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How Do Cells Exocytose Nanoparticles?

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Cite This: *Biochemistry* 2023, 62, 2229–2231 Read Online

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When designing nanomedicines, understanding their clearance from mammalian cells into culture medium following initial cellular entry is desirable. Nanomedicines require a sufficiently long intracellular residency time to exert their efficacy, yet their prolonged intracellular accumulation may induce cytotoxicity, inflammatory response, or cell stress. Yet, past studies on nanoparticle (NP) exocytosis were uncommon partly because most internalized NPs hardly exit the cell. Rather, NPs may experience degradation by intracellular nucleases or oxidases, entrapment inside acidic compartments (late endosomes or lysosomes), or aggregation that disfavors exocytosis. Quantitative studies on the percentage of NP exocytosis (Exo%; i.e., fraction of internalized NPs exocytosed by the cell) from different cell types remained scarce.

In 2020, we reported in *Biochemistry* an NP-based platform for quantifying the extent of NP exocytosis.[1] This platform contains a gold (Au) NP core that allows for measuring the cellular contents of Au by inductively coupled plasma mass spectrometry (ICP-MS) and imaging their subcellular localization by label-free confocal reflectance microscopy and transmission electron microscopy (TEM). We used this platform for probing the Exo% values on three different cell types (macrophages, endothelial cells, and cancer epithelial cells), and we surface-modified the platform with 12 different types of hydrocarbyl groups (straight-chain alkane, branched-chain alkane, cycloalkane, alkene, and aromatic groups; Figure 1). A key design aspect of our platform is its overall hydrophilicity, enabling us to decouple the effects of a given hydrocarbyl group and hydrophobicity. Our interest in hydrocarbyl groups stems from their prevalence in contemporary building blocks of nanomedicines, such as cell-penetrating peptides to deliver Cas9 protein and gRNA for genome editing and lipid NPs that form the basis of mRNA-based COVID-19 vaccines. Thus, insights into how hydrocarbyl groups affect NP−cell interactions are valuable. Also, we previously reported that the dodecyl group, when attached to the surfaces of NPs at low coverage (4 mol %), can boost the cellular entry of NPs by 30-fold in vitro[2] and drive the preferential association of inhaled NPs to alveolar macrophages in vivo.[3] Remarkably, we observed a strong dependence of Exo% on cell type and hydrocarbyl group. Of all hydrocarbyl groups tested, NPs with cyclododecyl groups exhibit high Exo% on macrophages and endothelial cells yet low Exo% on cancer cells. We postulate that NPs with cyclododecyl groups are attractive NP-based drug carriers; in the body, they can effectively exit scavenging cells (e.g., macrophages and endothelial cells in the liver) and

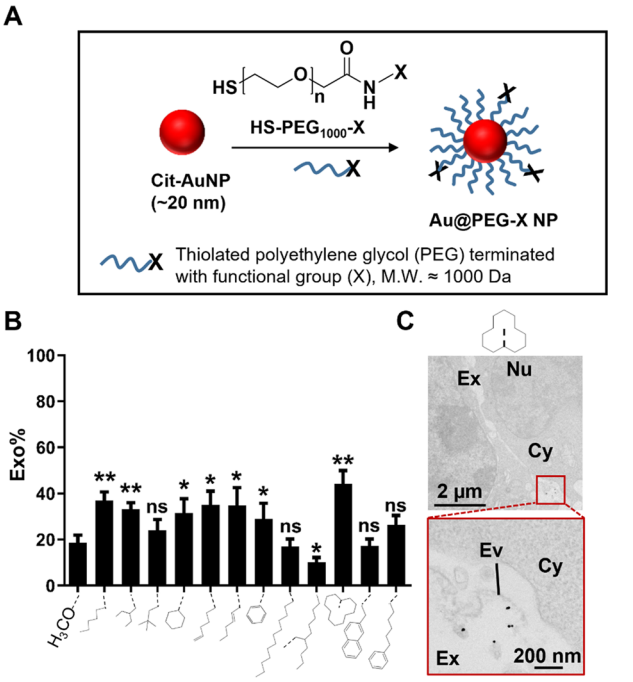

Figure 1. Exocytosis of hydrocarbyl functional group (X)-terminated, polyethylene-glycol-coated gold NPs (Au@PEG-X NPs) by mouse RAW 264.7 macrophages. (A) Schematic illustration of the preparation of Au@PEG-X NPs by conjugating thiol- and X-terminated bifunctional PEG strands ($HS\text{-}PEG_{1000}\text{-}X$) to 20 nm citrate-capped AuNPs (cit-AuNPs). (B) Cells were incubated with 1 nM NPs for 6 h, followed by allowing them to exocytose NPs to fresh NP-free medium for 24 h. The amounts of NPs exocytosed to the medium were measured by ICP-MS. Exo% is the percentage of exocytosis. Statistical analysis compared to Au@PEG-methoxy NPs (without any hydrocarbyl groups) was determined by one-way analysis of variance. No significance (ns); $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$. Error bar denotes the SD from three experiments. (C) Representative TEM images of extracellular Au@PEG-cyclododecyl NPs exocytosed from RAW264.7 cells. The lower image (red border) shows the enlargement of the boxed area of the upper image. Legend: Nu, nucleus; Cy, cytosol; Ex, extracellular space; Ev, extracellular vesicle. Reprinted with permission from ref 1. Copyright © 2020, American Chemical Society.

Received: June 16, 2023
Published: July 18, 2023

https://doi.org/10.1021/acs.biochem.3c00312
Biochemistry 2023, 62, 2229−2231

remain inside target cancer cells. There were two limitations in our report. Our data were phenomenological, as they did not address the biological mechanism for NP exocytosis, say the pathway of exocytosis and cellular trafficking machinery involved. Also, our data were based on techniques that utilize fixed or dead cells, so capturing the dynamic movement of NPs or materials secreted to the medium from the cell (or re-entering the cell from the medium) was challenging.

Subsequently, we addressed the first limitation by probing the exocytosis mechanism of NPs that were surface-modified with dodecyl groups and investigated the extracellular vesicles (EVs) that were secreted alongside the exocytosed NPs.[4] We envisaged that EVs would offer crucial clues to the mechanism for NP exocytosis because NPs typically reside in intracellular vesicles before exocytosis. Notably, we discovered that an even lower dodecyl coverage density of 0.5 mol % not only enhanced Exo% by up to 4 times but also upregulated genes related to exocytosis and vesicles upon treatment with cells. After trafficking to late endosomes (and multivesicular bodies), such NPs exit the cell predominantly via enhanced secretion of sub-100 nm, CD81-enriched exosomes, as confirmed by TEM imaging and Western blot analysis of the secreted EVs. Conversely, when we increased dodecyl coverage density to 4 mol %, Exo% dropped severely, and such NPs mostly traffic to lysosomes before exiting the cell via lysosome-mediated exocytosis, as verified by TEM imaging and pharmacological stimulation of lysosomes that triggered more EV secretion. Thus, we concluded that dodecyl loading affects the probability and pathway for NP exocytosis. Our work provides evidence of EVs as a pathway of NP exocytosis, besides their established roles in cell−cell communication and drug delivery. From a bioengineering perspective, our results suggest that treating cells with different coverages of hydrocarbyl groups on the NP surface will lead to the secretion of different amounts and types of EVs. In theory, we may harness the cells as "factories" by using NPs as triggers for producing subpopulations of EVs for specific biomedical applications, say exosomes for wound healing. Still, manufacturers and users of EVs should appreciate that the profile of EVs produced will depend on the cell type; our *Biochemistry* work reveals that different cell types could exocytose NPs modified with the same functional group at different probabilities.

To tackle the second limitation, we later developed a molecular probe to visualize the secretion of EVs from a living cell in situ (in cell-seeded medium without the need for prior harvesting of EVs) (Figure 2).[5] Our boron dipyrromethene (BODIPY) probe fluoresces under acidic conditions (acidic organelles and EVs), but it assumes a nonfluorescent, leuco-BODIPY form under alkaline conditions (medium); such pH sensitivity enabled selective imaging of intracellular precursor EVs in the cytosol and EVs in the medium with minimal cytotoxicity or false positive signals. After the probe enters the cell by diffusion, it will accumulate inside acidic organelles and emit fluorescence. Next, we detected by confocal imaging the real-time secretion of extracellular multivesicular bodies and, when paired with immunofluorescence labeling of the cytoskeleton, the transport of EVs along cellular pseudopodia before secretion via microtubules. Our probe will inspire more studies on dissecting the pathway of NP exocytosis.

Moving forward, understanding how cells process and clear NPs in vivo will not only yield critical insights into the safety and efficacy of nanomedicines but also establish the translatability of past in vitro reports on NP exocytosis. With studies on how certain cell types (e.g., renal tubules) exocytose NPs in vivo on the horizon, we encourage the cell biology, nanomaterials, and imaging communities to jointly unravel the complex yet exciting roles of exocytosed materials (both injected NPs and secreted EVs) in therapy and pathophysiology.

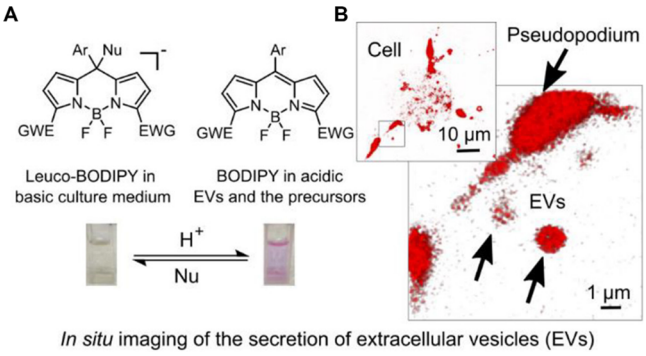

Figure 2. In situ detection of EVs in cell culture medium by a pH-reversible fluorescent probe. (A) Reversible transformation between nonfluorescent leuco-BODIPY in near-neutral culture medium (pH ≈ 7.4) and fluorescent BODIPY upon acidification to pH 7. Nu: nucleophile. (B) Real-time formation of EVs as detected by this probe. HeLa cells were treated with leuco-BODIPY and imaged by confocal microscopy to generate time-series images. The lower image shows the enlarged image of the boxed area in the upper image. Arrows indicate real-time accumulation of EVs. Reprinted with permission from ref 5. Copyright © 2021, American Chemical Society.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was in part supported by the Research Matching Grant Scheme and a General Research Fund (no.: 14300120) from the Research Grants Council and a Vice-Chancellor Discretionary Fund from The Chinese University of Hong Kong (CUHK). C.H.J.C. acknowledges a Croucher Innovation Award from the Croucher Foundation and the Chow Yuk Ho Technology Centre for Innovative Medicine from CUHK.

https://doi.org/10.1021/acs.biochem.3c00312
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