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Materials and Methods

Reagents

Antibody was principally purchased from Santa Cruz (EGFR sc-03), Cetuximab (Bristol Mayer Squib), IgG (Jacson Laboratory), Carboplatin (Bristol Mayer Squib), Para formaldehyde (Electron Microscopy Science), Cy3 and Cy5 (GE Healthcare), mouse plasma (Equitech Bio Inc, Kerrville, TX)

Cell culture

Ovarian cancer cell lines (OVCAR5, OV202) and lung cancer cell lines (A549 and H157) was purchased from American Type Culture Collection and cultured using DMEM and RPMI 1640 with L-glutamine (Cellgro Mediatech, Inc.) supplemented with 10 % FBS and 1% antibiotics (penicillin-streptomycin).

Synthesis of gold nanoconjuagtes

Gold nanoparticles (AuNPs) were synthesized from tetrachloroauric acid by wet chemical methods using sodium borohydride as a reducing agent as previously described.[1] AuNPs thus formed was characterized using UV-Vis and TEM confirming ~ 5 nm size spherical nanoparticles formed by this method. Cetuximab (C225) and Immunoglobin (IgG) was added as (4 μg/mL) to a solution of AuNP to prepare the AuC225 and AuIgG conjugate to study the intracellular targeting (Fig S1). The nanoconjugates were centrifuged at 20,000 rpm at 10 ^{0}C for 1h. The conjugates were collected as pellets and characterized by UV-Vis and TEM.

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Synthesis of anti-EGFR antibody labeled with Cy3 or Cy5 dye and Immunofluorescence Microscopy: The monoclonal anti-EGFR antibody cetuximab (C225) was labeled with mAB labeling dye Cy5 & Cy3 respectively, according to reported procedure (GE healthcare, PA33001 and PA33005).[1] Briefly, antibody (2mg/mL) was diluted with PBS (1:1) and then incubated with Cy3/Cy5 dye for 30 minutes. Excess unlabeled dye was separated by a filtration column provided in the kit. Labeled antibody (C225-Cy3) thus obtained was used for cell culture assay. OVCAR5 and A549 cells ($2x10^4$/well) were plated in a 4 well chamber slides. Next day, the cells were treated either with C225-Cy5/Cy3 (4 μg/mL) for 30 minutes at 4 ^{0}C followed by washing with ice cold PBS (3 times). Cells were fixed in 2 % paraformaldehyde in PBS (15 minutes), and washed with PBS at room temperature before adding mounting media (Vectashield) containing nuclear stain DAPI for confocal microscopy.[1] .

In vitro targeting of Au-C225 to cancer cells expressing EGFR

Confluent OVCAR5 and A459 cells in 100-mm dishes were treated with the purified nanoconjugates (AuC225, AuIgG and similarly for AuC225CP and AuIgGCP) at a dose of 4 μg/mL for 2 h. After the treatment, cells were washed with PBS, trypsinized, and thoroughly washed in PBS to remove any unbound conjugates. Finally, cells were centrifuged at 1,300 rpm to collect the pellet to analyze gold content by instrumental neutron activation analysis (INAA) analysis, as reported previously.[2]

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Western Blot Analysis

The EGFR expression in OVCAR5, OV202, A549, and H157 cells was determined by western blot analysis. Confluent cells were lysed by using NP-40 lysis buffer. Protein concentration in the lysates was determined by Bradford assay (Bio-rad). The proteins were loaded on a 7.5% SDS-PAGE gel for separation and transferred to a PVDF membrane. The membrane was incubated with EGFR antibody (Santa Cruz, sc-03, 1:250 dilution) in 3% milk. Blot was washed thrice with TBS-Tween 20 (0.1 %) and incubated with secondary antibody conjugated with peroxidase (HRP) (1:10,000 dilutions) in TBST. Beta-actin (mouse-IgG) was used as a control for protein loading.

Inductively Coupled Plasma-MS Analysis (ICP) for Carboplatin quantification

Antibody (355 μM) was preincubated with carboplatin (450.0 μM) under shaking in a 1.0 mL of a total solution. After 3 h of mixing, an aliquot of 390 μL was taken from the reaction mixture and added to 25.0 mL of AuNP solution. The incubation was continued for 2 h under shaking at room temperature. The resulting solution was centrifuged at 20,000 rpm (50 2T I) for 1 h at 10 ^{0}C. The pellets were collected and purified by washing once with DI water followed by ultracentrifugation at 30,000 rpm (50 2T I) for 30 min at 10 ^{0}C. The purified pellet thus obtained was used to quantify the carboplatin content using ICP-MS as reported.[3] Carboplatin concentration in AuC225CP was found to be 44.0 μg/ml (118.6 μM). We quantified the number of C225 bind to each GNP surface is ~ 2 [4] and the number of carboplatin bound to each GNP is 63.

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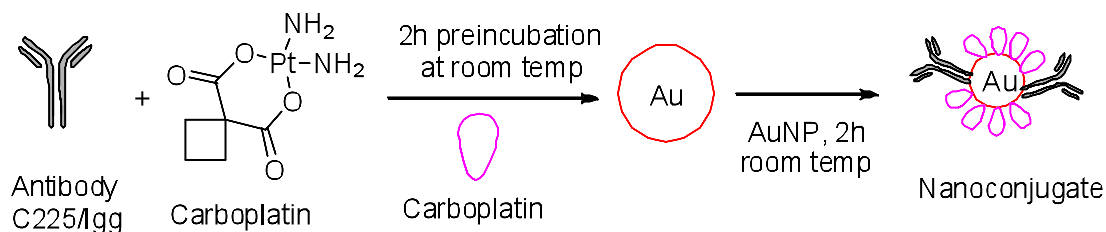

Scheme 1: Synthesis of nanoconjugated carboplatin

Transmission electron microscopy (TEM) to visualize intracellular localization of AuNP.

OVCAR5 and A549 cells were treated with AuC225 and AuIgG for 2h, then trypsinized and pelleted at 1300 rpm for 5 min. The pellet thus obtained were further washed with PBS (twice) and fixed in trump's solution containing 1% gluteraldehyde and 4 % formaldehyde in 0.1 M phosphate buffer (pH 7.2). Thin sections of (80 nm) were processed as described before and images were taken on a TECHNAI 12 operating at 120KV. [1]

Release study of Carboplatin from AuC225CP in Mouse Plasma

Gold conjugates containing carboplatin (AuC225CP) were incubated with mouse plasma at 37 ^{0}C at various time points (0 h, 6 h, 24 h) in a total volume of120 µL. After the final incubation, the resulting solutions were centrifuged at 30,000 rpm for 30 min at 10 ^{0}C. Supernatants thus obtained were analyzed using ICP-MS to quantify the release of carboplatin. A control sample (without any mouse plasma) was also analyzed to determine the concentration of Pt in the original solution.

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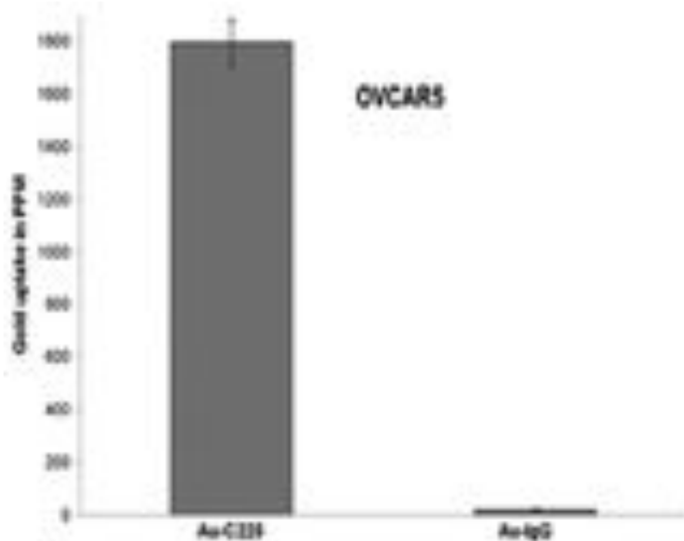

***Figure S1*:** Targeting efficiency was determined by the measurement of gold content in the cells using INNA. (A) The figure demonstrates the gold content in OVCAR5 cells after treatment with 4 μg/ml of either AuC225 or AuIgG at 37 ^{0}C for 2 h. The Figure shows increased gold uptake when cells were treated with gold nanoconjugates in a targeted (AuC225) vs. non-targeted (AuIgG) fashion.

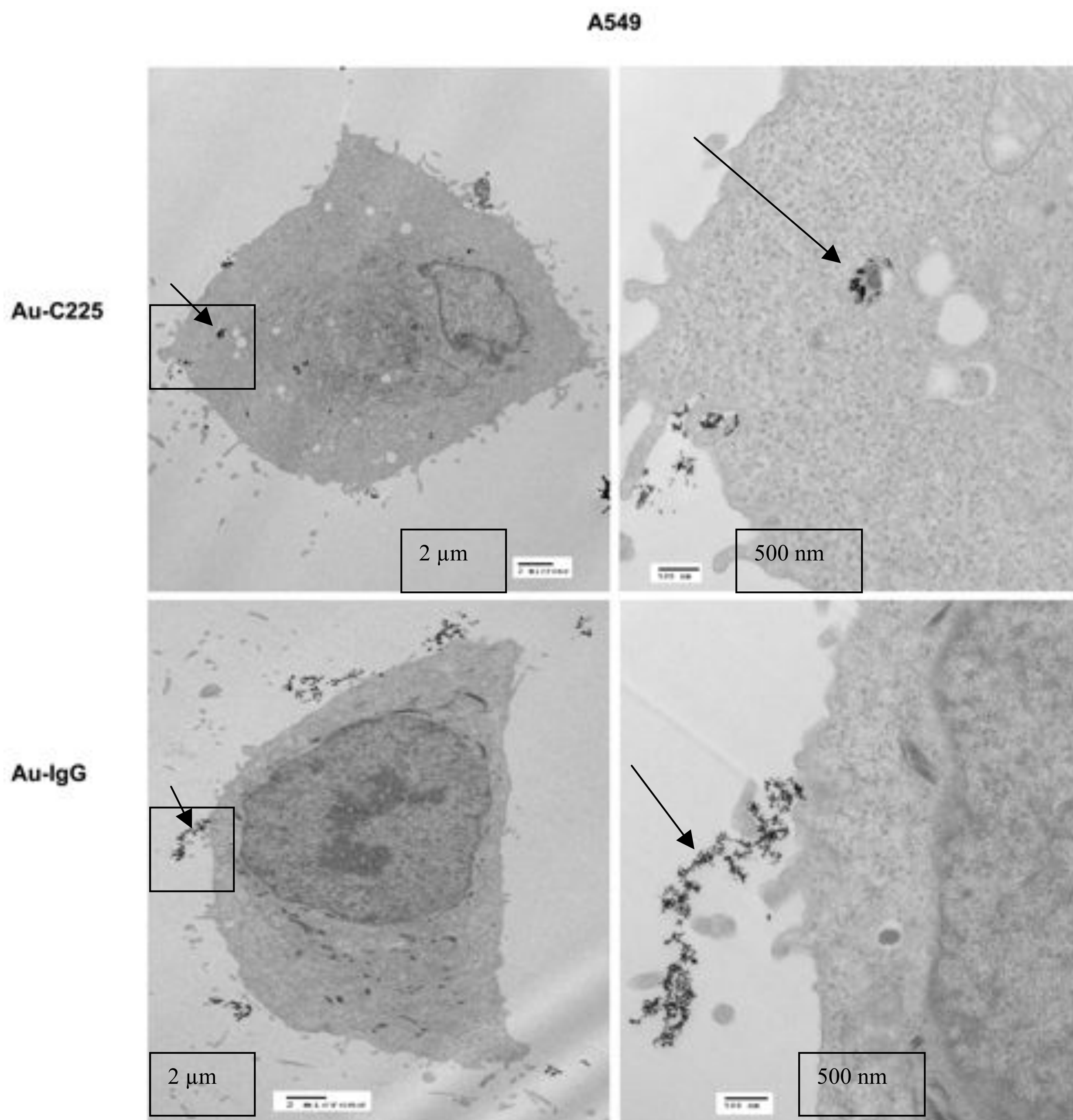

Figure S2: TEM analysis of the A549 cell as obtained by treating the cell with AuC225 and AuIgG nanoconjugates at 37 ^{0}C for 2 h, showing the higher gold uptake inside the cell (the upper panel from left to right) when administered in targeted way while most of the gold conjugates are outside the cell when treated with non targeted fashion, AuIgG (the lower panel from left to right).

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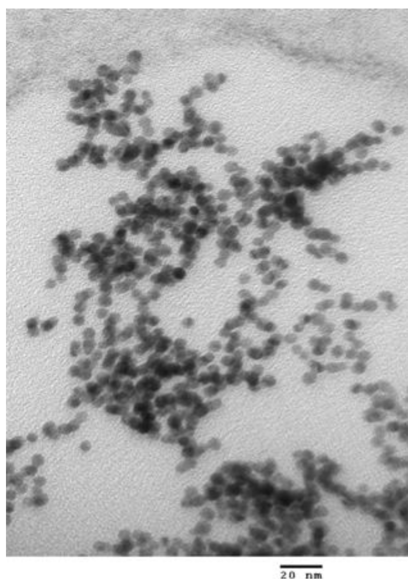

Figure S3: A high magnification image of the 5 nm size gold nanoparticles in the nanoconjugate.

Reference:

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