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Proteomics

Identification of Drug Targets In Vitro and in Living Cells by Soluble-Nanopolymer-Based Proteomics**

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

Herein we introduce a proteomic strategy based on soluble nanopolymers for the identification of drug targets in vitro and in cultured cells. Soluble nanopolymers, such as dendrimers, are highly branched nanomolecules with attractive properties as drug-delivery vehicles and imaging contrast agents. Dendrimers have excellent solubility, high structural homogeneity, controlled surface functionalities, cell-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 different functional groups.[6–8] Herein, we present the first use of drug-conjugated dendrimers in combination with proteomic analysis to identify drug targets from cells in culture.

The proteomic strategy includes a two-step procedure based on a novel drug-conjugated nanopolymer (Figure 1 A). The soluble dendrimer is multifunctionalized with drug candidates intended to promote a specific interaction with protein targets, and with "handle" groups that facilitate final isolation through a highly efficient conjugation. In the first step, the drug-conjugated nanopolymer is incubated with cells for the amount of time required for efficient delivery. In the second step, the cells are lysed, and proteins bound to the drug are isolated on a solid support. The proteins are then identified by mass spectrometric analysis.

We chose generation 4.0 (G4) poly(amidoamine) (PAMAM) dendrimer, which contains 64 amine groups and has a theoretical diameter of 4.5 nm. It thus has a size similar to that of many folded proteins but still possesses adequate reactive groups for conjugation. For proof-of-principle experiments, the anticancer drug methotrexate (MTX), an antimetabolite and antifolate drug used in the treatment of cancer and autoimmune diseases through inhibition of the metabolism of folic acid,[9] was conjugated to the dendrimer. We also functionalized the reagent with hydroxyamine as the "handle" group, which enables us to isolate drug targets by using aldehyde–agarose beads (Figure 1 B). The density of MTX and the hydroxyamine groups can be readily controlled during the synthesis; for example, the current reagent has 10 MTX molecules and 10 hydroxyamine groups per dendrimer. In order to monitor whether the synthesized dendrimer reagents could be delivered efficiently into living cells, we also functionalized the dendrimer with fluorescein isothiocyanate (FITC). A flowchart for the whole synthesis is shown in Figure S1 of the Supporting Information; details of the synthetic steps can also be found in the Supporting Information. We analyzed the intermediates and the final product by UV/Vis spectroscopy to confirm the successful functionalization of the dendrimer with MTX and FITC (see Figure S2).

We first carried out in vitro studies with a complex whole-cell extract to examine the ability of the dendrimer–MTX reagent to target specific proteins (see Figure S3). As the protein dihydrofolate reductase (DHFR) is a well-known

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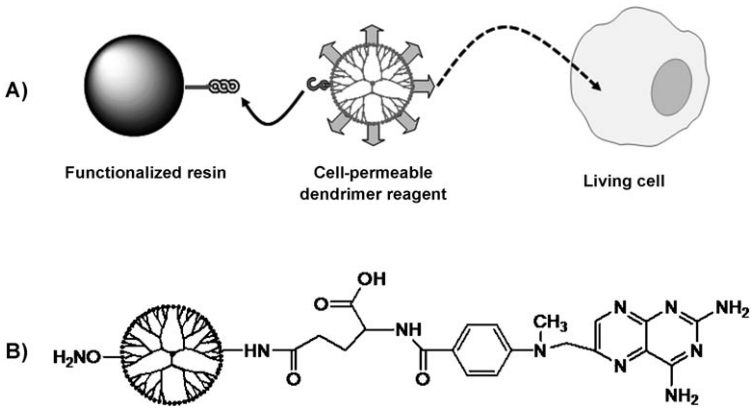

Figure 1. A) Schematic representation of the soluble-nanopolymer-based approach to the identification of drug targets. B) Structure of the dendrimer–MTX reagent.

MTX target, we used DHFR to evaluate the effectiveness of our reagent. To ensure that DHFR was captured through a specific interaction with MTX, free MTX at different concentrations was added to the cell lysate as the competitive control during affinity enrichment, and the assay was monitored by western blotting (Figure 2 A). At concentrations above 10 μM, free MTX could almost completely outcompete bound MTX, and DHFR was barely detectable by western blotting.

Qualitative analysis of the MTX-bound complex resulted in the identification of several hundred proteins, as is typical in affinity-based proteomics.[10,11] In this study, we combined quantitative proteomics with dendrimer–MTX enrichment to identify specific MTX targets in vitro. We used a metabolic isotope-labeling method—stable isotope labeling with amino acids in cell culture (SILAC)—to introduce stable isotopes differentially and enable quantitative measurements.[12,13] Human DG-75 B cells were grown in "light" (with amino acids of natural isotope abundance) and "heavy" media (with $^{13}C_6$-bearing versions of arginine and lysine) in parallel. This experiment resulted in a 6 Da mass shift for tryptic peptide containing either $^{13}C_6$-Arg or $^{13}C_6$-Lys. In the present study, the "light" cell lysate was used for direct incubation with the dendrimer–MTX reagent, whereas the "heavy" cell lysate was first mixed with free MTX before 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 and then analyzed by mass spectrometry.

The SILAC experiments (Figure 2 B) enabled us to differentiate those nonspecific, highly abundant proteins present in a close to 1:1 ratio in the "light" and "heavy" samples. The differentially enriched proteins in the "light" sample were 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), was also identified in a "light"-to-"heavy" ratio of 5:1, and was thus a strong candidate. Deoxycytidine kinase is an enzyme that plays an important role in the salvage pathway of nucleotide biosynthesis, and it was recently reported that MTX can specifically regulate dCK activity in this pathway.[14] We also found several other proteins with high "light"-to-"heavy" ratios. For example, aspartate aminotransferase and trifunctional purine biosynthetic protein adenosine-3 have been reported to be involved in MTX-related biosynthetic pathways.[15,16] The results demonstrated that the combination of SILAC with drug–dendrimer conjugates was an effective approach for the identification of specific drug targets; however, the specific interactions were observed under in vitro conditions and may thus not reflect intracellular events.

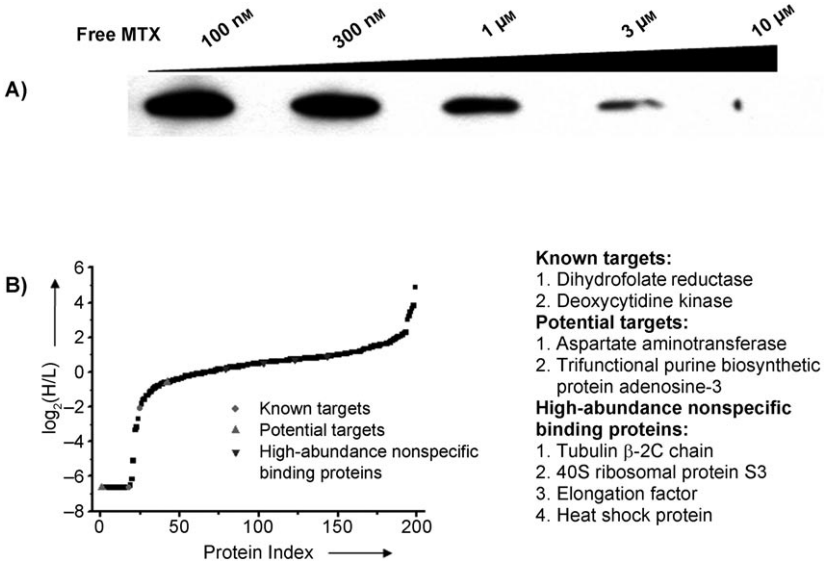

Figure 2. A) Western-blotting analysis of in vitro dendrimer–MTX targets with an anti-DHFR antibody. Free MTX was added at different concentrations as the competitive binding agent. B) Profiling of proteins identified in the SILAC experiment against their $\log_2$(H/L) value (H and L are the peak areas of the "heavy" and "light" peptides).

The ultimate utility of a drug-functionalized dendrimer is its intended ability to permeate cell membranes. Once we had characterized the dendrimer–MTX reagent in vitro, we further investigated whether the reagents could be effectively delivered into living cells. The delivery experiments were carried out with two cell types: a suspension cell line (human B cells DG-75) and an adherent cell line (HeLa cells). The time course for uptake of the dendrimer reagent into DG-75 cells is shown in Figure S4; a steady increase in the fluorescence signal was observed during an 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 by flow cytometry experiments (Figure 3 A) and fluorescence microscopy imaging (Figure 3 B).

Having identified the amount of time required for intracellular delivery of the

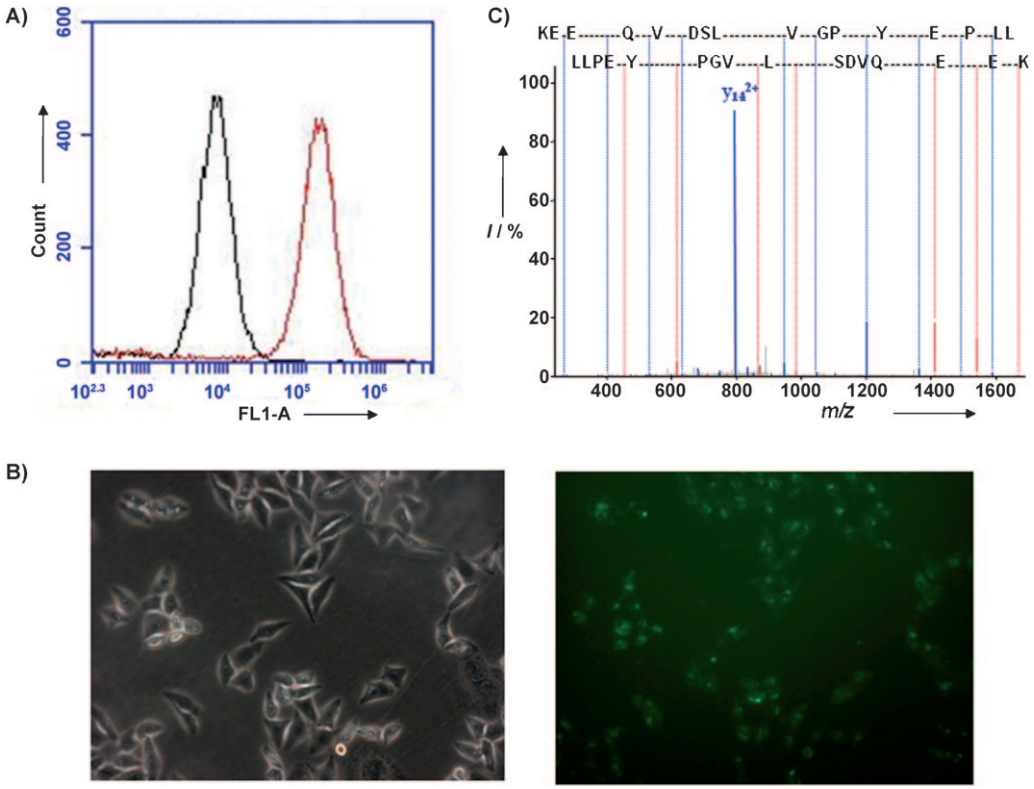

Figure 3. A) Flow cytometry and B) fluorescence microscopy imaging analysis after delivery of the dendrimer reagent into HeLa cells. C) MS/MS spectrum of a peptide identified from living cells: LLPEYPGVLSDVQEEK from DHFR. FL1-A = fluorochrome A detected by FL1 channel. Black curve = cells without dendrimer treatment. Red curve = cells with dendrimer reagents.

dendrimer–MTX reagent, we coupled the experiment with a proteomic study to identify the interacting protein targets of methotrexate in living cells. DG-75 cells were washed with phosphate-buffered saline (PBS) to remove excess reagent and were subsequently lysed. Aldehyde–agarose beads were then 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 both in vitro and from living cells are listed in the Supporting Information. Proteins that were identified in vitro but not in vivo are also highlighted. Two proteins known to interact with methotrexate, DHFR and dCK, were identified by this approach. This result confirms the ability of our reagent to successfully capture proteins from living cells through their interaction with drugs. The peptide LLPEYPGVLSDVQEEK from DHFR was identified by tandem mass spectrometry (MS/MS; Figure 3C). To the best of our knowledge, no dendrimer has been used previously as a drug carrier in living cells with the purpose of retrieving proteins on the basis of their interaction with a drug.

Our strategy based on multifunctionalized soluble nanopolymers demonstrates that dendrimer-based nanomedicine has great potential to successfully probe drug-target proteins in vitro and in living cells. The 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 clear advantages over existing methods: 1) dendrimers provide us with multiple sites of attachment, which facilitates the synthesis of intracellular probes; 2) hydrophobic or negatively charged drugs or prodrugs can be immobilized on dendrimers to improve their bioavailability, as long as these drugs remain bioactive on the dendrimer; 3) the combination of mass spectrometry and functionalized dendrimers provides an unprecedented opportunity for the sensitive, fast identification of proteins of interest in the most physiologically relevant environment. Currently, we are studying a phosphopeptide–dendrimer system as a prodrug for the inhibition of kinase. We anticipate the broad application of this new strategy in many important biological systems.

Experimental Section

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

In vitro capture of proteins that interact with MTX: The synthesized dendrimer–MTX reagent (20 μL, 20 nmol) was incubated for 10 min with the appropriate amount of cell lysate to form protein–drug conjugates in the solution. A slurry of aldehyde–agarose beads (20 μL) was then added to capture the whole complex, and the mixture was incubated for a further 10 min. The beads were washed three times with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1 % NP-40, 1 × Mini Complete Protease Inhibitor Cocktail (Roche), pH 7.5; Tris = 2-amino-2-hydroxymethylpropane-1,3-diol, EDTA = ethylenediaminetetraacetic acid) to remove nonspecific proteins, and the bound proteins were then eluted with gel-loading buffer (20 μL). The eluate was subjected to electrophoresis on a 12 % SDS gel for detection by either western blotting or silver staining followed by in-gel digestion and mass spectrometric analysis.

SILAC experiments: "Light" and "heavy" lysates (2 mg each) were used for affinity enrichment and for the control experiment (with additional free MTX (500 μM) as a competitive reagent in the lysate), respectively. After incubation, the two sets of beads were combined for on-bead digestion with trypsin, followed by nano-LC–MS/MS analysis of the resulting peptides on a high-resolution hybrid dual-cell linear ion trap–orbitrap mass spectrometer (LTQ Orbitrap

Velos, Thermo Fisher) coupled to an Eksigent nanoflow HPLC system.

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

Capture of drug targets in living cells: The reagent was incubated with living cells for 5 h, and then the cells were harvested, washed three times with PBS, and lysed in the lysis solution for 20 min on ice. The lysate was then centrifuged at 13200*g* for 10 min, and the supernatant was collected. Aldehyde–agarose beads (20 μL slurry) were added to capture the dendrimer reagent with bound proteins. Following on-bead digestion, the protein targets from living cells were identified by nano-LC–MS/MS analysis.

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