Cell Nucleus Targeting for Living Cell Extraction of Nucleic Acid Associated Proteins with Intracellular Nanoprobes of Magnetic Carbon Nanotubes

Yi Zhang†,‡ Zhengyan Hu,†‡ Hongqiang Qin,†‡ Fangjie Liu,†‡ Kai Cheng,†‡ Ren’an Wu,*† and Hanfa Zou*†

†CAS Key Lab of Separation Sciences for Analytical Chemistry, National Chromatographic R&A Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, 116023, China
‡Graduate School of Chinese Academy of Sciences, Beijing 100049, China

Supporting Information

ABSTRACT: Since nanoparticles could be ingested by cells naturally and target at a specific cellular location as designed, the extraction of intracellular proteins from living cells for large-scale analysis by nanoprobes seems to be ideally possible. Nucleic acid associated proteins (NAaP) take the crucial position during biological processes in maintaining and regulating gene structure and gene related behaviors, yet there are still challenges during the global investigation of intracellular NAaP, especially from living cells. In this work, a strategy to extract intracellular proteins from living cells with the magnetic carbon nanotube (oMWCNT@Fe3O4) as an intracellular probe is developed, to achieve the high throughput analysis of NAaP from living human hepatoma BEL-7402 cells with a mass spectrometry-based proteomic approach. Due to the specific intracellular localization of the magnetic carbon nanotubes around nuclei and its strong interaction with nucleic acids, the highly efficient extraction was realized for cellular NAaP from living cells, with the capability of identifying 2383 intracellular NAaP from only ca. 10 000 living cells. This method exhibited potential applications in dynamic and in situ analysis of intracellular proteins.

Intracellular proteins are highly diverse and dynamic in both spatial and temporal dimensions. Molecular probes such as fluorescent dyes, fluorescent proteins, quantum dots, etc. have achieved great success in tracing the amounts, location, and substrates of proteins from living cells in real-time and/or in situ. Unfortunately, most such cellular probes are optical and only available for the investigations of single proteins or a small group of proteins, technologies based on which yet suffer from the difficulties in high throughput exploration of complex cellular proteins. Conventional mass spectrometry (MS)-based proteomic strategies could directly present fine-grained details for global intracellular proteins including protein turnover and protein post-translational modifications (PTMs), which however are usually carried out with sample preparation technologies in vitro that might be improper to explore the dynamic changes of proteins in cells. Up to date, it is still a great challenge to exhibit the intracellular proteins from living cells with high throughput.

Due to the intracellular uptake of nanoparticles by living cells, molecular probes at nanoscale (nanoprobes) seem to be very promising to extract intracellular molecules from living cells. Such as for intracellular proteins, the dendraimers, linked with an anticancer drug molecule (methotrexate, MTX), have been applied for the detection of MTX substrates of four proteins from living cells using the quantitative proteomic method; the antibody-conjugated magnetic nanoparticles have also been utilized to harvest the endocytosis vesicles for the profiling of proteins involved during the native process of cellular uptaking. Carbon nanotubes (CNTs) and functional-CNTs (f-CNTs) with good biocompatibility could easily be internalized by cells and thus have been used as intracellular probes for in vivo imaging, drug delivery, gene delivery, etc. Also, CNT-based nanomaterials could interact with nucleic acids (NA) through the hydrophobic interaction and π−π stacking, making them widely employed in the determination of NA in vitro. Ideally, it is possible to use CNTs and f-CNTs as nanoprobes to extract intracellular NA and their associated proteins. Previously, the nucleic acid associated proteins (NAaP), which bridge genome and proteome and take crucial roles in regulating gene structure and function, have been successfully extracted using the magnetic multiwall carbon nanotube (oMWCNT@Fe3O4) in an in vitro approach. Combining the nuclear internalization of CNT-based nanomaterials, NAaP in live cells might be targeted by the nanoprobe of oMWCNT@Fe3O4. Moreover, taking advantages of the nuclear targeting, the living cell extraction would probably present a higher efficiency than the in vitro method. Herein, to achieve the high throughput investigation of proteins from living cells, a strategy of using the magnetic carbon nanotube as the intracellular probe has been developed.
for the exploration of NAaP. As illustrated in Figure 1, oMWCNT@Fe₃O₄ was incubated with living human hepatoma BEL-7402 cells; with the cellular internalization of oMWCNT@Fe₃O₄ by the living cells, the probe of oMWCNT@Fe₃O₄ localized around nuclei. After cell lysis, the intracellular NA along with the associated NAaP were harvested with the centrifugation and the help of a magnet; finally, the obtained NAaP were identified using a nano-LC-MS/MS system.

**EXPERIMENTAL SECTION**

Synthesis of oMWCNT@Fe₃O₄. The synthesis of oMWCNT@Fe₃O₄ was performed according to the previous method. Briefly, 1.0 g of multiwall carbon nanotube (MWCNT) was oxidized using the solution of concentrated HNO₃ and H₂SO₄ (30 mL/90 mL) at 120 °C for 30 min under refluxing. Then, the oxidized MWCNT (oMWCNT) was collected and dried at 60 °C overnight. For the growth of Fe₃O₄, 0.1 M FeCl₃ was first employed to incubate with the oMWCNT at 25 °C for 2 h in order to load Fe³⁺ on the surface of oMWCNT; then, the oMWCNT@Fe³⁺ was collected and dried. After being dispersed in 50 mL of ethylene glycol solution containing 3.6 g of NaAc and 1.5 g of 1,6-diaminohexane, oMWCNT@Fe₃O₄ was synthesized using the solvothermal synthesis at 200 °C for 8 h. The products were washed with ethanol and water for 3 times, vacuum-dried at 60 °C overnight, and dissolved in a mixed solvent containing 98% (v/v) water and 2% (v/v) dimethyl sulfoxide (DMSO) as a stock solution (1 mg/mL) for further applications.

Living Cell Extraction Strategy for NAaP. The cells were first washed with PBS for 3 times to fully remove the proteins in the culture medium (RPMI-1640). Then, the solution of oMWCNT@Fe₃O₄, which was diluted with RPMI-1640, was added into the culture dishes and incubated with cells. After incubation, the cells were washed with PBS for 3 times to get rid of the redundant materials. Then, cells were collected and washed with PBS for another 3 times. Then, cells were cracked with the lysis buffer (250 mM sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM ethylene glycol bis(2-aminoethyl ether)-N,N,N′,N′′-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2% protease inhibitor Cocktail). The mixtures of oMWCNT@Fe₃O₄ and NAaP on NA were gathered with centrifugation at 30 000g for 10 min and purified against H₂O with a magnet for 3 times. The entire extraction processes were performed on ice.

Protein Digestion and MS Identification. The digestion and analysis of proteins were performed according to previous works. Briefly, the extracted protein and NA mixtures (from ca. 1 000 000 cells) were resuspended in 150 µL of denature buffer (8 M urea, 50 mM Tris-HCl, pH 7.4) and treated with intense sonication (Scientz Biotechnology, China) at 400 W for 5 s on ice to break the NA chains and assist the following protein digestion process. The proteins were reduced with dithiothreitol (DTT, 2 µmol) and alkylated with indole-3-acetic acid (IAA, 4 µmol). Then, the protein solution was diluted with 1 mL of Tris-HCl (50 mM, pH 8.0), and 4 µg of trypsin was subsequently added for protein digestion at 37 °C for 16 h. The digested peptides were desalted through a reverse phase C18 SPE column and eluted using a mixed solution of 80% (v/v) acetonitrile (ACN) and 20% (v/v) water containing 0.1% trifluoroacetic acid (TFA). By lyophilization, the peptides were stored at ~80 °C for the following nano-LC-MS/MS analysis. The MS-based identification was technically repeated twice for each sample. Detailed parameters during peptides separation and protein identification with the nano-LC-MS/MS system were listed in the Supporting Information.

**RESULTS AND DISCUSSION**

The probe of oMWCNT@Fe₃O₄ was synthesized through the oxidation of MWCNTs and the subsequent growth of Fe₃O₄ nanoparticles on the oxidized MWCNTs (oMWCNTs). The sizes of the Fe₃O₄ nanoparticles grown on oMWCNTs were under 10 nm (Supporting Information, Figures S-1a,b). As intracellular nanoprobe should be biocompatible for living cells, the viability of BEL-7402 cells treated with the probe of oMWCNT@Fe₃O₄ was measured by the Cell Counting Kit-8 (CCK-8) assay (Figure 2). It was observed that the oMWCNT@Fe₃O₄ was highly biocompatible for the BEL-7402 cells with the concentration of oMWCNT@Fe₃O₄ below 150 µg/mL.

To achieve the extraction of NAaP from living cells, the internalization of the nanoprobe of oMWCNT@Fe₃O₄ into...
cells would be an important step to be addressed. In this work, to trace the location of the functionalized-oMWCNT in living cells, the oMWCNTs were labeled with the fluorescent group of fluorescein isothiocyanate (FITC) as FITC-oMWCNT. As observed by laser scanning confocal microscopy (LSCM), the majority of FITC-oMNCT was localized at the nuclei (Figure 3a–e) consistent with the previous report, enabling the probe of oMWCNT@Fe3O4 to have a reliable opportunity to capture NA and NAaP around cell nuclei.

Before the high throughput investigation of NAaP from living cells, we need to confirm the feasibility of the strategy. During the extraction processes, nonspecific adsorption on both oMWCNT@Fe3O4 and extracted NA would involve superior disturbances for the final results. To verify the selectivity of oMWCNT@Fe3O4 in the extraction of NAaP from living cells, two well-known NAaP of histone H3 (bound to DNA and involved in forming chromosome) and the eukaryotic translation initiation factor 4E (eIF-4E, linked with mRNA, and directed the linking of ribosome toward mRNA) were chosen as the target NAaP. Meanwhile, β-actin, which was a composition of the cytoskeleton, was selected as the impurity protein. The histone H3 and eIF-4E were extracted from living cells using the nanoprobe of oMWCNT@Fe3O4 and analyzed by the Western blotting assay, which were then compared with those in the whole cellular proteins (Supporting Information, Figure S-3). The amounts of histone H3 and eIF-4E remained constant as compared with the whole cell proteins, while the amount of extracted β-actin was greatly suppressed. Some residual β-actin was detected with the target NAaP, and it might come from the complexes associated with DNA or RNA which formed during the transcription.

The NAaP are of great significance to maintain, manipulate, and regulate the gene structure, replication, and expression during cellular processes, some of which are realized by regulating the expression levels of NAaP under different statuses of cells. For instance, it has been reported that the expression of eIF-4E increases under the stimulation of serum or growth factors (such as the platelet-derived growth factor, PDGF). Thus, to evaluate the utility of living cell extraction by the nanoprobe of oMWCNT@Fe3O4, the expression levels of eIF-4E and histone H3 under different cell culture conditions were investigated, using BEL-7402 cells treated with low concentration of bovine serum (BS, 0.5%) as the starving group and treated with high concentration of BS (10%) as the normal group (schematically illustrated in Figure 4a). NAaP from both starving and normal groups were extracted using oMWCNT@Fe3O4, which were also compared with the whole cell proteins from both groups (Figure 4b). The amounts of histone H3 stayed constant, while the amounts of eIF-4E were reduced significantly for cells treated with 0.5% BS when using the probe of oMWCNT@Fe3O4 and the whole cell proteins. This presented an attractive potential of oMWCNT@Fe3O4 as the intracellular probe to display the regulation information of proteins from living cells. All of the above results indicated the feasibility and utility of this living cell extraction strategy based on intracellular nanoprobe, which made the following high throughput identification of the collected proteins reliable.

To ensure the high throughput profiling of intracellular proteins from living cells, the extraction efficiency was of essential importance. Thus, intracellular extraction efficiencies for NA and NAaP using oMWCNT@Fe3O4 were profiled, which were further compared with a reported in vitro method. First, the extraction efficiency for NA (using ca. 1 000 000 cells) was surveyed by comparing the residual NA in the supernate before and after extraction with living cells or cell lysates (in vitro method) using agarose gel electrophoresis.

Figure 3. LSCM images of BEL-7402 cells (a−c) without the treatment of FITC-oMWCNT, (d−f) with the treatment of a 9 h incubation with FITC-oMWCNT; (a and d) were excited at 488 nm to observe intracellular FITC-oMWCNT and (b and e) were excited at 405 nm to observe DAPI-dyed nuclei; (c) was merged by (a) and (b), and (f) was merged by (d) and (e). (Scale bar: 10 μm). (Bright field images of cells treated with or without FITC-oMWCNT were listed in Supporting Information, Figure S-2.)
the living cells or the cell extracts. The estimated extraction efficiencies for NA by counting the intensities of NA bands on gel were ca. 95.3 ± 1.0% for the living cell extraction and 93.5 ± 2.3% for the in vitro extraction, respectively, both presenting the highly efficient extraction for NA. Second, the extracted

NAaP with both living cell extraction and in vitro extraction (from ca. 1 000 000 cells) were quantified by the bicinchoninic acid (BCA) assay (Figure 5b). Interestingly, 20.7 ± 1.4 μg of NAaP was extracted from living cells, while only 7.6 ± 2.0 μg of NAaP was extracted from cell lysates, presenting a more effective extraction of using the nanoprobe of the oMWCNT@Fe3O4 from living cells. During living cell incubation, oMWCNT@Fe3O4 could interact with intracellular NA more equally than that during the in vitro extraction, which are usually only on the surface of the aggregated NA from cell lysates. Thus, it was estimated that intracellular NAaP could be superiorly preserved with the living cell extraction, the majority of which might contribute to the NAaP weakly binding on NA. In this case, more information of intracellular NAaP would probably be exhibited by using the developed living cell extraction strategy.

With a 2D-nano-LC MS/MS system, 4118 NAaP were identified from ca. 1 000 000 cells and 2383 NAaP were identified from only ca. 10 000 cells (Supporting Information, Tables S-1 and S-2), which were far more than that identified by the in vitro method (2595 proteins from ca. 1 000 000 cells and 803 proteins from ca. 10 000 cells,23 Figure 6a). This was actually consistent with the amounts of NAaP extracted from living cell extraction and the in vitro extraction. Plus, through counting, 1.63 times more spectra of identified NAaP (from ca. 1 000 000 cells) were obtained with the living cell extraction (590 156 spectra) than with the in vitro extraction (362 064 spectra). After normalization, 91.2% (1974 proteins) of the overlapped part (2165 proteins) from the identified NAaP with both living cell extraction and in vitro extraction shared the differences less than 5-fold (Supporting Information, Figure S-7), which was believed to be not significant.23 Thus, living cell extraction would present reliable results of identified NAaP as the in vitro method while having a higher efficiency.

To further assess the living cell analysis strategy using the nanoprobe of oMWCNT@Fe3O4, intracellular location and binding substrates of the identified NAaP were annotated with GoMiner,26 which were compared with that obtained by the in vitro method previously (Figure 6b, number of annotated NAaP was listed in Supporting Information, Table S-3). The majority of the identified NAaP with this live extraction were annotated to localize in the nucleus or ribosome and bind with NA or proteins, which might have contact with NA directly or indirectly by forming protein complexes, such as the ribosome complexes. Also, the annotated NAaP with living cell extraction were much more than those with the in vitro extraction. Therefore, by internalization at the nucleus and binding with NA, not only were the intracellular environments potentially maintained with the living cell extraction, but also high efficiency and sensitivity for the analysis of NAaP was achieved. In this case, the sample size in future applications could be extremely lowered (commonly ca. 10 000 000 cells during the conventional proteomic analysis),27 and it would be very useful for precious and limited samples, such as the stem cells.

CONCLUSIONS

In summary, a nanoprobe-based intracellular extraction strategy was developed to profile the proteome of NAaP from living cells. The nanoprobe of magnetic oMWCNT@Fe3O4 was supposed to be internalized by cells and localized around the nucleus. On the basis of the interaction of NA on CNTs and the association of NAaP with NA, the living extraction of NAaP from BEL-7402 cells was fulfilled, demonstrating promising
opportunities to gain the dynamic cellular protein information with a high efficiency. In total, 4118 NAaP from ca. 1 000 000 cells and 2382 NAaP from only ca. 10 000 cells were identified. To the best of our knowledge, it was the first trial to identify the NAaP proteome from living cells with an intracellular nanoprobe, which was believed to be of great potential in revealing intracellular protein networks in gene related actions and regulations. Moreover, by developing novel nanoprobe owning biocompatibility and specific intracellular location, proteomes and other cellular components such as metabolites, organelles, etc. would be analyzed from living cells, which, aiming toward real-time and in situ information with high throughput, might assist one in depicting the diverse intracellular regulation networks during highly dynamic physiological processes.

**ASSOCIATED CONTENT**

**Supporting Information**

Supplementary experimental procedures, optimization of the extraction processes, TEM images of oMWCNT and oMWCNT@Fe₃O₄, bright field images of BEL-7402 cells with LSCM analysis, selectivity for NAaP with living cell extraction using oMWCNT@Fe₃O₄, semiquantification of results from the Western blotting assay, spectra counting analysis of MS results from the in vitro method and living cell strategy, uptake amounts of FITC-oMWCNT by cells and identified amounts of NAaP using oMWCNT@Fe₃O₄ under different conditions, lists of identified and annotated NAaP. This material is available free of charge via the Internet at http://pubs.acs.org/.

**AUTHOR INFORMATION**

*Corresponding Author*

*E-mail: hanfazou@dicp.ac.cn* (H.Z.); *wurenan@dicp.ac.cn* (R.W.).

*Notes*

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

Financial support from the Creative Research Group Project of NSFC (21021004), the National Natural Science Foundation of China (Nos. 21235006, 21175134, 81161120540), the China State Key Basic Research Program Grant (2013CB911202, 2012CB910601), National Key Special Program on Infection Diseases (2012ZX10002009-011), and the Analytical Method Innovation Program of MOST (2009IM031800, 2010IM030500) are gratefully acknowledged.

**REFERENCES**

