Supplementary Information

Disulfide Bond Formation of Thiols by Carbon Nanotubes

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1. Materials and Methods

1.1. Chemicals. CNTs synthesized by the high-pressure catalytic CO (HiPco) decomposition method were purchased from Nano-Integris. CNTs synthesized by the arc plasma jet (APJ) method and by the enhanced direct injection pyrolytic synthesis (eDIPS) method (MEIJO eDIPS EC2.0) were purchased from Meijo Nano Carbon Co., Ltd. Sodium dodecyl sulfate (SDS), sodium deoxycholate, ammonia solution, acetic acid, L-cysteine (Cys), L(−)-cystine (Cys-Cys), (+/−)-dithiothreitol (red-DTT), glutathione (GSH and GSSG), formic acid, methanol, guanidine hydrochloride, urea and lysozyme from egg white were obtained from Wako Pure Chemical Industries, Ltd. The oxidized form of dithiothreitol, i.e., *trans*-4,5-dihydroxy-1,2-dithiane, designated ox-DTT, was purchased from Sigma-Aldrich Co. LLC. Ethylenediamine-*N,N,N′,N′*-tetraacetic acid, disodium salt, dehydrate (EDTA) was obtained from Dojindo Laboratories.

1.2. Redox Reaction of Dispersant-Free HiPco CNTs with Thiols Using Ultrasonication. The HiPco CNTs were used to examine the reaction of CNTs with thiols. First, the raw HiPco CNTs were dried under vacuum to remove coexisting volatile impurities such as organic solvents. Subsequently, 1 mg of the CNTs was mixed with 1 mL of 0.1% v/v ammonium acetate buffer solution (pH 4.5) and 10 μL of 100 mM thiols dissolved in the buffer solution using an ultrasonic processor (Nanoruptor NR-350, Cosmo Bio Co., Ltd.) for 15 min at a power of 350 W to create 1-mL suspensions containing 1 mg/mL CNTs and 1 mM thiols. After mixing, these samples were centrifuged at 3,000 rpm for 10 min (H-103N, Kokusan Co. Ltd.). The upper 400-μL aqueous fractions obtained from the supernatants were then filtered using a polytetrafluoroethylene (PTFE) syringe filter membrane (As One Corp.) with a 0.22-μm pore

size to collect products from the thiols. The collected products were subjected to the absorption measurement.

To form the samples under nitrogen atmosphere, the raw HiPco CNTs dried by the vacuum process were further exposed to nitrogen gas, and then they were mixed with the buffer solutions (pH 4.5). The mixtures were subsequently bubbled with nitrogen gas in a closed vial for deoxygenation. After that, 10 μL of 100 mM thiol in the buffer solution was injected into the mixtures to make samples containing 1 mg/mL CNTs and 1 mM thiols. Finally, the same procedures mentioned above, i.e., ultrasonication, centrifugation and filtration, were applied to the samples.

1.3. Collection of Absorption Spectra. The absorption spectra of the thiols and the CNTs were recorded with a UV–vis–NIR spectrophotometer (UV-3600, Shimadzu Corp.) using a quartz cell with a path length of 10 mm. Disulfide bonds generated a spectral peak or shoulder in the UV region. The absorption peaks of the CNTs at approximately 940–1350 nm and 620–940 nm were assigned to the first optical transition (S_{11}) and the second optical transition (S_{22}) of the semiconducting species, respectively, for the HiPco CNTs. The absorption peak at approximately 400–620 nm was assigned to the first optical transition (M_{11}) of the metallic species.

1.4. Collection of Electrospray Ionization Mass Spectra. Each thiol solution or its product solution was subjected to electrospray ionization mass spectrometry (ESI-MS) to identify the solution components and their chemical structures. Sample solutions containing Cys, Cys-Cys, GSH or GSSG were diluted 10-fold into (100:0.3) methanol/formic acid solution. Sample solutions containing DTT were diluted 1.6-fold into 0.1 wt% ammonium acetate solution at pH

9.5 to adjust their pH to approximately 7; they were then diluted 10-fold into (100:0.2) methanol/formic acid solution. Finally, they were filtered through a 0.22-μm pore size PTFE membrane and subjected to ESI-MS. The ESI mass spectrometer (ZQ2000, Nihon Waters K.K.) was operated in positive-ion mode for Cys, Cys-Cys, GSH and GSSG and in negative-ion mode for DTT.

1.5. On-Column Redox Reaction of Dispersant-Free HiPco CNTs with Thiols Using a Liquid Chromatography System. The redox reaction of the CNTs with the thiols was examined using a high-performance liquid chromatographic column, which was filled with CNT-immobilized silica matrices. The matrices (Chemcobond NH2, Chemco Plus Scientific Co., Ltd.) have amino groups, enabling physical adsorption of the CNTs onto their surfaces. The chromatographic system (Shimadzu Corp.) comprised a degasser (DGU-20A), a pump (LC-20AT), a photodiode array detector (SPD-M20A) and an autosampler (SIL-20AC). The on-column redox reaction using the CNTs on the matrices was conducted at a flow rate of 0.05 mL/min at room temperature using a 0.1% v/v ammonium acetate buffer solution (pH 4.5) as a running buffer, where 20 μL of 500 μM thiol solutions dissolved in the same buffer solutions were loaded into the columns.

1.6. Collection of Absorption Spectra of the SDS-Dispersed CNTs in the Presence of Thiols. The HiPco CNTs dispersed in 1 wt% sodium dodecyl sulfate (SDS) solution (pH 4.5) were reduced by the addition of different concentrations of the thiol solutions containing 1 wt% SDS. An aliquot of 30 mg of HiPco CNTs was suspended in 30 mL of 1 wt% SDS (1 mg/mL) using an ultrasonic processor (Nanoruptor NR-350) for 1 min at a power of 350 W as a

preliminary dispersion treatment. The suspension was further dispersed for 1 h at a power density of *ca.* 20 W cm^{-2} using an ultrasonic homogenizer (Sonifire 250D, Branson) equipped with a 0.5-in flat tip. The glass vial containing this dispersion was immersed in a water bath at 18°C to prevent an increase in the temperature of the dispersions during the ultrasonication treatment. The dispersed sample was subsequently centrifuged at 210,000 × *g* for 1 h using an ultracentrifuge (himac CS100GXII and S80AT3 rotor, Hitachi Koki Co., Ltd.) to remove the residue of catalytic metal particles, CNT bundles, and other impurities. The upper 70% of the supernatant was collected as a purified CNT dispersion containing 1 wt% SDS. The CNT sample was then diluted into 1 wt% SDS solution to adjust the absorbance of the dispersion at 600 nm to 0.28, which corresponds to 0.13 mg/mL; it was then further diluted 15-fold into a 1 wt% SDS solution. The pH value of the dispersion was subsequently adjusted to 4.5 using 0.1 M HCl. Additionally, 0–20 mM thiol solutions (pH 4.5) containing 1 wt% SDS were prepared by dissolving the thiols into SDS solutions and by adding HCl or NaOH for pH adjustment. Finally, aliquots of the CNT dispersions (375 μL), which had been incubated for several days for equilibration, were mixed with the thiol solutions (125 μL) to prepare samples containing 0.0065 mg/mL CNTs, 0–5 mM thiols and 1 wt% SDS. The absorbances of these samples at 1250 nm at various time periods are shown in Figure S7.

2. Results

2.1. Absorption Spectra of Cys, Cys-Cys, GSH and GSSG.
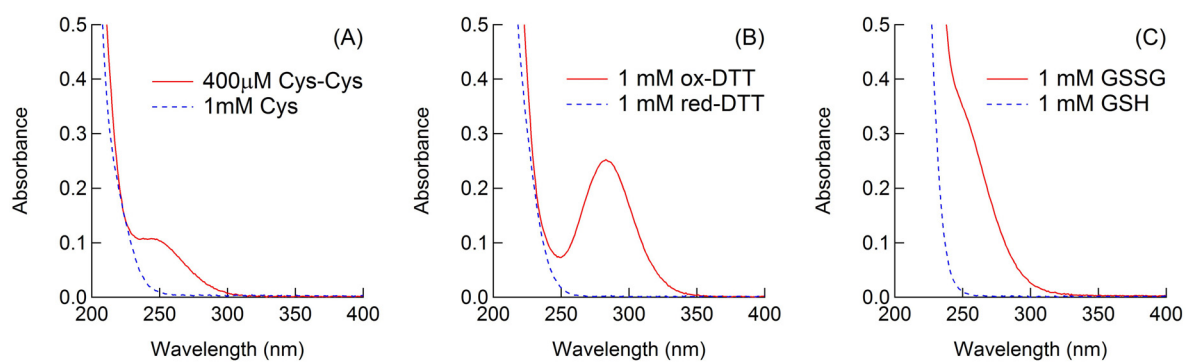
Figure S1. Absorption spectra of the thiols in 0.1% v/v ammonium acetate buffer (pH 4.5).

2.2. Redox Reaction Using Highly Purified HiPco CNTs at pH 4.5. Highly purified HiPco CNTs were used to exclude effects of any other impurities on the redox reaction. First, highly dispersed HiPco CNTs were prepared by the same method described in section 1.6 to remove the residue of catalytic metal particles. The highly dispersed CNTs obtained from the supernatant were subsequently washed with methanol, purified water and then 0.1% v/v ammonium acetate buffer solution (pH 4.5) on a membrane filter with a 0.2-μm pore size (Omnipore membrane filter, Merck Millipore Corp.) using vacuum filtration to remove SDS molecules and other soluble impurities. One-milliliter fractions of the highly purified CNT mixture were then mixed with 10 μL of 100 mM thiols dissolved in the same buffer solution (pH 4.5) using an ultrasonic processor (Nanoruptor NR-350) for 15 min at a power of 350 W to prepare 1-mL suspensions containing approximately 1 mg/mL of CNTs and 1 mM thiols. The suspensions were subsequently centrifuged and then filtered by the same method as that described in section 1.2. The absorbance of the collected products was then measured.
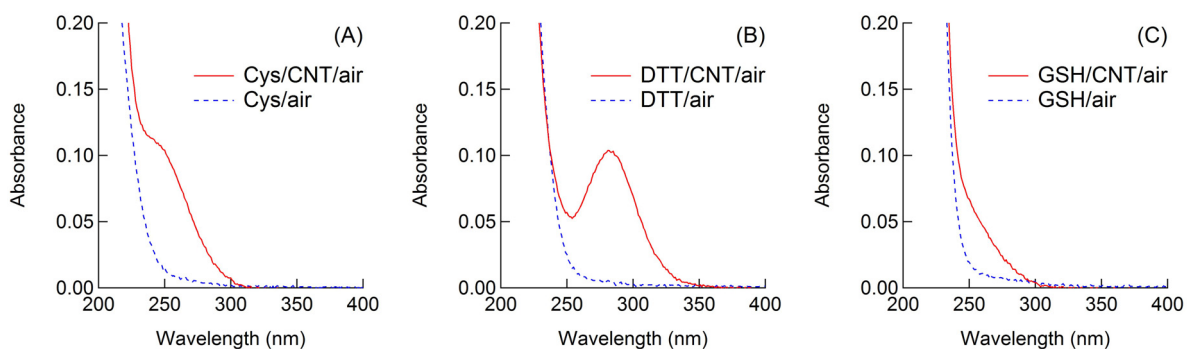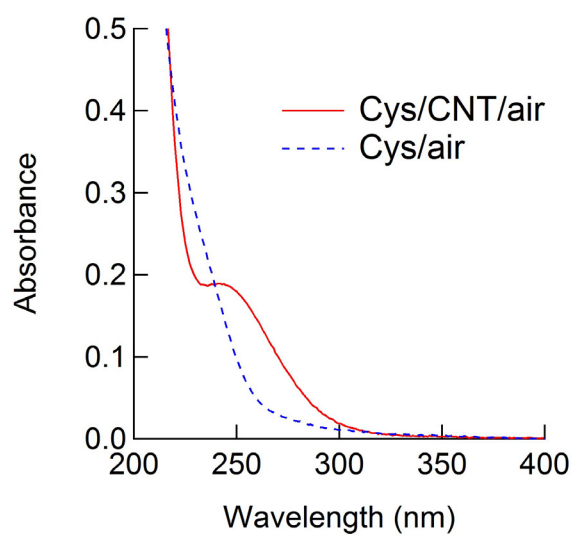

Figure S2. Absorption spectra of the products from the thiols in the presence and absence of the highly purified CNTs at pH 4.5.

2.3. Redox Reaction Using Highly Purified HiPco CNTs at Neutral pH Values. A procedure similar to that mentioned in section 2.2 was performed in the absence of the buffer solution to examine pH changes in the mixture of the CNTs and thiols; that is, the washing process described in section 2.2 was completed using a sufficiently large amount of purified water, resulting in a sample pH of approximately 6. The highly purified buffer-free CNT sample was mixed with buffer-free thiol solution at approximately pH 6 (adjusted to that level using NaOH) using an ultrasonic processor (Nanoruptor NR-350) for 15 min at a power of 350 W to prepare 800-µL suspensions containing approximately 0 or 1 mg/mL of CNTs and 1 mM thiols. The suspensions were subsequently centrifuged at 3000 rpm for 10 min using a centrifuge (H103n). pH values of the supernatants were measured to examine pH changes in the presence and absence of the CNTs. Finally, the supernatants were filtered through a PTFE membrane with a 0.22-µm pore size and then subjected to the absorption measurement.

The pH values were found to be 6.0 ± 0.2 and 7.1 ± 0.2 in the presence and absence of the CNTs, respectively. The absorption spectra of the filtered supernatants are shown in Figure S3. The spectrum of the sample collected from the mixture in the presence of CNTs exhibited a shoulder at approximately 250 nm, indicating the presence of the disulfide bond. By contrast, the spectrum of the sample collected from the mixture in the absence of CNTs showed no such spectral shoulder. This result indicates that formation of the disulfide bond can be induced by the CNTs even at neutral pH. Incidentally, the sample in the absence of CNTs showed a weak absorption at approximately 225–300 nm, which was different from that of Cys-Cys. This weak absorption is ascribable to deprotonation of the thiol group of Cys, which is available at neutral pH values.[1] Thus, the deprotonation can be prevented by the electron transfer from the thiols into the CNTs in the presence of the CNTs.
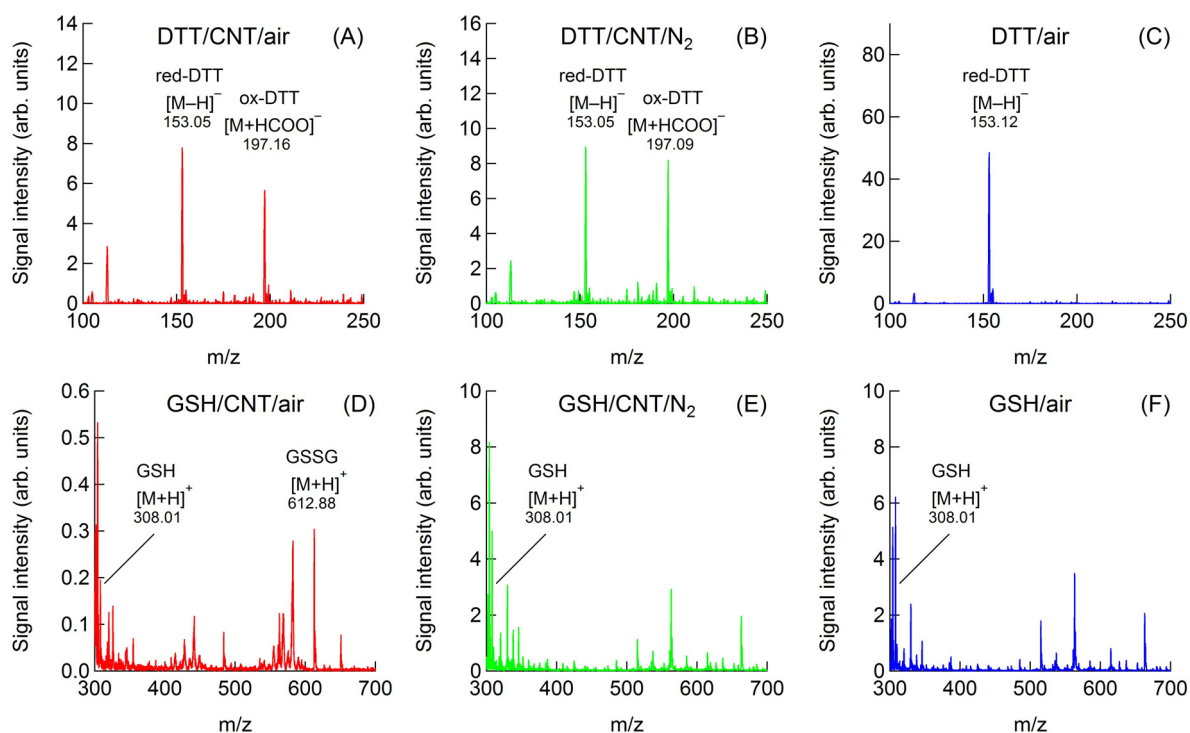
Figure S3. Absorption spectra of the products from cysteine in the presence and absence of the highly purified CNTs in buffer-free solution.

2.4. Redox Reaction of Dispersant-Free HiPco CNTs with Thiols Using Ultrasonication
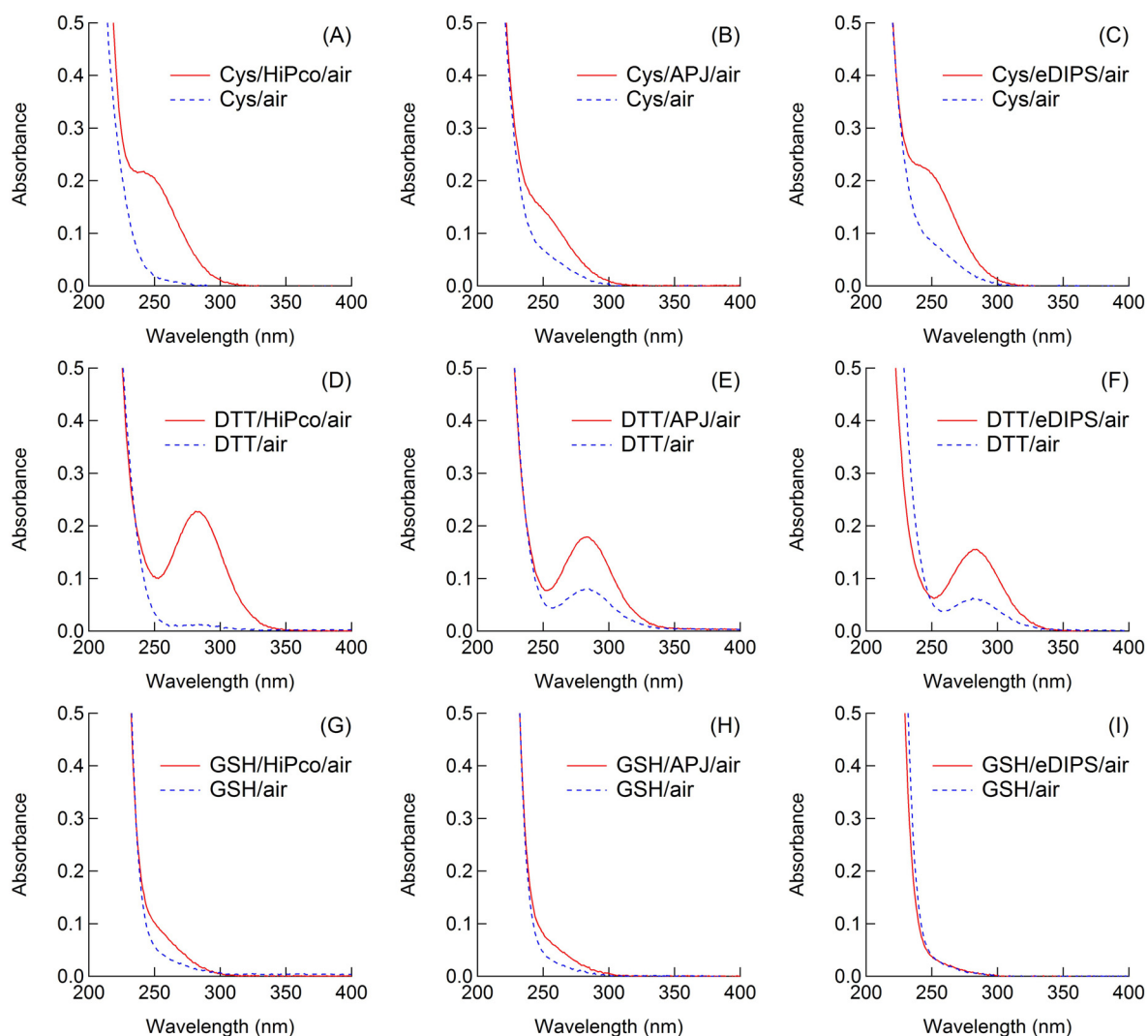

Figure S4. Mass spectra of the products from DTT (A-C) or GSH (D-F) under different conditions.

2.5. Comparison between Different CNT Types in the Redox Reaction. Different types of CNTs were used to examine their redox reactions with thiols. CNTs synthesized by the APJ method and by the eDIPS method as well as by the HiPco method were used as model carbon nanotubes. The APJ CNTs have diameters of 1.4 ± 0.1 nm, and the eDIPS CNT have diameters of 2.0 ± 0.8 nm. Each 5-mg CNT sample was mixed with 6 mL of 0.1% v/v ammonium acetate buffer solution (pH 4.5) using an ultrasonic homogenizer (Sonifire 250D) equipped with a 0.25-in flat tip for 10 min at a power density of *ca.* 20 W cm^{-2} as a preliminary dispersion treatment. Subsequently, each 6-mL mixture was centrifuged at $10,000 \times g$ for 10 min using an ultracentrifuge (himac CS100GXII and S80AT3 rotor). The upper 2-mL fractions of the centrifuged 6-mL samples were then removed to obtain 4 mL of CNT mixtures as the samples used for the redox reaction with the thiols. Additionally, the upper 4-mL fractions of the other centrifuged 6-mL samples were collected to obtain 4 mL of CNT-free solutions as the control samples; note that these control samples were used to examine effects of coexisting soluble impurities on the redox reaction. Each 4-mL sample was mixed with 1 mL of 10 mM thiols in 0.1% v/v ammonium acetate buffer solution (pH 4.5) using the ultrasonic homogenizer for 1 h at a power density of *ca.* 20 W cm^{-2} to prepare 5-mL suspensions containing 0 or 1 mg/mL of CNTs and 2 mM thiols. After mixing, each sample was centrifuged at $10,000 \times g$ for 10 min using the ultracentrifuge. The upper 1-mL aqueous fractions were obtained from the supernatants to collect products from the thiols and were then subjected to an absorption measurement (Figure S5). Table S1 listed the product yields from the thiols under the respective conditions; note that the mixing treatment in this experiment were performed for 2 mM thiols using the ultrasonic homogenizer equipped with the flat tip, which is different from the method shown in Section 1.2.

Figure S5. Representative absorption spectra of the products from 2 mM thiol solutions mixed with or without different types of CNTs, i.e., HiPco, eDIPS, or APJ, under ambient air: (A-C) Cys; (D-F) DTT; (G-I) GSH.

Table S1. Product yields of the oxidized forms of the thiols in the presence of the CNTs.

CNT	Yield (%)		
	Cys	DTT	GSH
HiPco	75	44	24
APJ	27	39	16
eDIPS	84	21	4

## 2.6. Absorption spectra of the different types of CNTs

The absorption spectra of the CNTs used in this study were shown in Figure S6; the CNTs were dispersed in 1 wt% sodium deoxycholate solution for 1 h at a power density of *ca.* 20 W cm^{-2} using an ultrasonic homogenizer (Sonifire 250D) equipped with a 0.25-in flat tip.
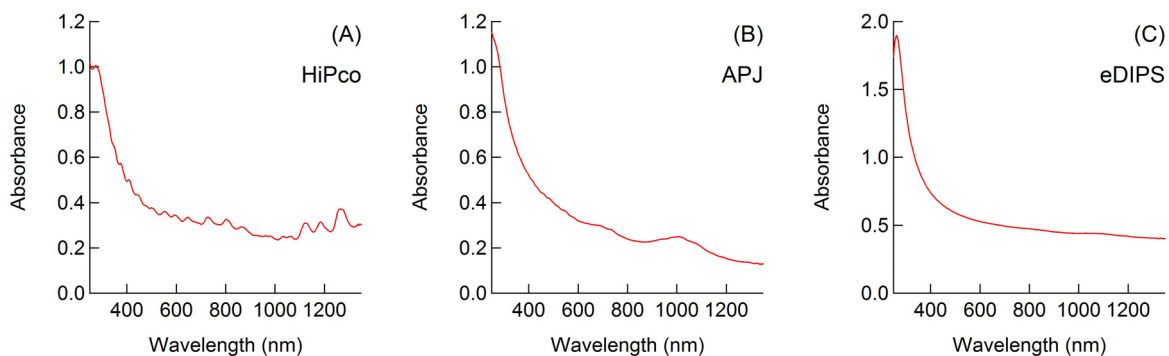
Figure S6. Absorption spectra of the different types of CNTs, i.e., HiPco, eDIPS, or APJ, dispersed in 1 wt% sodium deoxycholate.

2.7. Reduction Kinetics of the SDS-Dispersed CNTs by the Addition of Thiols.
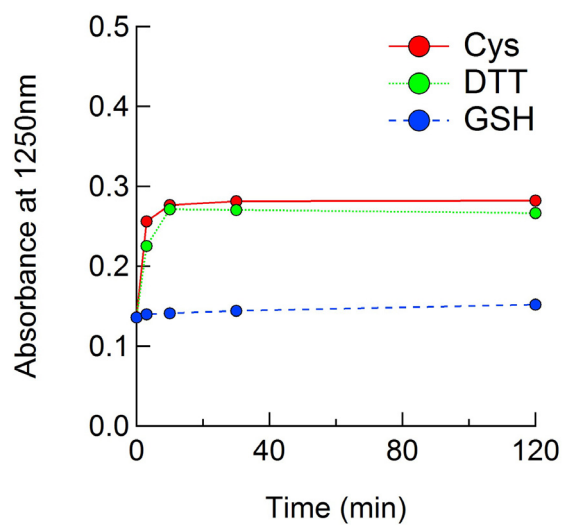
Figure S7. Time-dependent absorbance changes of the SDS-dispersed CNTs at 1250 nm in the presence of 250 μM thiols.

2.8. Molecular Dynamics Simulations of Thiol Molecules around an Oxidized CNT.

All-atom molecular dynamics (MD) simulations of Cys and GSH with a charged CNT in an explicit water box were performed. The charged (6,6) CNT was used as a model of an oxidized CNT, as described in our previous study.[2] The CNT has a total charge of +5, which corresponds to one positive charge per 92 carbon atoms of the CNT. The net charge was evenly divided among all carbon atoms of the CNT. The system contained a CNT, five chloride ions and 3444 water molecules for Cys or 3420 water molecules for GSH in addition to the thiol molecules—specifically, three Cys molecules, two Cys-Cys molecules, three GSH molecules or two GSSG molecules. As an initial state, the thiol molecules were placed in the vicinity of the CNT. The structure of the (6,6) CNT with 48-Å length was generated using TubeGen 3.4.[3] The CNT was modeled using the general AMBER force field (GAFF),[4] and the thiols were modeled using the Amber99SB force field.[5] The water molecules were modeled as TIP3P water.[6] Chloride ions were modeled using the monovalent anion parameters by Joung and Cheatham.[7] Simulations were conducted with the NPT ensemble at a temperature of 298 K and a pressure of 1.0 bar in a box (approximately $48 \times 48 \times 48$ Å^3). To prevent the CNT from protruding from the periodic boundary box, the CNT was fixed along the z-axis at the center of the box by a harmonic restraint with a spring constant of $k = 2.390$ kcal mol^{-1} Å^{-2} (= 1000 kJ mol^{-1} nm^{-2}), and scaling of the box size in the z-axis direction was omitted. The temperature was controlled by a Langevin thermostat with an inverse friction constant of 0.5 ps^{-1}. The pressure was controlled by a Parrinello–Rahman barostat[8] with a relaxation time of 2.0 ps. The electrostatics were treated by using the particle mesh Ewald (PME) method[9] with a 10.0 Å cut-off distance. The van der Waals interactions were expressed by the cut-off method with 10.0 Å cut-off distances. The covalent bonds for the hydrogen atoms in the thiol molecules were constrained by

the linear constraint solver (LINCS).[10] The covalent bonds in the water were constrained by using the SETTLE algorithm.[11] The integration time step was 2 fs. The simulations were conducted using the GROMACS 4.6.7 simulator.[12]
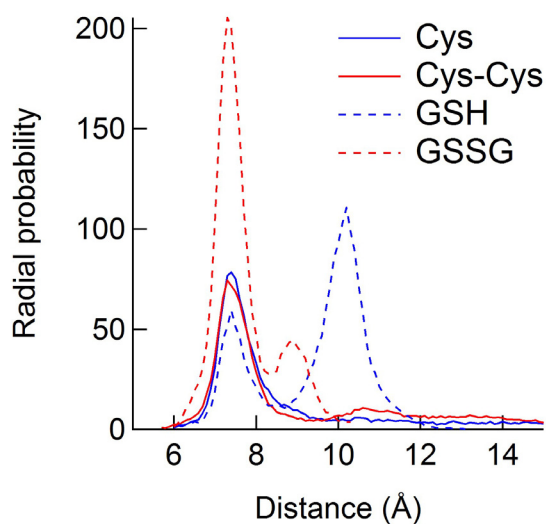

Figure S8. Radial probability distributions for the sulfur atom of Cys, Cys-Cys, GSH and GSSG around the sidewall of the (6,6) CNT. The probability values are scaled by the average probability.

2.9. Reduction of the Surfactant-Dispersed CNTs by the Addition of Lysozyme. Highly dispersed HiPco CNTs were prepared by the same method described in section 1.6 and then diluted into 1 wt% SDS solution to adjust the absorbance of the dispersion at 600 nm to 0.28, which corresponds to 0.13 mg/mL. Reduced lysozyme from egg white was prepared as follows. Native lysozyme was dissolved into a solution containing guanidine hydrochloride, DTT and EDTA to make a 50 mg/mL lysozyme solution in the presence of 6 M guanidine hydrochloride 30 mM DTT and 2 mM EDTA. Subsequently, the lysozyme solution was dialyzed against 2 mM EDTA solution using dialysis tubing (Visking tubing, MWCO 12,000–14,000). The resulting precipitate of lysozyme was dissolved into a solution containing 6 M urea and 2 mM EDTA. The dissolved lysozyme solution was filtered through a 3,000 NMWL membrane (Amicon Ultra-15 Centrifugal Filter Units with Ultracel-3 membrane, EMD Millipore Corp.) by the centrifugation at 3,000 rpm (H-103N, Kokusan Co. Ltd.) to remove DTT and then the concentrated sample was diluted five-fold into a solution containing 6 M urea and 2 mM EDTA; this process was repeated five times. Finally, the reduced lysozyme concentration was adjusted to 100 μM using a solution containing 6 M urea and 2 mM EDTA. Native lysozyme was also dissolved into a solution containing 6 M urea and 2 mM EDTA and used as a control sample. The CNT and lysozyme samples prepared above were diluted into a solution containing urea, EDTA and SDS to prepare samples containing 0.0065 mg/mL CNTs, 1 μM lysozyme, 0.6 M urea, 2 mM EDTA and 0.05 wt% SDS. The absorption spectra of these samples are shown in Figure S9.
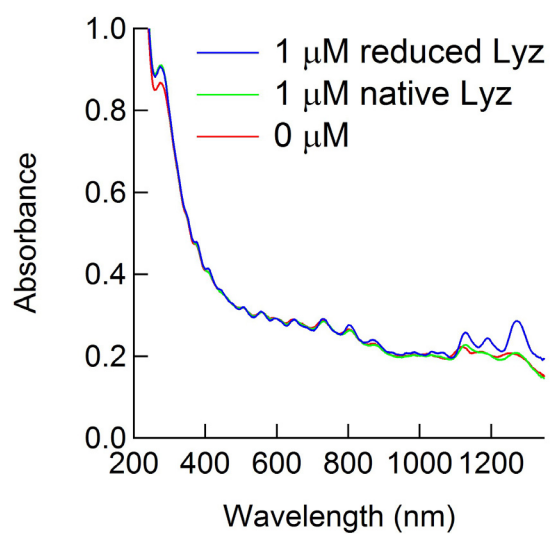
Figure S9. Absorption spectra of HiPco CNTs in the presence and absence of the reduced and native lysozyme (Lyz).

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