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Synthesis of an Artificial Cell Surface Receptor that Enables Oligohistidine Affinity Tags to Function as Metal-Dependent Cell-Penetrating Peptides

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Cell-penetrating peptides and proteins (CPPs) have emerged as important new tools for the delivery of impermeable molecules into living mammalian cells. These delivery systems have been constructed from basic segments of HIV Tat,[1] oligoguanidine-containing peptoids,[2] β-peptides,[3] and other oligocationic motifs.[4] The mechanism of cellular penetration by CPPs is primarily thought to involve endocytosis,[5] particularly with larger cargo,[6] but varies depending on the delivery system and the cell type.[7]

Oligohistidine peptides are often fused to proteins to provide affinity tags ("His tags") that facilitate protein purification. To enable these peptides to function as CPPs, we synthesized an artificial cell surface receptor (**1**) comprising the plasma membrane anchor *N*-alkyl-3β-cholesterylamine linked to the metal chelator nitrilotriacetic acid (NTA). *N*-Alkyl-3β-cholesterylamine derivatives can function as prosthetic molecules active on the surface of living mammalian cells because this steroid can insert into cellular plasma membranes, project linked headgroups from the cell surface, and rapidly cycle between the plasma membrane and intracellular endosomes, similar to many naturally occurring cell surface receptors.[8,9] The NTA motif binds tightly to nickel, cobalt, copper, and zinc dications, and immobilized metal chelate chromatography (IMAC) with NTA-linked supports is widely used to purify proteins fused to His tags.[10] Other lipids[11,12] and polymers[13] linked to NTA headgroups have also been reported.

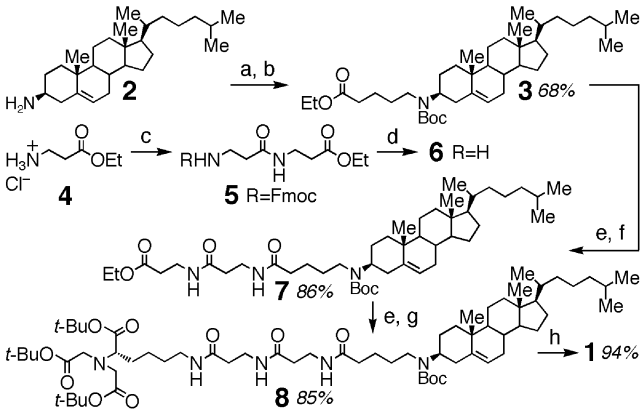
Receptor **1** was synthesized from 3β-cholesterylamine (**2**)[9] as shown in Scheme 1. Alkylation of **2** with ethyl 5-bromovalerate, followed by Boc protection to afford **3**, provided an improved route to protected *N*-alkyl derivatives of the membrane anchor. Addition of a linker shown to increase the population of these compounds on the cell surface,[9] acylation with a protected NTA derivative, and final deprotection afforded **1** in 47% overall yield.

As representative His-tagged proteins, we overexpressed in *E. coli* the monomeric green fluorescent protein AcGFP fused to C-terminal $(His)_6$ and $(His)_{10}$ peptides and purified these proteins by IMAC. The $(His)_{10}$ peptide binds Ni−NTA complexes ~6-fold more tightly than $(His)_6$. For example, $(His)_6$ and $(His)_{10}$ peptides fused to $5HT_3R$ serotonin receptors exhibit affinities for Ni−NTA derivatives of $K_d = 1.05$ μM and $K_d = 166$ nM, respectively.[14]

To qualitatively examine the ability of receptor **1** to mediate the cellular uptake of His-tagged proteins, human Jurkat T-lymphocytes were treated with **1** (10 μM) for 1 h to load this compound into the outer leaflet of the cellular plasma membrane. These cells were washed and subsequently treated with a solution of $AcGFP(His)_{10}$ (3.2 μM) and $Ni(OAc)_2$ (100 μM) for an additional 4 h. As shown in Figure 2 (panel A), examination of these cells by confocal laser scanning microscopy revealed fluorescent protein both on cell surface and in defined intracellular compartments. Washing the cells with disodium NTA (400 μM) as a chelator removed the bound protein from the cell surface, facilitating examination of the intracellular distribution (Figure 2, panel B). Delivery of $AcGFP(His)_6$ under these conditions provided similar results, but the cells were 4-fold less fluorescent (Figure 3, panel D). The fate of internalized $AcGFP(His)_{10}$ was examined by coadministration with DiI-loaded low-density lipoprotein (LDL), a protein internalized by receptor-mediated endocytosis, as a red fluorescent marker of endosomes and lysosomes.[15] Substantial colocalization of red and green fluorophores was observed under these conditions (Supporting Information). This delivery to endosomes and lysosomes represents a common destination of proteins fused to CPPs.[16]

Quantitative analysis by flow cytometry of synthetic receptor-mediated uptake is shown in Figure 3. Intracellular delivery of $AcGFP(His)_{10}$ >600-fold above basal levels of endocytosis was achieved by preloading the cellular plasma membrane with receptor **1** (10 μM) for 1 h followed by addition of the protein (3.2 μM) and $Ni(OAc)_2$ (100 μM). Higher receptor concentrations, addition

Scheme 1 [a]



[a] Reagents and conditions: (a) ethyl 5-bromovalerate, K_2CO_3, DMF, 60 °C; (b) $(Boc)_2O$, DIEA, CH_2Cl_2; (c) *N*-Fmoc-β-Ala, EDC, HOBt, DIEA, CH_2Cl_2; (d) piperidine, DMF; (e) $LiOH{\cdot}H_2O$, MeOH/THF/H_2O (3:2:1); (f) **6**, EDC, HOBt, CH_2Cl_2; (g) N^{α},N^{α}-bis[(*tert*-butoxycarbonyl)methyl]-L-Lys *tert*-butyl ester, EDC, HOBt, CH_2Cl_2; (h) TFA, CH_2Cl_2.

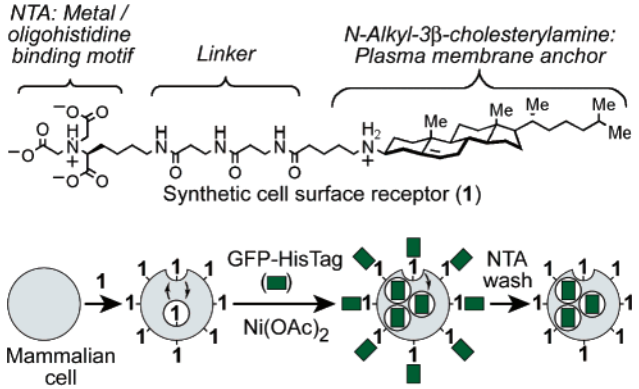


Figure 1. Top: Structure of the synthetic cell surface receptor (**1**) that binds metals and His tags. Bottom: Strategy for delivery of His-tagged proteins into mammalian cells by synthetic receptor-mediated endocytosis.

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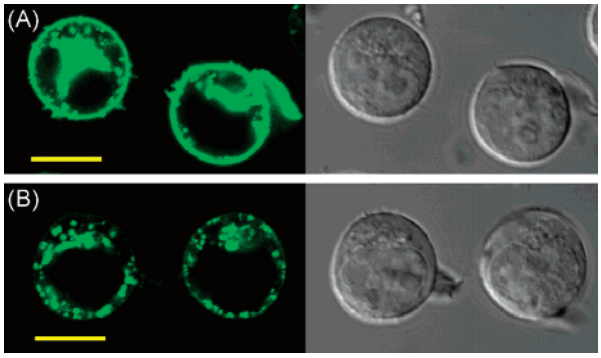

Figure 2. Confocal laser scanning (left) and differential interference contrast (right) micrographs of living Jurkat lymphocytes. Cellular plasma membranes were preloaded with receptor **1** (10 μM) for 1 h at 37 °C, cells were washed with fresh media, and media containing AcGFP(His)$_{10}$ (3.2 μM) and Ni(OAc)$_2$ (100 μM) was added for an additional 4 h at 37 °C. Cells shown in panel A were imaged immediately after this treatment. Cells shown in panel B were washed with NTA (400 μM, 30 min) prior to microscopy to remove noninternalized protein from the cell surface. Scale bar = 10 μm.

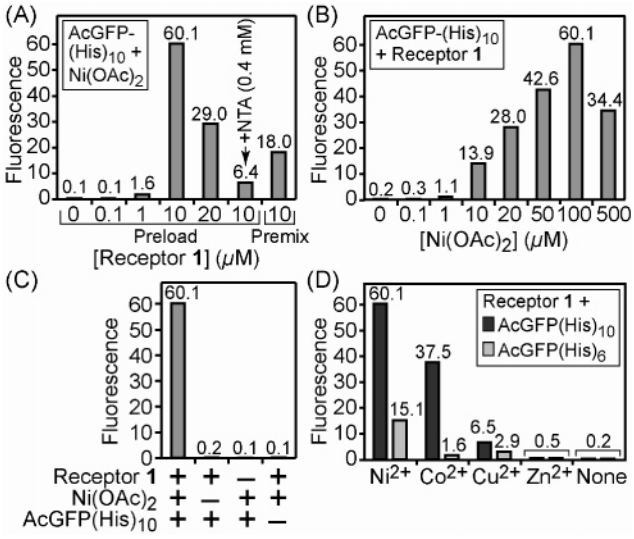

Figure 3. Analysis of cellular uptake of His-tagged AcGFP proteins by flow cytometry. Each bar represents the average fluorescence of 10 000 cells. Unless otherwise noted, cellular plasma membranes of Jurkat lymphocytes were preloaded with receptor **1** (10 μM) for 1 h at 37 °C, cells were washed with fresh media, and His-tagged AcGFP (3.2 μM)/metal diacetate solution (100 μM) was added for 4 h at 37 °C. Prior to analysis, cells were washed with NTA (400 μM, 30 min) in PBS (pH 7.4) to remove bound surface protein. Panel A: Dose dependence of receptor-mediated uptake. Premix conditions: A solution containing receptor **1**, Ni(OAc)$_2$, and AcGFP(His)$_{10}$ was added to cells for 4 h. Panel B: Dependence on [Ni(OAc)$_2$]. Panel C: Omission control experiments. Panel D: Uptake of (His)$_6$ and (His)$_{10}$ fusion proteins promoted by different metal diacetates.

of disodium NTA as a competitor, or premixing the receptor with the protein and metal resulted in lower levels of uptake (panel A). Preloading the plasma membrane may facilitate uptake due to the high surface density of immobilized metal chelate.[17,18] Significantly higher or lower concentrations of Ni(OAc)$_2$ were less effective mediators of uptake (panel B), and omission control experiments confirmed the importance of all three components (panel C). Comparison of diacetates of Ni, Co, Cu, and Zn revealed that Ni(OAc)$_2$ was the most effective metal for delivery of both (His)$_6$ and (His)$_{10}$ fusion proteins.

Consistent with the mechanism of synthetic receptor-mediated endocytosis,[9] uptake of His-tagged AcGFP proteins was blocked by cooling cells to 4 °C (data provided in the Supporting Information). These conditions enabled quantification of the number of synthetic receptors on the cell surface. Jurkat lymphocytes were treated with **1** (10 μM) for 1 h at 37 °C, the cells were washed, chilled to 4 °C, and excess AcGFP(His)$_{10}$ (19 μM) and Ni(OAc)$_2$ (100 μM) were added to saturate the surface-bound NTA groups. Comparison of these cells with fluorescent bead standards by flow cytometry revealed an average of ~45,000,000 synthetic receptors per cell surface. Previous studies of related compounds suggest that a similar population of synthetic receptors resides in intracellular endosomes.[9]

Heavy metals, such as Ni^{2+}, exhibit dose-dependent toxicity to biological systems. To examine the toxicity of this metal acetate and protein delivery, cellular viability was examined by flow cytometry. In these experiments, Jurkat lymphocytes were treated with receptor **1** (10 μM, 1 h), followed by AcGFP(His)$_{10}$ (3.2 μM), and Ni(OAc)$_2$ (100 μM, 4 h) to enhance protein uptake by 600-fold. Cells were washed with disodium NTA to remove cell surface protein and were cultured for an additional 48 h. Cellular viability was determined by light scattering and staining the nuclei of membrane-compromised dead cells with the fluorophore propidium iodide. Under these conditions, 97% of the cells were viable, compared with 99% of untreated controls, and no effects on cellular morphology were observed. Other studies have similarly reported low toxicity of nickel salts at ≤160 μM in cell culture.[19] By enabling common oligohistidine affinity tags to function as cell-penetrating peptides, synthetic receptor **1** provides a potentially versatile new probe of cellular biology.

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Supporting Information Available: Supporting figures (S1−S3), compound characterization data, and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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