Chemical Decaging

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Chemical Remodeling of Cell-Surface Sialic Acids through a Palladium-Triggered Bioorthogonal Elimination Reaction**

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Dedicated to Professor Qing Huang

Abstract: *We herein report a chemical decaging strategy for the in situ generation of neuramic acid (Neu), a unique type of sialic acid, on live cells by the use of a palladium-mediated bioorthogonal elimination reaction. Palladium nanoparticles (Pd NPs) were found to be a highly efficient and biocompatible depropargylation catalyst for the direct conversion of metabolically incorporated N-(propargyloxycarbonyl)neuramic acid (Neu5Proc) into Neu on cell-surface glycans. This conversion chemically mimics the enzymatic de-N-acetylation of N-acetylneuramic acid (Neu5Ac), a proposed mechanism for the natural occurrence of Neu on cell-surface glycans. The bioorthogonal elimination was also exploited for the manipulation of cell-surface charge by unmasking the free amine at C5 to neutralize the negatively charged carboxyl group at C1 of sialic acids.*

Sialic acids (Sias) are a family of nine-carbon-atom monosaccharides that often reside at the outmost end of cell-surface glycans.[1] Sias have more than 50 naturally occurring forms, among which Neu5Ac is the most abundant in humans.[1,2] Found at all cell surfaces of vertebrates as well as certain bacteria, Sias play critical roles in diverse cellular processes, including cell differentiation, host–pathogen interactions, and signaling transduction.[3] Furthermore, the highly prevalent Sias create negative charges on the cell membrane.[4] For example, metastatic cancer cells often express glycoproteins containing a high density of negatively charged Sias. The resulting repulsion between these late-stage cancer cells facilitates their entry into the blood stream.[5] Neu5Ac can be modified at the C5 position or at the C4, C7, C8, and C9 hydroxy groups, thus contributing to the diversity of the Sia family.[1] Besides these modifications, Neu5Ac could potentially be de-N-acetylated to give the core Neu. Recent studies supported the presence of this monosaccharide in certain cell types, particularly nerve cells and cancer cells.[6] Nevertheless, Neu is rarely encountered in nature, and whether it exists as a constituent of cell-surface oligosaccharides remains debatable. Neu is chemically unstable and thus unlikely to be enzymatically installed into glycans by the biosynthetic machinery like Neu5Ac.[7] Alternatively, Neu might be directly generated from Neu5Ac on cell-surface glycans through de-N-acetylation, presumably by certain de-N-acetylases.[7] Although this mechanism seems plausible, the existence of such enzymes remains elusive. A means to generate Neu on cell surfaces would facilitate the elucidation of the biological functions and biosynthetic mechanism of Neu. Therefore, we sought to develop such a strategy by exploiting palladium-mediated elimination reactions and methodology for metabolic glycan labeling (Figure 1).

Palladium is a powerful transition metal in catalyzing diverse chemical transformations ranging from carbon–carbon bond formation to versatile cleavage reactions.[8] Recently, some palladium-mediated elimination reactions have been exploited for unmasking an array of chemically caged molecules, including fluorophores,[9] nucleotides,[10] and amino acid side chains of intact proteins,[11] within live cells. Such methods provided a powerful bioorthogonal activation strategy for: I) turning on the fluorescence of chemically masked fluorophores (e.g. propargyl-derived fluorescein) in living cells,[9] II) spatially controlled activation of nucleotide prodrugs (e.g. 5-fluoro-1-propargyluracil) with enhanced local therapeutic effects,[10] and III) intracellular activation of enzymes to probe the downstream signaling pathways (e.g. chemically caged phosphothreonine lyase).[11] Together, these palladium-catalyzed biocompatible cleavage reactions have expanded the scope and applications of bioorthogonal reactions,[12] which have been mainly focused on biocompatible conjugation reactions in the past decade. We envisioned that such a decaging strategy could be exploited to chemically generate Neu from a metabolically incorporated caged-Neu precursor on cell-surface glycans.

The metabolic glycan-labeling method exploits the underlying biosynthetic machinery to metabolically incorporate chemically modified unnatural sugars into cellular glycans for

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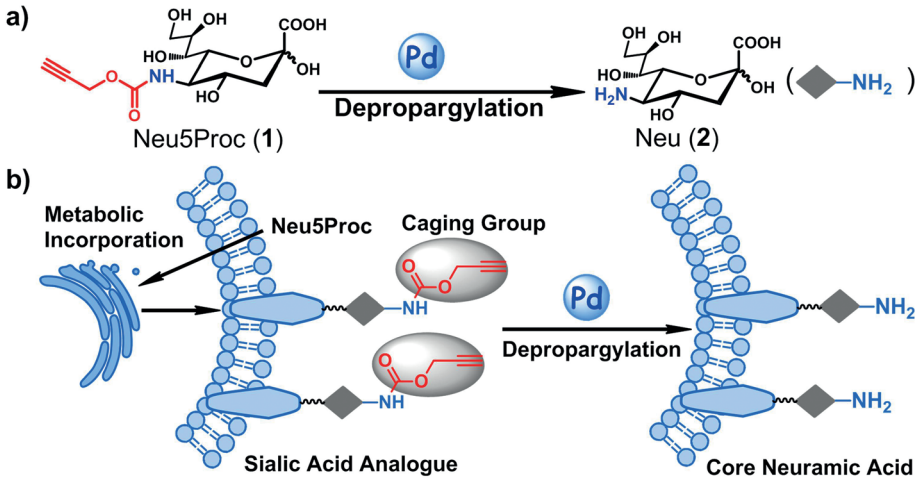

Figure 1. A chemical decaging strategy based on palladium-mediated elimination and metabolic glycan labeling for generating Neu on live cells. a) Neu5Proc serves as an analogue of Neu5Ac that can be converted into the core neuramic acid by a palladium-mediated depropargylation reaction. b) Neu5Proc can be metabolically incorporated into cell-surface sialylated glycans. Upon the addition of a Pd catalyst, the Proc group is cleaved to form Neu in situ.

diverse labeling purposes.[13] For example, the Neu5Ac biosynthetic pathway is permissive to Neu5Ac analogues containing functional groups such as azides and alkynes at the *N*-acyl position.[14] In many cases, the same labeling handles can be installed by use of the corresponding analogues of *N*-acetylmannosamine (ManNAc), the biosynthetic precursor of sialic acid.[15] The incorporated unnatural sugars can be subsequently conjugated with fluorescent probes for glycan imaging by bioorthogonal chemistry (e.g. click chemistry). Instead of bioconjugation, we sought to eliminate the *N*-acyl substituent at C5 of Neu5Ac analogues to generate Neu. In the search for an appropriate Neu5Ac analogue, we turned our attention to a ManNAc analogue, *N*-(propargyloxycarbonyl)mannosamine (ManNProc), which was recently demonstrated to be metabolically converted into the corresponding sialic acid (Neu5Proc) with high efficiency.[16] Realizing that Neu5Proc also contains a Proc group that is sensitive to the Pd catalyst, we envisioned that the cell-surface Neu5Proc could be chemically unmasked to generate Neu in situ, in a process which may resemble the aforementioned de-*N*-acetylation process (Figure 1b).

We first prepared peracetylated ManNProc (Ac_4ManNProc) by organic synthesis[16] and Neu5Proc by enzymatic conversion with recombinant *Escherichia coli* K12 aldolase. The metabolic incorporation of the resulting compounds was examined in CHO cells: Cells treated with Neu5Proc for 24 h or Ac_4ManNProc for 72 h at 37 °C were labeled with biotin azide by copper-catalyzed azide–alkyne cycloaddition (CuAAC) and stained with Alexa Fluor 488–streptavidin. A robust fluorescence signal was detected by flow cytometry in a dose-dependent manner. The incorporation of Neu5Proc increased sharply as its concentration was increased from 0.025 to 5 mM; the incorporation efficiency reached saturation at 3 mM (see Figure S1 in the Supporting Information). When Ac_4ManNProc was used, highly efficient incorporation was observed at 50 μM (see Figure S2). We therefore used Neu5Proc (3 mM) or Ac_4ManNProc (50 μM) to introduce Neu5Proc onto cell-surface glycans throughout the rest of our study.

We then searched for an efficient palladium-based depropargylation catalyst by using the *N*-Proc-caged fluorogenic coumarin dye **3** as a fluorogenic reporter. Compound **3** is virtually nonfluorescent, but can be converted into the highly fluorescent compound **4** by the cleavage of its Proc group (Figure 2a). The increased fluorescence signal from decaged coumarin **4** was recorded to obtain a kinetic curve and the decaging efficiency. Because this Proc group can be eliminated by palladium species with all possible oxidation states (Pd^0, Pd^{II}, and Pd^{IV}), we surveyed the depropargylation activity of a series of Pd catalysts in all three oxidation states.[9b,17] A total of eight Pd reagents were surveyed: C1 stands for Pd^0 nanoparticles (Pd NPs), which were prepared according to the method based on reduction with sodium borohydride and characterized by transmission electron

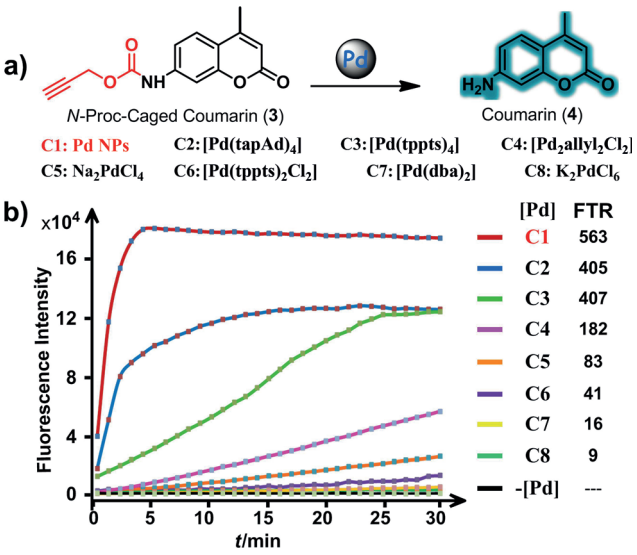

Figure 2. Catalyst screening for the palladium-triggered depropargylation reaction. a) Coumarin-based fluorogenic reporter for the depropargylation reaction. The *N*-Proc-caged coumarin derivative **3** exhibits quenched fluorescence that can be reactivated upon the removal of the Proc group to yield the coumarin dye **4** (λ_{ex} = 380 nm, λ_{em} = 450 nm). Palladium species examined in this study are shown below the scheme. For water-insoluble palladium species, stock solutions (10 mM) in dimethyl sulfoxide were prepared and were subsequently diluted into an aqueous buffer before usage. tapAd: 1,3,5-triaza-7-phosphaadamantane, tppts: tris(3-sulfophenyl)phosphine trisodium salt, [$Pd_2allyl_2Cl_2$]: allylpalladium(II) chloride dimer, dba: dibenzylideneacetone. b) Depropargylation activity of palladium catalysts (100 μM) as determined by the increase in fluorescence (corresponding to the conversion of **3** (100 μM) into compound **4**). FTR: fluorescence turn-on ratio.

microscopy (see Figure S3); C2 and C3 are two active Pd^0 catalysts with water-soluble phosphine ligands; C4 is a Pd^0 precursor, whereas C7 is an air-stable Pd^0 catalyst; C5 and C6 are two additional Pd^{II} catalysts without or with a phosphine ligand; C8 is a widely used and commercially available Pd^{IV} catalyst. C1–C3 exhibited higher reactivity than the other catalysts (Figure 2b). In particular, Pd NPs (C1) demonstrated the highest reactivity, with a fluorescence turn-on ratio (FTR) as high as 563-fold that of the control, and reached saturation within 4 min. The high cleavage efficiency may be due to active Pd atoms or Pd clusters that have been leached from this heterogeneous catalyst. The depropargylation activity of these Pd species was next examined directly on Neu5Proc by LC–MS analysis (see Figure S4). As expected, Pd NPs exhibited the best cleavage efficiency (> 95% yield within 4 min). Finally, a toxicity study indicated that CHO cells and Jurkat cells had high viability even in the presence of Pd NPs at a concentration of 500 μM (10 times of our working concentration; see Figures S5 and S6). Taken together, these results show that Pd NPs are highly efficient and biocompatible catalysts for the depropargylation of Neu5Proc.

We next applied Pd NPs for the direct conversion of Neu5Proc into Neu on cell-surface glycans. We developed a fluorescence assay to characterize the depropargylation reaction on cell surfaces by monitoring the diminishment of the terminal alkyne (Figure 3a). The cells incorporating Neu5Proc were treated with biotin azide and stained with Alexa Fluor 488–streptavidin. Analysis by flow cytometry showed that the fluorescence intensity of palladium-treated cells was significantly lower than that of those without Pd treatment. In particular, Pd NPs (C1) exhibited the highest cleavage efficiency of the Pd catalysts tested (Figure 3b; see also Figure S7), in agreement with the in vitro assay results described above. Furthermore, the depropargylation on cell surfaces was imaged by confocal fluorescence microscopy. Significantly lowered fluorescence was observed when the CHO cells incubated with Ac_4ManNProc were treated with Pd NPs, in agreement with the results of flow cytometry (Figure 3c). When CHO cells treated with peracetylated *N*-(4-pentynoyl)mannosamine (Ac_4ManNAl), a ManNAc analogue containing a noncleavable terminal alkyne moiety, was used as a negative control, no significant fluorescence change was observed upon treatment with Pd NPs (Figure 3c; see also Figure S8). Similar results were observed in HeLa cells (see Figure S9). These results collectively confirmed that the fluorescence decrease was caused by palladium-mediated depropargylation rather than damage of the alkynyl group by Pd species.

To further confirm the reaction products of palladium-triggered Neu5Proc depropargylation on cell surfaces and quantify the reaction efficiency, we developed a LC–MS analysis method (Figure 4a). The cell-surface Sia could be released from sialylated glycoproteins and quantified by LC–MS. For Neu, we designed a MS tag, 4-(dimethylamino)benzoic acid succinimidyl ester (DMABA-NHS), to facilitate detection (Figure 4b). DMABA-NHS forms an amide bond with Neu to block the free amino groups, therefore avoiding side reactions of amines with ketones and aldehydes in the acidic hydrolysis medium used for releasing Neu from

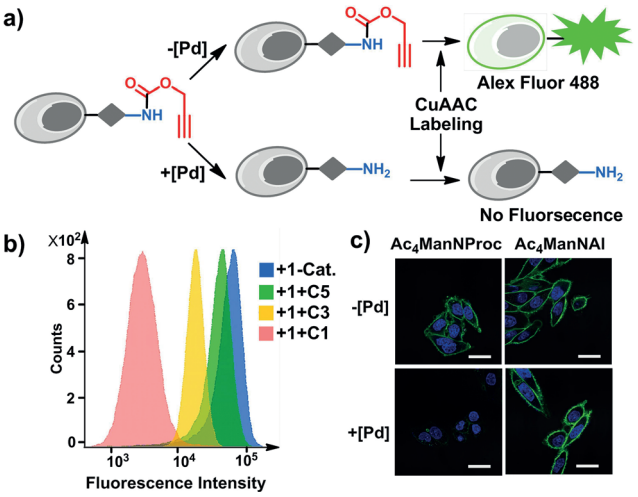

Figure 3. Palladium-mediated Neu5Proc depropargylation on live cells. a) Scheme describing the fluorescence assay for Neu5Proc depropargylation. The cells with incorporated Neu5Proc were treated with a Pd catalyst or with a vehicle. The presence of alkynes on the surface of cells was then assessed by CuAAC-mediated fluorescence labeling, followed by flow cytometry and confocal fluorescence imaging. b) CHO cells were incubated with Neu5Proc (3 mM) for 24 h, followed by treatment with a Pd catalyst or a vehicle for 5 min. The cells were then treated with biotin azide, stained with Alexa Fluor 488–streptavidin, and analyzed by flow cytometry. c) CHO cells were treated with Ac_4ManNProc (50 μM) or Ac_4ManNAl (50 μM) for 72 h, followed by treatment with Pd NPs or a vehicle for 5 min. After CuACC-mediated fluorescence labeling, the cells were imaged by confocal fluorescence microscopy. The nuclei were visualized by staining with Hoechst 33342 (blue signal). Scale bars: 20 μm.

glycoconjugates (see Figure S10). Moreover, DMABA-derivatized Neu showed a distinct MS peak due to the presence of the dimethylamino group. CHO cells displaying Neu5Proc were depropargylated by Pd NPs and treated with DMABA-NHS. Cells were then lysed, and Sias were released and collected for LC–MS analysis in the selected-ion recording (SIR) mode. The single quadrupole detector was set for recording only the anion of interest (*m*/*z* 348: $[Neu5Proc\text{-}H]^-$), and the ion intensity of $[Neu5Proc\text{-}H]^-$ decreased 2.4-fold after depropargylation triggered by Pd NPs on Neu5Proc-displaying CHO cells (Figure 4c). The same sample was then subjected to selected-cation LC–MS analysis for $[(Neu \approx MS\ tag)+H]^+$ (*m*/*z* 415; Figure 4d). The $[(Neu \approx MS\ tag)+H]^+$ peak from CHO cells treated with Pd NPs increased by a factor of 2.6 after depropargylation as compared to the control samples. The decrease in the $[Neu5Proc\text{-}H]^-$ anion peak and the increase in the $[(Neu \approx MS\ tag)+H]^+$ cation peak both confirmed that the depropargylation reaction occurred through the desired process. The cleavage efficiency from two independent experiments was averaged to 71% according to the decline in the $[Neu5Proc\text{-}H]^-$ peak of cell-surface glycans.

Finally, we sought to demonstrate that the palladium-mediated conversion of Neu5Proc into Neu could be utilized to manipulate the cell-surface charge. The negatively charged carboxyl groups on Sias create negative charges on the plasma membrane;[4] these charges are particularly important for producing strong cell–cell repulsion among Sia-overexpress-

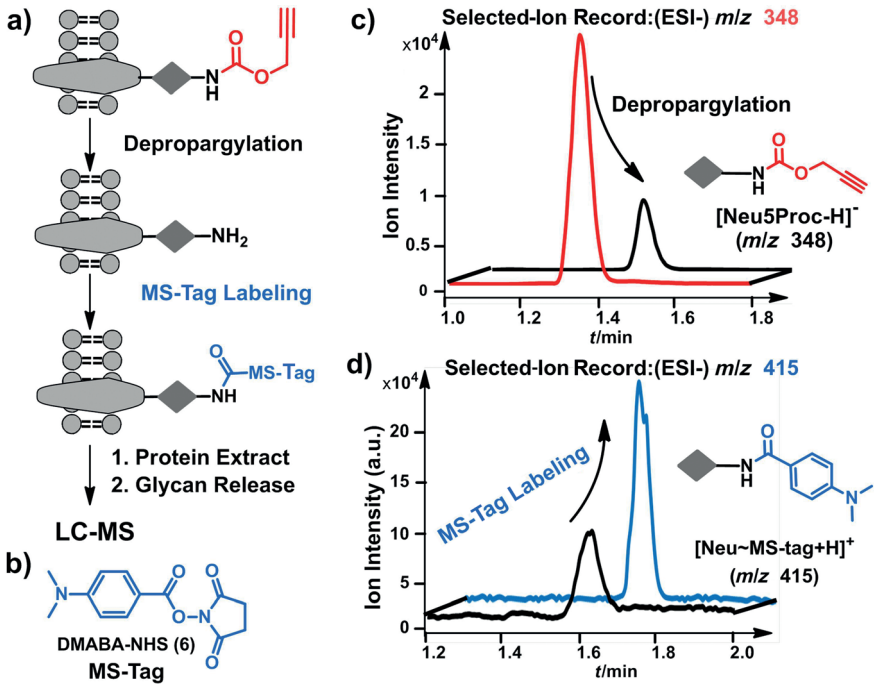

Figure 4. Quantification of Neu5Proc-depropargylation efficiency on the surface of live cells by LC–MS. a) Work flow for quantifying the efficiency of palladium-mediated Neu5Proc depropargylation by LC–MS analysis. Compound **6**, which can block the liberated free amine after the depropargylation of Neu5Proc, was used as the MS tag. A selected-ion recording mode was then used to remove the MS signals due to unwanted molecules. b) Structure of the DMABA MS tag. c,d) Chromatographic signal of selected ions ([Neu5Proc-H]$^-$ and [NeuNH$\approx$MS-tag+H]$^+$). CHO cells were incubated with Ac_4ManNProc (50 μM) for 72 h, and the surface Neu5Proc and Neu (with MS tag) were detected by LC–MS after the depropargylation process.

ing cancer cells.[5] We envisioned that the depropargylation of Neu5Proc ($pK_a = 2.74$; see Figure S11) would produce Neu containing a free amine at the C5 position with strong proton affinity ($pK_{a1} = 2.44$, $pK_{a2} = 9.33$; see Figure S11), thus partially neutralizing the carboxyl-derived negative charge on cell surfaces (Figure 5a). We tested this hypothesis on K20 cells, a subclone of the human B lymphoma cell line BJA-B that is deficient in the de novo biosynthesis of Sias.[18] K20 cells therefore contain no Sias on the cell surface and are able to uptake Neu5Ac or its analogues from the culture medium and incorporate them into cell-surface glycans. K20 cells incorporating Neu5Proc were treated with Pd NPs, and the ζ potential was measured before and after the Pd-NP-triggered depropargylation. The cell-surface ζ potential was shifted to a less negative value, thus indicating the generation of free amine groups (Figure 5b, top). As a control, K20 cells with no Neu5Proc supplementation exhibited a less negative ζ potential, which was not affected by treatment with Pd NPs (Figure 5b, bottom). The modulation of cell-surface charge was also possible in Jurkat cells (see Figures S12 and S13). Interestingly, neutralization of the negative charge may render the cells prone to clustering (Figure 5a). As expected, Jurkat cells incorporating Neu5Proc formed significantly larger clusters after treatment with Pd NPs (Figure 5c; see also Figure S14), whereas for cells without Neu5Proc incorporation, the treatment with Pd NPs did not alter clustering. Taken together, these results show that the palladium-mediated depropargylation on Neu5Proc could liberate a free amine in the negatively charged Sias, thus offering a chemical strategy for the manipulation of cell-surface charge and cell clustering.

In summary, bioorthogonal decaging strategies offer a facile approach for the in situ activation or liberation of biomolecules under physiological conditions. Although the photodecaging strategy is another efficient and non-invasive method for the rapid uncaging of biomolecules, such as proteins,[19] the bulky photocaging groups are usually not compatible with metabolic engineering, which relies on the limited tolerance of the cellular biosynthetic machinery to modifications of natural substrates.[2] In contrast, the Proc group utilized in this study is small in size and tolerated by the sialic acid biosynthetic pathway. Proc is chemically inert and becomes sensitive only in the presence of a palladium catalyst. To our knowledge, the reported in situ conversion of Neu5Proc into Neu is the first example of the creation of the core nine-carbon-atom sugar Neu on the surface of live cells. This transformation may facilitate the study of the functional roles of Neu under various physiological and/or pathological conditions. Notably, Neu5Ac is a key residue on cell-surface glycans that mediates the interaction between influenza virions and host cells.[20] Whether Neu on host cells can be directly recognized by the Neu5Ac-binding proteins of the virion coat remains to be investigated. Finally, metabolic engineering of cell-surface sialic acids has been previously exploited for modulating cell–cell interactions[21] and controlling stem-cell fate.[22] Our strategy provides a simple and straightforward approach to trigger cell clustering by manipulating the cell-surface charge, with temporal control enabled by chemical decaging. Future applications of our method include cell and tissue engineering for regenerative medicine.[23]

Keywords: bioorthogonal elimination · cell surfaces · metabolic oligosaccharide engineering · palladium · sialic acids

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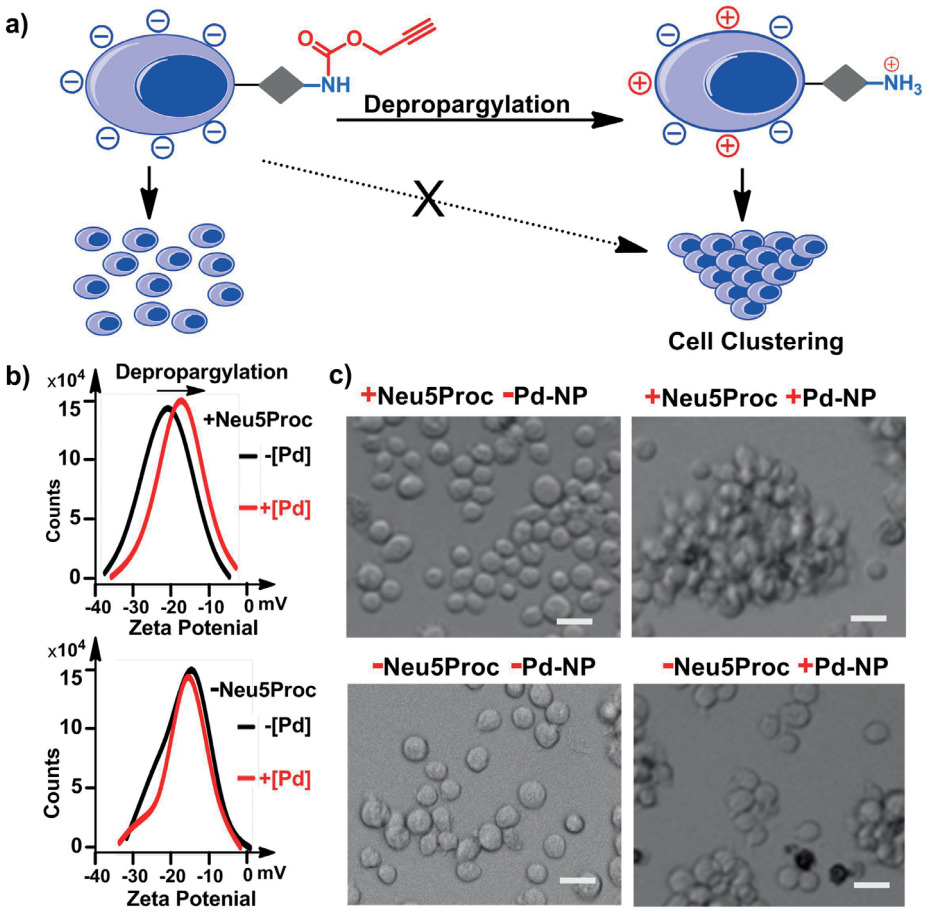

Figure 5. Palladium-triggered Neu5Proc decaging for the manipulation of cell-surface charge and cell clustering. a) The palladium-mediated in situ conversion of Neu5Proc into Neu could generate a free amine at C5 and thus alter cell-surface charge and cause increased propensity for cell clustering. b) Measurement of the ζ-potential distribution of Neu5Proc-incorporating (top) and native (bottom) BJA-B K20 cells to determine their surface-charge variation before and after treatment with Pd NPs. c) Confocal microscopy imaging of Neu5Proc-incorporating (top) and native (bottom) Jurkat cells before and after treatment with Pd NPs. Palladium-mediated depropargylation of surface-displayed Neu5Proc caused significant cell clustering that was not observed without Pd NPs or Neu5Proc. Scale bars: 10 μm.

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