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Biomolecular interaction analysis for carbon nanotubes and for biocompatibility prediction

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ABSTRACT

The interactions between carbon nanotubes (CNTs) and biologics have been commonly studied by various microscopy and spectroscopy methods. We tried biomolecular interaction analysis to measure the kinetic interactions between proteins and CNTs. The analysis demonstrated that wheat germ agglutinin (WGA) and other proteins have high affinity toward carboxylated CNT (f-MWCNT) but essentially no binding to normal CNT (p-MWCNT). The binding of f-MWCNT–protein showed dose dependence, and the observed kinetic constants were in the range of 10^{-9} to 10^{-11} M with very small off-rates (10^{-3} to 10^{-7} s^{-1}), indicating a relatively tight and stable f-MWCNT–protein complex formation. Interestingly in hemolysis assay, p-MWCNT showed good biocompatibility, f-MWCNT caused 30% hemolysis, but WGA-coated f-MWCNT did not show hemolysis. Furthermore, the f-MWCNT–WGA complex demonstrated enhanced cytotoxicity toward cancer cells, perhaps through the glycoproteins expressed on the cells' surface. Taken together, biomolecular interaction analysis is a precise method that might be useful in evaluating the binding affinity of biologics to CNTs and in predicting biological actions.

Carbon nanotubes (CNTs) [1–3] are important for biomedical engineering with extraordinary optical, electrical, mechanical, and thermal properties [4,5]. However, undesirable side effects such as cardiopulmonary diseases, inflammation, and fibrosis [6] have been reported for several CNTs. Data from atomic force microscopy, transmission electron microscopy, fluorescence spectroscopy, and molecular dynamics stimulation methods [3,7–10] indicated that the causes might be the differences in roughness, surface charge, and surface group distribution of CNTs [11]. It was proposed that if the surface roughness and diameter of CNTs were equivalent to those of the neural cells, the interactions between CNTs and the cells were the strongest. This was thought to contribute to the nerve cells anchoring to a CNT basement membrane [12], the maturation and differentiation of neural stem cells, and the repairing of the damaged nerve tissues.

Recently, many groups have prepared modified CNTs [13,14] to overcome the disadvantages. For example, CNTs were coated with pulmonary surfactant to improve their chemical properties for biological application [15,16]. Perfluorooctanesulfonyl fluoride (PFOSF) was used to form a super hydrophobic surface that provided CNTs with enhanced antibacterial and mechanical properties [17]. Using the layer self-assembly technique, laminin CNTs formed basement membranes that could successfully induce the differentiation and maturation of the neural stem cells [18]. Primary rat hippocampal neurons that were cultured on 4-hydroxynonenal (4-HNE) protein-coated CNT basement grew a greater number of axons and branches than those in the control group [19]. In addition, serum-coated CNTs could significantly decrease the CNT toxicity [20,21].

However, there are various types of CNTs with different roughness, surface charge, and surface groups. How to quickly screen and rationally select a suitable CNT for modification is an emerging question of intense interest. Biomolecular interaction analysis is a common method used to measure the binding affinity of antibodies or small molecules to their biologic targets. We aimed to apply this

Abbreviations used: CNT, carbon nanotube; p-MWCNT, nonfunctional Multi-walled CNT; f-MWCNT, carboxylated Multi-walled CNT; WGA, wheat germ agglutinin; BSA, bovine serum albumin; NHS, *N*-hydroxysuccinimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; UV, ultraviolet; APS, aminopropylsilane; FKBP12, FK506-binding protein 12; PBS, phosphate-buffered saline.

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method for CNT property screening and coating evaluation. Described here are biomolecular interaction analysis of the kinetic on- and off-rates and the correlation with biological actions of the nonfunctional CNT (p-MWCNT) and commonly used carboxylated CNT (f-MWCNT). Wheat germ agglutinin (WGA) protein was used as a model protein due to its interesting properties such as selective binding to the carbohydrate side chains of glycoproteins that are commonly expressed on the surface of tumor or stem cells [12,22–27]. The biocompatibility of WGA-coated materials was tested by hemolysis and cell growth inhibition assays.

Materials and methods

Reagents

p-MWCNT (purity > 97%, outer diameter 10–20 nm, 5–15 μm in bundle length) and f-MWCNT (carboxylic functionalized MWCNT, MWCNT–COOH, purity > 97%, outer diameter 10–20 nm, 5–15 μm in bundle length) were purchased from Shenzhen Nano Port Inc. (Shenzhen, China). Bovine serum albumin (BSA) was obtained from Shanghai Boao Biological Technology Inc. (Shanghai, China). Coomassie Brilliant Blue G-250 (Bradford reagent) from KEYGEN BIOTECH. Inc. (Jiangsu, China). Leghorn hens were purchased from the Department of Laboratory Animals, Shanghai Academy of Agricultural Sciences, and fresh chicken blood was collected from a vein beneath the wings, treated with anti-coagulant heparin sodium, diluted by saline to 50 ml, and centrifuged at 1600 rpm for 5 min to collect the red blood cells for the hemolysis experiment. EZMTT reagent for cell viability assay was obtained from Hangzhou Jennifer BioTech. Inc. (Hangzhou, China). *N*-Hydroxysuccinimide (NHS)/1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was obtained from ThermoScientific (USA). Quantification of WGA binding was performed with a UV-1000 ultraviolet (UV) spectrophotometer. Biomolecular interaction analysis was performed using a ForteBio OctetRed 96 instrument with aminopropylsilane (APS) biosensors and Plexera's PlexArray SPRi instrumentation. Cells were observed using an Olympus CKX41 microscope (Japan).

WGA/CNT complex formation

The WGA/CNT complexes were prepared in water solution by shaking a 1:1 (volume) mixture of WGA solution and CNT suspension at room temperature for 60 min. The resulting pellet containing the WGA/CNT complexes was collected by centrifugation at 4000 rpm for 10 min, and the excess amount of WGA in the supernatant was measured by UV analysis at 280 nm. The bindings of WGA to CNTs were measured via the subtraction method comparing the WGA in the supernatants from factions with CNTs added against the ones without CNTs added. After removing the supernatant, the pellet containing the WGA/CNT complexes was freeze-dried and stored at 4 °C.

Dose-dependent binding of WGA to CNTs was performed by mixing a series of WGA solution (0–5 mg/ml, 5 ml) with p-MWCNT or f-MWCNT suspension (2 mg/ml, 5 ml), whereas dose-dependent binding of CNTs to WGA was done by mixing a series of p-MWCNT or f-MWCNT suspension (0–5 mg/ml, 5 ml) with WGA solution (2 mg/ml, 5 ml). Each experiment was repeated three times, and the average protein concentrations (by UV at 280 nm) were used for further data analysis.

To test whether a stable WGA/CNT complex can be formed, we washed the WGA/CNT complexes thoroughly with water five times. After washing, the complex pellets were tested using Bradford protein assay to measure the residual WGA protein that was still bound to the MWCNTs in the complex.

Kinetic measurement of protein binding to immobilized f-MWCNT

Biomolecular interaction analysis of protein binding to MWCNT was performed using a ForteBio OctetRed 96 instrument with APS biosensors. MWCNTs were loaded onto APS biosensors by dipping the sensor into samples containing 50 μg/ml MWCNTs in phosphate-buffered saline (PBS) in a 96-well plate for 5 min. The measurement of protein binding to MWCNTs was performed by first dipping the MWCNT-coated sensor into PBST buffer (PBS + 0.02% Tween 20) for 2 min to obtain the baseline signal, then into a sample for 5 min to measure the on-rate, and finally into the PBST buffer again for 3–5 min to obtain the dissociation rate. Proteins such as FK506-binding protein 12 (FKBP12; 0, 65, 194, or 583 nM) and WGA (0, 111, or 333 nM) were used as samples.

For further confirmation, the molecular interactions were tested on Plexera's PlexArray SPRi instrumentation. The p-MWCNT or f-MWCNT (50 μg/ml, 50 μl) was spotted in triples separately and cross-linked on a three-dimensional sensor chip along with positive (Rapamycin, 10 mM) and negative (dimethyl sulfoxide, DMSO) controls to verify the immobilization and to monitor the binding kinetics, respectively. Proteins (FKBP12 and WGA) in buffer (PBS) were injected at a flow rate of 2 μl/s for 300 s association time and 300 s dissociation time. Regeneration between injections was performed using 10 mM glycine–HCl (pH 2.0, 900 μl). All of the data were visualized using Instrument Control software (ICS) and analyzed using Plexera Data Explorer software.

Erythrocyte preparation and hemolysis assay

The damage to erythrocytes was tested by hemolysis assay. The erythrocytes were isolated from the anti-coagulant added fresh chicken blood (20 ml) by centrifugation at 2500 rpm for 10 min. After the upper plasma and the leukocyte layer were removed, the erythrocyte pellet was washed five times by repeated resuspension and centrifugation using physiological saline solution until the supernatant became colorless. Erythrocytes (2%) in normal saline (volume) were prepared for the test. Briefly, a 1:1 mixture of the 2% erythrocyte solution and samples (CNTs in saline, WGA–CNTs in saline, water to lyse the erythrocytes, and used as positive control) was incubated at 37 °C for 3 h and then centrifuged at 2500 rpm for 10 min. The supernatants along with the no-cell control were taken for UV absorbance measurement at 545 nm to determine the hemolysis rate. Each experiment was repeated at least three times, and the hemolysis rate was calculated as follows:

$$\text{Hemolysis rate}(\%) = \frac{\text{OD value of sampletube}}{\text{OD value of positive controltube}} \times 100\%$$

where OD is optical density.

Viability of cancer cell lines

WGA-mediated cytotoxicity was tested using cancer cell growth inhibition assay using cell lines (PC12, A549, and T24) in media (MEM, F12-K, and RPMI, respectively) containing 5% fetal calf serum. WGA–CNTs were prepared using the NHS/EDC cross-link method. Mixtures (1:1 in volume) of 2000 cells and CNTs (0–250 μg/ml) or WGA–CNTs (0–250 μg/ml) were plated in 96-well tissue culture (TC)-treated plates. After growth in 5% CO_2 at 37 °C for 24 or 72 h, cell morphology changes were observed under microscope, cell death was tested using 0.04% Trypan blue solution, and cell growth was measured by NADPH/NADH quantification [11] using a tetrazolium salt containing EZMTT reagent. Each experiment was repeated at least three times. For cell growth assay, the