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Time-Resolved Proteomic Visualization of Dendrimer Cellular Entry and Trafficking

Linna Wang,[†,⊥] Li Yang,[†,⊥] Li Pan,[‡] Naveen Reddy Kadasala,[§] Liang Xue,[†] Robert J. Schuster,[‡] Laurie L. Parker,[‡,#] Alexander Wei,[*,§,∥] and W. Andy Tao[*,†,‡,∥]

[†]Department of Biochemistry, [‡]Department of Medicinal Chemistry & Molecular Pharmacology, and [§]Department of Chemistry, Purdue University, West Lafayette, Indiana 47907, United States

[∥]Center for Cancer Research, Purdue University, West Lafayette, Indiana 47907, United States

Supporting Information

ABSTRACT: Our understanding of the complex cell entry pathways would greatly benefit from a comprehensive characterization of key proteins involved in this dynamic process. Here we devise a novel proteomic strategy named TITAN (Tracing Internalization and TrAfficking of Nanomaterials) to reveal real-time protein−dendrimer interactions using a systems biology approach. Dendrimers functionalized with photoreactive cross-linkers were internalized by HeLa cells and irradiated at set time intervals, then isolated and subjected to quantitative proteomics. In total, 809 interacting proteins cross-linked with dendrimers were determined by TITAN in a detailed temporal manner during dendrimer internalization, traceable to at least two major endocytic mechanisms, clathrin-mediated and caveolar/raft-mediated endocytosis. The direct involvement of the two pathways was further established by the inhibitory effect of dynasore on dendrimer uptake and changes in temporal profiles of key proteins.

Nanoparticles are being actively studied and developed due to their capabilities to penetrate physiological barriers.[1] Our understanding of nanoparticle internalization and cellular trafficking would greatly benefit from a detailed, time-resolved characterization of key proteins involved in this dynamic process. However, it remains challenging to obtain the comprehensive information at the molecular level using traditional technologies.[2] Imaging methods coupling with perturbations of cells with inhibitors, siRNA silencing, or mutations have been used to show that endocytosis provides a major route for cellular entry for different types of nanoparticles.[3,4] There have also been many attempts to decipher mechanisms of nanoparticle uptake using surface modification,[5−7] such as by fluorescent or spectroscopic tracking of surface labels.[8,9] More recently, proteomic studies have been employed to study cellular response to nanoparticles as well as their trafficking.[10−13] However, a systematic quantitative analysis of temporal changes in nanoparticle−protein interactions would be critical for an improved understanding of this highly dynamic and complex cellular process.

Although dendrimers have been extensively studied as nanosized drug delivery vehicles, the molecular information on dendrimer uptake remains limited and mostly characterized at the organelle level. For example, immunohistochemical methods have identified a handful of biomarkers that colocalize with dendrimers during uptake.[14] Previous studies based on flow cytometry and imaging approaches also suggested the cellular internalization of dendrimers through clathrin-mediated and caveolar/raft-mediated endocytosis.[15,16] Here we introduce a novel proteomic strategy termed TITAN (Tracing Internalization and TrAfficking of Nanomaterials) to reveal the spatiotemporal distribution of the key proteins involved in the pathways for dendrimer entry and trafficking. We modified polyamidoamine generation 3 (PAMAM G3) dendrimers with a fluorescent tag, a photoreactive cross-linker, and a covalent "handler" to track, capture, and isolate interacting proteins in a time-resolved manner throughout the course of endocytosis (Figure 1A). This multifunctional reagent spontaneously assembles into nanosized aggregates (Supporting Information) and enables us to obtain information on nanoparticle uptake at the cellular, subcellular, and molecular levels by tandem optical microscopy and mass spectrometric analysis.

As illustrated in Figure 1B, engineered dendrimers were added to HeLa cells and incubated for set intervals ranging from 0.5 to 2 h, as determined by confocal microscopy (Figure S1) as well as earlier studies.[17] Proteins directly involved in nanoparticle uptake and trafficking were covalently captured with dendrimers upon UV irradiation, and isolated and purified under conditions optimized for minimum contamination by non-cross-linked proteins (SI and Figure S2). We reasoned that cross-linkers with a spacer length of 12.5 Å only react with proteins in direct contact with the dendrimer, which can subsequently withstand vigorous washing conditions. Specifically, covalent cross-linking and aldehyde-hydrazide bioconjugation can withstand treatments with 2% SDS, 8 M urea, and 3 M NaCl, all used in the removal of nonspecifically bound proteins. The tryptic peptides derived from enriched samples were then analyzed by nanoflow HPLC coupled to high-resolution mass spectrometry. Label-free quantification was achieved with a library of 160 synthetic peptides spiked as internal standards to differentiate the interacting proteins cross-linked with the dendrimer from nonspecific ones.[18] Proteins with at least twice the abundance in UV-treated samples relative to negative controls (without UV irradiation) were considered as proteins cross-linked with the dendrimer, thus the specific interacting proteins (SI and Table

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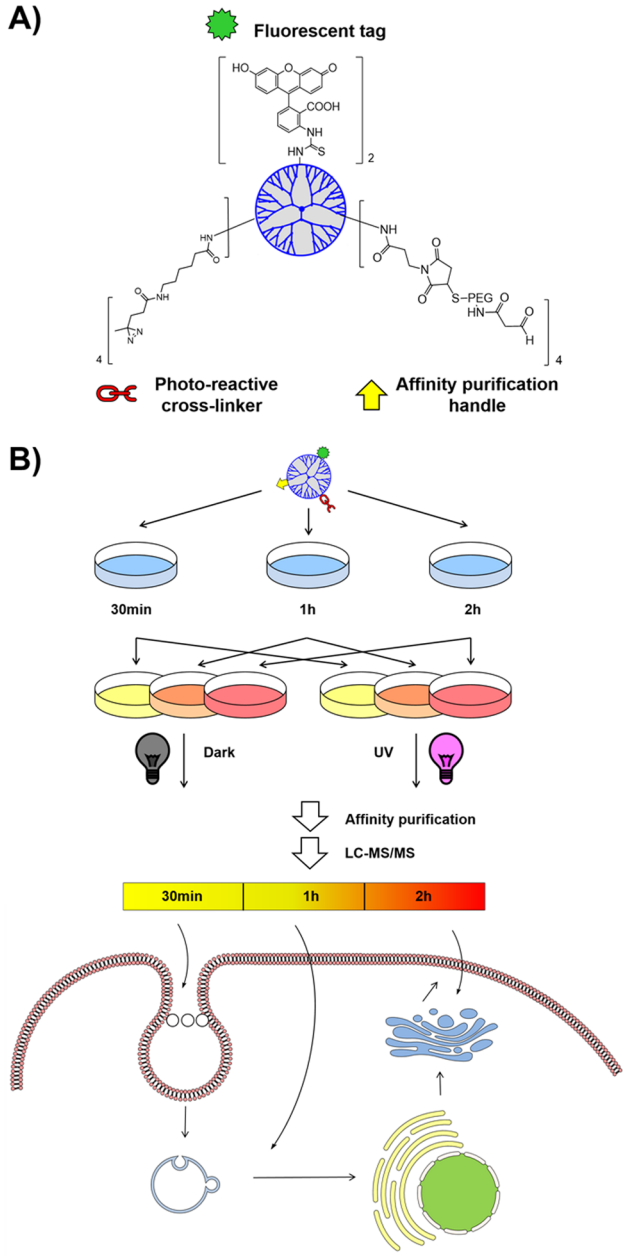

Figure 1. (A) Schematic representation of the functionalized dendrimer. (B) Experimental workflow for TITAN analysis.

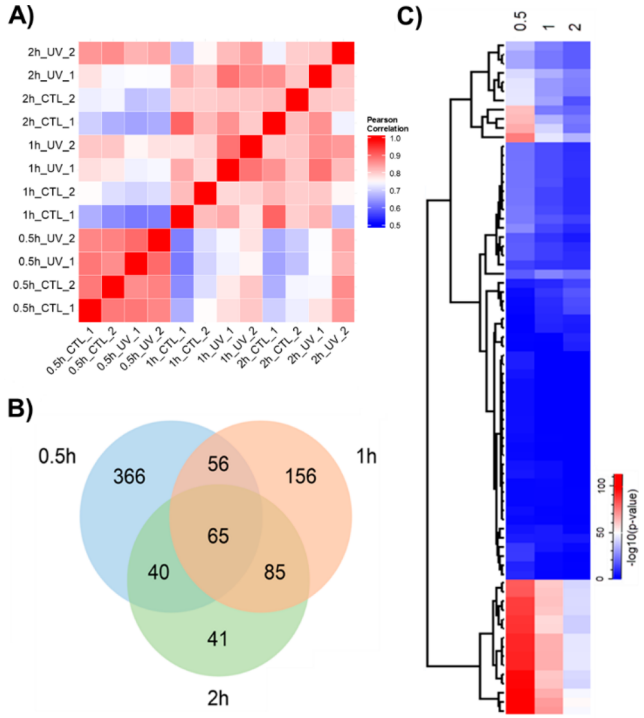

Figure 2. (A) Pearson correlations between the two biological replicates. (B) Venn diagram of the cross-linked proteins at the three time points. (C) Statistical overrepresentation test based on Gene Ontology Cellular Component.

S1). Our label-free quantitative data from the two independent biological replicates correlated well for each time point (Figure 2A), and a large majority of the cross-linked proteins were found in the overlaps between the replicates (Figure S3). In order to increase the confidence level of the cross-linked proteins being true dendrimer-interacting proteins, we only chose those overlapped proteins for follow-up data mining.

In total, TITAN analysis identified 809 cross-linked proteins in biological replicates over the three time points during dendrimer internalization into HeLa cells. Among them, 366 (45.2%), 156 (19.3%), and 41 (5.1%) proteins were exclusively captured at 0.5, 1, and 2 h, respectively, suggesting that TITAN analysis was able to reveal the temporal distribution of the interacting proteins cross-linked with the dendrimer among the three time points (Figure 2B and Table S2).

To further investigate whether TITAN analysis was also capable of correlating spatial information with the captured cross-linked proteins, we performed the PANTHER test, which determines whether there is statistical overrepresentation of specific genes or proteins in the sample relative to a reference list (e.g., the entire human genome), based on Gene Ontology Cellular Component (GOCC).[19] As shown in Figure 2C and Table S3, the proteins with the highest levels of enrichment originated from extracellular regions (including vesicles, organelles, and exosomes) or from membrane-bound vesicles or organelles, especially at 0.5 and 1 h.

Previous studies using flow cytometry and imaging techniques showed decreased dendrimer uptake upon inhibition of clathrin or caveolar, suggesting the involvement of the clathrin- and caveolar/raft-mediated endocytosis.[15,16] Therefore, we carefully examined our proteomic data for all known proteins involved in the two endocytic mechanisms for dendrimer internalization.

The most commonly observed endocytic pathway, clathrin-mediated endocytosis (CME), is correlated with the constitutive internalization of the transferrin receptor (TFRC)[20] and is also characterized by the formation of clathrin-coated pits (CCPs) on the membrane surface, induced by proteins such as AP2 adaptor complex, clathrin, and adaptor protein disabled-2 (DAB2). The CCPs are then released from the plasma membrane by the scission of dynamin, followed by the decoating of clathrin via the action of auxilin and heat shock protein 8 (HSPA8), leading to the formation of clathrin-coated vesicles (CCVs).

Since clathrin-coated pits and vesicles form rapidly,[21] the proteins involved in this process are expected to cross-link with the dendrimer at an early stage. TITAN indeed showed that transferrin receptor (TFRC) and proteins involved in CCP formation such as clathrin (CLTC) and adaptor proteins (AP2A1 and DAB2) were captured exclusively at the 0.5 h

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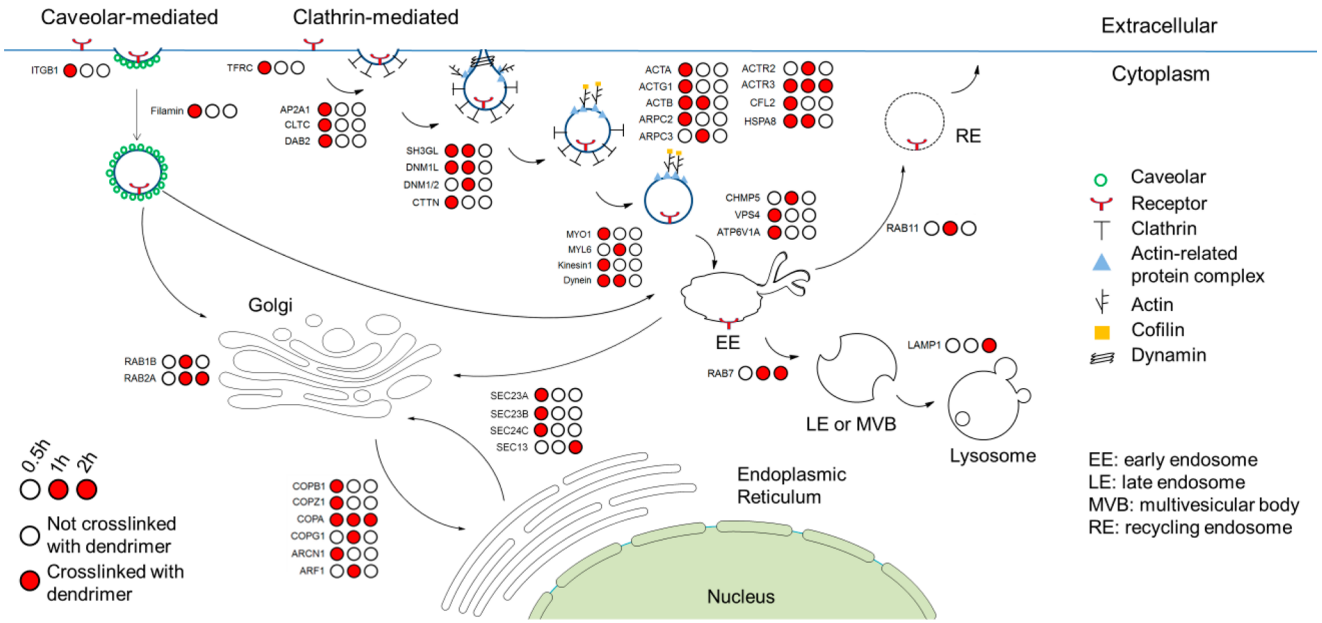

Figure 3. Spatiotemporal information for dendrimer-interacting proteins involved in the clathrin- and caveolar/raft-mediated endocytic pathways revealed by TITAN analysis.

time point. In addition, proteins involved in the scission of CCPs (e.g., dynamin (DNM1L and DNM1/2), endophilin (SH3GL), and cortactin (CTTN)), and the decoating of clathrin (e.g., HSPA8) were also captured at 0.5 or 1 h (Figure 3). Actin and actin-associated proteins are well-known to play essential roles in the movement of the plasma membrane and the formation of CCPs or CCVs.[21] Consistently, TITAN analysis was able to find actin proteins (e.g., ACTA, ACTG1, and ACTB), actin related proteins (e.g., ARPC2, ARPC3, ACTR2, and ACTR3), and actin capping protein CFL2 at earlier time points (0.5 or 1 h).

Caveolar/raft-mediated endocytosis, another common pathway for cell internalization, was also examined as a mechanism of dendrimer uptake. Although caveolin itself was not identified in our experiments, other proteins intimately associated with the pathway pointed to its early involvement in dendrimer uptake (Figure 3). Proteins detected at 0.5 h included: (a) integrin beta-1 (ITGB1), a plasma membrane receptor that is internalized by caveolar/raft-mediated endocytosis, (b) COPI complex proteins (COPB1, COPZ1, and ARCN1),[22] associated with the caveosome during transportation to the endoplasmic reticulum (ER), and (c) COPII complex proteins (SEC23A, SEC23B, and SEC24C), responsible for exporting caveolae from the ER to the Golgi.[22]

Both CCVs and caveosomes are trafficked into early endosomes (EEs), where they are either recycled back to the cell surface via recycling endosomes (REs), delivered into the trans-Golgi network, or matured into multivesicular bodies (MVBs) or late endosomes (LEs) that eventually fuse with lysosomes for degradation. Although the protein marker of quick-recycling endosomes, RAB4, was not identified, the slow-recycling endosome marker RAB11 was observed by TITAN analysis at 1 h.[23] Other RAB family members localized at Golgi (RAB1B and RAB2A) were found at later time points (1 and 2 h, respectively).

The conversion of EEs into MVBs or LEs involves the endosomal sorting complexes required for transport (ESCRT) proteins. Consequently, the detection of ESCRT-III proteins VPS4 and CHMP5, at 0.5 and 1 h, respectively, provided strong evidence of dendrimer trafficking to MVBs or LEs during these periods. In addition, Ras-related protein 7 (RAB7), a key regulator for early to-late endosomal maturation, was found at later time points (1 and 2 h). TITAN analysis was also able to pinpoint the fusion of these matured endosomes with lysosomes by the temporal appearance of lysosomal-associated membrane protein 1 (LAMP1), detected exclusively at 2 h. Spatiotemporal information for all interacting proteins involved in clathrin- and/or caveolar/raft-mediated endocytic pathways is presented in Figure 3.

TITAN also enabled us to identify key proteins involved in the regulation of endocytosis. For example, retention factors in the PDZ protein family stabilize membrane proteins at the cell surface by suppressing endocytosis.[24] TITAN analysis found multiple PDZ proteins (PDLIM7, AHNAK2, and SYNPO) exclusively at 0.5 h, suggesting the inhibitory effect of PDZ family proteins on dendrimer uptake. The role of phosphorylation has also been widely implicated in the regulation of endocytosis. Again, TITAN identified 18 kinases at different time points in our study; among them, 12 have been previously identified as regulators in clathrin- and caveolar/raft-mediated endocytic pathways.[25] Interestingly, six of these regulatory kinases (AK1, CKB, CSK, PFKP, PRKACA, and PRKDC, detected at 0.5 or 1 h) were also implicated in the early stage viral infection of mammalian cells,[25,26] which suggests similar roles in dendrimer uptake.

Ubiquitination is another post-translational modification extensively involved in endocytosis. The ubiquitination of membrane proteins serve as an internalization signal that triggers the endocytosis of cargo proteins, and also for EE sorting and for regulating the transport of cargo proteins to MVBs or LEs.[27] TITAN analysis was able to identify multiple ubiquitin ligases (UBR4, RBBP6, STUB1, RBX1, TRIM25, and UBA1) and deubiquitinating enzymes (UBB and USP14) at all three time points, which is consistent with the global roles of ubiquitination during the entire endocytic process.

Finally, we used the TITAN method to study the effects of dynasore, a low-molecular weight inhibitor of dynamin, on

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endosomal trafficking in HeLa cells. The effect of dynasore on dendrimer internalization was first characterized by flow cytometry, which confirmed decreased uptake in dynasore-treated cells over a 2 h period (Figure S4). We hypothesized that dynasore suppressed dendrimer uptake by inhibiting dynamin-mediated release of CCPs and caveosomes from the cell membrane. As expected the target protein dynamin was cross-linked only at 0.5 h without dynasore treatment. After the treatment, dynamin was no longer detectable based on the quantitative proteomic data. The relative levels of other representative proteins involved in CCP/CCV formation such as clathrin, HSPA8, and ACTB, also decreased upon dynasore inhibition (Figure S5); similar patterns were found for COPI complex proteins, RAB7 and LAMP1. Altogether, these results further demonstrated the direct involvement of the clathrin- and caveolar/raft-mediated endocytic pathways in the internalization of dendrimers.

In summary, we present a time-resolved, quantitative proteomic strategy to trace nanoparticle cellular uptake and transportation. TITAN analysis enabled the elucidation of key proteins involved in dendrimer uptake by HeLa cells via two major endocytic pathways, clathrin- and caveolar/raft-mediated endocytosis, as well as proteins with regulatory roles in endosomal uptake and trafficking in a temporal manner. The direct involvement of both pathways was further confirmed by the inhibition of dynamin. We note that while different types of nanoparticles may be internalized by other routes or require longer delivery times, TITAN provides a general point of entry for spatiotemporal proteomic analysis during nanoparticle cellular uptake and can uncover key proteins involved in nanoparticle internalization or trafficking.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b07875.

Materials, detailed procedures for the preparation and characterization of functionalized dendrimers, cell culturing, in vitro optimization of cross-linking and washing conditions, in vivo cross-linking and sample preparations for mass spectrometric analysis, dynasore inhibition experiments, flow cytometry, confocal microscopy, LC-MS/MS analysis, label-free quantification, MS data analysis, and annotation enrichment analysis (PDF)
Label-free quantification of TITAN analysis (XLSX)
Complete list of crosslinked proteins (XLSX)
Statistical overrepresentation test (XLSX)

■ AUTHOR INFORMATION

Corresponding Authors

*watao@purdue.edu
*alexwei@purdue.edu

Present Address

[#]Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, Minnesota 55455, United States.

Author Contributions

[⊥]These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

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