Supporting Information

Ge et al. 10.1073/pnas.1105270108

SI Text

Concentration and Function of Several Abundant Proteins in Blood Circulation. BSA, bovine fibrinogen (BFG), gamma globulin (Ig), and transferrin (Tf) are four of the most abundant proteins in blood circulation. Serum albumin concentration in plasma from adults is 35–55 g/L, whereas total protein concentration in the plasma is 67–86 g/L (1). Serum albumin is synthesized in the liver and present in all body fluids, which has a good binding capacity for many different types of ions and molecules like fatty acid, hormones, bilirubins, and drugs, and thus acts as a transport protein in plasma (2). Normal fibrinogen concentration in the plasma of adults is 2.0–4.0 g/L. Fibrinogen plays overlapping roles in blood clotting, fibrinolysis, cellular and matrix interactions, the inflammatory response, wound healing, and neoplasia (3). Gamma globulin concentration in plasma is about 8.3–23.0 g/L, which plays an important role in immune defense mechanisms. Gamma globulin contains five species of immunoglobulin (Ig): IgG, IgA, IgM, IgD, and IgE, and among them the main component is IgG (75%) produced by B lymphocytes to identify and neutralize foreign objects such as bacteria and viruses. Transferrin concentration in plasma from healthy subjects is 2.2–4.0 g/L. The fundamental role of transferrin is to control the levels of free iron in body fluids by binding, sequestering, and transporting Fe3+ ions (4). In addition, transferrin can bind to many other metals, including gallium and cobalt (5). These proteins may accumulate in blood plasma and function as a diagnostic marker for a certain disease. These blood plasma proteins exhibit a wide variety of functions and have different structural properties (2).

Single-Wall Carbon Nanotubes (SWCNTs) Induced the Changes of Protein Secondary Structure. It is known that CD spectra in the far-UV region (180–250 nm) probes the secondary structures of proteins, and in the near-UV region (usually from 250 to 350 nm) monitors the side-chain tertiary structures of proteins (6, 7). In the far-UV region, the secondary structures of protein have 192, 208, and 222 nm characteristic CD peaks of α-helix and β-sheet, respectively. In the near-UV region, the tertiary structures have 255, 261, and 268 nm characteristic CD peaks for Phe, 277 nm for Tyr, and 279, 284, and 291 nm for Trp. These characteristic CD spectra provide an important tool to monitor the change of secondary and tertiary structures of a protein.

We hence examined the protein secondary-structure changes with and without interactions with SWCNTs. The CD results demonstrated that, after incubation with SWCNTs, those characteristic peaks of amino acids in the studied proteins had noticeable shifts with decreased peak intensities (Fig. 1*G*). The protein binding-induced CD changes were more significant for BFG and Ig interacting with SWCNTs (Fig. 1*G*). On the basis of secondary-structure calculations using the Yang method (8), these changes correlated to 10% (Tf) and 40% (BFG and Ig) decreases in the α-helix structure, 20% (BFG) and 15% (Ig) decreases in random coil, a 15% (Ig) reduction in turns, and a concomitant 60% (BFG and Ig) increase in the β-sheet structure, respectively. Other SWCNTs binding-induced CD changes and the related calculations on secondary structure are summarized in this *SI Text* (Table S2). The near-UV CD spectra showed no obvious change of the position and intensity of feature peaks, which indicates that some of the local tertiary structure remains intact (see Fig. 1*G*, *Inset*).

Competitive Binding of Individual Protein onto SWCNT Analyzed by SDS-PAGE. To confirm the findings mentioned above, we attempted to semiquantitatively analyze protein adsorption by SDS-PAGE (Fig. S4–S6). The free protein in the supernatant after incubation with SWCNT was subjected to SDS-PAGE. For BFG, free protein in the supernatant and adsorbed protein released from SWCNTwere analyzed, respectively. After 5 min of incubation, the band intensity of each protein rapidly changed from dark to light (Fig. S4), which indicates that protein content in the supernatant became much smaller due to protein adsorption onto SWCNT molecules. Fibrinogen, the principal protein of vertebrate blood clotting, is a hexamer containing two sets of three different chains (α, β, and γ), linked to each other by disulfide bonds. If making a comparison of the band intensity before and after 5 min of incubation, it was obvious that the band intensity of β-chain and γ-chain of BFG had the most notable changes, and Ig was the second, Tf the third, and BSA the smallest. All band intensities changed significantly, indicating all protein molecules were adsorbed to SWCNT relatively fast, which was consistent with the fluorescence results. With increasing of incubation time, the band intensity became lighter and lighter. After 30 min of incubation, no obvious color change was observed, indicating that the adsorption of proteins by SWCNTs had reached saturation. However, atomic force microscope images show that it could take up to 5 ho to reach the well-ordered rod-like final structures for BFG and Ig, indicating there are some structural reconfiguration and reordering going on for these proteins.

Subsequently, we investigated the protein mixtures interacting with the SWCNTs. We chose the mixture of BFG and BSA firstly. After coincubation of SWCNTs with mixed proteins BFG and BSA, the supernatant was analyzed by SDS-PAGE. After 5 min incubation, the band intensity of each protein rapidly became light (Fig. S4*C*), especially for BFG, whereas the change of band color of BSA was not so notable for the first 60 min. This finding implied that, in the mixed system, the BFG is more preferred in binding with the SWCNTs. After incubation for 60 min, the two bands of β- and γ-chains of BFG were hardly visible, but the band color of BSA showed almost no change. Therefore, these results indicated that similar to the single protein case, BFG was still preferred in the binding to SWCNTs in the protein mixtures of BSA and BFG. Incubation SWCNTs with FBS also exhibited similar behavior, with different proteins adsorbed at different time scale (Fig. S5). Further incubation SWCNTs with fresh rat plasma demonstrated that fibrinogen, complement factor B and H precursor and albumin were bound to SWCNTs (Fig. S6), especially the protein bands of β- and γ-chains are much clearer than native plasma (Fig. S6, lane 4). The competitive binding within multiple protein molecules seems to depend on their respective hydrophobic capacity within each protein.

Toxicity Evaluation of Highly Packed Protein-Coated SWCNTs. The cytotoxicity of pristine SWCNTs and protein-coated SWCNTs was evaluated in two human cell lines, human acute monocytic leukemia cell line (THP-1) and human umbilical vein endothelial cells (HUVECs) (ATCC). THP-1 was first derived from the peripheral blood. The pristine SWCNTs exhibited a dose-dependent toxicity to THP-1 and HUVECs, in which we chose 30 μg/mL SWCNTs for following evaluation (Fig. S7*A*). Cell viability of SWCNTs with fewer BFG proteins adsorbed was obviously less than those with saturation adsorption of BFG in both THP-1 and HUVEC cells (Fig. S7*B*).

Molecular Dynamics Computational Methods. The blood proteins simulated in this work were taken from the Protein Data Bank

(PDB) and Swiss-Model Repository as BFG with PDB ID 1DEQ (P4, P5, and P6 chains were chosen to be the monomer without the modified groups), Ig with PDB ID 3HR5 (one heavy and one light chain were chosen from the multimer structure), Tf with PDB ID 2HAV, and BSA with SWISS-MODEL ID 432779d395a52bfc9f6574bc3e98afcd_1. All SWCNTs used in the molecular dynamics (MD) simulations for the adsorption of CNT for proteins BFG, Ig, Tf, and BSA were armchair CNT (14, 14) with diameters around 2.0 nm, which were consistent with the experimental ones. The geometrical coordinate parameters of CNT were generated by using Nanotube Modeler software.

MD simulations were performed by NAMD (9) version 2.6. The various blood proteins were modeled by CHARMM 32b1 force field (10). Carbon atoms of (14, 14) SWCNTs were assumed to be type carbon atoms with Lennard–Jones parameters kilocalorie per mole, angstrom. The interaction between these carbon atoms of SWCNTs and other atoms were generated by CHARMM 32b1 force field. The various blood proteins were well separated from SWCNTwith the minimum distance of larger than 5.0 Å initially. The length of the SWCNT in each system was as long as about one and a half the size of the corresponding protein. All systems were solvated in periodic TIP3P modeled water box (11) and neutralized by adding sodium and chloride ions with 0.2 M salt concentration as physiological condition. The periodic simulation box is large enough with the distance between its boundary and the solute being more than 5.0 Å in each system. The numbers of atoms were 247,977; 35,021; 56,148; and 61,706 for the SWCNT adsorption system with BFG, Ig, Tf, and BSA, respectively.

Simulations were performed at a constant temperature of 310 K and pressure of 1 atm, with the Particle Mesh Ewald method (12) for long-range electrostatic interactions and time step of 2 fs. The cutoff for the van der Waals interaction was set to 12 Å. All systems were simulated in the NPT (fixed number of atoms N, pressure P, and temperature T) ensemble for more than 120 ns.

Inductively Coupled Plasma (ICP)-MS and Transmission Electron Microscope (TEM) Characterization. TEM characterization of SWCNTs was performed by Tecnai G2 F20 U-TWIN TEM, and AFM characterization of SWCNTs was performed by the Dimension Icon AFM from Veeco. ICP-MS (Thermo Elemental X7) was used to provide a quantitative analysis of the metal impurities in SWCNTs as described previously (52).

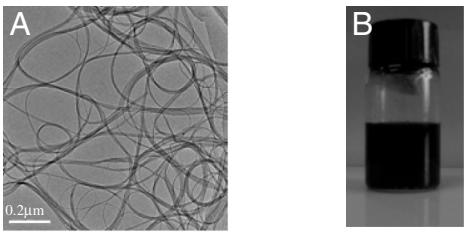
Quantitative Analysis of Protein by Fluorescence Spectroscopy. A 200 μg/mL SWCNTs suspension was prepared by dispersing SWCNTs powder in PBS (10 mmol/L, pH 7.4) using ultrasonication instead of any surfactant (to avoid adverse interference from surfactants because they also bind to SWCNTs), and a 100 μg/mL protein solution was prepared in PBS as well. Equal volumes of SWCNTs suspension and protein solution were mixed and incubated at 37 °C for 0 min–3 h, which were constantly rotated to ensure good mixing. For each experiment with different incubation time (0, 5, 30, 60, 120, and 180 min), three independent repeats were performed for statistics (see Fig. 4). Following incubation, the mixtures were immediately centrifuged at 15,000 × *g* for 10 min. The protein content of supernatant was detected using fluorescence spectroscopy (excitation wavelength at 280 nm, emission wavelength at 332 nm). By deducting the protein content of supernatant from the total protein quantity, we can infer protein quantity bound to nanotubes. In order to eliminate interference from proteins aggregate and precipitate, the same procedure was employed for the protein PBS solution without CNT as a control. Thus, a kinetic analysis of interaction between proteins and SWCNTs was performed by monitoring the absorbance of aqueous solution at 332 nm for different lengths of time using fluorescence spectrometry (Tecan Infinite M200). Each native protein at different concentrations was used to obtain a standard curve. The content of absorbed proteins was therefore calculated by the subtraction of protein content remaining in the supernatant from the total protein.

Qualitative Analysis of Protein by SDS-PAGE. The above supernatant and the SWCNT–protein sediment resuspended in PBS were incubated at 100 °C for 5 min in sample buffer (0.25 M Tris·HCl, 5% urea, 10% SDS, 50% glycerine, 0.5% bromophenol blue). A 20-μL sample was loaded on a 12% Bis-Tris SDS-PAGE gradient gel, and then separated by electrophoresis for 60 min at 100 V using Bio-Rad. Proteins bands were stained with Coomassie blue. The resulting protein bands were read by a densitometer (Bioimaging system Image Quant 300). The rat plasma was incubated with SWCNTs, respectively. By centrifugation the supernatant was discarded and the sedimentation was reconstituted in loading buffer that was finally analyzed by SDS-PAGE.

Analysis of Protein–SWCNT Conjugates by Circular Dichroism Spectroscopy and Atomic Force Microscopy. Equal volumes of 100 μg/mL protein solution and 200 μg/mL SWCNTs suspension were mixed. After incubation for 5, 10, 30, and 60 min, respectively, the mixed system was determined immediately by Circular Dichroism Spectrometer. Morphology of protein–SWCNT conjugates by AFM. The SWCNTs powder was dispersed in *N,N*-dimethylformamide (DMF) solution. Before use, the SWCNTs DMF solution was diluted five times by PBS. Equal volumes of 100 μg/mL protein solution and 200 μg/mL SWCNTs were mixed. About 30 μL SWCNT–protein adducts suspension after 10 min and 5 h were taken out and deposited on a fresh mica surface, and then the mica was measured by AFM, respectively.

Live/Dead Viability/Cytotoxicity Assay. Live/Dead Viability/Cytotoxicity assay was used to distinguish the live and dead cells. Cytotoxicity was measured using a LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes) for staining with calcein AM (live cells fluoresce green) and ethidium homodimer (dead cells fluoresce red). THP-1 cells were treated with 30 μg/mL SWCNTs with/without protein-bindings for 12 h. After removal of SWCNTs, cells were washed with PBS and stained with LIVE/DEAD assay kit reagent as specification and imaged by fluorescence microscopy.

1. Kasper DL, Braunwald E, Fauci AS, Hauser SL, Longo DL, Jameson JL (2004) *Harrison's Principles of Internal Medicine* (McGraw–Hill, New York), pp 966–972.
2. Schaller J, Gerber S, Kaempfer U, Lejon S (2008). *Human Blood Plasma Proteins: Structure and Function* (Wiley, New York), pp 14–45.
3. Mosesson M (2005) Fibrinogen and fibrin structure and functions. *J Thromb Haemost* 3:1894–1904.
4. Baker E (1994) Structure and reactivity of transferrins. *Adv Inorg Chem* 41:389–464.
5. Smith T (2005) Human serum transferrin cobalt complex: Stability and cellular uptake of cobalt. *Bioorg Med Chem* 13:4576–4579.
6. Kelly SM, Jess TJ, Price NC (2005) How to study proteins by circular dichroism. *Biochim Biophys Acta* 1751:119–139.
7. Lynch I, Dawson KA (2008) Protein-nanoparticle interactions. *Nano Today* 3:40–47.
8. Yang J, Wu C, Martinez H (1986) Calculation of protein conformation from circular dichroism. *Methods Enzymol* 130:208–269.
9. Phillips JC, et al. (2005) Scalable molecular dynamics with NAMD. *J Comput Chem* 26:1781–1802.
10. Feller SE, MacKerell AD, Jr (2000) An improved empirical potential energy function for molecular simulations of phospholipids. *J Phys Chem B* 104:7510–7515.
11. Jorgensen WL, Chandrasekhar J, Madura JD, Impey RW, Klein ML (1983) Comparison of simple potential functions for simulating liquid water. *J Chem Phys* 79:926–935.
12. Essmann U, et al. (1995) A smooth particle mesh Ewald method. *J Chem Phys* 103:8577–8593.



C

Cr	Fe	Mo	Co	Ni	Mn	V
11,233±10	4,626±37	3,074±14	1811±20	182±1.5	77±0.3	50±0.2
Ti	Cu	Cd	La	As	Se	
45±4	35±2	12±0.1	9.2±0.7	6.7±0.2	1.9±0.1	

Fig. S1. Characterization of SWCNT. (*A*) Transmission electron microscope images of SWCNT aqueous suspension. (*B*) Optical image of SWCNT suspension in PBS. (*C*) Inductively coupled plasma MS analysis of metal residues in SWCNTs (in micrograms per gram).

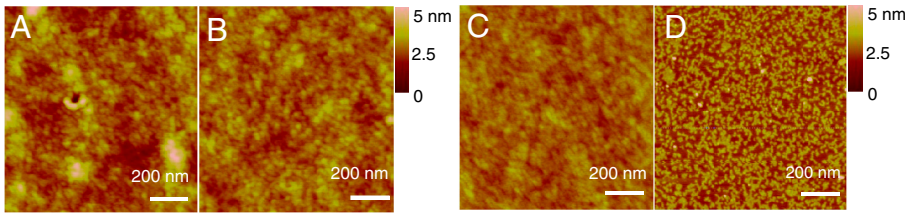


Fig. S2. Atomic force microscope (AFM) images of proteins. AFM images of Ig (*A*), BFG (*B*), Tf (*C*), and BSA (*D*).

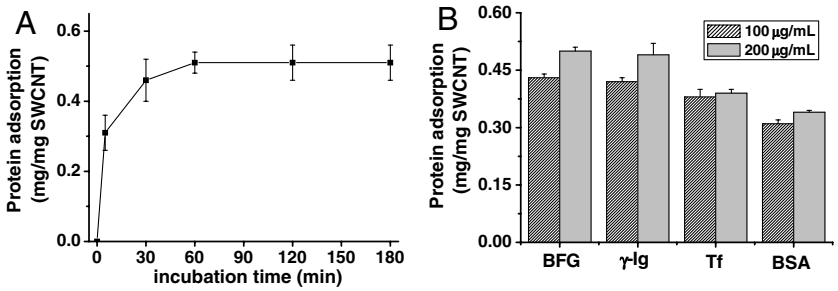


Fig. S3. Quantitative analysis of the protein in FBS adsorbed by SWCNTs. (*A*) The kinetics curves of protein in 1% FBS adsorbed by 200 mg/mL SWCNTs from 0 to 180 min. (*B*) The adsorption of different species of proteins by SWCNTs at two concentrations ($N = 5$; error bars are SD).

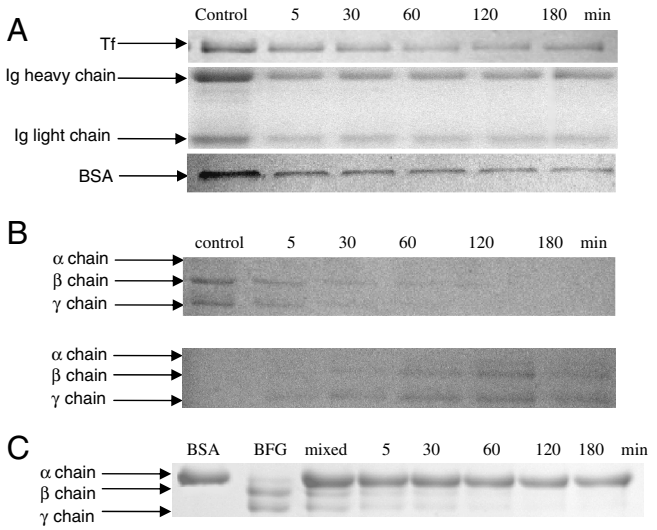


Fig. S4. SDS-PAGE analysis of interaction between SWCNTs and individual or mixed protein. (*A*) SDS-PAGE analysis of supernatant from incubation SWCNTs, respectively, with Tf, Ig, and BSA. Lanes: 1, control: protein alone; 2–6, supernatant from incubation SWCNTs with protein for 5, 30, 60, 120, and 180 min in sequence. (*B*) SDS-PAGE analysis of supernatant (*Upper*) and sediment (*Lower*) from incubation SWCNTs with BFG. Lanes: 1, control: protein alone; 2–6, supernatant from incubation SWCNTs with protein for 5, 30, 60, 120, and 180 min in sequence. (*C*) SDS-PAGE analysis of supernatant from incubation SWCNTs with mixed proteins of BSA and BFG (note, the supernatant was concentrated by five times). Lanes: 1–3, control: BSA, BFG, and mixed proteins in sequence; 4–8, protein supernatant from incubation SWCNTs with mixed proteins of BSA and BFG for 5, 30, 60, 120, and 180 min in sequence. Lane 2, BFG has been divided into three monomers: α, β, γ. The molecular weight of α-chain is 63.5 kDa, where black arrows indicate the presence of β-chain and γ-chain at 56 and 50 kDa, and the monomer of BSA is 68 kDa.

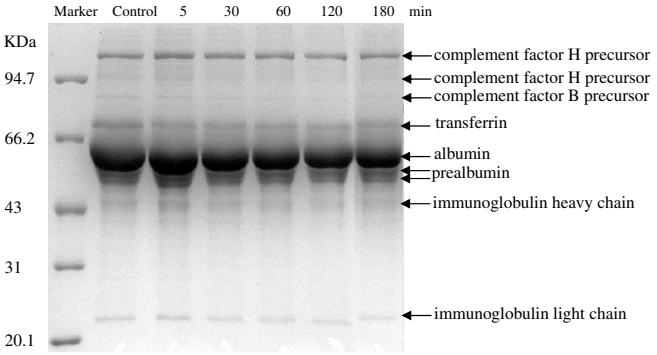


Fig. S5. SDS-PAGE analysis of interaction between SWCNTs and FBS. SDS-PAGE was used to analyze the supernatant from incubation of SWCNTs with FBS. Lanes: 1, molecular weight markers; 2, control (8% FBS); 3–7, the supernatant of FBS after incubation with SWCNTs for 10, 30, 60, 120, and 180 min. Rows: 1, complement factor H precursor (molecular mass 140 kDa); 2, complement factor H precursor (97 kDa); 3, complement factor B precursor (85 kDa); 4, transferrin (77 kDa); 5, albumin (69 kDa); 6 and 7, prealbumin (54 kDa); 8, immunoglobulin heavy chain (51 kDa); 9, immunoglobulin light chain (25 kDa).

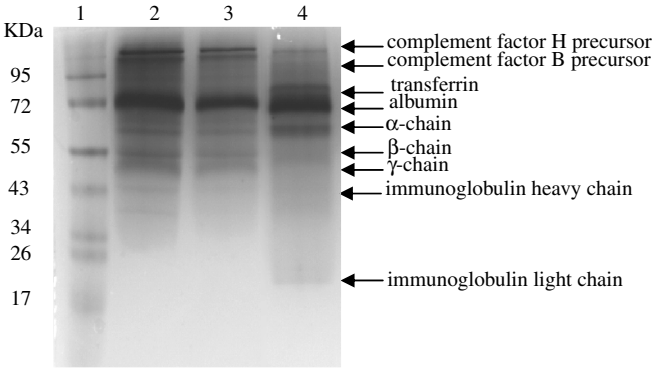


Fig. S6. SDS-PAGE analysis of interaction between SWCNTs and plasma protein. SDS-PAGE was used to analyze the SWCNT-binding proteins from incubation of SWCNTs with plasma. Lane 1, molecular mass markers; 2, 5% plasma incubation with SWCNTs; 3, 2.5% plasma incubation with SWCNTs; 4, control: 2.5% plasma.

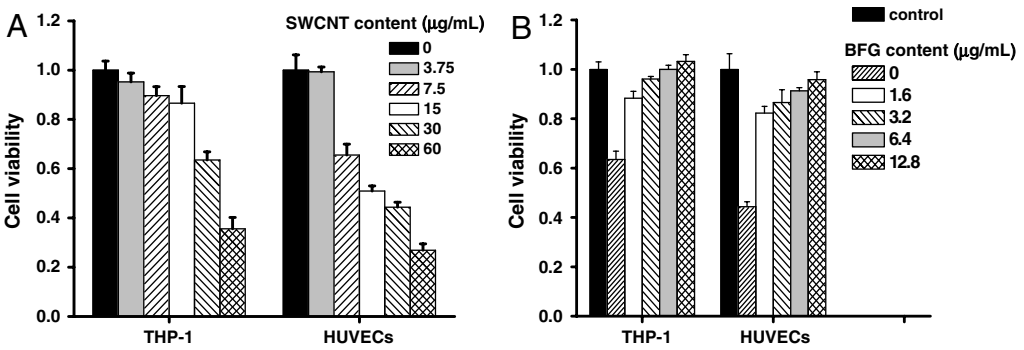


Fig. S7. Cytotoxicity of SWCNTs alone and SWCNT adsorbed protein. (*A*) The cytotoxicity of SWCNTs at different concentrations. (*B*) The cytotoxicity of SWCNTs preincubation with BFG at different concentrations.

Ge et al. www.pnas.org/cgi/doi/10.1073/pnas.1105270108

Table S1. The number of each hydrophobic amino acid in a protein molecule and the total number of hydrophobic amino acids in a protein molecule

Protein species	Hydrophobic amino acid	No. of amino acid in a protein molecule	Total no. of amino acid in a protein molecule	No. of hydrophobic amino acid on the surface of protein	Protein species	Hydrophobic amino acid	No. of amino acid in a protein molecule	Total no. of amino acid in a protein molecule	No. of hydrophobic amino acid on the surface of protein
	alanine	132				alanine	61		
	isoleucine	144				isoleucine	15		
	leucine	198				leucine	65		
	methionine	62				methionine	10		
BFG	proline	154	1,120		Tf	proline	32	293	
	valine	154				valine	48		
	tryptophan	70		14		tryptophan	8		1
	tyrosine	102		47		tyrosine	26		13
	phenylalanine	104		27		phenylalanine	28		15
	alanine	82				alanine	47		
	isoleucine	34				isoleucine	15		
	leucine	108				leucine	65		
	methionine	12				methionine	5		
IgG	proline	98	578		BSA	proline	28	252	
	valine	130				valine	38		
	tryptophan	28		2		tryptophan	3		1
	tyrosine	52		25		tyrosine	21		9
	phenylalanine	34		10		phenylalanine	30		10

The number of hydrophobic amino acids containing benzene exposed on the surface of protein molecule and the total number of hydrophobic amino acids in a protein molecule including tryptophan, tyrosine, and phenylalanine.

Table S2. Calculation of protein secondary structure before and after incubation with SWCNTs for 10 and 60 min

	BFG, %	BFG+SWCNT, 10 min, %	BFG+SWCNT, 60 min, %
Helix	50.30	25.60	0.00
Beta	0.00	54.30	68.10
Turn	0.00	0.00	4.10
Random	49.70	20.10	27.80
Total	100.00	100.00	100.00
	IgG, %	*IgG+SWCNT, 10 min, %*	*IgG+SWCNT, 60 min, %*
Helix	44.10	23.10	7.20
Beta	0.00	26.80	67.20
Turn	24.00	28.10	8.00
Random	31.80	21.90	17.60
Total	100.00	100.00	100.00
	Tf, %	*Tf+SWCNT, 10 min, %*	*Tf+SWCNT, 60 min, %*
Helix	23.80	23.00	18.60
Beta	49.40	54.60	56.40
Turn	3.00	0.00	0.00
Random	23.90	22.40	25.00
Total	100.00	100.00	100.00
	BSA, %	*BSA+SWCNT, 10 min, %*	*BSA+SWCNT, 60 min, %*
Helix	46.10	24.80	51.20
Beta	15.60	38.70	11.30
Turn	8.30	8.80	0.00
Random	30.00	27.60	37.50
Total	100.00	100.00	100.00