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Dispersion of Cisplatin-Loaded Carbon Nanohorns with a Conjugate Comprised of an Artificial Peptide Aptamer and Polyethylene Glycol

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Abstract: Hydrophobic single-wall carbon nanohorns (SWNHs) were dispersed in aqueous media by noncovalently modifying their surfaces with conjugate molecules comprised of polyethylene glycol (PEG) and a peptide aptamer (NHBP-1) that specifically bind to the surfaces of the SWNHs. The conjugates were synthesized by coupling PEG (average molecular weights of 20,000 and 5000) to the N-terminus of NHBP-1 to produce 20PEG-NHBP and 5PEG-NHBP, respectively. Oxidized SWNHs (oxSWNHs) mixed with 20PEG-NHBP or 5PEG-NHBP were well dispersed in water and passed through a gel filtration column, whereas the oxSWNHs treated with PEG stuck to the top of the column. Although the presence of salts in the media significantly impaired the dispersibility of the oxSWNHs, the oxSWNHs/20PEG-NHBP complexes were well dispersed in both the phosphate-buffered saline (PBS) and cell culture medium. The amount of 20PEG-NHBP bound to the oxSWNHs was estimated to be 0.32 g/g of oxSWNHs, and a dynamic light scattering analysis revealed the diameter of the oxSWNHs/20PEG-NHBP complex to be approximately 210 nm. We then showed that CDDP@oxSWNHs/20PEG-NHBP, in which the cancer chemotherapy drug cisplatin (CDDP) was loaded inside the oxSWNHs, was well dispersed in both the PBS and culture medium and exerted a potent cytotoxic effect against cancer cells. The good dispersion of drug-loaded carbon nanomaterials, like that seen here, is a prerequisite for the clinical application of such materials.

Keywords: Anticancer agent; biocompatibility; carbon nanotubes; drug delivery systems; nanomedicine

Introduction

Carbon nanomaterials have been attracting attention as novel biomaterials potentially useful in the field of nanomedicine.[1] The significant characteristics of the carbon nanomaterials include their stability, inertness, and large surfaces area (providing high loading capacity for guest molecules), all of which suggest the potential utility of these materials as carriers in drug delivery systems (DDSs).[2,3] Unfortunately, their

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inherent hydrophobicity poses an impediment to their clinical application. In an aqueous environment, such as the blood, hydrophobic nanomaterials have an inclination to form large agglomerates, which could clog capillary vessels and lead to serious consequences. Thus, endowing carbon nanomaterials with a molecular dispersibility under aqueous conditions is currently a foremost challenge in this field.

Among the various carbon nanomaterials, we have been focusing on single-wall carbon nanohorns (SWNHs).[4] These members of the single-wall carbon nanotube family usually exist as spherical aggregates of nanotubes whose ends are closed with a cone-shaped cap (horn). SWNHs are prepared by vaporizing graphite rods via CO_2 laser ablation in Ar at ambient temperature; thereby, SWNHs are absolutely free of metal contamination, which is an advantage over most carbon nanotubes from the viewpoint of their clinical applications. SWNHs, nanotubes with a diameter of 2–5 nm, protrude from the surface of a spherical aggregate, the diameter of which is approximately 80–100 nm. This size makes SWNHs particularly attractive as a carrier in DDSs, because, at that size, SWNHs would likely exert "enhanced permeation and retention (EPR)" effects[5] which allow the nanoparticles to preferentially penetrate and accumulate within solid tumors having neovascularization.

SWNHs have large surface areas, and their surfaces area can be further increased by oxidation to make holes (nanowindows) in their walls[6] through which small molecules, such as N_2, Ar, C_{60}, $Fe(CH_2COO)_2$, and cisplatin [*cis*-diammine-dichloroplatinum (CDDP)], among others, are able to infiltrate into the inner space.[7,8] We previously showed that the oxygen-treated SWNHs (oxSWNHs) can load dexamethasone,[9] CDDP,[8] and a polyethylene glycol–doxorubicin (PEG–DXR) conjugate[10] and that the loaded dexamethasone or CDDP is slowly released from the complexes into the medium. All of these experimental data, as well as the physicochemical properties of the SWNHs, suggest the potential utility of these materials as drug carriers in DDSs. Before clinical application, however, the poor dispersibility of these materials must be resolved.

A wide variety of approaches to making carbon nanomaterials dispersible in water have been reported, i.e., both covalent (chemical) and noncovalent modifications.[11] For example, the solubility of SWNHs is improved by amination (covalent modification).[12,13] In comparison with such covalent modification, noncovalent modification has other advantages. (i) The inherent properties of carbon nanomaterials would be preserved after the modification, and (ii) the dispersion procedure is rather simple. In the noncovalent modification approach, amphiphilic agents, i.e., molecules having both hydrophobic and hydrophilic moieties, have been used as surfactants to improve the solubility of the SWNHs.[14] In this case, the hydrophobic portion of the amphiphilic molecule binds to the surface of the SWNH while the hydrophilic portion endows the material with compatibility in an aqueous environment. Block copolymers are examples of materials in which the amphiphilicity is explicitly built in. Nucleic acids as well as polysaccharides have also been used as dispersants for carbon nanotubes, although their amphiphilic nature is implicit. Among the possible amphiphilic block copolymers, with regard to clinical applications, polyethylene glycol (PEG) is attractive for use with SWNHs because PEG has no toxicity, is properly stable, and has a low immunogenicity.[15] In that regard, Murakami et al.[10] showed that a conjugate comprised of a hydrophobic drug, doxorubicin (DXR), and PEG (PEG–DXR) can serve as a surfactant to disperse oxSWNHs in aqueous solution.

(1) Bekyarova, E.; Ni, Y.; Malarkey, E. B.; Montana, V.; MaWilliams, J. L.; Haddon, R. C.; Parpura, V. Applications of carbon nanotubes in biotechnology and biomedicine. *J. Biomed. Nanotechnol.* **2005**, *1*, 3–17.

(2) Bianco, A.; Kostarelos, K.; Prato, M. Applications of carbon nanotubes in drug delivery. *Curr. Opin. Chem. Biol.* **2005**, *9*, 674–679.

(3) Shiba, K. Functionalization of carbon nanomaterials by evolutionary molecular engineering: Potential application in drug delivery system. *J. Drug Targets* **2006**, *14*, 512–518.

(4) Iijima, S.; Yudasaka, M.; Yamada, R.; Bandow, S.; Suenaga, K.; Kokai, F.; Takahashi, K. Nano-aggregates of single-walled graphitic carbon nano-horns. *Chem. Phys. Lett.* **1999**, *309*, 165–170.

(5) Matsumura, Y.; Maeda, H. A new concept for macromolecular therapeutics in cancer chemotherapy: Mechanism of tumoritropic accumulation of proteins and the antitumor agent Smancs. *Cancer Res.* **1986**, *46*, 6387–6392.

(6) Fan, J.; Yudasaka, M.; Miyawaki, J.; Ajima, K.; Murata, K.; Iijima, S. Control of opening in single-wall carbon nanotubes and single-wall carbon nanohorns using oxygen. *J. Phys. Chem. B* **2006**, *110*, 1587–1591.

(7) Yudasaka, M.; Ajima, K.; Suenaga, K.; Ichihashi, T.; Hashimoto, A.; Iijima, S. Nano-extraction and nano-condesation for C60 incorporation into single-wall carbon nanotubes in liquid phases. *Chem. Phys. Lett.* **2003**, *380*, 42–46.

(8) Ajima, K.; Yudasaka, M.; Murakami, T.; Maigné, A.; Shiba, K.; Iijima, S. Carbon nanohorns as anticancer drug carriers. *Mol. Pharm.* **2005**, *2*, 475–480.

(9) Murakami, T.; Ajima, K.; Miyawaki, J.; Yudasaka, M.; Iijima, S.; Shiba, K. Drug-loaded carbon nanohorns: Adsorption and release of dexamethasone in vitro. *Mol. Pharm.* **2004**, *1*, 399–405.

(10) Murakami, T.; Fan, J.; Yudasaka, M.; Iijima, S.; Shiba, K. Solubilization of single-wall carbon nanohorns using a PEG-doxorubicin conjugate. *Mol. Pharm.* **2006**, *3*, 407–414.

(11) Klumpp, C.; Kostarelos, K.; Prato, M.; Bianco, A. Functionalized carbon nanotubes as emerging nanovectors for the delivery of therapeutics. *Biochim. Biophys. Acta* **2006**, *1758*, 404–412.

(12) Isobe, H.; Tanaka, T.; Maeda, R.; Noiri, E.; Solin, N.; Yudasaka, M.; Iijima, S.; Nakamura, E. Preparation, purification, characterization, and cytotoxicity assessment of water-soluble, transition-metal-free carbon nanotube aggregates. *Angew. Chem., Int. Ed.* **2006**, *45*, 6676–6680.

(13) Tagmatarchis, N.; Maigné, A.; Yudasaka, M.; Iijima, S. Functionalization of carbon nanohorns with azomethine ylides: Towards solubility enhancement and electron-transfer processes. *Small* **2006**, *2*, 490–494.

(14) Zhang, M.; Yudasaka, M.; Miyawaki, J.; Fan, J.; Iijima, S. Isolating single-wall carbon nanohorns as small aggregates through a dispersion method. *J. Phys. Chem. B* **2005**, *109*, 22201–22204.

(15) Veronese, F. M.; Pasut, G. PEGylation, successful approach to drug delivery. *Drug Discovery Today* **2005**, *10*, 1451–1458.

However, the use of DXR diminishes the utility of this approach in clinical applications. To better generalize the noncovalent dispersal of SWNHs, in this study, we used a peptide aptamer as the SWNH-binding block (NHBP-1)[16] and synthesized a PEG–peptide aptamer conjugate for dispersal of the SWNHs. We found that surface-modified oxSWNHs are able to load CDDP onto their inner space to produce well-dispersed CDDP-loaded carbon nanohorns (CDDP@oxSWNHs/20PEG-NHBP).

Experimental Section

Synthesis of oxSWNHs and PEG–NHBP. The oxidized SWNHs (oxSWNHs) were prepared from the "dahlia" type SWNHs as previously described.[4,6] The PEG derivatives that have an *N*-hydroxysuccinimide (NHS) ester at one end (20PEG–NHS and 5PEG–NHS, average molecular weights of 20,000 and 5000 respectively) were purchased from NOF Corp. (Tokyo, Japan). The peptide aptamer NHBP-1 (DYFSSPYYEQLF) was previously isolated as a SWNH binder using a peptide phage system.[16] NHBP-1 was synthesized by the Anygen Co., Ltd. (Gwang-ju, Japan). To synthesize the PEG–NHBP conjugate, 40 mg of 20PEG–NHS or 10 mg of 5PEG-NHS in 1 mL of acetonitrile was first mixed with 3.2 mg of the NHBP-1 peptide dissolved in 1 mL of *N*,*N*-dimethylformamide (DMF), after which triethylamine was added. This reaction mixture was stirred for 3–4 days, after which the reaction product (PEG–NHBP) was purified by reverse-phase HPLC (LC-2010C, Shimadzu, Kyoto, Japan) using a Cosmosil 5C18-AR-II column (Nacalai Tesque, Kyoto, Japan), and then the fractionated PEG–NHBP was lyophilized for storage. The molecular weights of the fractioned samples were analyzed by matrix-assisted laser desorption time-of-flight mass spectrometry (PBS-II, Ciphergen Biosystems) to confirm that the conjugates were synthesized.

Preparation of a Dispersed Solution of oxSWNHs. To obtain well-dispersed oxSWNHs using PEG–NHBP, the oxSWNHs were first dispersed in 0.2 mL of dimethyl sulfoxide (DMSO) to a concentration of 10 mg/mL. After sonication (Bioruptor UCD-200TM, Cosmo Bio, Tokyo, Japan; power = 130 W), 10 μL of the oxSWNH suspension was mixed with 15 μL of PEG–NHBP (20 mg/mL in DMSO), then briefly sonicated again, and diluted via addition of 975 μL of water (purified by Biocell A10, Millipore Corp., Billerica, MA). The final concentrations of the oxSWNHs, PEG–NHBP, and DMSO in this mixture were 0.1 mg/mL, 0.3 mg/mL, and 2.5%, respectively. The ratio of PEG–NHBP to the oxSWNHs in the preparation procedure was determined by our pilot tests in which several conditions were investigated (data not shown). Finally, the mixture was vortexed for 2–5 h at room temperature to obtain the well-dispersed oxSWNHs.

(16) Kase, D.; Kulp, J. L., III; Yudasaka, M.; Evans, J. S.; Iijima, S.; Shiba, K. Affinity selection of peptide phage libraries against single-wall carbon nanohorns identifies a peptide aptamer with conformational variability. *Langmuir* **2004**, *20*, 8939–8941.

Evaluation of Dispersion of the oxSWNHs/PEG–NHBP Complex. We evaluated the dispersibility of the oxSWNHs/PEG–NHBP complex using (1) gel chromatography, (2) absorption measurements, and (3) DLS (see below). For gel chromatography (Figure 2), we used a PD-10 column into which 8.3 mL of Sephadex G-25 had been prepacked (GE Healthcare UK, Ltd., Buckinghamshire, U.K.). Purified water served as the eluant. In the visual inspection experiments (Figure 3), the oxSWNHs/PEG–NHBP complex was diluted in PBS [10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl (pH 7.4)] or culture medium [RPMI 1640 containing 5% fetal bovine serum (FBS), 100 μg/mL penicillin, and 100 units/mL streptomycin] at concentrations of 50 μg/mL oxSWNHs, 150 μg/mL PEG–NHBP, and 1.25% DMSO. For the absorption measurements (Figure S1 of the Supporting Information), 10 μg/mL oxSWNHs, 30 μg/mL PEG–NHBP, and 0.25% DMSO were used. The samples (0.3 mL) were then stored in cuvettes, and the absorbance at 800 nm was monitored using a Shimadzu UV-2550 spectrophotometer.

Estimation of the Amount of PEG–NHBP Bound to oxSWNHs. To remove the unbound PEG–NHBP, the oxSWNHs/PEG–NHBP solution was filtered using a Microsep centrifugal device (MWCO of 300K; PALL Life Sciences, Ann Arbor, MI). The residual PEG–NHBP on the membrane was then removed with three washes of purified water. The recovered solution of the oxSWNHs/PEG–NHBP complex was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, 15 to 25% gradient, Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan). The separated PEG–NHBP was visualized after being stained with a solution containing 1% iodine and 2% potassium iodide and quantified using a densitometer. The concentration of the oxSWNHs was quantified on the basis of the adsorption at 800 nm using a standard curve.

Dynamic Light Scattering (DLS) Measurements. The DLS measurements were taken using a FPAR-1000 (Otsuka Electronics, Osaka, Japan) at 25 °C. The oxSWNHs/PEG–NHBP complex was filtered using a Microsep centrifugal device (MWCO of 300K; PALL) and diluted to 30 μg/mL in water.

Preparation of CDDP@oxSWNHs/20PEG–NHBP. CDDP was loaded into the oxSWNHs as described by Ajima et al.[8] The loaded CDDP in the oxSWNHs was observed as CDDP nanoclusters by TEM (Figure S2 of the Supporting Information). The quantity of CDDP within the CDDP@oxSWNHs was estimated to be 0.22 g/g of CDDP@oxSWNHs using thermogravimetric (TG) analysis (data not shown). The CDDP@oxSWNHs were then dispersed with 20PEG–NHBP as described above with slight modifications. In this experiment, we used ethanol instead of DMSO because DMSO is known to inactivate CDDP. In addition, to prevent the possible leakage of the CDDP from the oxSWNHs into the medium, the CDDP@oxSWNHs and 20PEG–NHBP were mixed by sonication for 5 s instead of vortexing for hours. The CDDP@oxSWNHs were dispersed in a 50% ethanol/water mixture to a concentration of 2 mg/mL. After a quick

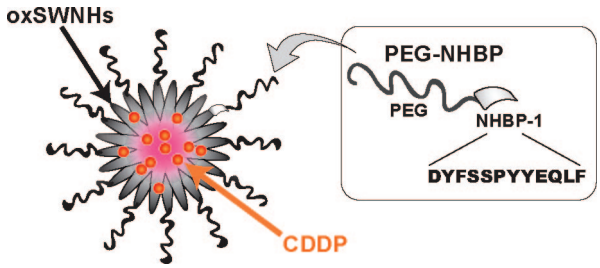

Figure 1. Schematic representation of CDDP@oxSWNHs/20PEG–NHBP. Initially, the anticancer drug CDDP was loaded into the oxidized single-wall carbon nanohorns (oxSWNHs) through their nanowindows. The surface of the resultant CDDP@oxSWNHs was then modified by a conjugate comprised of polyethylene glycol (PEG) and an artificial peptide aptamer against the SWNHs (NHBP-1).

sonication, they were rapidly diluted with a 20PEG–NHBP solution and sonicated for 5 s. Despite this short mixing time, the obtained CDDP@oxSWNHs/20PEG–NHBP complex exhibited a good dispersibility (see below). The absorption measurements carried out as described above gave concentrations of 30 μg/mL CDDP@oxSWNHs, 90 μg/mL 20PEG–NHBP, and 0.8% ethanol.

Cytotoxicity Assay. NCI-H460 human non-small cell lung cancer cells were cultured in RPMI 1640 containing 5% FBS, 100 μg/mL penicillin, and 100 units/mL streptomycin at 37 °C under a humidified atmosphere of 5% CO_2. The cytotoxicity of the CDDP@oxSWNHs/20PEG–NHBP complex was estimated by measuring the metabolic activity of the viable cells using the Cell Proliferation Reagent WST-1 (Roche Diagnostics GmbH, Mannheim, Germany). The cells were seeded onto a 96-well plate (Asahi Technology, Chiba, Japan) to a density of 2.5×10^3 cells/well in 100 μL of medium and incubated overnight. The medium in each well was then replaced with 100 μL of a new medium containing various concentrations of CDDP@oxSWNHs/20PEG–NHBP. After being incubated for 53 h, the medium was aspirated, and 110 μL of fresh medium containing the WST-1 solution was added. Optical microscopic observations were made after 50 h, but before the medium containing the CDDP@oxSWNHs was aspirated.

Results and Discussion

Figure 1 shows the design for the CDDP@oxSWNHs/20PEG–NHBP complex. NHBP-1 is a 12-amino acid peptide aptamer (DYFSSPYYEQLF) that was isolated as a SWNH binder using a peptide phage system.[16] A conjugate comprised of PEG and NHBP-1 (PEG–NHBP) binds to the surfaces of the CDDP@oxSWNHs via the peptide moiety, and the PEG moiety endows the resultant complex with a good dispersibility. Because CDDP is situated inside the oxSWNHs, it does not interfere with the interaction between the NHBP-1 and oxSWNHs and is slowly released from the complex.

To make the PEG–NHBP conjugate, the N-terminal amino group of the peptide was reacted with a preactivated carboxyl

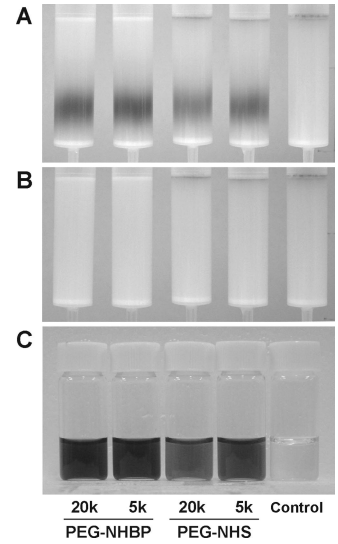

Figure 2. Gel filtration chromatography of dispersed solutions of the oxSWNHs/PEG–NHBP complex in water. Whereas the oxSWNHs dispersed with PEG–NHBP passed through the column, other samples did not; therefore, the residues remained near the top of the column: (A) samples running in columns, (B) columns after elution, and (C) eluted samples.

group at one end of the PEG chain (PEG–NHS). Two types of PEG–NHBPs were prepared, 20PEG–NHBP and 5PEG–NHBP, in which the average molecular weights of the PEG chain were 20,000 and 5000, respectively. The PEG–NHBP conjugates were mixed with the oxSWNHs in water to obtain the dispersed oxSWNHs/PEG–NHBP complexes.

To assess the dispersibility of the oxSWNHs/PEG–NHBP complex, we then attempted to pass dispersed solutions of the complex through a PD-10 gel filtration column (Sephadex G-25). Nanoparticles will pass through a gel filtration column only if they are well dispersed.[10] We found that the oxSWNHs treated with 5PEG–NHBP or 20PEG–NHBP passed completely through the column and were recovered as black solutions (Figure 2), indicating that they were well dispersed in the water. In contrast, the oxSWNHs with no additives were entirely retained at the top of the column. In addition, most of the oxSWNHs treated with PEG–NHS, which lacks the NHBP-1 peptide, also stuck to the top of the column, even though they appeared to be well dispersed before the gel filtration. The NHBP-1 peptide itself did not exhibit any surfactant activity with the SWNHs (data not shown). Thus, our gel filtration data indicated that the PEG–NHBP conjugates, but not the PEG or the NHBP peptide, endow the oxSWNHs with an enhanced dispersibility.

As often observed with colloidal particles, low concentrations of salts in the medium increase the level of formation of large agglomerates of carbon nanomaterials.[17] For that reason, we next estimated the stabilities of the dispersed oxSWNHs/PEG–NHBP solutions in the presence of salts. Bearing the potential clinical applicability of SWNHs in

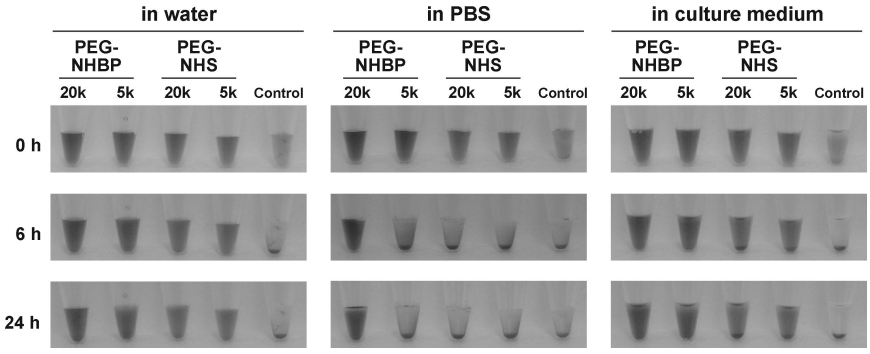

Figure 3. Visual inspection of the oxSWNHs/PEG–NHBP complexes in water, PBS, and culture medium. Whereas the oxSWNHs/20PEG–NHBP complex remained dispersed after being incubated for 24 h in PBS, the other four preparations precipitated.

mind, we tested two media, i.e., phosphate-buffered saline (PBS) and cell culture medium containing 5% fetal bovine serum (FBS). The dispersibility was then scored by visual inspection 0, 6, and 24 h after the oxSWNHs/PEG–NHBP complexes had been mixed into the media. As shown in Figure 3, in PBS, only the oxSWNHs treated with 20PEG–NHBP remained dispersed after incubation for 24 h. They also maintained a good dispersion in the water or culture medium. The oxSWNHs treated with 5PEG–NHBP precipitated within 6 h of their addition to PBS. When the oxSWNHs were treated with PEG–NHS (which does not contain the NHBP-1 peptide), they retained a fair dispersibility in water over the 24 h observation period, but in PBS, most of the oxSWNHs precipitated within 6 h, confirming the conclusion drawn from the gel filtration experiments that PEG–NHS does not endow the SWNHs with a good dispersibility. The oxSWNHs with no additive rapidly precipitated in water, PBS, or culture medium. Thus, only 20PEG–NHBP endowed the oxSWNHs with a good dispersibility in the salt solution or culture medium. Apparently, 5PEG–NHBP was not enough to suppress this salt-derived aggregation of the SWNHs.

We found that formation of the agglomerate was suppressed in the culture medium, even though the medium contains a variety of salts. It is noteworthy that although a visual inspection suggested that PEG–NHS had some ability to mediate a dispersion, a quantitative analysis of the absorbance at 800 nm clarified the differences in the abilities of PEG–NHBP and PEG–NHS to mediate the dispersion (Figure S1 of the Supporting Information). Given that a culture medium contains bovine serum as well as various small molecules, it may have been that these molecules somehow contribute to the suppression of the precipitation in the absence of NHBP. Interestingly, Cherukuri et al.[18] recently reported that surfactant molecules on the surfaces of carbon nanotubes are replaced by serum albumin as soon as they are intravenously injected into mice.

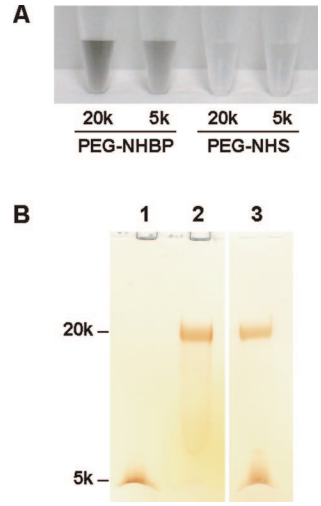

Figure 4. Estimation of the amount of PEG–NHBP bound to oxSWNHs. (A) oxSWNHs/PEG–NHBP solutions after unbound PEG–NHBP had been removed by ultrafiltration. In the control experiment (right), the oxSWNHs treated with PEG–NHS were not recovered after filtration, indicating that the PEG–NHS did not endow the oxSWNHs with a good dispersibility. (B) Bound 5PEG–NHBP (lane 1) and 20PEG–NHBP (lane 2) were separated by SDS–PAGE (shown at the 5k and 20k migration points, respectively) and stained with iodine for quantification. The oxSWNHs were stuck to the top of the gel. In lane 3, a mixture of 5PEG–NHBP and 20PEG–NHBP was run as the control.

We then estimated the amount of PEG–NHBP bound to the oxSWNHs. For this purpose, the dispersed oxSWNH solutions (in water) were ultrafiltered through a membrane with a MWCO of 300,000 to remove the unbound PEG derivatives. The oxSWNHs treated with PEG–NHBP were then recovered from the membrane and resuspended in water as a black-colored solution (Figure 4A). On the other hand, the oxSWNHs treated with PEG–NHS were tightly bound

(17) Sano, M.; Okamura, J.; Shinkai, S. Colloidal nature of single-walled carbon nanotubes in electrolyte solution: The Schulze-Hardy rule. *Langmuir* **2001**, *17*, 7172–7173.

(18) Cherukuri, P.; Gannon, C. J.; Leeuw, T. K.; Schmidt, H. K.; Smalley, R. E.; Curley, S. A.; Weisman, R. B. Mammalian pharmacokinetics of carbon nanotubes using intrinsic near-infrared fluorescence. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 18882–18886.

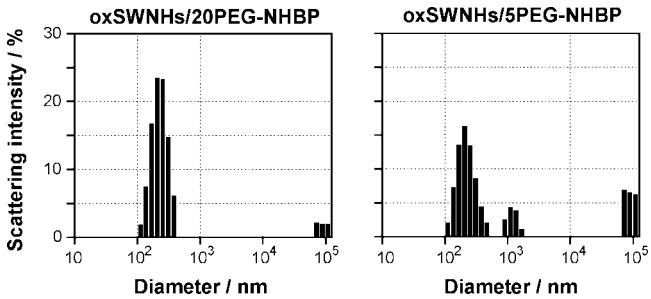

Figure 5. DLS analysis of oxSWNHs/20PEG–NHBP (left) and oxSWNHs/5PEG–NHBP (right).

to the ultrafiltration membrane (probably via a hydrophobic interaction) and were not recovered, which again confirmed that, by itself, PEG is not able to endow the oxSWNHs with a good dispersibility. The recovered oxSWNHs/PEG–NHBP complexes were then subjected to SDS–PAGE during which the PEG–NHBP became detached from the oxSWNHs and separately electrophoresed (Figure 4B). Because the PEG moieties can be semiquantitatively stained with iodide, we were able estimate the amount of bound PEG–NHBP per oxSWNHs. From these analyses, we calculated that approximately 0.32 g (±0.02 g) of the 20PEG–NHBP was bound to 1 g of oxSWNHs, which means that each oxSWNH particle was ornamented with approximately 6000 PEG–NHBP molecules. In the iodide staining experiments, we found that some oxSWNHs/5PEG–NHBP complex (but not the oxSWNHs/20PEG–NHBP complex) was irreversibly trapped on the ultrafiltration membrane. Because of this unwanted adsorption onto the membrane, we were unable to quantify the binding of 5PEG–NHBP to the oxSWNHs.

We used dynamic light scattering (DLS) to estimate the sizes of the oxSWNHs/20PEG–NHBP and oxSWNHs/5PEG–NHBP complexes (Figure 5). When the complexes are in water at a concentration of 0.03 mg/mL, the average diameter of both was calculated to be 210 nm, which agrees fairly well with the value obtained using oxSWNHs/PEG–DXR.[10] The oxSWNHs/5PEG–NHBP sample contained a small fraction whose average diameter was 1 μm, which is indicative of the poorer dispersibility of 5PEG–NHBP.

In our earlier studies, we were able to load two drugs (dexamethasone and CDDP) into the oxSWNHs, and the loaded drugs were then slowly released in a biologically active form.[8,9] However, in these cases, the dispersibility of the SWNHs was poor, so agglomerates were formed when the oxSWNHs were applied to the cell cultures. For our study, we chose the CDDP-loaded oxSWNHs to test the dispersibility mediated by PEG–NHBP. This was because Ajima et al.[8] previously showed that the loaded CDDP exists in the form of nanoclusters in the interior of the nanohorns. In contrast, dexamethasone, which is much larger (major axis of dexamethasone is ~1.5 nm, whereas that of CDDP is ~0.5 nm, and the average size of a nanowindow is ~2–3 nm) and much more hydrophobic than CDDP, would be expected to bind to the exterior surfaces of the SWNHs and interfere with the binding of PEG–NHBP.

To prepare CDDP@oxSWNHs/20PEG–NHBP, we first loaded CDDP into the oxSWNHs and observed the CDDP nanoclusters described by Ajima et al.[8] (Figure S2 of the Supporting Information). The quantity of CDDP within the CDDP@oxSWNHs was estimated to be 0.22 g/g of the CDDP@oxSWNHs using thermogravimetric (TG) analysis (data not shown), which agreed well with our earlier report. We next mixed the CDDP@oxSWNHs with 20PEG–NHBP in water as described above with slight modifications; for example, we used 2.5% ethanol instead of DMSO because DMSO is known to inactivate the CDDP, and the mixing time was shortened to avoid any possible leakage of the CDDP from the oxSWNHs. Thereafter, the dispersibility of the prepared CDDP@oxSWNHs/20PEG–NHBP in water, PBS, and culture medium was estimated by spectroscopic

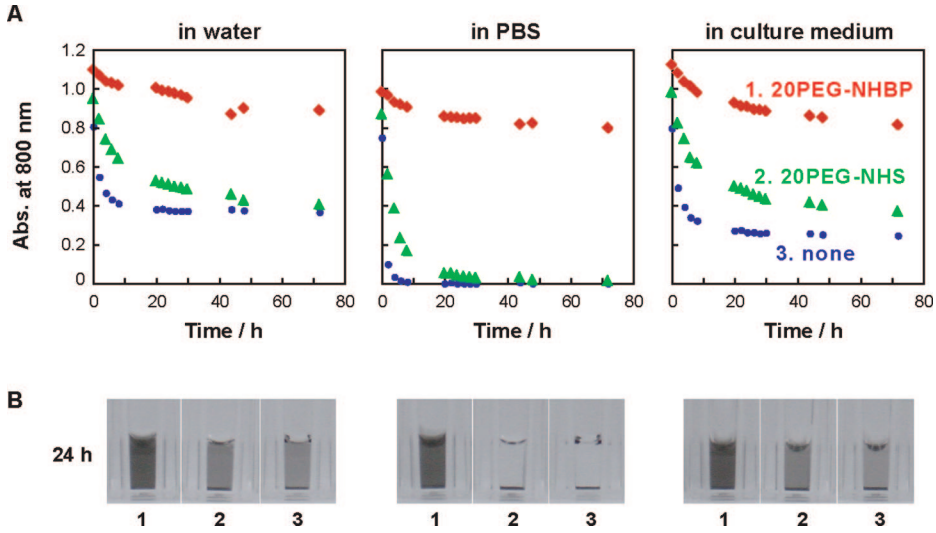

Figure 6. Dispersibility of CDDP@oxSWNHs/20PEG–NHBP in water, PBS, and cell culture medium. (A) The dispersibility was monitored on the basis of the absorbance at 800 nm. CDDP@oxSWNHs/20PEG–NHS and CDDP@oxSWNHs served as controls. (B) Visual inspection of solutions of CDDP@oxSWNHs/20PEG–NHBP (tube 1), CDDP@oxSWNHs/20PEG–NHS (tube 2), and CDDP@oxSWNHs (tube 3) after incubation for 24 h.

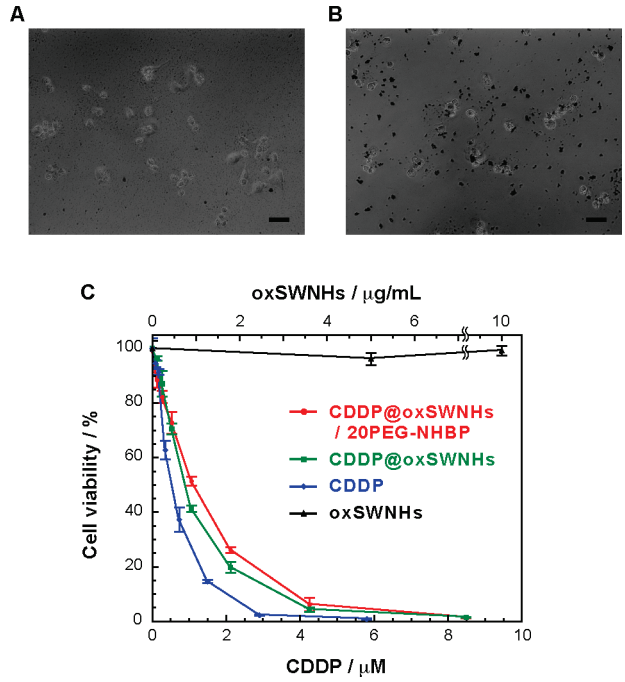

Figure 7. Cytotoxicity of CDDP@oxSWNHs/20PEG–NHBP. (A and B) Phase-contrast micrographs of NCI-H460 cells incubated for 50 h with CDDP@oxSWNHs/20PEG–NHBP (A) or CDDP@oxSWNHs (B). Many black dots of the agglomerated form of the oxSWNHs can be observed in panel B. Bars represent 100 μm. (C) Viabilities of NCI-H460 cells treated with various concentrations of CDDP@oxSWNHs/20PEG–NHBP (red), CDDP@oxSWNHs (green), CDDP (blue), or oxSWNHs (black). Concentrations were standardized to CDDP alone (except for the oxSWNHs where the amounts of the oxSWNHs are shown at the top). Viabilities were measured in WST-1 assays.

analysis. As shown in Figure 6, the CDDP@oxSWNHs/20PEG–NHBP complex retained a good dispersibility, even in PBS. Thus, the loading of CDDP into the oxSWNHs did not interfere with the surface modification by PEG–NHBP, which is consistent with the CDDP molecules being in the interior of the oxSWNHs.[8] If CDDP did bind to the exterior surface of the oxSWNHs, we expect that they would compete with PEG–NHBP and reduce the dispersibility of the oxSWNHs. For example, doxorubicin, which we know binds to the exterior surface of the SWNHs, does reduce the solubility of DXR@oxSWNHs/20PEG–NHBP (our unpublished results).

To show that the loaded CDDP is released as an active drug, we added CDDP@oxSWNHs/20PEG–NHBP to the cultures of NCI-H460 cells (a human lung cancer cell line). Subsequent microscopic observations revealed that the CDDP@oxSWNHs quickly formed micrometer-sized agglomerates, as we previously observed. In contrast, there was little agglomeration of CDDP@oxSWNHs/20PEG–NHBP, even after incubation for 3 days, which confirms the good dispersibility of CDDP@oxSWNHs/20PEG–NHBP (Figure 7A,B). When we estimated the cytotoxicity by measuring the metabolic activity of viable cells using the WST-1 assays, we found that CDDP@oxSWNHs/20PEG–NHBP exerted a dose-dependent cytotoxic effect on the NCI-H460 cells (Figure 7C). Moreover, the oxSWNHs/20PEG–NHBP complexes themselves had no effect on cell proliferation (data not shown), suggesting that the cytotoxicity was caused by the released CDDP. Apparently, modification of the surface of CDDP@oxSWNHs using 20PEG–NHBP did not perturb the release of CDDP from the complex. In accordance with the observations, we confirmed that the release kinetics of CDDP[8] was essentially the same between CDDP@oxSWNHs and CDDP@oxSWNHs/20PEG–NHBP (data not shown).

We have shown the possible use of dispersible SWNHs as a cisplatin carrier. As carriers of drug delivery systems, various nanoparticles, including polymer micelles, liposomes, among others, have been proposed, and some of them have already been used in clinical applications.[19] The stability of particles is one of the important factors required for nanocarriers because it would facilitate various modifications of the particles and would furnish them with a long circulation in the body. In this regard, carbon nanotubes and carbon nanohorns are attractive molecules.[1–3] Now, extensive research studies, including pharmacokinetic analyses, are in progress in our group and other groups to explore the possible uses of carbon nanomaterials in the DDS field.[20,21]

Conclusion

We have demonstrated that the noncovalent modification of SWNHs using a conjugate comprised of PEG and a peptide aptamer (NHBP-1) endows hydrophobic oxSWNHs with a good dispersibility under aqueous conditions. We also showed that modification of the surfaces of the oxSWNHs by PEG–NHBP does not interfere with their ability to load and release CDDP. Our achievement represents another step toward the clinical application of nanocarbon materials.

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Supporting Information Available: A spectroscopic analysis result of the oxSWNHs dispersion (Figure S1) and a TEM image of CDDP@oxSWNHs (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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(19) Portney, N. G.; Ozkan, M. Nano-oncology: Drug delivery, imaging, and sensing. *Anal. Bioanal. Chem.* **2006**, *384*, 620–630.
(20) Miyawaki, J.; Yudasaka, M.; Imai, H.; Yorimitsu, H.; Isobe, H.; Nakamura, E.; Iijima, S. In vivo magnetic resonance imaging of single-walled carbon nanohorns by labeling with magnetite nanoparticles. *Adv. Mater.* **2006**, *18*, 1010–1014.
(21) Liu, H.; Cai, W.; He, L.; Nakayama, N.; Chen, K.; Sun, X.; Chen, X.; Dai, H. In vivo biodistribution and highly efficient tumour targeting of carbon nanotubes in mice. *Nat. Nanotechnol.* **2007**, *2*, 47–52.