5.0 x 10^3 cells/well and incubated for 24 hours before Cetuximab-**1**-AuNP bioconjugates (NPC3, NPC6 and NPC8) and control bioconjugates (NPaR) were added at a concentration of 15 nM, as well as different concentrations of free Cetuximab (6.1, 13.7, 18.2 and 100 μg/ml). Growth inhibitory effect was measured after 72 hours of

treatment using PrestoBlue and XTT assays. For PrestoBlue determinations 20 µl of reagent was added to each well and after 2 hours the fluorescence at 570 nm was determined. In the case of XTT assay the cell medium was replaced after incubation to avoid interference from AuNPs in the colorimetric analysis. Aliquots of 20 µl XTT solution (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) were added to each well and after 2 hours the colour formed was quantified by a spectrophotometric plate reader (Perkin Elmer Victor3 V) at 490 nm. Cell cytotoxicity was evaluated in terms of cell growth inhibition in treated cultures and expressed as % of control condition.

2.- Determination of labeling sites in treated Cetuximab mAbs.

Mol LyCH / mol Cetuximab			
Experiment 1	Experiment 2	Experiment 3	Average
1.1	1.3	1.5	**1.3 ± 0.2**

Table S1. Determination of labelling sites in Cetuximab mAbs with LYCH under optimized oxidation conditions (oxidation time = 30 minutes; solution pH = 7; periodate concentration = 10 mM; and reaction temperature = RT). LYCH reacts with the aldehyde groups formed on Cetuximab mAbs after oxidation to form fluorescent Cetuximab-LYCH conjugates.

3.- Differences into linker mediated conjugation and native Ab absorption onto NPs

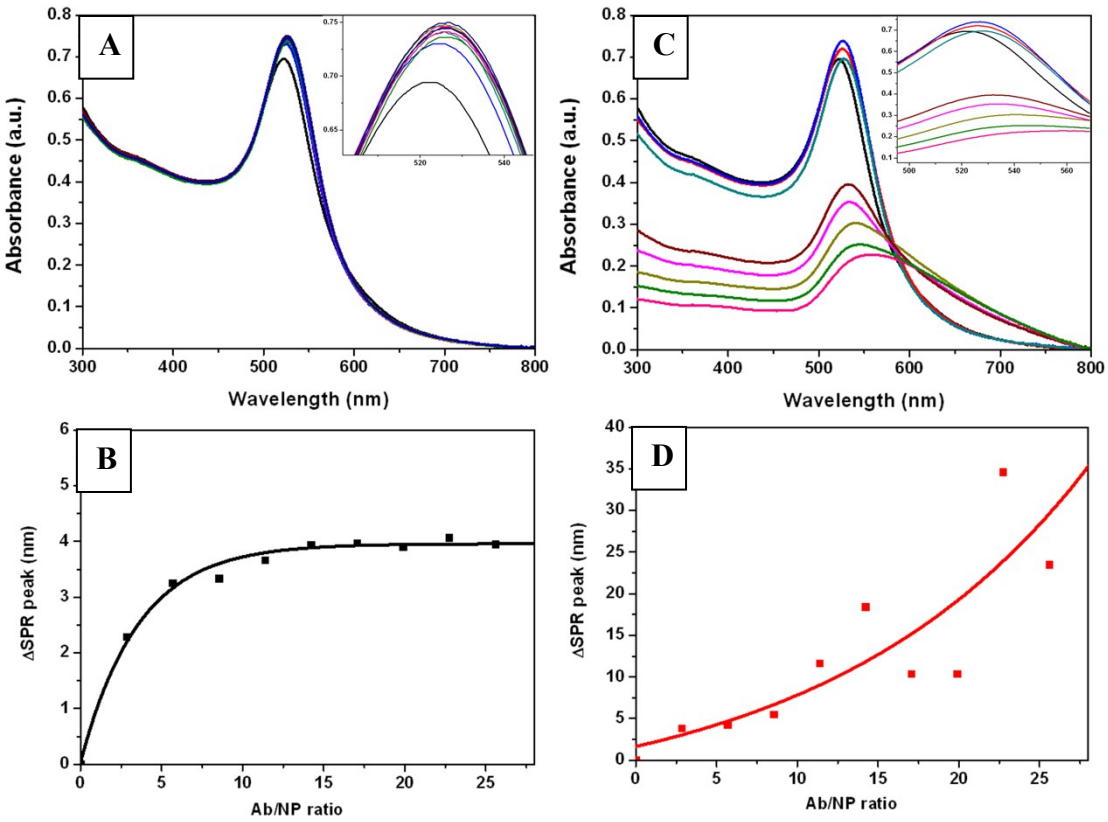

Figure S1. A) Normalized SP absorption bands from the Au NPs (17.2 ± 1.8 nm) with increasing amount of Cetuximab-**1** conjugates in solution. B) Shift of the SPR peak from Au NPs upon conjugation with Cetuximab mAbs. Experimental data was fitted to Boltzmann equation (R^2 = 0.984). C) the same as A but in the case of direct incubation of NPs with unmodified Cetuximab mAbs. D) the same as B but in the case of direct incubation of NPs with unmodified Cetuximab mAbs.

4. Quantification of Ab attached to the NPs.

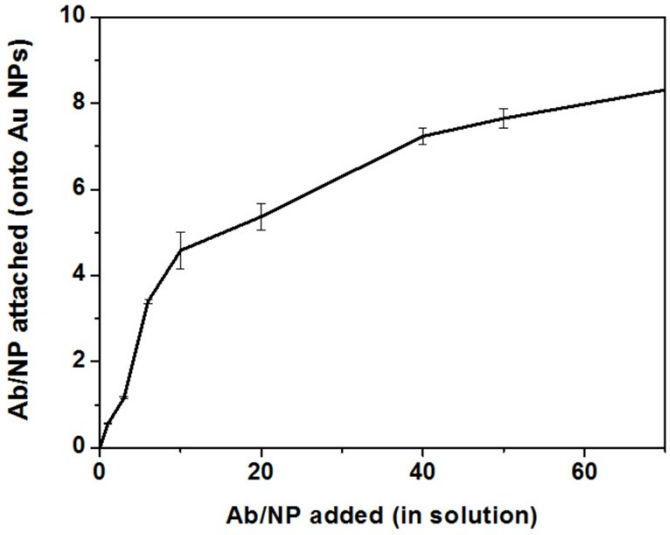

Figure S2. Quantification of Abs bound to 17.1 ± 2.4 nm Au NPs by using the conjugation strategy proposed. Ab-**1** (goat anti-rabbit Abs) were labelled with Alexa Fluor 488. Then, Alexa Fluor 488-Ab-**1** were added to the Au NPs at different ratios, and the fluorescence emission intensity from unbound Alexa Fluor 488-Ab-**1** was measured. The average amount of attached Abs to NPs was determined by subtracting the unbound Alexa Fluor 488-Ab-**1** from total.

5. Control of the stability of AuNP- non modified Ab conjugates.

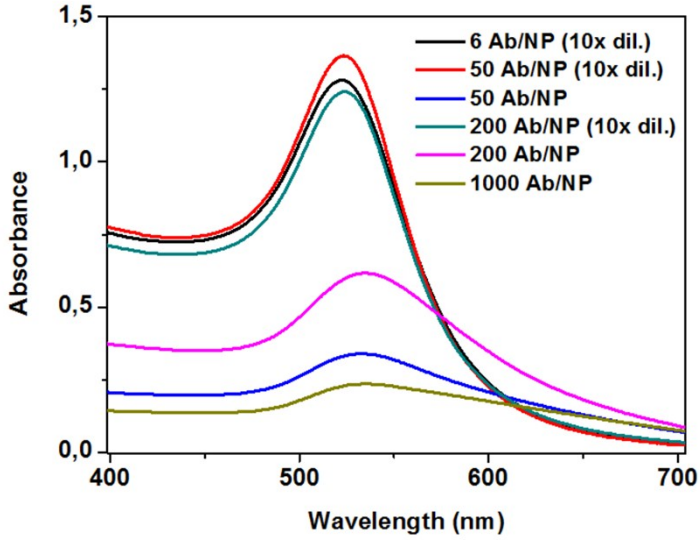

Figure S3. UV-visible spectra of 17.2 ± 1.8 nm Au NPs with increasing native goat anti-rabbit Ab/NP ratio (in solution) at the same concentration used for the previous experiments (~ 18 nM Au NPs) and a 10 times dilution after 1 hour of incubation.

6. 3D reconstruction of the AuNP-8mAb-AuNP structure.

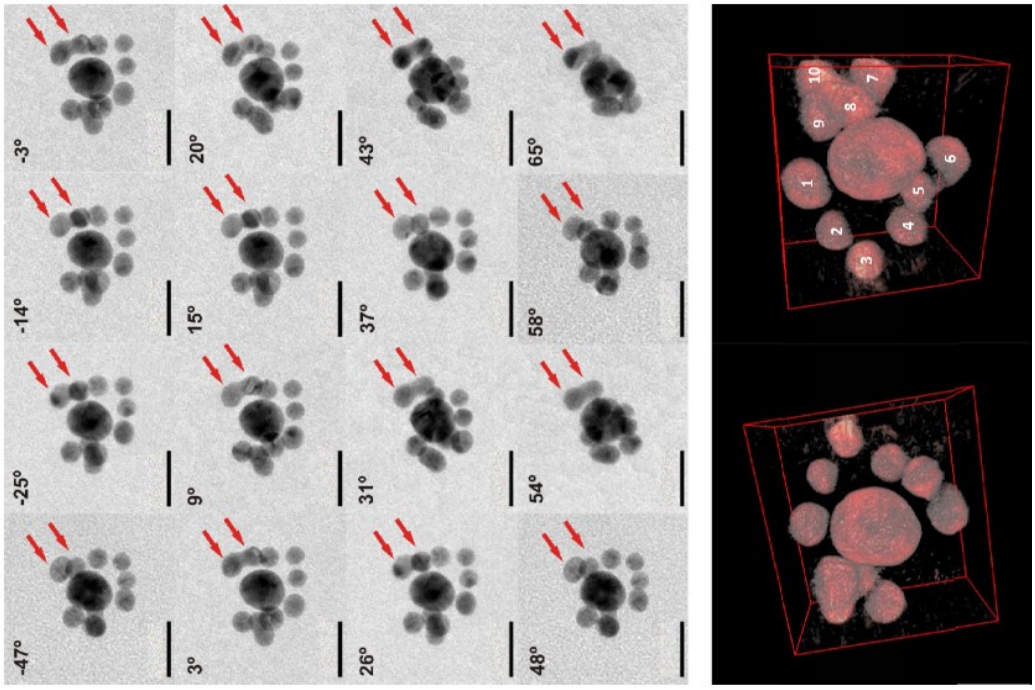

Figure S4. 3D reconstruction of NPC8 derived from tomography TEM images. Representative bright field TEM images series (from -69° to 65°) of a satellite shaped NP assembly based on specific Ab-antigen interactions between 8 ± 1.5 nm AuNPs coupled to rabbit anti-BSA Abs and 17.2 ± 1.8 nm AuNPs coupled to goat anti-rabbit Abs. There are 10 Au NPs of 8 nm surrounding the bigger 17 nm Au NPs. Notice that the two NPs marked with red arrows suffer a live process of coalescence during the 1 hour and 45 minutes tilt process. They start as separated NPs (see tilt images from -47° to -14°) and fuse when reaching approximately -3° (half of the process, approximately after 55 minutes under beam exposure). B) Front and back 3D reconstruction of the satellite system. Scale bars are 20 nm.

7. *Relative affinity measurements using ELISA:* The relative affinity of free Cetuximab and nanoparticle conjugates to EGFR was determined by the ELISA method, similarly as previously described[8]. Briefly, A431 cells were seeded at a concentration of 10^4 cells per well in 96-well plates and incubated for 24 h. After incubation cells were washed with PBS and fixed with a 3.7 % (v/v) paraformaldehyde solution for 10 min at 25 ºC. After incubation, cells were washed three times with PBS and blocked with 1 % (w/v) BSA in PBS for 2 h at 25 ºC. Different concentrations (ranging from 0 to 50 nM in PBS) of free Cetuximab, NPC6 or NPCRabbit were added to the wells. After 2 h incubation at 25 ºC, wells were washed three times with PBS. Then wells were incubated with an anti-human secondary antibody conjugated to Horseradish Peroxidase (HRP, 1.1000) for 2 h at 25 ºC. After incubation wells were washed extensively with PBS and, after washing, 100 µl of the colorimetric substrate TMB was added to each well. After 30 min reaction, the color formed on each well was measured at 450 nm using a Victor V3 plate reader. Kds were calculated using GraphPad software.

	Kd (nM)
Cetuximab	0.05 ± 0.02
NPC-6	1.46 ± 0.24
NPC-rabbit	N.D

Table S2. Kds of free Cetuximab, NPC-6 and NPC-Rabbit against EGFR determined by ELISA.

8.- Analysis of NP distribution on the cell membrane.

The specific functionality of Cetuximab-**1**-AuNP bioconjugates allows studying the distribution of EGF receptors in the different regions of the plasma membrane. With this aim, membrane-binding interactions of Cetuximab-**1**-AuNP bioconjugates with different Cetuximab loading (NPC3, NPC6 and NPC8) were compared by a combined TEM-statistical approach. Briefly, areas of the plasma membrane sheets were selected (800 x 800 nm) from TEM images and processed for determining the (x,y) coordinates of all AuNPs using Image J. Subsequent analysis of the NP coordinates was done using Ripley's univariate K-function, a statistic tool for point pattern analysis. A macro that carries out the analysis function was gently provided by Prior *et al*.[5, 6] The K-function is expressed as a linear transformation by the equation:

$$L(r) - r = \mathrm{sqrt}\left[(K(r) / \pi) - r\right]$$

Values of L(r)-r above 1.0 (normalization of 99% confidence interval (C.I.)) indicate significant clustering within the defined x-axis radius (r). Values below -1.0 indicate significant dispersal. And no deviation outside the C.I. indicates a random pattern.

Ripley's K-function analysis precisely determined the extent of clustering within the AuNP patterns in the obtained plasma membrane sheets, showing large clustering for NPC8 and NPC6 and a random pattern for NPC3 (Figure S4-A, right). A more clustered AuNP pattern was observed for higher multivalent NPs, NPC6 and NPC8. This effect could be attributed to the capacity of these NP bioconjugates to induce multiple EGFR crosslinking as suggested also by Bhattacharyya *et al.*[9] According to these authors, the same clustering effect was necessary for Cetuximab (C225) and the gold-Cetuximab nanoconjugates with high coverage (3 antibodies per nanoparticle) to be internalized by dynamin-2 dependent caveolar endocytosis. On the contrary, they did not observe

clustering of the partially covered nanoparticles (1 antibody per nanoparticle) and this resulted in a different endocytic pathway. This may have significant implications in the internalization process, which is strongly dependent on the inter ligand distance[10] and the diffusion rate of receptors on the plasma membrane.[11]

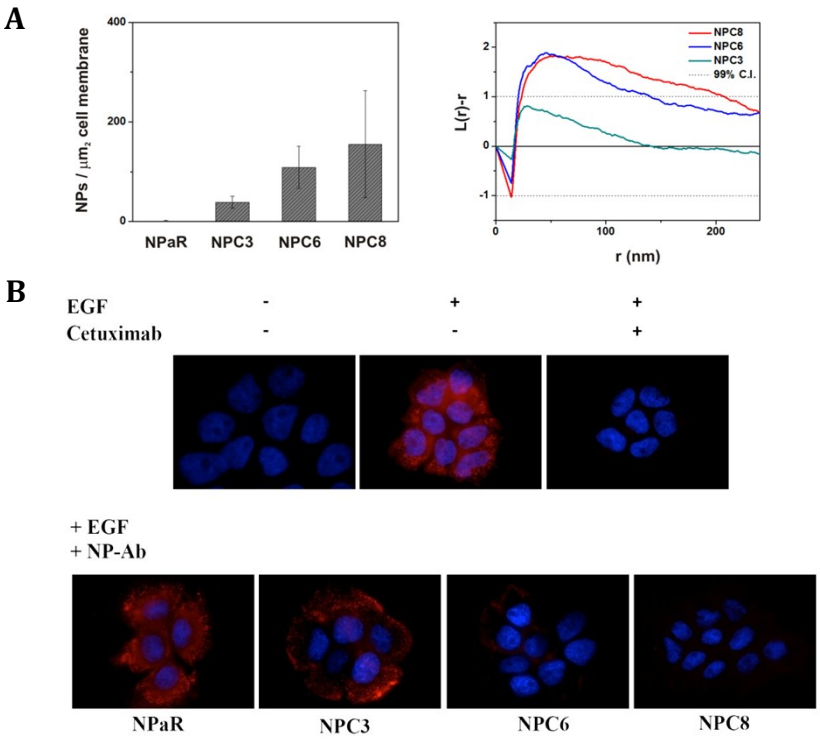

Figure S5. A) Mean number of AuNPs present in the plasma membranes measured from an area of 800 x 800 nm^2 in a total of 10 TEM images from different cells (left) and Ripley's K-function analysis (right). B) Fluorescence optical microscopy images of A431 cells after incubating Cetuximab and the Cetuximab-**1**-AuNP bioconjugates at a concentration of 8 nM for 2 hours at 37 ºC and staining with Alexa Fluor 555-EGF. Nuclei were stained with DAPI.

9. TEM images of A431 cells after incubation of NPaR bioconjugates

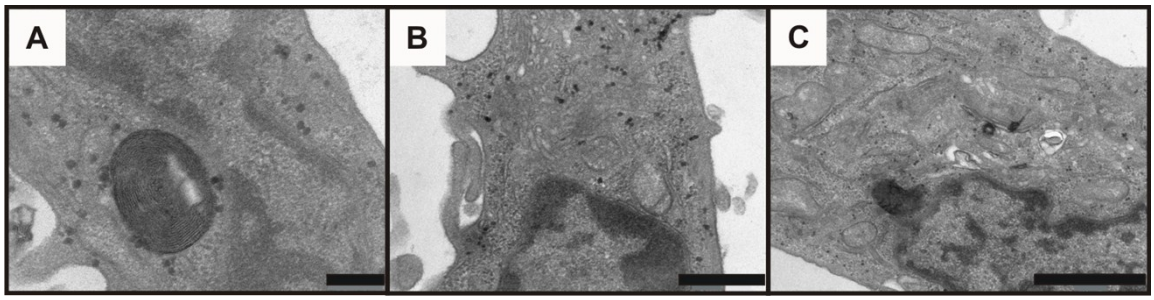

Figure S6. Representative TEM images of A431 cells after incubation of NPaR bioconjugates for 24 hours at 37 ºC. Scale bars are 200 nm (A), 500 nm (B) and 1 μm (C).

10. UV-vis spectra of NPC8 and NPC6 conjugates in the cell lysates.

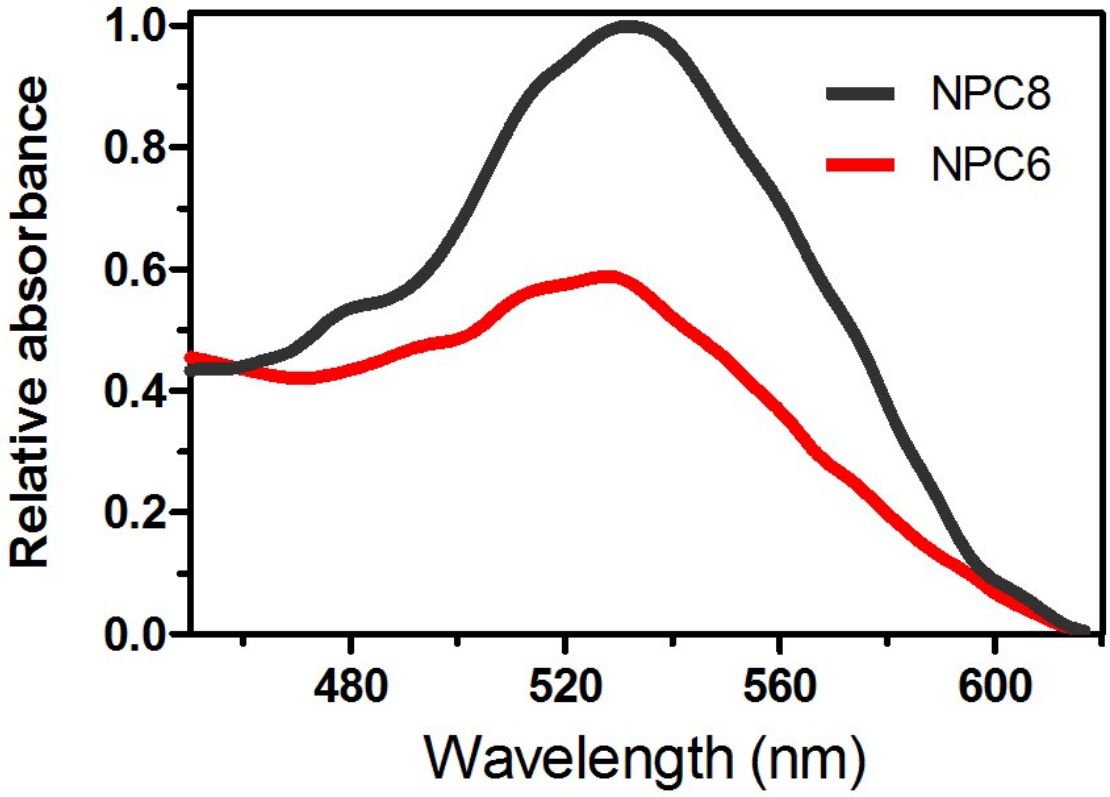

Figure S7. UV-vis spectra of NPC8 and NPC6 conjugates in the cell lysates. The unusual shape of the spectra is the result of subtracting the background from the cells. NPC3 and NPaR absorption spectra are confounded in the background

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11. Confocal Microscopy analysis of EGFR experssion.

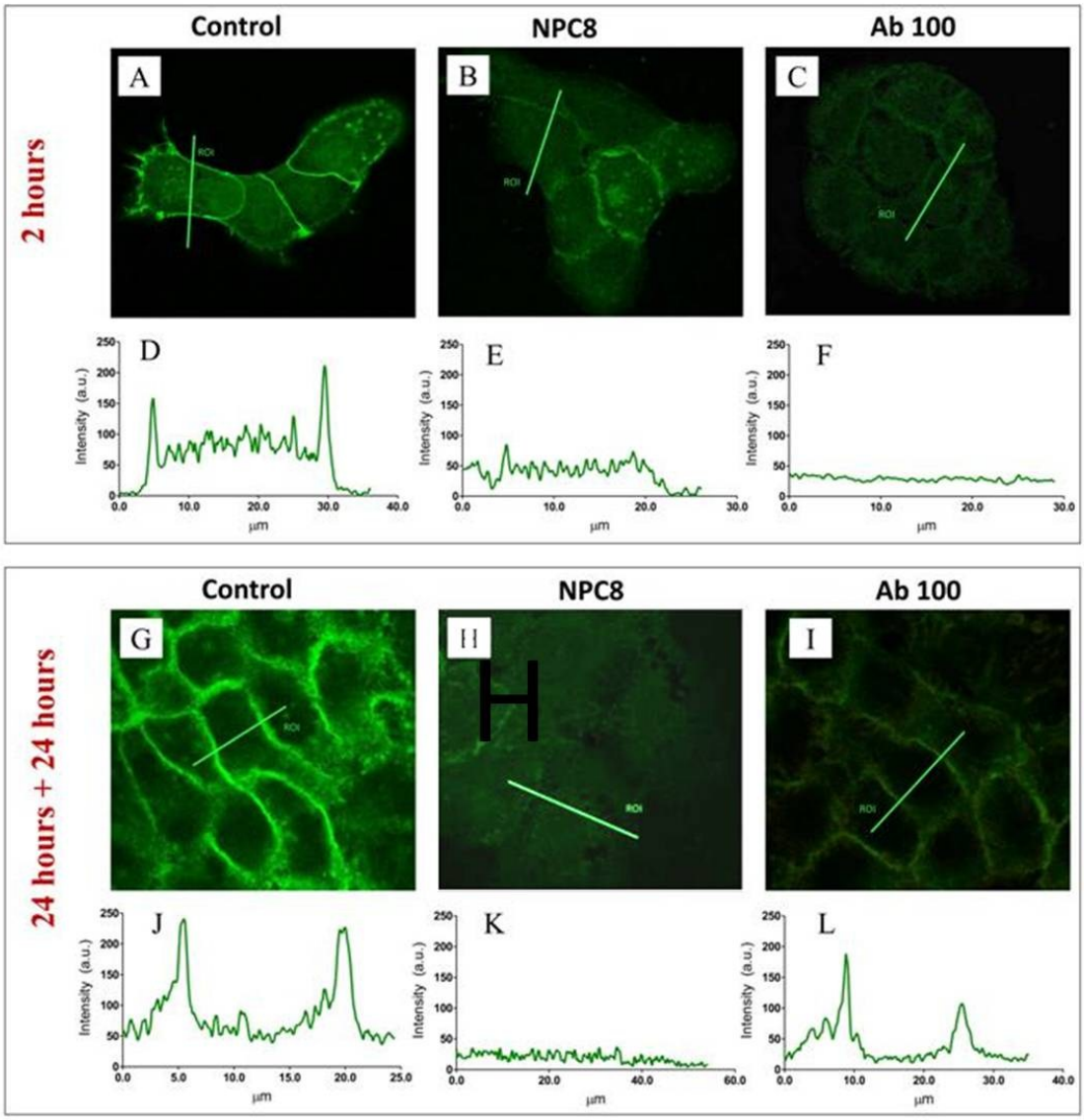

Figure S8. CLSM images and intensity profile analysis of control A431 cells (control) and the same cells incubated with bioconjugate NPC8 (NPC8) and free cetuximab (Ab100). Cells were incubated for 2 hours in absence (A and D) or presence (B and E) of NPC8 nanoparticles or for 24 +24 hours (24 hours incubation and additional 24 hours incubation with fresh medium at 37ºC) in absence (G and J) or presence (H and K) of NPC8 bioconjugate. As positive control condition, A431 cells were also incubated with 100 µg/ml of free cetuximab for 2h (C and F) or for 24 h+ 24 h (I and L). EGFR was detected using an anti EGFR antibody conjugated with FITC (green).

12. Cell Viability

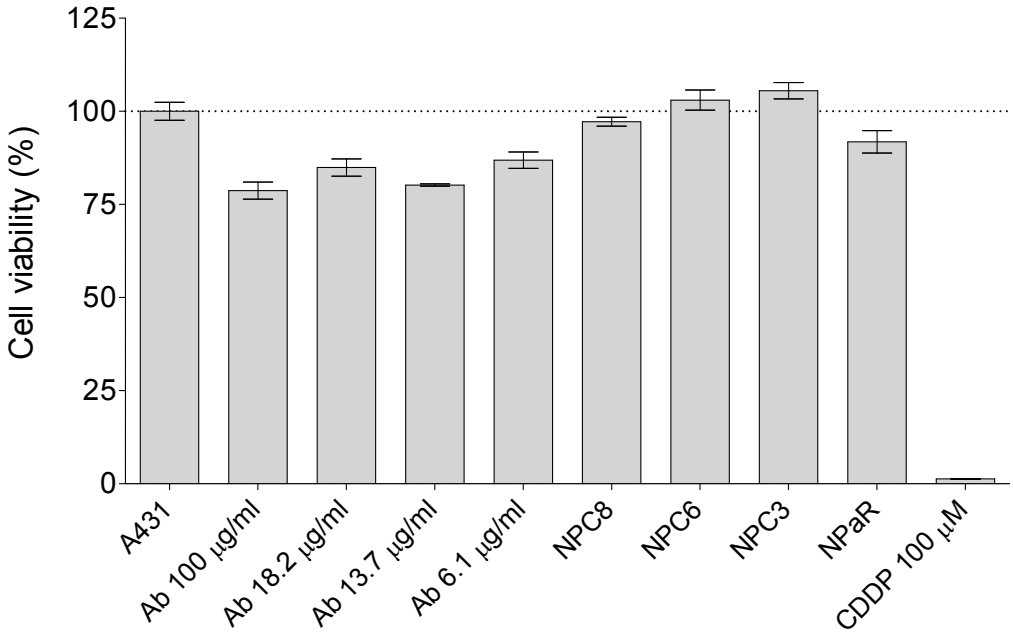

Figure S9. PrestoBlue cell viability assay of A431 cells after 72 hours incubation of free Cetuximab at 6.1, 13.7, 18.2 and 100 μg/ml, 15 nM of Cetuximab-**1**-AuNP bioconjugates, and 100 μM Cisplatin (CDDP) as a positive control of a cytotoxic compound.

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