Insight into the Mechanisms of Combined Toxicity of Single-Walled Carbon Nanotubes and Nickel Ions in Macrophages: Role of P2X₇ Receptor

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S Supporting Information

ABSTRACT: Coexistence of nanomaterials and environmental pollutants requires in-depth understanding of combined toxicity and underlying mechanism. In this work, we found that coexposure to the mixture of noncytotoxic level of single-walled carbon nanotubes (SWCNTs) (10 $\mu\text{g/mL}$) and Ni^{2+} (20 μM) induced significant cytotoxicity in macrophages. However, almost equal amount of intracellular Ni^{2+} was detected after Ni^{2+} /SWCNT coexposure or Ni^{2+} single exposure, indicating no enhanced cellular uptake of Ni^{2+} occurred. SDS-PAGE analysis revealed 50% more SWCNTs retained in Ni^{2+} /SWCNT exposed cells than that with SWCNT exposure alone, regardless of the exposure sequence (coexposure, Ni^{2+} pre- or post-treatment), suggesting inhibited SWCNT exocytosis by Ni^{2+} . The increased cellular dose of SWCNTs could quantitatively account for the elevated toxicity of Ni^{2+} /SWCNT mixture to cells. It was then found that agonist (ATP) and antagonist (o-ATP) of P2X₇R could regulate intracellular SWCNT amount and the cytotoxicity accordingly. In addition, inhibition of P2X₇R by P2X₇-targeting siRNA diminished the inhibitory effect of Ni^{2+} . It was therefore concluded that Ni^{2+} impeded SWCNT exocytosis by inhibiting P2X₇R, leading to higher intracellular retention of SWCNTs and elevated cytotoxicity. Our work identified exocytosis inhibition as an important mechanism for SWCNT/ Ni^{2+} toxicity, and revealed the crucial role of P2X₇R in mediating such inhibitory effect.

INTRODUCTION

Chemicals released into the environment usually coexist with other environmental pollutants as complex mixtures.^{1–3} Concurrent or sequential human exposure to these chemicals dictates the necessity of risk assessment of chemical mixtures. From both the public health and regulatory perspectives, combined toxic effects among mixture components are of particular concerns. Recently, the rapid development of nanotechnology has brought about large amount of nanomaterials that not only improve the quality of life but also raise great concerns on their potential risks on environment and human health. It has been demonstrated that coexposure of some nanomaterials and contaminants induce altered toxicity in living organisms, and the interactive outcomes generally fall into the following three categories. First of all, copresence of nanomaterials and contaminants results in no increased toxicity.⁴ Second, nanomaterials alleviate the toxicity of contaminants compared to the exposure of individual contaminant.^{5–7} The lowered toxicity is often interpreted by the adsorption of contaminants on nanomaterials and

consequently reduced bioavailability of contaminants in living organisms. Third, of the most importance and environmental significance, nanomaterials aggravate the toxicity of contaminants in living organisms. This combined toxicity has been observed in binary mixtures composed of either fullerene C₆₀ or nano-TiO₂ and a variety of chemicals such as PAHs,^{8,9} PBDEs,¹⁰ TBT,¹¹ and heavy metals.^{12,13} In most of the studies, the combined toxicity is attributed to the so-called “Trojan Horse” effect of nanomaterials, in which they facilitate the ingestion of contaminants by the organisms, increase the cellular dose and therefore amplify the deleterious effects of these toxicants to organisms. The mechanism is also applied to explain the combined effects of carbon nanotubes (CNTs) and a variety of contaminants.^{14–18} In an alternative mechanism, TiO₂ nanoparticles were shown to accelerate the biotransfor-

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mation of pentachlorophenol to more toxic metabolites, thus enhance the overall toxicity.¹⁹ These studies have made great progress in understanding the impacts of nanomaterials on the toxicity of environmental contaminants. However, the alteration of nanomaterials toxicity by the chemical contaminant in the mixtures is mostly ignored, and deserves equal attention.

In recent years, single-walled carbon nanotubes (SWCNTs), due to their unique physical, chemical, and electronic properties, have been widely used in the fields of biomedicine, biotechnology, and electronics.^{20,21} With ever increasing range of applications and production rates, SWCNTs will inevitably enter the environment during their life circle. Lines of evidence revealed that SWCNTs could enter organisms via the respiratory or intestinal tract and accumulate in living organisms for as long as three months.^{22–25} In vivo studies showed that CNTs through inhalation (intratracheal instillation and pharyngeal aspiration) could induce acute inflammation, progressive granulomas and fibrosis in animals.^{25,26} In vitro, CNTs were shown to cause lower cell viability and depressed cell proliferation in human lung-tumor and keratinocyte cells^{27,28} and decrease in cell-adhesion ability and altered cell-cycle regulation in embryo kidney cells.²⁹ The toxicities have also been detected in other cell types, namely mesenchymal stem cells,³⁰ and epithelial cells.³¹ Most recently, our studies and others showed that SWCNTs could readily enter macrophages and interfere with the biogenesis and gene expression of lysosome, proteasome and mitochondrial respiration chains, inducing inflammatory response,³² resulting in suppressed engulfing function of primary monocytes/macrophages.^{33,34}

Nickel is ubiquitously present in the environment, and the exposure to low doses of Ni compounds is unavoidable. In the field of nanotechnology, nickel is often used as a catalyst for the synthesis of commercial SWCNTs, or as a coating material on the surface of nanoparticles, which lead to the coexistence of nanoparticles and nickel. The presence of nickel in these products might be toxic as it can form reactive oxygen species (ROS) that is detrimental to cells. As a Group I carcinogen to humans,³⁵ environmental nickel has already raised health concern stemming from the knowledge of its toxicity and pathogenic properties derived from occupational epidemiology and animal studies including carcinogenicity, genotoxicity, and neurotoxicity.^{36–38} Especially, nickel ions (II) were shown to inhibit the activity of an important purinergic receptor, P2X₇R,^{39,40} which mediates the production and release of inflammasomes.^{41,42} Most importantly, this receptor was reported to mediate the secretion of lysosomes,^{43,44} where most carbonaceous nanomaterials including SWCNTs are localized after cellular internalization.^{45–47} Normally, at the cellular level, the toxic properties and extent of SWCNTs depend on the amount of SWCNTs inside cells, which are determined by a variety of cell processes including exocytosis. It is possible that SWCNTs and other contaminants such as nickel ions interact with each other, resulting in altered toxicological profile. Thus, we hypothesized that nickel ions might interfere with toxicokinetics and thus the toxicity of SWCNTs.

To test the hypothesis, monocytic/macrophage cells were employed based on two considerations. Macrophages are abundant in P2X₇R expression.⁴⁸ They are also pivotal players in both innate and adaptive immunity. As a part of the first line of defense against invading foreign substances including nanoparticles, macrophages are the primary target for nano-

particle toxicity.^{32,34} Therefore, we compared the toxicity of SWCNTs and nickel ions both alone and in combination on macrophage survival, and investigated the molecular mechanisms underlying the combined effects.

MATERIALS AND METHODS

Materials. The murine macrophage cell line Raw264.7 cells (ATCC: TIB-71) and human monocytic cell line THP-1 cells (ATCC: TIB-202) were acquired from American Type Culture Collection. All ingredients for the culture media were purchased from Gibco, Invitrogen (UK). SWCNTs (CNTs purity >95%, SWCNT purity >90%, ash <5 wt %) synthesized by chemical vapor deposition (CVD) method were originally obtained from Chengdu Organic Chemicals Co., Ltd. (SiChuan, China) in high purity. The detailed information can be found on the company Web site: <http://www.timesnano.com/>. The Alexa Fluor488 probe was purchased from Invitrogen (USA). Nickel chloride, ATP (adenosine 5'-triphosphate), oATP (oxidized adenosine 5'-triphosphate) and Hoechst 33342 were purchased from Sigma-Aldrich (USA). Rabbit anti-mouse P2X₇ and β -actin antibody were purchased from Cell Signaling Technology (U.S.). IRDye 680RD Goat anti-Rabbit IgG (secondary antibody) was from LI-COR company (U.S.).

Preparation of Single-Walled Carbon Nanotubes.

Preparation of SWCNTs solution was performed according to the procedure described previously.³² In detail, 10 mg SWCNTs were suspended in 40 mL of a 3:1 mixture of concentrated H₂SO₄/HNO₃ in a 200 mL flask and sonicated in a water bath (KQ-500DV, 40 kHz) for 24 h at 40–50 °C. The resultant suspension was then diluted with 200 mL deionized water and filtered through a membrane (pore size 0.22 μ m), followed by washing with 50 mL deionized water on the membrane. The SWCNTs were resuspended in sterilized deionized water at a concentration of 1 mg/mL with brief sonication (KQ-500DV, 40 kHz). The acid-functionalized SWCNTs suspension was black, well dispersed, and had neutral pH.

Characterization of SWCNTs. The morphology and structure were imaged with a HitachiH-7500 transmission electron microscope (TEM, Tokyo, Japan). Specifically, the sample was diluted to 0.5 mg/mL in water. Thereafter, the suspension was precipitated onto a copper net and dried at room temperature and then subject to TEM examination (Figure S1). In addition, the infrared spectra of SWCNTs were collected by using a FT-IR spectrometer (JASCO, Inc., Easton, MD) (Figure S1). Zeta potential (ZP) and dynamic light scattering of SWCNTs were measured by Zetasizer Nano (Malvern Instruments, Malvern, UK). Raman measurement was performed via using Renishaw Raman spectroscope (Wotton-under-Edge, UK) with excitation wavelength at 532 nm (Figure S1).

Cell Culture and Exposure. Raw264.7 and THP-1 cells were cultured at 37 °C in fully humidified atmosphere containing 5% CO₂ in complete culture medium (cRPMI) consisting of RPMI-1640 and 10% heat deactivated fetal bovine serum (FBS) supplemented with 20 mM L-glutamine and 100 U mL⁻¹ penicillin/streptomycin. For all experiments of SWCNTs quantification, cells were seeded in 6-well plates (Corning, U.S.) at a density of 5×10^5 cells/mL in cRPMI and allowed to attach for overnight. The stock solution of 1 mg/mL SWCNTs was prepared by sonicating in a cold water bath (KQ-500DV, 40 kHz) for 5 min before usage. Thereafter, the

stock solutions were diluted in cRPMI to desired concentrations for exposure.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) Analysis of Ni^{2+} . To measure Ni^{2+} content inside and outside cells, Raw264.7 cells (5×10^5 cells/mL) were treated with $20 \mu\text{M}$ Ni^{2+} and mixture of $10 \mu\text{g/mL}$ SWCNTs and $20 \mu\text{M}$ Ni^{2+} (brief mixing for 30 s) for 24 h. After then, the extracellular supernatants were collected. Meanwhile, the cells were washed three times with PBS and digested with $\text{HNO}_3/\text{H}_2\text{O}_2$ (1:1). After that, the levels of Ni^{2+} inside cells and in the supernatant were assessed using the ICP-MS (Agilent 7500). In addition, to detect the absorption of Ni^{2+} onto SWCNTs in cells, we further centrifuged the cell lysate from mixture treated group to isolate SWCNTs and the Ni^{2+} amount pelleted with SWCNTs was measured by ICP-MS.

SDS-PAGE and UV-vis-NIR Quantification of SWCNTs in Cells and Supernatants. To quantify SWCNTs amount inside macrophages, the cells were treated with SWCNTs or the mixture of SWCNTs and Ni^{2+} for indicated times. After treatment, the intracellular SWCNTs were quantified by using SDS-PAGE method, as described previously.⁴⁹ In detail, the cells were lysed with $160 \mu\text{L}$ lysis buffer (1% SDS, 1 mM MgCl_2 , and 1 mM CaCl_2) for 3 min, then $20 \mu\text{L}$ cell lysates were subjected to SDS-PAGE electrophoresis on a standard BD Mini Vertical Gel (10×8 cm) with only 4% stacking gel as SWCNTs loading substrate. The samples were electrophoresed at 120 V for 2 h, and SWCNTs would deposit on the loading well forming a dark band. After that, loading wells were sealed with 4% stacking gel. Next, UMAX scanner was used to scan SWCNTs bands on the gel and the intensity of the bands were analyzed using Gelpro software (Media Cybernetics, U.S.). To ensure the loaded samples were from same cells number, the protein amount of each sample was quantified with BCA protein assay kit (KangWei Co. Ltd., China). Simultaneously, the corresponding supernatant in each treatment group was collected for UV-vis-NIR (Varian Cary 5000, USA) analyses.

SWCNTs Labeling and Tracking within Raw264.7 Cells. To track SWCNTs internalization in macrophages, We labeled SWCNTs with a fluorescent dye Alexa Fluor 488 (SWCNT-Alexa Fluor 488) and then compared the distribution pattern of SWCNTs in cells treated with SWCNTs or SWCNTs/ Ni^{2+} . The cells were exposed to Alexa Fluor 488 labeled SWCNTs for 24 h or mixture of Alexa Fluor 488 labeled SWCNTs/ Ni^{2+} , and then SWCNTs outside cells was washed off with PBS. The internalization of SWCNTs was performed via confocal laser scanning microscopy (Leica, Mannheim, Germany). The Alexa Fluor 488 labeled SWCNTs was prepared by mixing SWCNTs with an amine-containing fluorescent dye, Alexa Fluor488 cadaverine, and through EDC/NHS mediation (1:1) in MES buffer, Alexa Fluor 488 was covalently linked with the carboxyl groups of SWCNTs. The labeled SWCNTs were further purified and concentrated by centrifugation at 12000 rpm using ultrafiltration tubes (Millipore, Germany) for 4 cycles and then diluted to the desired concentration before exposure to cells for confocal microscopy imaging.

Flow Cytometric Assay. To further confirm Ni^{2+} influence on SWCNTs exocytosis, we analyzed fluorescence intensity of Alexa Fluor 488-SWCNTs within cells by using flow cytometer to evaluate the relative amount of intracellular SWCNTs. Raw264.7 cells were incubated with $5 \mu\text{g/mL}$ Alexa Fluor 488-SWCNTs or mixture of $5 \mu\text{g/mL}$ Alexa Fluor 488-SWCNTs and $20 \mu\text{M}$ Ni^{2+} for 24 h. The cells were then washed three

times with PBS before trypsinizing and resuspended in medium. The fluorescent intensity of Alexa Fluor 488-SWCNTs were analyzed using a flow cytometer (BD Biosciences, San Jose, CA) by collecting 10 000 events. The data files were saved and analyzed using Cell Quest software (BD Biosciences).

P2X₇ Receptor Silencing. Raw264.7 cells were transfected with small interfering RNA (siRNA) to silence P2X₇ receptor expression. P2X₇R-targeting oligonucleotides were designed and generated from full-length mouse P2X₇R by Shanghai GeneChem Co., Ltd. (Shanghai, <http://www.genechem.com.cn>). After testing knockdown efficiencies, stem-loop DNA oligonucleotides were synthesized by Shanghai GeneChem Co. Ltd. (sense, 5'- CCG G GC GGA AAG AGC CTG TTA TCA GCT CGA GCT GAT AAC AGG CTC TTT CCG CTT TTT G-3'; antisense, 5'- AAT TCA AAA AGC GGA AAG AGC CTG TTA TCA GCT CGA GCT GAT AAC AGG CTC TTT CCG C-3') and cloned into the lentivirus-based RNAi vector GV248. A nontargeting stem-loop DNA GV248 vector was also generated for use as a negative control. Lentiviral particles were prepared according to protocol provided by GeneChem Co. Ltd. Raw264.7 cells were then infected with P2X₇R-RNAi-lentivirus or negative control virus in the presence of ENI solution (GeneJikai, Shanghai, China) (MOI = 100). Then, the cells were treated with the mixed solution for 10 h at 37 °C in fully humidified atmosphere containing 5% CO_2 . After removing infection solution, the cells were further cultured in fresh medium for another 2 days. Part of the cells were then collected to extract total RNA for quantitative reverse transcription polymerase chain reaction (QRT-PCR) and proteins for Western blot analyses to confirm the effectiveness of knock-down expression of P2X₇R. Then, the cells were used for different experiments.

P2X₇ Receptor Gene Expression and mRNA Transcription. The mRNA levels of P2X₇ receptors in cells after P2X₇R knock-down were measured by real-time RT-PCR (LightCyclerC480, Roche, Germany). The comparative C_t method for relative quantification of gene expression ($\Delta\Delta C_t$ method) was used, and gene expression levels of P2X₇ receptors were normalized to gene expression of GAPDH (as a housekeeping gene control) using the following equation: P2X₇R mRNA normalized expression = $2^{[\Delta C_t(\text{GAPDH}) - \Delta C_t(\text{P2X}_7\text{R})]}$.

Western Blot Assay. Cell lysates were separated by SDS-PAGE with 10% separation gel. The samples were transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore, Germany), and the membrane was blocked with 5% nonfat milk (TBS) and washed three times with TBST (containing 0.1% Tween 20). Thereafter, the membrane was incubated with primary antibodies (1:1000 dilution) against P2X₇ and β -actin (Cell Signaling Technology, MA) at 4 °C for overnight, and then washed with TBST again. The membrane was incubated with corresponding secondary antibody (IRDye 680RD Goat anti-Rabbit, 1:5000 dilutions) for 1 h at room temperature in the dark, followed by three washes with TBST and two washed with TBS. The bands of target proteins were observed using an Odyssey CLx instrument (LI-COR, U.S.). Quantitative analysis of the band intensities was performed using Gel-pro image software (Media Cybernetics, U.S.).

Cytotoxicity Assay. Cell viability was assessed by an aqueous soluble tetrazolium/formazan assay (WST-1 assay) based on the conversion of a tetrazolium salt into water-soluble formazan product, which is mediated by cellular dehydrogenases present only in living cells. Therefore, the intensity of

dye converted is directly proportional to the number of living cells.⁵⁰ The cells were exposed to the tested samples at series concentrations. Cells without treatment were set as control. After treatment, the cells were washed with PBS twice and incubated with the WST-1 assay reagents for 2 h and absorbance was measured at 450 nm with a Varioskan Flash microplate reader (Varioskan Flash, Thermo Scientific, Waltham, MA). Cell viability was expressed as the percentage of the control group.

Statistical Analysis. Data were expressed as mean $\pm$ SD., and the difference between groups was evaluated using two-tails Student's *t* test, with the significance level set at **p* < 0.05, ***p* < 0.01.

RESULTS AND DISCUSSION

Combined Toxicity of SWCNTs and Nickel Ions (II).

Well-dispersed suspension of SWCNTs in aqueous solution was synthesized following the protocol described in the [Materials and Methods](#) section. The resulting CNT suspension contains 0.056 wt % of iron and no nickel and cobalt was detected, as we reported previously.³² The approach enables insertion of carboxyl and hydroxyl groups around the sidewalls and at the tips of the SWCNTs, which makes the tubes well dispersed in aqueous medium for days ([Figure S1](#)). In addition, hydrodynamic size and surface charge were analyzed by dynamic light scattering assessment ([Table 1](#)). Hydrodynamic

Table 1. Hydrodynamic Size (HDS, nm) and Zeta Potential (ZP, mV) of SWCNTs in Water, Serum-Free Medium (SFM), Serum-Supplemented Medium (SSM)

SWCNTs	Water	SFM	SSM
HDS	86.4 $\pm$ 4.4	1132 $\pm$ 69	112 $\pm$ 6.2
ZP	-47.1 $\pm$ 1.6	-23 $\pm$ 0.7	-8.4 $\pm$ 0.2

diameters and surface charges of nanomaterials changed in SSM and SFM compared to water because in serum free medium, the ions would be neutralizing the surface charge of SWCNTs and causes aggregation, but in serum medium, the serum protein would facilitate the dispersion of SWCNTs, resulting lower hydrodynamic size, compared with serum free medium, but still larger than that in water.

Subsequently, we investigated the combined toxicity of SWCNTs and Ni²⁺. As shown in [Figure 1](#), macrophages did not undergo cell viability loss when exposed to 5 and 10 μ g/mL SWCNTs for 24 h, which is consistent with our previous studies showing that 10 μ g/mL SWCNTs poses no cytotoxicity on Raw264.7 cells or murine peritoneal macrophages, as determined by LDH release, live/dead staining and apoptosis analyses.^{32,34,47} Under the same experimental condition, 20 μ M Ni²⁺ was noncytotoxic, but there was about 21% viability loss when cells were exposed to a mixture of 20 μ M Ni²⁺ and 10 μ g/mL SWCNTs ([Figure 1](#)). Furthermore, a mixture of 20 μ g/mL SWCNTs and 20 μ M Ni²⁺ caused approximately 26% cell death, whereas 20 μ g/mL SWCNTs alone induced 14% cell death, indicating that Ni²⁺ at 20 μ M dramatically aggravated the cytotoxicity of SWCNTs by almost 2-fold ([Figure 1](#)). The toxicity of SWCNTs and Ni²⁺ mixture to macrophages is obviously higher than the additive effect of SWCNTs and Ni²⁺ toxicity alone. The cytotoxicity of Ni²⁺ with higher concentrations was not further investigated because higher concentrations caused significant cell death ([Figure S2](#)). Interestingly, we did not observe any toxicity to macrophages upon

Figure 1. Cell viability after treatment with SWCNTs or Ni²⁺, alone or in combination. Raw264.7 cells were exposed to 5, 10, and 20 μ g/mL SWCNTs, 20 μ M Ni²⁺, and mixture of SWCNTs and Ni²⁺. Then, the cell viability was determined by WST-1 assay.

SWCNTs/Ni²⁺ treatment at lower SWCNTs concentration (5 μ g/mL) ([Figure 1](#)), suggesting that SWCNTs-induced cytotoxicity only occurs when internalized SWCNTs exceed certain threshold. Taken together, these data demonstrated that coexposure of SWCNTs and Ni²⁺ resulted in elevated toxicity in macrophages.

Nickel Ions (II) Quantification by ICP-MS. The above result supports the idea that coexposure of SWCNTs and Ni²⁺ could lead to elevated toxicity in macrophages. Nonetheless, the underlying mechanism is still unknown. One possibility for the elevated toxicity might be that SWCNTs could act as a carrier to transport more Ni²⁺ into cells. To address the question, we measured the levels of Ni²⁺ both in cells and in supernatants by using ICP-MS. As shown in [Figure 2](#), the intracellular Ni²⁺ amount for SWCNTs/Ni²⁺ treatment group was approximately the same to that of Ni²⁺ treatment group, indicating that the presence of SWCNTs did not change the internal amount of Ni²⁺ in macrophages. Meanwhile, the Ni²⁺ contents in the supernatants of these two groups showed similar results as well. In addition, because the Ni²⁺ could be

Figure 2. Quantitative analysis of Ni²⁺ by inductively coupled plasma mass spectrometry (ICP-MS). Raw264.7 cells were treated with 20 μ M Ni²⁺ and mixture of 10 μ g/mL SWCNTs and 20 μ M Ni²⁺ for 24 h. After then, the cells lysates and the supernatants were analyzed with ICP-MS (*n* = 3). The level of Ni²⁺ in cells without treatment was set as control.

Figure 3. Quantification of SWCNTs in cells and supernatants and the localization pattern of SWCNTs in macrophages upon treatment with SWCNTs or a mixture of SWCNTs and Ni^{2+} . (A) SDS-PAGE gel (numeric data for upper panel and gel image for middle panel) and UV-vis-NIR analyses (lower panel) of SWCNTs amount in macrophages and supernatants after 24 h coexposure of SWCNTs and Ni^{2+} . (B) SDS-PAGE gel and UV-vis-NIR analyses of SWCNTs amount in macrophages and supernatants after the cells were post-treated with Ni^{2+} . Raw264.7 macrophage were exposed to 10 $\mu\text{g/mL}$ SWCNTs for 8 h, followed by removing SWCNTs solution, and further incubated with 10 μM and 20 μM Ni^{2+} for 16 h. (C) SDS-PAGE gel and UV-vis-NIR analyses of SWCNTs amount in macrophages and supernatants after the cells were pretreated with Ni^{2+} . Macrophages were pretreated with 10 and 20 μM Ni^{2+} for 16 h, followed by removing Ni^{2+} and incubation with 10 $\mu\text{g/mL}$ SWCNTs for another 8 h. The intensity of the SWCNTs bands was analyzed using Gelpro software ($n = 3$). (D) Representative confocal images of Raw264.7 cells upon exposure to 0 (control), 10 $\mu\text{g/mL}$ SWCNTs, and mixture of SWCNTs (10 $\mu\text{g/mL}$) and Ni^{2+} (20 μM) for 24 h. Scale bar = 30 μm . (E) Confocal images showing the localization pattern of SWCNT-Alexa Fluor 488 (green) in macrophages (Arrows: Red, cell membrane; pink, cell nucleus; cyan, cytoplasm) and (F) the fluorescence intensity of Alexa Fluor 488 of cells receiving SWCNT-Alexa Fluor 488 or SWCNT-Alexa Fluor 488/ Ni^{2+} exposure, as determined by flow cytometry analyses.

adsorbed on SWCNTs to some extent, we detect the adsorption capacity of SWCNTs for Ni^{2+} and found that only 3.5% of Ni^{2+} was adsorbed on SWCNTs but 96.5% Ni^{2+} entered cells in free status, which constitute neglectable effect in cell lysate (Figure 2). Together, the combined data ruled out

the possibility that the elevated toxicity was caused by increased Ni^{2+} transportation into cells.

Nickel Ions (II) Inhibits Exocytosis of SWCNTs. After ruling out the possibility that the elevated toxicity of SWCNTs and Ni^{2+} in macrophages was due to SWCNT-mediated transport of more Ni^{2+} into cells, we hypothesized another

Figure 4. Quantification of intracellular SWCNTs accumulation and reduced viability of macrophages upon exposure to SWCNTs and SWCNTs/ Ni^{2+} . (A) Concentration-dependent increase of intracellular SWCNT accumulation in macrophages exposed to 10, 20, 30, and 50 $\mu\text{g/mL}$ SWCNTs alone, and mixture of 10 $\mu\text{g/mL}$ SWCNTs and 20 μM Ni^{2+} for 24 h. Lower panels show corresponding representative SWCNTs bands in gel. The intensity of the dark band of the SWCNTs was analyzed using Gelpro software. (B) Cell viability change of macrophages upon exposure to SWCNTs alone and SWCNTs/ Ni^{2+} .

possibility that Ni^{2+} inhibits exocytosis of SWCNTs resulting in increased accumulation of SWCNTs in cells, leading to higher toxicity. To validate the hypothesis, we compared the intracellular SWCNTs in macrophages after exposure of SWCNTs alone or the mixture of SWCNTs/ Ni^{2+} for 24 h. Simultaneously, the corresponding supernatants were collected for UV-vis-NIR analysis. As showed in Figure 3A, compared to the SWCNTs alone group, coexposure with Ni^{2+} increased the amount of intracellular SWCNTs (Figure 3A, upper panel) and decreased the extracellular SWCNTs amount (Figure 3A, lower panel) in a Ni^{2+} concentration-dependent manner, indicating that Ni^{2+} inhibited SWCNTs exocytosis from cells. However, the significant increase of intracellular SWCNTs after Ni^{2+} coexposure also raised the question as to whether the increase was due to increased SWCNT uptake or not. To exclude the possibility, the cells were first exposed to SWCNTs for 8 h so as to take up equal amount of SWCNTs for both treatment groups. Then the SWCNTs solution was removed, and Ni^{2+} was added to the test group for further incubation for 16 h. Subsequently, we measured the intracellular and extracellular SWCNTs amount by SDS-PAGE and UV-vis-NIR, respectively. Similarly, we found that intracellular SWCNTs (Figure 3B, upper panel) increased and extracellular SWCNTs (Figure 3B, lower panel) decreased in a Ni^{2+} concentration dependent manner as well, which verified the inhibitory effect of Ni^{2+} on SWCNTs exocytosis from macrophages. Besides, pretreatment of Ni^{2+} also induced a large increase of intracellular SWCNTs in macrophage cells (Figure 3C). All data demonstrated that Ni^{2+} indeed inhibited SWCNTs exocytosis and as a result, led to more SWCNTs accumulation in cells. Furthermore, by using confocal microscopy, we further showed that, compared to SWCNTs group, more SWCNTs accumulation was observed in SWCNTs/ Ni^{2+} treated cells (Figure 3D).

Normally, biological effects of nanoparticles are closely related to their cellular transportation and localization inside cells. We found that SWCNTs were mostly accumulated in cytoplasm and perinuclear area, and Ni^{2+} did not influence the distribution pattern of SWCNTs (Figure 3E). More SWCNTs

were shown to be retained within cells, as shown by the flow cytometer data (Figure 3F), which further supported our SDS-PAGE and UV-vis-NIR results.

Equivalent External Exposure Concentration and Toxicity of SWCNTs Due to Inhibition Effect of Ni^{2+} .

The toxicity of nanomaterials to cells, in fact, is determined by intracellular amount of nanoparticles, which, however, is to some extent dependent on external exposure concentration. Hence, to further delineate the relationship between inhibitory extent of Ni^{2+} and subsequent toxicity, we compared intracellular SWCNT amounts in macrophages receiving mixture exposure with those in cells exposed to different concentrations of SWCNTs ranging from 10 to 50 $\mu\text{g/mL}$. As shown in Figure 4A, after 24 h exposure, the SWCNTs amount inside cells increased with increasing SWCNT exposure concentration. The intracellular SWCNTs amount for SWCNTs/ Ni^{2+} treatment group was approximately equivalent to that of 25 $\mu\text{g/mL}$ SWCNTs treatment group, indicating that the presence of Ni^{2+} indeed influenced the internal amount of SWCNTs in macrophages. More importantly, the cell viability assay showed similar toxicity of these two groups, with 23% and 21% reduction of cell viability, respectively (Figure 4B, $p < 0.01$), suggesting the elevated toxicity of SWCNTs/ Ni^{2+} was elicited due to increased SWCNTs accumulation in cells.

P2X₇R Mediates the Inhibitory Effects of Ni^{2+} on SWCNTs Exocytosis.

In our recent study, we demonstrated that P2X₇ receptor is involved in the regulation of SWCNTs exocytosis in lysosomes.⁵¹ Therefore, we hypothesized that SWCNTs exocytosis might be suppressed by Ni^{2+} through inactivating P2X₇ receptor. To verify our hypothesis, an agonist (ATP) and a specific inhibitor (oATP) of P2X₇ receptor were used. The agonist and inhibitor had no significant influence on cell viability (Figure S3). As illustrated in Figure 5A, ATP activation of P2X₇R promoted exocytosis of internalized SWCNTs from cells, leading to less retention of SWCNTs in cells compared to the control group (SWCNTs treatment alone). In contrast, oATP inhibition of P2X₇R suppressed exocytosis of SWCNTs, resulting in more SWCNTs retained in

Figure 5. P2X₇R modulation on the intracellular accumulation of SWCNTs and cytotoxicity and influence of P2X₇R gene silencing on SWCNTs accumulation and cell survival. (A) SDS-PAGE quantification of intracellular SWCNTs in macrophages and (B) Corresponding cell viability of macrophages upon exposure to SWCNTs and SWCNTs/ Ni^{2+} in the presence of P2X₇R agonist and antagonist, as determined by WST-1 assay. Raw264.7 cells were pretreated with P2X₇R agonist (ATP, 2 mM) and P2X₇R inhibitor (oATP, 2 mM) for 1 h, followed by exposure of 10 $\mu\text{g/mL}$ SWCNTs or mixture of 10 $\mu\text{g/mL}$ SWCNTs and 20 μM Ni^{2+} for 24 h. (C) Amount of intracellular (upper panel) and extracellular (lower panel) SWCNTs in cells with (labeled with P2X₇ siRNA) or without P2X₇R knock-down, and (D) Corresponding cell survival after the same treatments, as determined by WST-1 assay. Quantification of SWCNTs was performed using SDS-PAGE and UV-vis-NIR. Dark bands intensity of SWCNTs in gel image was analyzed using Gelpo image software.

cells, with dramatic reduction of cell viability in comparison to the control (SWCNTs treatment only) group (Figure 5B). Similar inhibitory effect and cytotoxicity were observed in macrophages treated with Ni^{2+} (Figure 5A,B), indicating that

Ni^{2+} probably targets the same receptor (P2X₇ receptor) as oATP on macrophages. Additionally, the increased SWCNT accumulation in SWCNTs/ Ni^{2+} group could be dramatically alleviated by ATP pretreatment (Figure 5A, green; Figure 5B,

Figure 6. Cell viability, SWCNTs amount in cells and supernatants after treating THP-1 cells with SWCNTs or a mixture of SWCNTs and Ni²⁺. (A) THP-1 cell viability after treatment with SWCNTs or Ni²⁺, alone or in combination. (B) SDS-PAGE gel (numeric data for upper panel and gel image for lower panel) and (C) UV-vis-NIR analyses of SWCNTs amount in THP-1 cells and supernatants after 24 h coexposure of SWCNTs and Ni²⁺, respectively. The intensity of the SWCNTs bands was analyzed using Gelpro software ($n = 3$).

green), suggesting that ATP played a detoxification role by activating P2X₇R and promoting SWCNTs exocytosis. Together, these findings revealed that Ni²⁺ induced inhibition of SWCNTs exocytosis and the elevated toxicity of SWCNTs/Ni²⁺ was probably mediated by P2X₇ receptor.

In an attempt to elucidate the crucial role of P2X₇ receptor mediating combined toxicity of SWCNTs/Ni²⁺, the macrophages were transfected with a P2X₇ receptor-specific small interfering RNA (siRNA) to knockdown P2X₇ receptor expression. The siRNA treatment had no significant effect on cell viability (Figure S4) but decreased the expression of P2X₇R mRNA by 84% and the protein level by 88% (Figure S5). The resulting cells were used in the following experiments. To determine whether Ni²⁺ inhibited SWCNTs exocytosis is through targeting and blocking P2X₇R, we compared the intracellular and extracellular SWCNT amount between the normal cells (without P2X₇R siRNA treatment) and silenced cells (P2X₇R siRNA-treated cells) after exposing them to SWCNTs or SWCNTs/Ni²⁺ for 24 h. The SDS-PAGE analyses showed that SWCNTs amount inside the silenced cells was over 2-fold more than that in the normal cells (Figure 5C), and SWCNTs exposure induced higher toxicity to the silenced cells than to the normal cells (Figure 5D). This further verified the essential role P2X₇R plays in SWCNTs exocytosis. Furthermore, for the silenced cells, the addition of Ni²⁺ did not change the intracellular or extracellular SWCNTs amount and cytotoxicity in comparison to the SWCNTs treatment group (Figure 5C,D). All these data unequivocally support our hypothesis that P2X₇ receptor mediated Ni²⁺-induced inhibition of SWCNT exocytosis and the elevated toxicity of SWCNTs/Ni²⁺ mixtures.

Confirming the Combined Toxicity and Ni²⁺ Inhibitory Effect on SWCNTs Exocytosis by THP-1 Cells. We further confirmed the revealed mechanism in human monocytic/macrophage cell line, THP-1, which expresses P2X₇R similarly to that of RAW264.7. THP-1 cells were induced to macrophages with PMA for 18 h (1 μ g/mL) before exposure and the macrophage cells showed no cell death upon treatment of SWCNTs (<20 μ g/mL) and Ni²⁺ (<10 μ M) (Figure S6), but there was about 14% viability loss when cells were exposed to a mixture of 10 μ M Ni²⁺ and 10 μ g/mL SWCNTs (Figure 6A), indicating that Ni²⁺ at 10 μ M

dramatically aggravated the cytotoxicity of SWCNTs. In addition, the determination of intracellular and extracellular SWCNTs showed that, compared to the SWCNTs alone group, coexposure with Ni²⁺ increased the amount of intracellular SWCNTs (Figure 6B) and decreased the extracellular SWCNTs amount (Figure 6C) in a Ni²⁺ concentration-dependent manner, which further confirmed our findings that Ni²⁺ inhibited SWCNTs exocytosis and resulted in elevated toxicity.

The “Trojan Horse” effect^{8–13} was previously elucidated with a focus on the influence of nanomaterials on the toxicity/bioavailability of environmental contaminants, while ignoring the effect of contaminants on the toxicity of nanomaterials. In contrast to previous studies, our work excluded the possibility of “Trojan Horse” effect of SWCNTs on Ni²⁺ toxicity, but still observed elevated toxicity of SWCNTs in the presence of Ni²⁺ at nontoxic doses. In this work, we revealed a novel mechanism for the combined toxicity of nanomaterial/chemical mixtures in which Ni²⁺, by blocking the activation of P2X₇R, inhibited SWCNTs exocytosis, resulting in increased intracellular accumulation of the nanotubes and higher toxicity. Our study suggests that, in the evaluation of the combined toxicity of nanomaterials and chemicals, modulation on the toxic-kinetics of chemicals by nanomaterials as well as the nanomaterials by chemicals need to be investigated in a balanced fashion so as to gain a full picture of the underlying mechanisms.

In a way, the interactive effects of nanomaterial/chemical mixtures are similar to drug–drug interactions in pharmaceutical science, in which one pharmaceutical chemical interacts in vivo with another one and attenuates either the therapeutic efficacy or clinical toxicity of the latter. The drug interactions occur through the alteration of either drug-metabolizing enzymes or cell membrane transporters. Until now, two major classes of transporters have been found to mediate drug interactions, that is, ABC family (such as P-glycoprotein) and SLC family (such as OATP1B1).⁵² The most important finding of this study is that we identified another membrane protein, P2X₇R, as the mediator involved in SWCNTs–Ni²⁺ interactions. P2X₇R is an extracellular ATP-gated ion channel protein highly expressed on the plasma membrane of neurons and immune cells including T cells, dendritic cells, and monocytes/macrophages.⁴⁸ This unique receptor was shown

to mediate the inflammatory response as well as the release of secretory lysosomes in macrophages.^{41,42} In addition to Ni²⁺, some other heavy metals including copper and cadmium ions were both shown to inhibit P2X₇R.^{39,53} Therefore, the mechanism revealed in this work with the SWCNTs/Ni²⁺ mixtures may be also applicable to Cu²⁺, Cd²⁺, and possibly other chemicals which inhibit P2X₇R.

Nickel and nickel compounds are used in many industrial products such as stainless steel and nickel alloys, catalysts, and pigments.^{54,55} Some evidence demonstrated that metal-containing SWCNTs are likely more toxic than the metal-free nanotubes.^{27,56} A well accepted explanation is that transition metals (including nickel) react with hydrogen peroxides in cells and produce large amount of reactive oxygen species (ROS), which are detrimental to cells. Here, our findings would suggest that residual metal ions released from SWCNTs during SWCNT modification such as acid purification, might act as an exocytosis inhibitor of SWCNTs and aggravate the toxicity of SWCNTs.

The present study has verified that Ni²⁺ altered the biological fate of SWCNTs -exocytosis in macrophages through blocking membrane receptor-P2X₇R. However, the precise mechanisms have not been clearly recognized to date. We speculate that there are three possibilities. First of all, Ni²⁺ binds directly to the extracellular domain of P2X₇R and induces allosteric change of P2X₇R, resulting in P2X₇R malfunction. Alternatively, Ni²⁺ interferes directly with the downstream signaling of P2X₇R such as Ca²⁺ influx. And third, Ni²⁺ competes the receptor binding sites with ATP, an activator and natural ligand of P2X₇R, thus numbs P2X₇R. The answer to these questions would provide a better understanding of the initial molecular events for the inhibition of P2X₇R, which warrants detailed investigation in the future.

In summary, the findings of this work demonstrated that noncytotoxic level of Ni²⁺ and SWCNTs exhibited elevated toxicity to macrophages in combination. The underlying mechanism was revealed to be that Ni²⁺ inhibits the activation of P2X₇ receptor, which is required for the exocytosis of SWCNTs, leading to accumulation of intracellular SWCNTs and higher toxicity. The findings highlight the importance of evaluating the risks of mixtures in addition to single chemical/contaminant, and suggest caution in case of possible coexistence or sequential exposure of Ni²⁺ and SWCNTs in environment because nontoxic exposure levels of Ni²⁺ and SWCNTs might induce adverse health outcomes in the mixture. Furthermore, our data underscore the challenge of predicting environmental risks posed by nanomaterials, which requires careful integration of complex interactions between nanomaterials and contaminants.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b03842

Figures showing the characterization of SWCNTs by TEM, FT-IR, Raman; cell viability under the treatment with a series concentrations of Ni²⁺; cell viability after treatment with ATP and oATP; cell viability after treatment with P2X₇R siRNA and negative control; and relative mRNA level and protein level of P2X₇R after P2X₇R siRNA treatment; cell viability of THP-1 cells under the treatment of Ni²⁺ and SWCNTs (PDF)

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Notes

The authors declare no competing financial interest.

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