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Nano LC-MS Based Proteomic Analysis as a Predicting Approach to Study Cellular Responses of Carbon Nanotubes

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Nano-bio interface has been paid much attention recently, though with the lack of methodology to predict the potential responses in biological systems such as cells induced by nanomaterials. In this study, we described a proteomic approach to investigate the proteome change in K562 cells exposed to oxidized single-walled carbon nanotubes (o-SWCNTs). 605 proteins were identified by semi-quantitative proteomic analysis (SQPA), including 29 significantly changed proteins with spectra count (SpC) ratios larger than 2 or less than 0.5. Three of them including HBA, CFL1 and LMAN2 were further validated by western blotting. The differential proteins were further classified by Ingenuity Pathways Analysis (IPA) to integrate them into a signaling network. Based on the information by this network, we predict that o-SWCNT treatment activated cell aggregation, decreased cell migration, but had no effect on cell death. And these cellular responses were further experimentally demonstrated. The protein signaling network established in this study would greatly benefit the studies on the bio-applications of o-SWCNTs and their toxicity studies. Our study demonstrated that proteomics could be used as a predicting tool to study nano-bio interface at cellular level.

Keywords: Leukemia Cell, Nanotoxicity, Liquid Chromatography, Mass Spectrometry.

1. INTRODUCTION

Carbon nanotubes (CNTs) are one of the most studied materials in nanoscience and nanotechnology. CNTs have wide applications in many fields because of their high conductivity, extraordinary tensile strength, and high efficiency in heat conduction.¹ However, pristine CNTs are difficult to be manipulated because of their low dispersibility in solution and poor chemical reactivity with other molecules.² In order to address these problems, carboxyl functionalized CNTs (o-CNTs) were synthesized in 1998,³ and then a variety of derivatives were prepared based on o-CNTs such as amine (NH₂), polyethylene glycol (PEG), and polyetherimide (PEI) modified CNTs.⁴ After functionalization, the surface activity and dispersibility of CNTs are greatly improved, and the o-CNTs as well as their derivatives were increasingly used as separation additives,⁵ imaging agents,⁶ detection sensor⁷ and drug carrier in chemotherapy⁸–¹⁰. The wide applications of o-CNTs have led to the increase of the exposure potential of CNTs to humans, which necessitate the studies on nano-bio interface.¹¹ Currently, CNTs has been shown to induce ROS generation,¹² NF-kappa B activation,¹³ production of inflammatory cytokines and growth factors.⁶,¹⁴ Besides, CNTs have been found to induce cell death by the residual heavy metal catalysts¹⁵ and pulmonary fibrosis in mice.¹⁶ Although considerable cellular responses of CNTs have been explored based on the existed injury pathways, there is no report aimed to predict the cellular responses induced by o-CNTs.

System biology is a new theme in biological science, aims for a system-level understanding of a biological system.¹⁷ It could provide comprehensive information of a complex biologically regulatory system. Proteomics, as an important branch of system biology, attempts to study
biological processes comprehensively by the systematic analysis of proteins expressed in cells or tissues. Recently, proteomic tool has been widely used to screen tumor biomarkers, identify cellular signaling pathway, and investigate protein–protein interactions. Thus proteomics are supposed to be an effective approach to predict CNT-induced cellular responses by determining the proteome changes in cells.

In this study, proteomic tool was employed to analyze the proteome changes in leukemia K562 cells treated with oxidized single-walled carbon nanotubes (o-SWCNTs). We established a protein signaling network based on the proteomic results to predict the cellular responses of o-SWCNTs in K562 cells. In addition, we validated our predictions by testing the cellular migration, aggregation and cell death because these cellular effects play important roles in the maintenance of cellular functions. This study has important implications from the perspectives of biosafety and bio-application of o-CNTs because leukemia K562 cells play an important role in defending the body against foreign materials and CNTs have exhibited great potential to be used as drug carriers to deliver molecular cargos into K562 cells.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents
2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) and 1-methoxy-5-methylphenazinium methylsulfate (PMS) were purchased from Dojindo laboratory (Kumamoto, Japan); The stromal cell-derived factor 1 (SDF-1) antibodies against HBA, CFL1 and LMAN2 were purchased from BD Biosciences (Franklin Lakes, NJ, USA); Single-walled carbon nanotubes (SWCNTs) were purchased from Sino-nano company (Beijing, China), and treated with the mixture of concentrated HNO3 and H2SO4 (1:3 v/v) to produce the oxidized single-walled carbon nanotubes (o-SWCNTs) as described previously. The prepared o-SWCNTs were rinsed sufficiently and lyophilized for future use.

2.2. Cell Culture
K562 cells were obtained from the Institute of Blood, Chinese Academy of Medical Sciences (Tianjin, China). Cells were seeded initially at a density of 1.0 × 10^4 cells/mL, cultured in 75-cm² vented culture flasks at 37 °C with RPMI-1640 medium containing 10% calf serum in 5% carbon dioxide atmosphere, and split every 2–3 days. The cells were harvested in the logarithmic phase and counted by a hemocytometer.

2.3. Cell Treatment
The K562 cells were equally seeded into 75-cm² vented culture flasks and pre-incubated for 24 h. Then cells were equally divided into two groups including EXP and CON. The EXP cells were exposed 30 μg/mL o-SWCNTs for 48 h, while CON cells were treated with the same method except the exposure to o-SWCNTs. Both cell samples were separated by centrifugation, washed with cold PBS and harvested for proteomic analysis.

2.4. Proteomic Analysis
Cell samples were suspended in 1.5 mL of lysis buffer (50 mM Tris-HCl buffer, pH 7.4, 0.1% v/v Triton-100, 1 mM PMSF, 1% DTT, 8 M urea, 1 mM Na2VO4, 1 mM EDTA, 10 mM NaF, 1/16 piece of protease inhibitor cocktail) and lyzed by a probe sonication. After centrifugation at 20, 000 g for 10 min, the lysis supernatant was collected and added to 8 mL extraction solute (acetone/ethanol/acetic acid 50:50:0.1) pre-cooled at −20 °C. After 24 h storage at 4 °C, the proteins were precipitated by centrifuging at 20 000 g for 30 min. After redissolution, the protein concentrations in both EXP and CON samples were equivalent by a Bradford method, and then the protein samples were denaturalized, digested and desalted by the method previously reported. Finally, the resulting peptide samples were re-dissolved in 200 μL 0.1% formic acid for nano- LC/MS/MS analysis.

The nano-LC/MS/MS system comprised a quaternary Surveyor pump and a LTQ linear ion trap mass spectrometer equipped with a nanospray source (Thermo, San Jose, CA, USA). The temperature of the ion transfer capillary was set at 200 °C, and the spray voltage at 1.8 kV. All MS and MS/MS spectra were acquired in a data-dependent mode with one full MS scan, followed by ten MS/MS scans. The nano-LC/MS/MS analysis was performed based on a previously reported method.

The acquired MS/MS spectra were searched against the Human database using the Turbo SEQUEST in the BioWorks 3.2 software suite (Thermo). Peptides were searched using fully tryptic cleavage constraints and up to two missed cleavages sites were allowed for tryptic digestion. The searching results were filtered with the parameters as reported by Roth et al. For semi-quantitative comparison of the proteins identified in EXP and the CON, SpC for each identified protein from each experiment were extracted, averaged, normalized, and compared. The protein network of the differential proteins as well as corresponding cellular functions involved was assigned using Ingenuity Pathway Analysis (http://www.ingenuity.com/).

2.5. Western Blotting (WB) Analysis
The protein extracts for proteomic analysis were re-dissolved in cell lysis buffer. The proteins were loaded on 12% gels (Bia-Rad, Hercules, CA, USA) to separate by SDS-PAGE. Then, the target bands were cut and the proteins were transferred to nitrocellulose membranes (Bia-Rad, Hercules, CA, USA). Membranes were blocked with...
5% nonfat milk, indicated with primary antibody (Santa Cruz, CA, USA), and developed with horseradish peroxidase (HRP)–conjugated secondary antibody (Santa Cruz, CA, USA) and enhanced chemiluminescence reagents (Bia-Rad, Hercules, CA, USA). β-actin was used as loading control.

2.6. Cell Migration Assay
The assay was conducted using 8.0-μm pore size and 6.5 mm diameter transwell filters (Costar, MA, USA). K562 cells were seeded in the upper chamber (2 × 10^5 cells/well), and starved for 24 h in serum-free medium. Then the upper chamber were combined with lower chamber that contains 500 μL RPMI 1640 medium (20% FBS and 100 ng/mL of SDF-1β) with 0 to 50 μg/mL o-SWCNTs. After 12-h incubation, cell numbers in the lower chamber were evaluated using WST-1 assay.

2.7. Toxicity Assay
Annexin V–FITC coupled with PI was used as double staining agent to investigate the short-term toxicity of o-SWCNTs by flow cytometry. The assay was performed as previous reported. In detail, K562 cells were exposed to 0, 10, 20 and 50 μg/mL o-SWCNTs for 2 h, and then the cells were collected. After exposure to annexinV-FITC and PI at room temperature for 15 min, and cell samples was diluted to a final volume of 500 μL and analyzed by flow cytometry from BD (Franklin Lakes, NJ, USA). WST-1 assay was used to test the viability of K562 cells incubated with o-SWCNTs for a long term (48 h). 100 μL aliquot of K562 cell culture in the logarithmic phase was seeded into a 96-well plate (10,000 cells/well). After overnight incubation, 100 μL aliquots of cell media with o-SWCNTs concentrations ranging from 0 to 50 μg/mL, were added into each well. After 48-hour incubation, the culture media in each well was refreshed with 200 μL cell medium containing 200 μM WST-1 and 2 μM PMS. The absorbance was read at 450 nm using a microplate reader (model 550 Bio-Rad Laboratories, Hercules, CA) as described previously.

3. RESULTS AND DISCUSSION
3.1. Characterization of o-SWCNTs
o-SWCNTs were prepared by an oxidation approach as reported previously. In detail, the raw SWCNTs were suspended in a mixed acid (HNO₃/H₂SO₄ 1/3) and reacted at 120 °C for 0.5 h. After sufficient rinse and dryness, the prepared o-SWCNTs were characterized by TEM and IR (Fig. 1). o-SWCNTs showed stretching vibration bands of O-H and –C=O from –COOH group at 3446 cm⁻¹ and 1637 cm⁻¹, respectively. The diameter of o-SWCNTs is at 2–5 nm and the length ranged from 500 to 800 nm.

3.2. Differential Proteins in K562 Cells Induced by o-SWCNTs
In order to identify the differential proteins in K562 cells induced by o-SWCNTs, two groups of K562 cell samples including EXP (cells incubated with o-SWCNTs) and CON (cells incubated without o-SWCNTs), were prepared (Fig. 2). Each group included two parallel cell samples. The cell samples were lysed to extract proteins, which were further digested, desalted and analyzed thrice on nano liquid chromatography coupled with tandem mass spectrometry (nano-LC/MS/MS). To improve the reliability of the identified proteins, stringent filter criteria were set for the data processing:

(i) at least two peptides identified per protein,
(ii) identified thrice in each of the three parallel analyses,
(iii) identified in both groups of samples.

Figure 1. TEM image and IR spectrum of o-SWCNTs. o-SWCNTs with 2–5 nm o.d. and 500–800 nm length, have two absorption peaks at 3446 and 1637 cm⁻¹ resulting from the –OH and O=C of –COOH on o-SWCNTs, respectively.
As a result, 605 proteins were identified as the candidates of differential proteins. Then a semi-quantitative proteomic analysis (SQPA) approach as reported previously\(^{28,29}\) was used to determine the spectra counts (SpC) of each proteins identified by nano-LC/MS/MS detection. The SpC ratios (R) of EXP versus CON were used to estimate the expression level of the differential proteins. Proteins with R values larger than 2 or less than 0.5 were considered as significantly changed proteins, and listed in Table I. Among them, 15 proteins were down regulated and 14 proteins were up regulated.

Then we randomly selected three proteins including HBA, CFL1 and LMAN2 from the 29 significantly changed proteins to validate the SQPA results by western blotting. As shown in Figure 3, the expression levels of HBA and CFL in the EXP group were significantly increased compared to CON group, while the LMAN2 in EXP group was lower. The western result is consistent with the SQPA data, which demonstrates the reliability of differential proteins identified by SQPA method.

### 3.3. Establishment of Signaling Network of the Changed Proteins Induced by o-SWCNTs in K562 Cells

To explore the cellular responses by o-SWCNTs, we first established a signaling network by classifying the changed proteins using software, Ingenuity Pathways Analysis (IPA), which is a widely used tool for protein network and signaling pathway analysis.\(^{31,32}\) As shown in Figure 4, the protein network includes up-regulated (red color), down-regulated (green), unchanged (gray) and some unidentified proteins (white). This network is closely related to four types of cellular responses including cellular assembly and organization, cellular function and maintenance, molecular transport and cell-to-cell signaling and interaction (Table II). The detailed information of proteins involved in each cellular function was not shown.

### 3.4. Predicting and Validating the Influences of o-SWCNTs to Cell Aggregation and Migration

Among these cellular effects, we are interested in cell aggregation and migration because these cellular responses...
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Table I. Significantly changed proteins induced by o-SWCNTs.

<table>
<thead>
<tr>
<th>R Values</th>
<th>ID</th>
<th>Symbol</th>
<th>Entrez gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.24</td>
<td>IP000299573</td>
<td>RPL7A</td>
<td>Ribosomal protein L7a</td>
</tr>
<tr>
<td>0.31</td>
<td>IP00024320</td>
<td>RBM3</td>
<td>RNA binding motif (RNP1, RRM) protein 3</td>
</tr>
<tr>
<td>0.31</td>
<td>IP00456887</td>
<td>HNRNPUL2</td>
<td>Heterogeneous nuclear ribonucleoprotein U-like 2</td>
</tr>
<tr>
<td>0.34</td>
<td>IP00007402</td>
<td>IPO7</td>
<td>Importin 7</td>
</tr>
<tr>
<td>0.38</td>
<td>IP00024719</td>
<td>HAT1</td>
<td>Histone acetyltransferase 1</td>
</tr>
<tr>
<td>0.38</td>
<td>IP000291946</td>
<td>USP10</td>
<td>Ubiquitin specific peptidase 10</td>
</tr>
<tr>
<td>0.40</td>
<td>IP00006980</td>
<td>C14ORF166</td>
<td>Chromosome 14 open reading frame 166</td>
</tr>
<tr>
<td>0.43</td>
<td>IP00012772</td>
<td>RPL8</td>
<td>Ribosomal protein L8</td>
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<tr>
<td>0.48</td>
<td>IP00215734</td>
<td>PRMT1</td>
<td>Protein arginine methyltransferase 1</td>
</tr>
<tr>
<td>0.48</td>
<td>IP00039181</td>
<td>RPL4</td>
<td>Ribosomal protein L4</td>
</tr>
<tr>
<td>0.48</td>
<td>IP00310132</td>
<td>DDX18</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 18</td>
</tr>
<tr>
<td>0.48</td>
<td>IP00055537</td>
<td>MRPL12</td>
<td>Mitochondrial ribosomal protein L12</td>
</tr>
<tr>
<td>0.48</td>
<td>IP00300127</td>
<td>NAT10</td>
<td>N-acetyltransferase 10 (GCN5-related)</td>
</tr>
<tr>
<td>0.48</td>
<td>IP00003949</td>
<td>UBE2N</td>
<td>Ubiquitin-conjugating enzyme E2N (UBC13 homolog, yeast)</td>
</tr>
<tr>
<td>0.48</td>
<td>IP00009950</td>
<td>LMN2</td>
<td>Lectin, mannose-binding 2</td>
</tr>
<tr>
<td>2.00</td>
<td>IP00005202</td>
<td>PGRMC2</td>
<td>Progesterone receptor membrane component 2</td>
</tr>
<tr>
<td>2.12</td>
<td>IP00010440</td>
<td>HAX1</td>
<td>HCLS1 associated protein X-1</td>
</tr>
<tr>
<td>2.23</td>
<td>IP00032830</td>
<td>REXO2</td>
<td>RNA exoribonuclease 2 homolog (S. cerevisiae)</td>
</tr>
<tr>
<td>2.30</td>
<td>IP00297084</td>
<td>DDOST</td>
<td>Dolichyl-diphosphooligosaccharide–protein glycosyltransferase</td>
</tr>
<tr>
<td>2.34</td>
<td>IP00180408</td>
<td>MYH15</td>
<td>Myosin, heavy chain 15</td>
</tr>
<tr>
<td>2.34</td>
<td>IP00034320</td>
<td>MAPRE2</td>
<td>Microtubule-associated protein, RPE1 family, member 2</td>
</tr>
<tr>
<td>2.36</td>
<td>IP00017381</td>
<td>RFC4</td>
<td>Replication factor C (activator 1) 4, 37kDa</td>
</tr>
<tr>
<td>2.41</td>
<td>IP00012011</td>
<td>CFL1</td>
<td>Cofilin 1 (non-muscle)</td>
</tr>
<tr>
<td>2.50</td>
<td>IP00289819</td>
<td>IGFR2</td>
<td>Insulin-like growth factor 2 receptor</td>
</tr>
<tr>
<td>2.62</td>
<td>IP00013891</td>
<td>TRA2A</td>
<td>Transform 2 alpha homolog (Drosophila)</td>
</tr>
<tr>
<td>2.72</td>
<td>IP00007401</td>
<td>SF3A1</td>
<td>Splicing factor 3a, subunit 1, 120kDa</td>
</tr>
<tr>
<td>2.91</td>
<td>IP00003174</td>
<td>RBM14</td>
<td>RNA binding motif protein 14</td>
</tr>
<tr>
<td>3.67</td>
<td>IP00453473</td>
<td>H4</td>
<td>Histone H4</td>
</tr>
<tr>
<td>9.38</td>
<td>IP00410714</td>
<td>HBA</td>
<td>Hemoglobin alpha subunit</td>
</tr>
</tbody>
</table>

Note: a. The identified proteins with the ratio of EXP and CON greater than 2 or less than 0.5 were listed here. All of the obtained data should meet the following criteria: 1. The minimum Xcorr of 1.9, 2.2 and 3.75 for singly, doubly and triply charged peptides, respectively; 2. The minimum △Cn cutoff values of 0.08; 3. More than or equal to 2 unique peptides identified per protein; 4. Being Identified in both group and the parallel samples; b. The raw spectral counts from one sample were firstly normalized (raw spectral counts for each identified protein were divided by the total spectral counts number after meeting the criteria shown in experimental source, then multiplied by 10,000) and averaged. Then the R value for each protein is the normalized value of EXP divided the value of CON, the proteins with R values more than 2 or less than 0.5 were selected.

are important for the use of o-SWCNTs in tissue engineering. We extracted the regulation pathway of cell aggregation and migration from the network map. Figures 5(A) and (B) showed the extracted cell aggregation and migration signaling pathway, respectively. The differential proteins induced by o-SWCNTs could increase the concentration of Ca$^{2+}$, which would further stimulate cell aggregation.\(^{33}\) Cell migration was closely combined with cell adhesion process. Seven proteins involved in the regulation pathway of cell adhesion and migration. Down regulation of LGALS1 could inhibit cell migration by modifying the organization of actin cytoskeleton and increasing small GTPase RhosA expression.\(^{34}\) IQGAP1, a target of Cdc42 and Rac1, is localized with E-cadherin and beta-catenin at sites of cell–cell contact. Down-expression of IQGAP1 would result in an increase

![Figure 3](https://example.com/fig3.png)

Figure 3. Expression levels of HBA, CFL1 and LMN2 by western blotting. After incubation with o-SWCNTs for 48 h, K562 cells were collected to prepare cell lysis solution. After boiling, the protein samples were overlaid to 12% SDS-PAGE gel and separated at 125 kV. Then the proteins were transferred to nitrocellulose membranes at 45 kV. After blocking, the membranes were incubated with primary antibody at 4 °C overnight, and then probed with HRP-conjugated secondary antibody.
in E-cadherin-mediated cell–cell adhesive activity, but a
decrease of adhesion between cell and extracellular matrix
(ECM). In addition, over-expression of CALR coupled
with matricellular protein thrombospondin, would inhibit
cell-ECM adhesion. Based on these two signaling
pathways, we predict that o-SWCNTs would stimulate cell
aggregation, but inhibit cell migration.

In order to validate this prediction, we experi-
mentally tested the aggregation and migration of K562 cell.
As shown in Figure 6(A), after treated with 30 μg/mL
of o-SWCNTs, the homogeneously growing K562 cells
aggregated into big agglomerates with diameter ranging
from 0.1 to 0.5 mm. This result suggested that o-SWCNTs
could stimulate cell aggregations. Then we used transwell
system to determine the influence of o-SWCNTs to cell
migration. K562 cells were first starved for 24 h, and
then seeded in the upper chambers of transwell. The lower
chambers were added with culture media (containing 20% FBS
and 100 ng/mL of SDF-1) containing 0 (control
group), 10, 20, 30 or 50 μg/mL o-SWCNTs. After 12 h
Table II. Cellular responses induced by o-SWCNTs.

<table>
<thead>
<tr>
<th>Category</th>
<th>Functions (cellular responses)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellular assembly and organization</td>
<td>Activation, aggregation, alignment, assembly, association, bending, binding, biogenesis, branching, morphogenesis, budding, cleavage, clustering, cross-linkage, depolarization, depolymerization, disruption, elongation, exocytosis, formation, fusion, growth, hyperpolarization, interaction, opening, organization, permeability, presence, priming, quantity, rearrangement, reassembly, release, reorganization, rescaling, secretion, segregation, shortening, size, sliding, stabilization, structural integrity, structure, surface pressure, trafficking, transport, tubulogenesis, turnover, uncoupling</td>
</tr>
<tr>
<td>Cellular function and maintenance</td>
<td>Assembly, autophagy, cell saturation density, clearance, contact growth inhibition, cytostasis, depolarization, destruction, differentiation, endocytosis, endoplasmic reticulum stress response, engulfment, exocytosis, function, generation, homeostasis, hyperpolarization, induction, ingestion, internalization, length, macropinocytosis, negative selection, organization, paired-pulse facilitation, phagocytosis, polarization, positive selection, presence, production, quiescence, reassembly, regulation, release, reorganization, repair, respiration, respiratory burst, secretion, size, turnover, vesiculation</td>
</tr>
<tr>
<td>Molecular transport</td>
<td>Accumulation, concentration, deposition, distribution, efflux, elimination, exchange, excretion, exocytosis, export, import, localization, mobilization, nuclear export, quantity, redistribution, release, reuptake, secretion, sequestration, translocation, transport, uptake</td>
</tr>
<tr>
<td>Cell-to-cell signaling and interaction</td>
<td>Activation, adhesion, allostimulatory capacity, attachment, attraction, binding, cell-cell adhesion, cell-cell contact, chemotraction, communication, contact growth inhibition, co-stimulation, deadhesion, delamination, detachment, disruption, excitatory postsynaptic potential, formation, fusion, gap junctional intercellular communication, induction, long term depression, migration, negative selection, paired-pulse facilitation, permeability, phagocytosis, positive selection, presence, priming, reassembly, recovery, recruitment, response, scattering, sensitization, signaling, stimulation, suppression, synaptic transmission, transepithelial electrical resistance</td>
</tr>
</tbody>
</table>

Figure 5. Cell aggregation and migration signaling pathways extracted from protein network. A) Stimulation mechanism of cell aggregation and B) inhibition mechanism of cell adhesion and migration in K562 cells treated with o-SWCNTs. The red and green arrows represent the positive and negative regulation, respectively.

incubation, we determined the number of cells migrating to the lower chambers. The cell number in lower chamber exposed to o-SWCNTs was divided by the cell numbers without tube expose to calculate the % change of migrated cells. As shown in Figure 6(B), the percentage of the migrated cells decreased to ca. 15% when the dose of o-SWCNTs was higher than 30 μg/mL, suggesting that o-SWCNTs could inhibit cell migration. And the inhibition showed a dose-dependent manner. It is high likely that the hydrophobic surface of o-SWCNTs is responsible for the inhibited cell migration and increased cell aggregation. o-SWCNTs could associate with cell membrane by hydrophobic interaction, and cells could anchor around the tubes, which will induce cell aggregation and inhibit cell migration. Taken together, all these results
well demonstrated the assumptions of cell aggregation and migration from protein signaling network.

3.5. Predicting and Validating the Influences of o-SWCNTs to Cell Death

Since cell toxicity is an important effect for the bio-application and toxicity studies on o-SWCNTs, we examined this effect on signaling network. We did not find any signaling changes related to cell death, suggesting that o-SWCNTs have limited effect on cell death. In order to demonstrate this assumption, two cellular viability assays including flow cytometry (FCM) and WST-1 assays were used to evaluate the toxicity of o-SWCNTs in K562 cells. K562 cells exposed to o-SWCNTs for 2 h were stained with Annexin V/PI to determine the short-term toxicity. As shown in Figure 7(A), each point in the figure corresponded to a single cell and its position on the graph represented the health status. The lower-left quadrant represented living cells and the other three quadrants of lower-right, upper-right and upper left represented the early apoptotic cells, advanced apoptotic cells and necrotic cells in the counter-clockwise direction, respectively. It was clearly shown that most cells exposed to o-SWCNTs were in the lower-left quadrant, suggesting that the short-term toxicity of o-SWCNTs was limited. WST-1 assay is based on cleavage of the tetrazolium salt WST-1 by the active mitochondria to produce a soluble colored formazan salt, which is water-soluble and can be photometrically quantified at 450 nm.\textsuperscript{37} We used WST-1 assay to determine the long-term toxicity of K562 cells exposed to o-SWCNTs. Figure 7(B) showed that K562 cells exposed to o-SWCNTs for 48 h were still alive, and did not induce significant cell death. Both the Annexin V/PI and WST-1 results indicated that o-SWCNTs have neither short-term nor long-term toxicities. These results are consistent with our assumption obtained from protein signaling network.

Figure 6. Experimental validation of cell aggregation and migration. A) Microscopy images of K562 cells treated with or without 30 µg/mL o-SWCNTs for 48 h. B) % changes of migrated cells. K562 cells were seeded in the upper chamber of transwell system and starved for 24 h. After that, the upper chamber was combined with the lower chamber that contains 0 to 50 µg/mL o-SWCNTs in RPMI 1640 medium supplemented with 20% FBS and 100 ng/mL of SDF-1β.* p < 0.05, compared to ctrl.

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4. CONCLUSIONS

In this study, o-SWCNTs induced differential proteins were identified by nano LC-MS based proteomic tool. These differential proteins were randomly selected and validated by western blotting, which proved the high reliability of the proteomic approach. By analyzing these differential proteins, we established a protein signaling network to predict the cellular responses of o-SWCNTs in K562 cells. Our predictions on cell aggregation, migration and death in K562 cells were experimentally validated. We found that o-SWCNTs showed stimulation effect on cell aggregation, inhibition on cell migration and no effect on cell death. The cellular responses of o-SWCNTs in K562 cells discovered in this study provide a comprehensive understanding for the bio-safety of o-SWCNTs and can potentially benefit the bio-application of CNTs in drug delivery, bio-imaging and tissue engineering. This proteomics based predictive tool could be also used to study the nano-bio interface of other nanomaterials.

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References and Notes

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