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Drug Delivery

Switching the Targeting Pathways of a Therapeutic Antibody by Nanodesign**

*Sanjib Bhattacharyya, Raman Deep Singh, Richard Pagano†, J. David Robertson, Resham Bhattacharya, and Priyabrata Mukherjee**

The application of nanomaterials in medicine is an emerging field with the potential to have a positive effect on human healthcare.[1] Nanoconjugates of monoclonal antibodies, such as the epidermal growth factor receptor (EGFR) antibody cetuximab (C225), are potential candidates for various biomedical applications, which include targeted delivery, detection/diagnosis, and imaging.[2,3] However, the mechanisms by which C225 and its nanoconjugates are transported into the cell and the molecular machineries that are involved in intracellular delivery are not well understood. A better understanding of the regulatory components that are involved in the endocytosis of nanoconjugates will aid in achieving specific intracellular targeting. To demonstrate that the mechanism of endocytosis of C225 in human pancreatic carcinoma (PANC-1) cells can be tailored by precise design of the nanoconjugates, we have designed two Au–C225 conjugates that have different numbers of antibodies. These conjugates are produced by exploiting the strong affinity of gold nanoparticles (AuNPs) for the thiol group in cysteine (Figure 1 a).[4] By using pharmacological inhibitors (Figure S1 in the Supporting Information), genetic approaches, and Cdc42-null cells we demonstrate that both C225 and gold-conjugated C225 with complete surface coverage (Au–C225-C; ca. three C225 units per NP) require clustering in the glycosphingolipid (GSL) domains at the plasma membrane to be internalized by dynamin-2 (Dyn-2) dependent caveolar endocytosis. However, the partially covered nanoconjugate (Au–C225-P; ca. one C225 unit per NP) is internalized by Cdc42-dependent pinocytosis/phagocytosis and requires the polymerization of actin. The ability to switch the endocytosis of C225 between caveolar and pinocytotic mechanisms by using an appropriate design may be useful in targeting specific intracellular pathways for better therapeutic intervention and fewer side effects.

To determine the mechanism and molecular machinery that are involved in the uptake of C225 and its nanoconjugates, we have designed two Au–C225 nanoconjugates as previously described: AuNPs with the surface partially covered by C225 (Au–C225-P) and AuNPs with the surface fully covered by C225 (Au–C225-C, Figure 1 a). The C225/AuNP ratio in Au–C225-P and Au–C225-C is approximately 1:1 and 3:1, respectively.[5] To study the interaction between C225 and its nanoconjugates with EGFR by confocal microscopy, PANC-1 cells were transfected with EGFR-green fluorescent protein (EGFR-GFP, see the Supporting Information) and treated with C225, Au–C225-P, or Au–C225-C for 1 h. The C225 units were labeled with Cy3. Both C225 and Au–C225-C co-localized with EGFR at the cell membrane to a similar extent, which indicated that the receptor-binding motif of C225 remained unaffected upon conjugation to AuNPs. However, the extent of co-localization of Au–C225-P with EGFR at the membrane was lower relative to that of C225 and Au–C225-C. This result suggests that the modes of interaction of Au–C225-P with the cell membrane are different to that of C225 and Au–C225-C (Figure S2 in the Supporting Information).

The internalization of C225 in PANC-1 cells is Dyn-2 dependent.[6] To investigate whether the Dyn-2-dependent internalization of C225 is affected by Au–C225 conjugates, PANC-1 cells were infected with wild-type Dyn-2 (Dyn-2-WT) or mutant Dyn-2 (Dyn-2(K44A)) adenoviruses and the internalization of Cy3-labeled C225, Au–C225-P, and Au–C225-C was monitored by confocal microscopy. The internalization of Au–C225-C was inhibited substantially in PANC-1 cells that were expressing Dyn-2(K44A) relative to cells that were expressing Dyn-2-WT (Figure 1 b,c). In contrast, the uptake of Au–C225-P in PANC-1 cells that were expressing Dyn-2(K44A), was not inhibited (Figure 1 b). Thus, the Dyn-2-dependent endocytosis of C225 can be altered by AuNPs with the appropriate design.

[*] Dr. S. Bhattacharyya, Dr. R. D. Singh, Dr. R. Bhattacharya, Dr. P. Mukherjee
Department of Biochemistry and Molecular Biology
Guggenheim 1311B, College of Medicine, Mayo Clinic
200 First St SW, Rochester, MN 55905 (USA)
E-mail: mukherjee.priyabrata@mayo.edu
Dr. R. D. Singh, Dr. R. Pagano
Thoracic Disease Research Unit, Mayo Clinic
Rochester, MN 55905 (USA)
Dr. J. D. Robertson
Department of Chemistry and University of Missouri Research Reactor
University of Missouri, Columbia, MO 65211 (USA)
Dr. P. Mukherjee
Biomedical Engineering, Mayo Clinic
Rochester, MN 55905 (USA)
Dr. P. Mukherjee
Cancer Center, College of Medicine, Mayo Clinic
Rochester, MN 55905 (USA)
[†] Deceased

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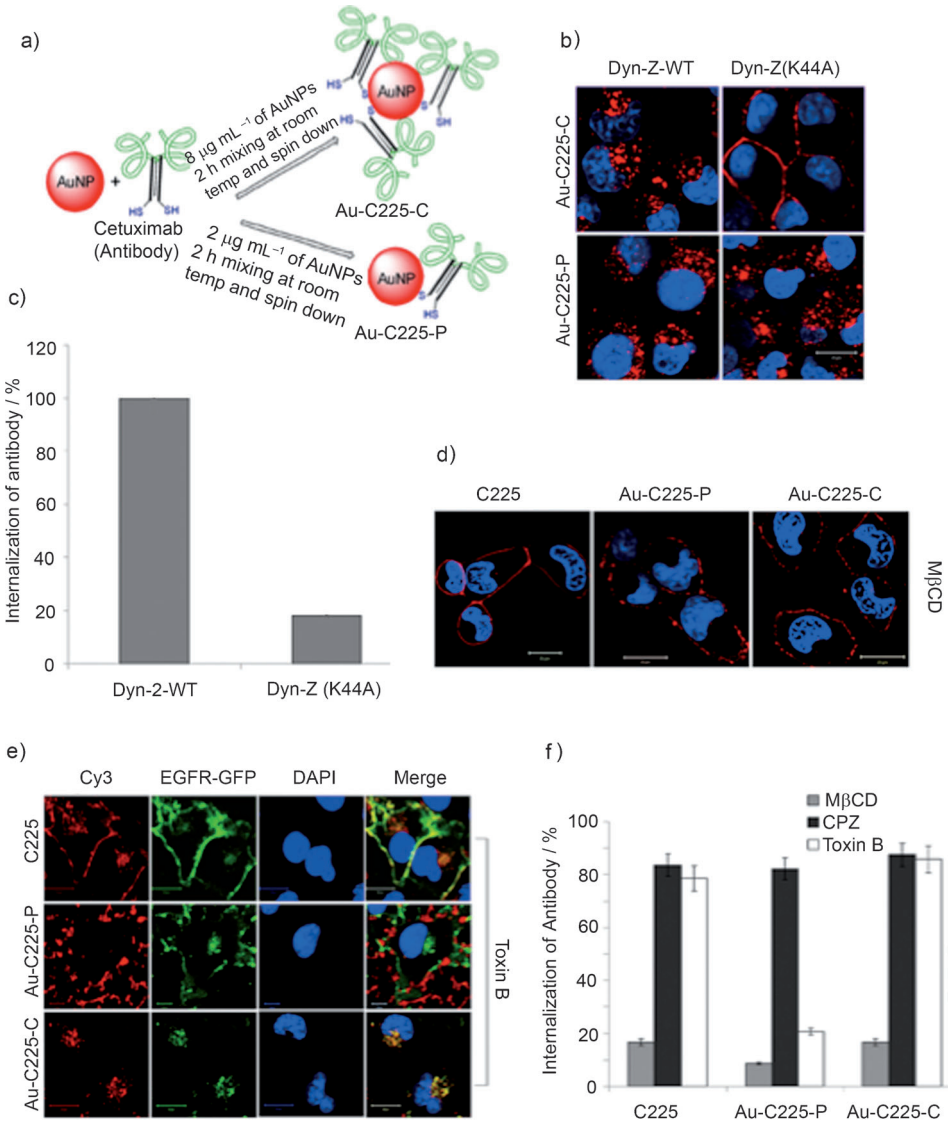

Figure 1. Effect of AuNP surface coverage on dynamin-2 requirement and mechanism of C225-nanoconjugate uptake; role of clathrin-mediated versus clathrin-independent endocytosis. a) Preparation and structure of the antibody nanoconjugates Au–C225-P and Au–C225-C. b) Fluorescence images showing switching of endocytosis mechanisms upon increasing AuNP surface coverage with C225, from Au–C225-P to Au–C225-C, in PANC-1 cells infected with Dyn-2-WT or Dyn-2(K44A) adenoviruses (scale bar: 20 μm). c) Quantification of the uptake of Au–C225-C in PANC-1 cells infected with Dyn-2-WT and Dyn-2(K44A) adenoviruses. d) Fluorescence images showing the inhibited internalization of Cy3-labeled C225, Au–C225-P, and Au–C225-C into PANC-1 cells (scale bar: 20 μm). e) Fluorescence images showing that uptake of Au–C225-P, but not C225 or Au–C225-C, was inhibited by treatment with toxin B (scale bar: 20 μm). f) Quantification of antibody uptake in the presence of different pharmacological inhibitors. CPZ = chlorpromazine.

As Dyn-2 is involved in both clathrin-mediated and clathrin-independent endocytosis, we wanted to further delineate the specific endocytotic pathways that are involved in the internalization of C225 nanoconjugates.[7] Chlorpromazine, an inhibitor of clathrin-mediated endocytosis, has no apparent effect on the internalization of C225, Au–C225-P, or Au–C225-C (Figure S3 in the Supporting Information) into PANC-1 cells.[8] However, as a positive control, the internalization of transferrin was inhibited (Figure S4 in the Supporting Information). These data clearly suggest that clathrin-mediated endocytosis is not the primary route for the internalization of C225 and its nanoconjugates into PANC-1 cells.

As C225 and its nanoconjugates are not internalized into PANC-1 cells by clathrin-mediated endocytosis, we investigated clathrin-independent pathways. To determine if a lipid raft is involved in the mechanism of uptake, we used β-methylcyclodextrin (MβCD), a reagent that extracts cholesterol from the plasma membrane, thereby inhibiting lipid raft dependent endocytosis.[8,9] Preincubation of PANC-1 cells with MβCD (5 mM) for 30 min blocked the internalization of C225 selectively (ca. 70%, Figure S5 in the Supporting Information), whereas a higher concentration of MβCD (10 mM) was required to inhibit the internalization of Au–C225-P and Au–C225-C to a similar extent (Figure 1d). Taken together, these data suggest that lipid raft mediated caveolar endocytosis may be involved in the uptake of Au–C225-P and Au–C225-C in PANC-1 cells. Preincubation of PANC-1 cells with another inhibitor of caveolar-mediated endocytosis, nystatin (50 $\mu g\,mL^{-1}$), also inhibited the internalization of both nanoconjugates (Figure S5 in the Supporting Information). This result provides further evidence for the involvement of lipid rafts in the caveolar endocytosis of C225 and its nanoconjugates.[8]

To investigate the involvement of clathrin-independent uptake pathways other than caveolar-mediated endocytosis, we used cytochalasin D, a fungal metabolite, that is known to block macropinocytotic/phagocytotic pathways by inhibiting actin polymerization.[10] Pretreatment of PANC-1 cells with cytochalasin D inhibited the internalization of Au–C225-P selectively, but did not inhibit the uptake of C225 or Au–C225-C (Figure S5 in the Supporting Information). Similarly, treatment of PANC-1 cells with *Clostridium difficile* toxin B inhibited the internalization of Au–C225-P, whereas toxin B also had no effect on the uptake of C225 or Au–C225-C (Figure 1e,f). Toxin B inhibits RhoA- and Cdc42-dependent uptake pathways.[8,11] These results suggest that RhoA- or Cdc42-dependent

pinocytosis and actin polymerization may be required for the uptake of Au–C225-P.

To further confirm the involvement of RhoA or Cdc42 in the internalization of Au–C225-P, we overexpressed the dominant negative RhoA (RhoAT19N) and Cdc42 (Cdc42T17N) mutants in PANC-1 cells and studied the internalization of C225, Au–C225-C, and Au–C225-P. Dextran particles, which require Cdc42 for internalization, were labeled with Alexa Fluor 594 and used as a positive control (Figure S6 in the Supporting Information).[12] The uptake of Au–C225-P was completely inhibited in the cells that were overexpressing Cdc42T17N, whereas the uptake of C225 and Au–C225-C remained unaffected (Figure 2a,b). However, the internalization of the C225 nanoconjugates was found to be independent of RhoA in the PANC-1 cells that were expressing the RhoA dominant negative mutant (Figure S7 in the Supporting Information). The results with cytochalasin D, toxin B, and the Cdc42 dominant negative construct independently demonstrate that endocytosis of Au–C225-P involves actin polymerization, and that entry into PANC-1 cells is primarily by Cdc42-dependent macropinocytosis. To visualize the endocytosis of C225, Au–C225-P, and Au–C225-C at the molecular level, we performed TEM on PANC-1 cells after the treatment with the C225 nanoconjugates. Figure 2c shows the induction of a caveolar structure at the plasma membrane after incubation with C225. Similarly, AuNPs were also found inside flask- and grape-shaped caveolar architectures in PANC-1 cells after treatment with Au–C225-C (Figure 2c), which further confirms the involvement of caveolar-mediated endocytosis for C225 and Au–C225-C. In contrast, AuNPs were found in close proximity to pinocytosis invaginations at the plasma membrane in PANC-1 cells that were treated with Au–C225-P (Figure S8 in the Supporting Information). The TEM image of Au–C225-P uptake at the pinocytosis morphology corroborates the results obtained from the Cdc42 dominant mutant and toxin B experiments.

As C225 and its nanoconjugates require lipid rafts for endocytosis, we investigated the involvement of specific lipid microdomains at the plasma membrane in caveolar-mediated endocytosis and other clathrin-independent pathways. PANC-1 cells were treated with the ceramide synthase inhibitor fumonisin B1 (FB1) to inhibit sphingolipid biosynthesis.[12,13] This treatment inhibited the internalization of C225 and both of the nanoconjugates (Figure 3a). Whereas the uptake of C225 and Au–C225-C could be restored by exogenous addition of ganglioside GM3, the internalization of Au–C225-P could not be restored by this method (Fig-

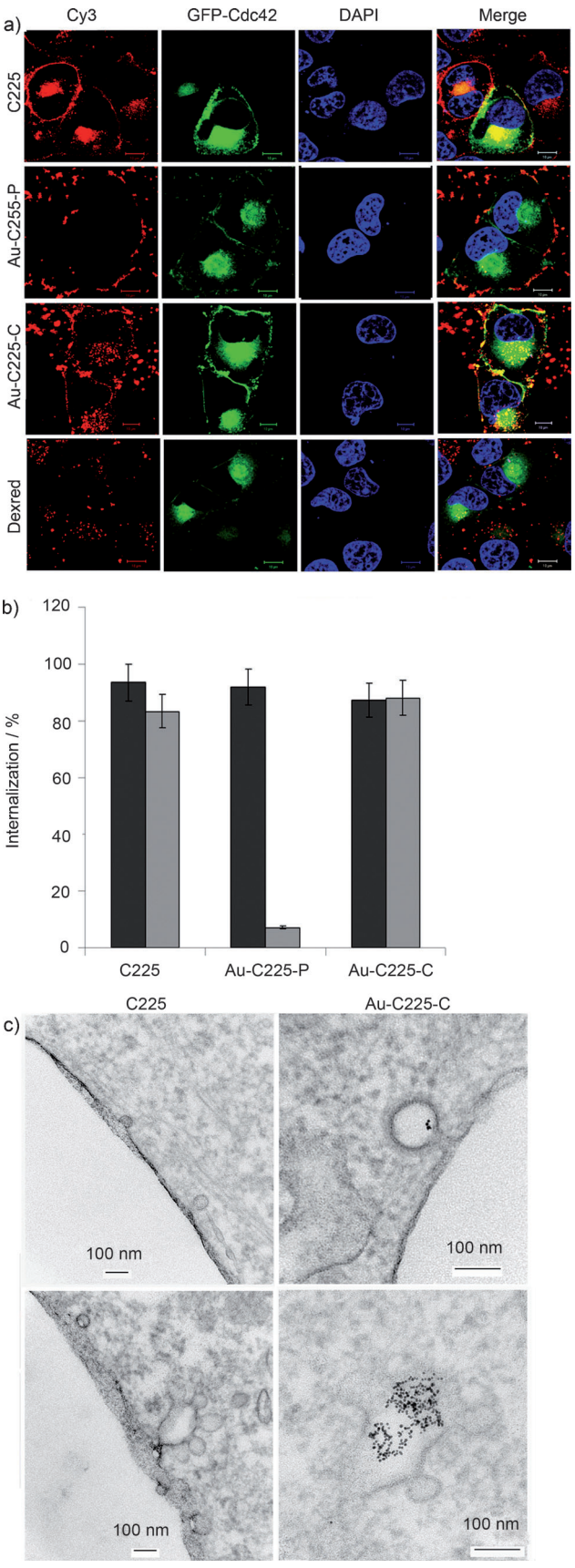

Figure 2. Role of Cdc42 GTPase on the internalization of C225, Au–C225-P, and Au–C225-C, and structural elucidation of clathrin-independent pathways. a) Fluorescence images of PANC-1 cells transfected with GFP-Cdc42T17N dominant negative mutants (scale bar: 10 μm) show that the uptake of Au–C225-P is inhibited, whereas the uptake of C225 and Au–C225-C remains unaffected. Lower panels are the images for cells treated with Dex-red (positive control). b) Quantification of nanoconjugate internalization into PANC-1 cells transfected with dominant negative mutant, GFP-Cdc42T17N (gray bars) versus wild type (black bars). c) TEM images of the arrest of the caveolar invagination of PANC-1 cells triggered by treatment with C225 or Au–C225-C (scale bar: 100 nm).

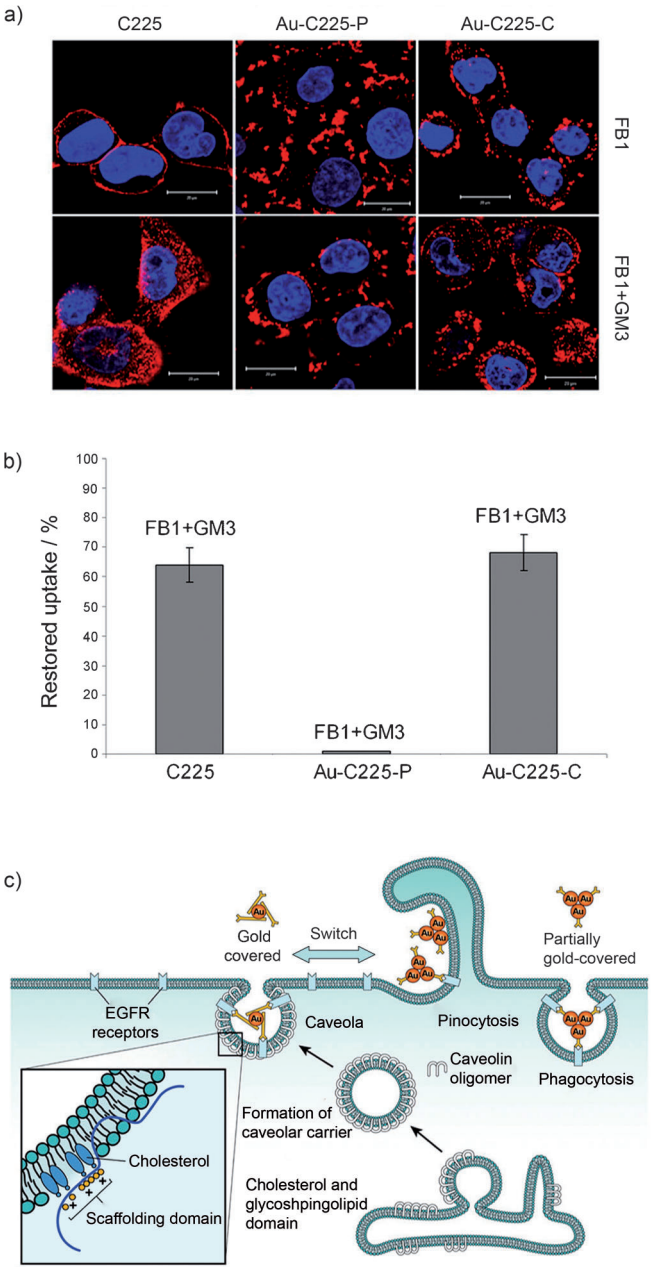

Figure 3. Lipid microdomains involved in the uptake of C225 nanoconjugates. a) Fluorescence images of PANC-1 cells preincubated with FB1 and subsequently treated with Cy3-labeled C225, Au–C225-P, or Au–C225-C. Lower panel images show the restoration of uptake of C225 and Au–C225-C, but not Au–C225-P, when incubated with GM3 (scale bar: 20 μm). b) Quantification of the restoration of uptake of C225, Au–C225-P, and Au–C225-C by the addition of GM3 to FB1-treated PANC-1 cells. c) Proposed mechanism of pathway switch as triggered by the antibody nanoconjugates Au–C225-P and Au–C225-C.

ure 3a, b).[13,14] These results suggest that C225 and Au–C225-C are internalized by a caveolar-mediated endocytosis mechanism that requires a GSL domain to build the endocytotic vesicles, whereas the uptake of Au–C225-P is primarily a fluid phase mediated process (Figure 3c).

Understanding the interaction of nanoconjugates with the cell is critical for the successful clinical translation of a nanomaterial-based, targeted drug-delivery system, and for minimizing side effects.[3b] C225 is primarily internalized into cells by a caveolar-mediated endocytosis mechanism that involves cholesterol and a GSL lipid raft microdomain at the plasma membrane.[15] Partial coverage of AuNPs with C225 allows the nanoconjugate to interact with the proteins at the plasma membrane through the available gold surface. Such interactions help the internalization of C225 through an alternative pathway that requires Cdc42 and the polymerization of actin. This hypothesis was further validated by quantifying the uptake of unconjugated AuNPs in Cdc42-null cells.[16] The uptake of unconjugated AuNPs was substantially inhibited in Cdc42-null cells (Figure S6 in the Supporting Information), which confirms the importance of an available gold surface during uptake by Cdc42-dependent pinocytosis/phagocytosis.

In conclusion, we have demonstrated that the mechanism of C225 uptake in PANC-1 cells can be shifted from a Dyn-2 dependent caveolar mechanism to a Cdc42-dependent pinocytosis/phagocytosis with properly designed nanoconjugates and appropriate loading of antibodies. Pinocytotic uptake of Au–C225-P can be reversed to the original Dyn-2-dependent caveolar pathway by fully coating the AuNP surface with C225 (Figure 3c). Tailoring the mechanism of antibody endocytosis might be useful in modulating intracellular signaling pathways and may be effective even if the cells have defects in their endocytosis and transport mechanisms.[17]

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