Published in final edited form as:

Angew Chem Int Ed Engl. 2011 April 26; 50(18): 4133–4136. doi:10.1002/anie.201006459.

Identification of Drug Targets *in vitro* and in Living Cells by Soluble Nanopolymer-based Proteomics**

Lianghai Hu^{a,b,c}, Anton Iliuk^{a,c}, Jacob Galan^{a,c}, Michael Hans^a, and Tao W. Andy^{a,c,d,e,*}

^aDepartments of Biochemistry, Purdue University, West Lafayette, IN, 47907

^bCollege of Life Science, Jilin University, Changchun 130012, PR China

^cCenter for Cancer Research, Purdue University, West Lafayette, IN, 47907

^dDepartment of Medicinal Chemistry & Molecular Pharmacology, Purdue University, West Lafayette, IN, 47907

^eDepartment of Chemistry, Purdue University, West Lafayette, IN 47907

Keywords

drug target; dendrimer; drug delivery; proteomics; mass spectrometry

High throughput drug discovery methods typically focus on protein targets which are screened *in vitro* against existing compounds for high specificity and affinity. This strategy, however, could result in unexpected or undetected off-targets effects, leading to high attrition rates in the later stages of drug development. Ideally, unbiased identification of proteins and associated complexes that bind to a drug or drug candidate would provide direct evaluation and therefore would be more appealing, allowing for valuable insight into target cellular functions.^[1] One of the most widely applied approaches to characterize proteins that bind specifically to candidate compounds is based on affinity chromatography combined with mass spectrometric identification.^[2] However, the strategy is typically based on a solid support that can only capture potential protein targets *in vitro* but not in living systems. To address this, activity-based protein profiling (ABPP) strategy has been successfully introduced to study enzyme families both *in vitro* and *in vivo*.^[3] The ABPP probes are based on either covalent reaction with the target proteins or photoaffinity labeling by incorporation of photoreactive groups. One important issue to consider is that a lot of important ligands are either hydrophobic or negatively charged, making direct delivery into living cells extremely challenging. Therefore, it is highly desirable to establish a general *in situ* approach to probe intracellular protein targets.

Here we introduce a proteomic strategy based on soluble nanopolymers in an attempt to identify drug targets *in vitro* and from cells in culture. Soluble nanopolymers such as dendrimers are highly branched nanomolecules with attractive properties as drug delivery vehicles and as imaging contrast agents. Dendrimers have excellent solubility, high structural homogeneity, controlled surface functionalities, cells permeation ability and low cytotoxicity.^[4, 5] We have previously used dendrimers as tools for isotope labeling-based quantitative proteomic and phosphoproteomic studies by chemically modifying them with

[**]This project has been funded in part by an NSF CAREER award, a 3M general fund, and by National Institutes of Health grants 5R21RR025802. We particularly thank a generous NIH high-end instrumentation grant, S10RR025044, for the purchase of LTQ-Orbitrap Velos mass spectrometer.

*Corresponding author: watao@purdue.edu.

different functional groups.^[6–8] Herein, we present the first time use of drug-conjugated dendrimers in combination with proteomic analyses to identify drug targets from cells in culture.

The proteomic strategy includes a two-step procedure based on a novel drug-conjugated nanopolymer (Fig. 1A). The soluble dendrimer is multi-functionalized with drug candidates intended to promote specific interaction with protein targets, and with “handle” groups that facilitate the final isolation through a highly efficient conjugation. In the first step, the drug-conjugated nanopolymer is incubated with cells for designated time to achieve efficient delivery. In the second step, the cells are lysed and proteins bound to the drug are isolated on a solid support, followed by mass spectrometric analyses for identification. We choose poly(amidoamine) (PAMAM) generation 4.0 (G4) dendrimer which contains 64 amine groups with a theoretical diameter of 4.5 nm and therefore has a size similar to many folded proteins while still possessing adequate reaction groups for conjugation. For proof-of-principle experiments, anti-cancer drug—methotrexate (MTX), an antimetabolite and antifolate drug used in treatment of cancer and autoimmune diseases by inhibiting the metabolism of folic acid,^[9] was conjugated to the dendrimer. In addition, we also functionalized the reagent with hydroxyamine as the “handle” group, which allows us to isolate drug targets using the aldehyde-agarose beads (Fig. 1B). The density of MTX and hydroxyamine groups can be easily controlled during the synthesis; for example, the current reagent has 10 MTX molecules and 10 hydroxyamine groups per dendrimer. To monitor whether the synthesized dendrimer reagents can be efficiently delivered into living cells, the dendrimer was also functionalized with fluorescein isothiocyanate (FITC). The whole synthesis flowchart is shown in Fig. S1, and the detailed synthesis steps can be found in the Supporting Information. The intermediates and final product were analyzed by UV/VIS spectrometry to confirm the successful functionalization of the dendrimer with MTX and FITC (Fig. S2).

In vitro studies using complex whole cell extract was first performed to examine the ability of the dendrimer-MTX reagent to target specific proteins (experimental flowchart is shown in Fig. S3). Because dihydrofolate reductase (DHFR) protein is a well-known MTX target, we used DHFR to evaluate the effectiveness of our reagent. To ensure that DHFR was captured through the specific interaction with MTX, free MTX at different concentrations was added into the cell lysate as the competitive control during the affinity enrichment and the assay was monitored by the Western blotting (Fig. 2A). At the concentration above 10 μ M, free MTX could almost completely compete off the bound MTX and DHFR was barely detectable by the Western blotting.

Qualitative analyses of MTX-bound complex resulted in the identification of several hundred proteins, typical in affinity based proteomics.^[10, 11] Here, we combined quantitative proteomics with the dendrimer-MTX enrichment to identify specific MTX targets *in vitro*. The metabolic isotope labeling method—stable isotope labeling with amino acids in cell culture (SILAC) was used to introduce stable isotopes differentially to achieve quantitative measurements.^[12, 13] Human DG-75 B Cells were grown in “light” (amino acids with natural isotope abundance) and “heavy” (¹³C₆-bearing versions of arginine and lysine) media in parallel, resulting in 6 Da mass shift of tryptic peptide containing either ¹³C₆-Arg or ¹³C₆-Lys. In the present study, the “light” cell lysate was used to directly incubate with the dendrimer-MTX reagent, while the “heavy” cell lysate was first mixed with free MTX before the capture by the reagent. After capturing and washing steps, the two sets of samples were combined and the bound proteins were directly digested on-bead with trypsin, followed by mass spectrometry analysis. The SILAC experiments (Fig. 2B) allowed us to differentiate those nonspecific, highly abundant proteins present at close to 1:1 ratio. The differentially enriched proteins in the “light” sample represent putative MTX targets. As

expected, the well-known MTX target protein DHFR was only detected in the “light” form. Another potential MTX target-deoxycytidine kinase (dCK) is also identified with “light”-to-“heavy” ratio of 5:1, representing a strong candidate. Deoxycytidine kinase is an enzyme that plays an important role in the salvage pathway of nucleotide biosynthesis, and it has been recently reported that MTX can specifically regulate dCK activity in this pathway.^[14] Additionally we found several other proteins with high “light”-to-“heavy” ratio. For example, aspartate aminotransferase and trifunctional purine biosynthetic protein adenosine-3 have been reported to involve in MTX-related biosynthetic pathway.^[15, 16] The data demonstrate that the combination of SILAC with drug-dendrimer conjugates was an effective approach to identify specific drug targets, but the specific interactions were under *in vitro* condition, which may not reflect the intracellular events.

The ultimate utility of a drug-functionalized dendrimer is its putative ability to permeate cell membranes. Once we characterized the dendrimer-MTX reagent *in vitro*, we further investigated whether the reagents can be effectively delivered into living cells. The delivery experiments were carried out with two types of cells, a suspension cell line—human B cells DG-75, and an adherent cell line—HeLa cells, respectively. The time course of cell uptake of dendrimer reagent into DG-75 cells is shown in Fig. S4, indicating the steady increase of the fluorescence signal with extended incubation time. This result indicated the continuous uptake of the dendrimer reagents by the cells. We further demonstrated successful delivery into the HeLa cells monitored by flow cytometry experiments (Fig. 3A) and fluorescence microscopy imaging (Fig. 3B).

Having identified the right amount and time for the intracellular delivery of dendrimer-MTX reagent, we coupled the experiment with proteomic study to identify the interacting protein targets of methotrexate in living cells. DG-75 Cells were washed with PBS to remove excess reagent and subsequently lysed. Then aldehyde-agarose beads were immediately added and incubated with the lysate at 4°C for 10 min to capture the dendrimer-protein complex. Finally, on-bead digestion was performed and the resulting peptides were analyzed by liquid chromatography-mass spectrometry. Proteins identified from both *in vitro* and living cells were listed in the Supporting Information. Proteins that were identified *in vitro* but not *in vivo* were also highlighted. Two known methotrexate-interacting proteins, DHFR and dCK, were identified by the approach, confirming the ability of our reagent to successfully capture drug-interacting proteins from living cells. Fig. 3C shows the identification of peptide, LLPEYPGVLSDVQEEK, from DHFR by tandem mass spectrum (MS/MS). To the best of our knowledge, this is the first time dendrimer has been used as a drug carrier in living cells with the purpose to retrieve drug-interacting proteins.

Our strategy based on multi-functionalized soluble nanopolymers demonstrated that the dendrimer-based nanomedicine has a great potential to successfully probe the drug target proteins *in vitro* and in living cells. The new strategy highlights chemical and technological approaches that seek to increase the quality of information obtained from high throughput experiments. The new approach has a number of obvious advantages over existing methods: First, dendrimers provide us with multiple sites of attachment, facilitating the synthesis of intracellular probes; Second, hydrophobic or negatively charged drugs or prodrugs can be immobilized on dendrimers to improve their bioavailability, as long as they remain bioactive on the dendrimer; Third, the combination of mass spectrometry and functionalized dendrimers provides unprecedented opportunity for sensitive, fast identification of proteins of interest in the most physiologically relevant environment. Currently we are studying a phosphopeptide-dendrimer system as a pro-drug to inhibit kinase. We anticipate broad applications of this new strategy in many important biological systems.

Experimental Section

Detailed experimental procedure is available in the Supporting Information. Annotatable datasets of identified peptides and proteins are accessible in a public domain.^[17]

In vitro capture of MTX-interacting proteins: 20 μ L (20 nmol) of the synthesized dendrimer-MTX reagent was incubated with designated amount of cell lysate to form protein-drug conjugates in the solution for 10 min, followed by the capture of the whole complex by adding 20 μ L slurry of aldehyde-agarose beads for another 10 min. After washing to remove non-specific proteins with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1x Mini Complete protease inhibitor cocktail (Roche), pH 7.5) three times, the bound proteins were eluted by 20 μ L of gel loading buffer. The eluate was run on a 12% SDS gel for either Western blotting detection or silver-staining followed by in-gel digestion and mass spectrometry analysis.

SILAC experiments: Equal amount of “light” and “heavy” lysates (2 mg each) were used for affinity enrichment and for control experiment (with additional free MTX (500 μ M) as competitive reagent in the lysate), respectively. After incubation, the two sets of beads were combined for on-bead digestion with trypsin, followed by nanoLC-MS/MS analysis of resulting peptides on a high resolution hybrid dual-cell linear ion trap orbitrap mass spectrometer (LTQ-Orbitrap Velos, Thermo Fisher) coupled to Eksigent nanoflow HPLC.

Delivery of the dendrimer-MTX reagent into living cells: The dendrimer-MTX reagent (50 μ M) was incubated in the culture media with living cells at 37°C for designated time (1–5 h). Free extracellular reagents were removed by washing with fresh medium three times and the cells were directly observed with fluorescence microscopy or analyzed by flow cytometry after fixation with 3.7% formaldehyde solution.

Capturing drug targets in living cells: After 5 h incubation of reagent with living cells, the cells were harvested, washed three times with PBS, and lysed in the lysis solution for 20 minutes on ice. Then the lysate was centrifugated at 13,200 $\times$ g for 10 minutes, and the supernatant were collected. Aldehyde-agarose beads (20 μ L slurry) were added to capture the dendrimer reagent with bound proteins. Finally on-bead digestion and nanoLC-MS/MS analyses were performed to identify the protein targets from living cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Lindsay MA. Nat. Rev. Drug Discov. 2003; 2:831–838. [PubMed: 14526386]
2. Katayama HY, Oda Y. J. Chromatogr. B. 2007; 855:21–27.
3. Cravatt BF, Wright AT, Kozarich JW. Annu. Rev. Biochem. 2008; 77:383–414. [PubMed: 18366325]
4. Astruc D, Boisselier E, Ornelas C. Chem. Rev. 110:1857–1959. [PubMed: 20356105]
5. Boas U, Heegaard PMH. Chem. Soc. Rev. 2004; 33:43–63. [PubMed: 14737508]
6. Iliuk A, Martin VA, Alicie BM, Geahlen RL, Tao WA. Mol. Cell. Proteomics. 2010; 9:2162–2172. [PubMed: 20562096]
7. Guo MJ, Galan J, Tao WA. Chem. Commun. 2007:1251–1253.
8. Tao WA, Wollscheid B, O'Brien R, Eng JK, Li XJ, Bodenmiller B, Watts JD, Hood L, Aebersold R. Nat. Methods. 2005; 2:591–598. [PubMed: 16094384]

9. Majoros IJ, Thomas TP, Mehta CB, Baker JR. *J. Med. Chem.* 2005; 48:5892–5899. [PubMed: 16161993]
10. Trinkle-Mulcahy L, Boulon S, Lam YW, Urcia R, Boisvert FM, Vandermoere F, Morrice NA, Swift S, Rothbauer U, Leonhardt H, Lamond A. *J. Cell Biol.* 2008; 183:223–239. [PubMed: 18936248]
11. Bantscheff M, Scholten A, Heck AJR. *Drug Discov. Today.* 2009; 14:1021–1029. [PubMed: 19596079]
12. Ong SE, Schenone M, Margolin AA, Li XY, Do K, Doud MK, Mani DR, Kuai L, Wang X, Wood JL, Tolliday NJ, Koehler AN, Marcaurelle LA, Golub TR, Gould RJ, Schreiber SL, Carr SA. *Proc. Natl. Acad. Sci. USA.* 2009; 106:4617–4622. [PubMed: 19255428]
13. Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M. *Mol. Cell. Proteomics.* 2002; 1:376–386. [PubMed: 12118079]
14. Uga H, Kuramori C, Ohta A, Tsuboi Y, Tanaka H, Hatakeyama M, Yamaguchi Y, Takahashi T, Kizaki M, Handa H. *Mol. Pharm.* 2006; 70:1832–1839.
15. Varshavsky A. *Proc. Natl. Acad. Sci. USA.* 1981; 78:3673–3677. [PubMed: 6943571]
16. Toledo-Sherman LM, Desouza L, Hosfield CM, Liao L, Boutillier K, Taylor P, Climie S, McBroom-Cerajewski L, Moran MF. *Clin. Proteomics.* 2004; 1:45–67.
17. The data associated with this manuscript may be downloaded from the ProteomeCommons.org Tranche network using the following hash: r4Zzf4wi7hsYJltwLzhr9V/qLLYwRxUrGX5Q4SLFQSLhUXve4IO6vvV2orYL9XZBtW4DlezLJgMNEsVw78RKg1fBBYQAAAAAABYA==

Figure 1.

A) schematic representation of soluble nanopolymer-based approach to identify drug targets;

B) Structure of the dendrimer-MTX reagents.

Figure 2.

A) Western blotting analyses of *in vitro* dendrimer-MTX targets using anti-DHFR antibody. Free MTX with different concentrations was added as the competitive binding agent; B) Profiling of proteins identified from SILAC experiment against their $\log_2(\text{H/L})$.

Figure 3.
3A) Flow cytometry and B) Fluorescence microscopy imaging analysis after delivery of the dendrimer reagent into HeLa cells; C) MS/MS spectrum of a peptide, LLPEYPGVLSVDVQEEK, from DHFR which is identified from living cells.