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Solubilization of Single-Wall Carbon Nanohorns Using a PEG−Doxorubicin Conjugate

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Abstract: A procedure for dispersing oxidized single-wall carbon nanohorns (oxSWNHs) in aqueous solution using a polyethylene glycol−doxorubicin (PEG−DXR) conjugate is described. In this procedure, oxSWNHs were first incubated with PEG−DXR in dimethyl sulfoxide (DMSO) or *N*,*N*-dimethylformamide (DMF), two organic solvents with relatively high electric dipole moments, after which the solvent was gradually changed to an aqueous one via addition of water until the final concentration of DMSO or DMF reached 10%. The PEG−DXR−oxSWNH complex that was obtained was able to pass through dextran-based chromatographic media (Sephadex G25) equilibrated with water. By contrast, untreated oxSWNHs and DXR-treated or PEG-treated oxSWNHs were unable to penetrate the column, indicating that the PEG−DXR conjugate endowed oxSWNHs with dispersibility in aqueous solution. In gel filtration experiments, the presence of free DXR had an inhibitory effect on the penetrability of PEG−DXR−oxSWNH complexes, which is consistent with the idea that PEG−DXR interacts with the surfaces of oxSWNHs via its DXR moiety. Quantitative analyses showed that the complex contained more than 250 mg of PEG−DXR for each gram of oxSWNHs. The average diameter of the dispersed complex was estimated to be approximately 160 nm using dynamic light scattering analysis. These results suggest that our method has the potential to open the way for the use of oxSWNHs as a clinically practical drug carrier.

Keywords: Nanomedicine; carbon nanohorns; drug delivery systems; dispersion; anticancer agent; biocompatibility

Introduction

Nanomedicine, which is an emerging bridge linking nanotechnology and advanced medical technology, involves the exploration of nanoscaled materials with the aim of developing novel types of drug carriers, imaging agents, sensors, etc.[1,2] Single-wall carbon nanohorns (SWNHs) are spherical, carbonaceous nanoparticles with an average diameter of 80−100 nm,[3] which suggests they could exhibit enhanced permeability and retention (EPR) effects[4,5] and thus

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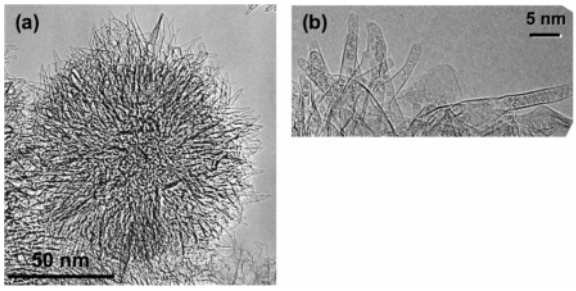

Figure 1. TEM images of oxSWNHs (a) and C_{60}-incorporated oxSWNHs (b).

accumulate within solid tumors showing neovascularization.[6,7] The constituent parts of SWNHs are graphene nanotubes that have closed ends with cone-shaped caps (horns) and diameters of 2−3 nm (Figure 1). SWNHs have extensive surface areas and multitudes of horn interstices, which enable large numbers of guest molecules to be adsorbed. In addition, oxidization or acid treatment can create pores, or "nanowindows" (<2 nm), in the walls of SWNHs,[8] through which small molecules [e.g., CH_4, H_2, N_2, Ar, fullerene (C_{60}), etc.] can infiltrate their interior space.[9−11] Such treatment also can be used to introduce functional groups (e.g., carboxyl and quinine groups) at the edges of the pores, to which various chemical compounds (e.g., biotin and polyethylene glycol) can be coupled to functionalize the surface.[12,13] Importantly, the synthesis of SWNHs does not require a metal catalyst, so extremely pure preparations can be produced with no potential for toxicity from contaminating metals.[14] Taken together, these properties suggest SWNHs have the potential for use as novel carriers in drug delivery systems.

We previously demonstrated that oxidized SWNHs (oxSWNHs) adsorb dexamethasone, an anti-inflammatory agent, at 200 mg/g, and that active dexamethasone is then slowly released from the complexes in phosphate-buffered saline (PBS).[15] In addition, Ajima et al.[16] recently reported that cisplatin [*cis*-diamminedichloroplatinium(II)], an anticancer agent, can be incorporated into the interior space of oxSWNHs and then slowly released into PBS. Despite these promising results, however, the hydrophobicity of SWNHs remains a problem to be solved before clinical application of SWNHs can become practical. Free oxSWNHs are poorly dispersed in an aqueous environment and readily self-assemble into agglomerates of micrometer-order size. Particles more than 4 μm in diameter might cause vascular occlusion in the human body.[17] Thus, for biocompatibility, it is essential that oxSWNHs be dispersible in aqueous media.

There have been numerous efforts to endow carbon nanotubes, which are also very hydrophobic, with dispersibility. These efforts can be categorized into two approaches: covalent modification[12,13,18−21] and noncovalent

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modification.[22−26] Noncovalent functionalization of carbon nanotubes with various polymers has been extensively studied to improve the dispersibility of the molecules. Some of these methods use carcinogenic or indiscriminately cytolytic molecules such as pyrene[23,27,28] or SDS.[26] Here we report that noncovalent modification of oxSWNHs using an amide-linked polyethylene glycol−doxorubicin (PEG−DXR) conjugate yielded well-dispersed PEG−DXR−oxSWNH complexes having diameters of approximately 160 nm, a size that would be expected to produce an EPR effect.

Experimental Section

Materials. *Dahlia*-like SWNHs and their oxidized form, oxSWNHs, were prepared as described previously.[8,29] Briefly, as-grown SWNHs prepared by laser ablation were oxidized for 10 min in oxygen (760 Torr) at 580 °C and then heated at 400 °C for an additional 2 h under ultrahigh vacuum (∼1 × 10^{-7} Torr).

Dimethyl sulfoxide (DMSO) (hybridoma-tested grade) was purchased from Sigma (St. Louis, MO). *N*,*N*-Dimethylformamide (DMF) (infinity pure grade), acetonitrile (HPLC grade), sodium dihydrogen phosphate, and doxorubicin hydrochloride (DXR) were from Wako (Osaka, Japan). Triethylamine (HPLC grade) was from Pierce (Rockford, IL). Formic acid (HPLC grade) was from Nacalai Tesque (Kyoto, Japan). Phosphotungstic acid was from Kanto Chemical (Tokyo, Japan). Physiological saline (0.9% NaCl) was from Ohtsuka Pharmaceutical (Tokyo, Japan). Polyethylene glycol (PEG) derivatives [$CH_3O(CH_2CH_2O)_n$-CO-CH_2CH_2-COO-NHS, *N*-hydroxysuccinimide] were from NOF Corp. (SUNBRIGHT ME-050CS and -200CS, Tokyo, Japan). PD-10 columns were from Amersham Bioscience (Uppsala, Sweden). Amicon Ultra (MWCO of 100 000) was from Millipore (Cork, Ireland). The *In Situ* Cell Death Detection Kit, Fluorescein was from Roche Molecular Biochemicals (Mannheim, Germany). TSK-GEL ODS-100S (4.6 mm × 150 mm) was from TOSOH (Tokyo, Japan). Fetal bovine serum (FBS) was from JRH Bioscience (Lenexa, KS). RPMI1640, Dulbecco's PBS, and trypsin-EDTA (0.05% trypsin and 0.53 mM EDTA-4Na) were from Invitrogen (Carlsbad, CA). Penicillin and streptomycin were from Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan), and Meiji Seika Kaisha, Ltd. (Tokyo, Japan), respectively. Cell culture dishes were from IWAKI (Tokyo, Japan). Lab-Tek Chamber Slides were from Nalge Nunc International (Naperville, IL).

Preparation of PEG−DXR. Eighty milligrams of 5K PEG−succinimidylsuccinate (5PEG) (average molecular weight of 5000) and 14 mg of DXR (1.5 molar equiv) were dissolved in 1 mL of DMF and 9 mL of DMF, respectively, containing 3.2 μL of triethylamine (TEA). The DXR solution was then added dropwise to the PEG solution with stirring, after which the reaction mixture was stirred while being shielded from light at 4 °C overnight. The mixture was then dialyzed against water for 36 h using a Spectra/Por CE membrane comprised of a 3.5 kDa MWCO and refreshing the external solution every 12 h. The resultant mixture was freeze-dried, dissolved in a small amount of water, and applied to a water-equilibrated LH-20 gel filtration column for further purification (ø, 1.5 cm × 12 cm). The 5PEG−DXR was eluted with water, after which the eluate was freeze-dried. For preparation of 20K PEG−DXR (20PEG−DXR), 160 mg of 20K PEG−succinimidylsuccinate (20PEG) and 7 mg of DXR were used.

Physicochemical Characterization of PEG−DXR. PEG−DXR polymers were analyzed by UV spectrophotometry at OD_{495} using a UV-2550 spectrometer (Shimadzu, Kyoto, Japan) with a 10% DMSO/90% H_2O mixture as the solvent. The degree of DXR−PEG conjugation was estimated to be 82% for 5PEG−DXR and 86% for 20PEG−DXR.

Preparation of PEG−DXR−OxSWNH Complexes. OxSWNHs were suspended in DMSO or DMF (2 mg/mL), after which the solution was sonicated at 30 s intervals for 5 min. The oxSWNH solution and PEG−DXR solution in DMSO or DMF (8 mg/mL) were mixed in a microtube followed by shaking using a MicroIncubator M-36 (TAITEC, Saitama, Japan) under shielding from light at room temperature for 1 h. The reaction mixture was stirred and diluted by dropwise addition of H_2O to a concentration of 10% in DMSO or DMF followed by stirring while being shielded from light at 4 °C overnight. To separate the PEG−DXR−

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oxSWNH complexes from the unreacted PEG−DXR and the organic solvents, the reaction mixture was transferred to an Amicon Ultra ultrafiltration device (100 kDa MWCO), diluted to 10 mL with H_2O, and then concentrated to less than 1 mL by centrifugation (1500*g*) at 4 °C. These sequential dilution and concentration procedures were repeated four times, yielding concentrated PEG−DXR−oxSWNH complexes (1 mg/mL oxSWNHs).

Dynamic Light Scattering (DLS) Analysis. To estimate the size of their hydrated diameters, samples of PEG−DXR−oxSWNH complexes (0.2 mg/mL) in distilled water or physiological saline (0.9% NaCl) were subjected to DLS analysis at 25 °C using a Photal FPAR-1000 dynamic laser scattering spectrometer (Otsuka Electronics, Tokyo, Japan) equipped with a semiconductor laser ($\lambda_0 = 660$ nm).

Transmission Electron Microscopy (TEM). TEM images were acquired using a Topcon (EM-002B) transmission electron microscope operated at 120 kV. PEG−DXR−oxSWNH complexes were negatively stained using a 2% (w/v) solution of phosphotungstic acid (PTA, pH 7.2) on a collodion-carbon-coated grid support.

Cell Culture. NCI-H460 human non-small cell lung cancer cells[30] were purchased from the National Cancer Institute (Frederick, MD) and maintained in RPMI1640[31] supplemented with 5% FBS, 100 μg/mL penicillin, and 100 units/mL streptomycin in continuous culture at 37 °C under a humidified atmosphere of 5% CO_2 and 95% air. The cells were passaged every 3−4 days.

Apoptosis Assay. Apoptosis induced by PEG−DXR−oxSWNH complexes was detected by terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling (TUNEL) using an *In Situ* Cell Death Detection Kit, Fluorescein (Roche) according to the manufacturer's protocol. Briefly, 12 500 cells in 500 μL of medium were seeded into the wells of Lab-Tek Chamber Slides (Nalge Nunc International) and incubated for 4 h at 37 °C, after which the medium was substituted with fresh media containing PEG−DXR−oxSWNH complexes (0.2 mg/mL oxSWNHs). After being incubated for an additional 72 h at 37 °C, the cells were fixed with 4% paraformaldehyde in PBS (pH 7.4), permeabilized with 0.1% Triton X-100 in sodium citrate, and incubated with the TUNEL reaction mixture. The label incorporated at sites of DNA damage was visualized using a LEICA (Solms, Germany) DM IER2 fluorescence microscope equipped with a LEICA DC300 FX digital camera. The percentage of apoptotic cells, which was defined as the ratio of the number of TUNEL-positive cells to the total cell number, was determined using data from three different visual fields.

Results and Discussion

Preparation of PEG−DXR-Modified Oxidized Carbon Single-Wall Nanohorns (PEG−DXR−OxSWNHs). PEG is one of the most biocompatible amphipathic polymers and has been extensively used for modification of pharmaceutical proteins and preparation of drug-carrying polymeric micelles. The properties conferred by PEG include increased water solubility, thermal stability, and resistance to proteolysis, decreased antigenicity and immunogenicity, and slower clearance from plasma.[32−34] DXR is a commonly used anticancer agent that has two aromatic rings in its structure[35] and, therefore, would be expected to interact with the hydrophobic surfaces of oxSWNHs via $\pi-\pi$ and hydrophobic interactions. The presence of an amino group in DXR enables its conjugation with PEG,[36,37] yielding an amphipathic molecule that would be expected to interact with the outer surface of oxSWNHs via its hydrophobic moiety and to endow oxSWNHs with dispersibility under aqueous conditions via its hydrophilic moiety. Furthermore, because the interaction between the conjugate and oxSWNHs is noncovalent, the conjugate could be used to exert a pharmacological effect upon release from the surface of oxSWNHs.

Figure 2 shows a scheme for the synthesis of PEG−DXR−oxSWNHs. DXR was linked to one end of PEG via its amide linkage. Because DMSO and DMF are good dispersion media for oxSWNHs (unpublished data), oxSWNHs were initially suspended in DMSO or DMF and then dispersed well by sonication. PEG−DXR (4 equiv weight) was then added to the dispersed oxSWNHs, and the mixture was shaken for 1 h at room temperature to allow the PEG−DXR molecules to adsorb onto the surfaces of the oxSWNHs. Next, the mixture was slowly diluted with 9 volumes of H_2O. It should be noted that both DMSO and DMF have relatively high electric dipole moments and, therefore, can be homogeneously mixed with H_2O. We expected that when the amount of organic solvent was reduced, PEG−DXR−oxSWNH complexes would form; i.e., core oxSWNH particles would be surrounded with PEG−DXR molecules such that the DXR moieties faced the surfaces of the

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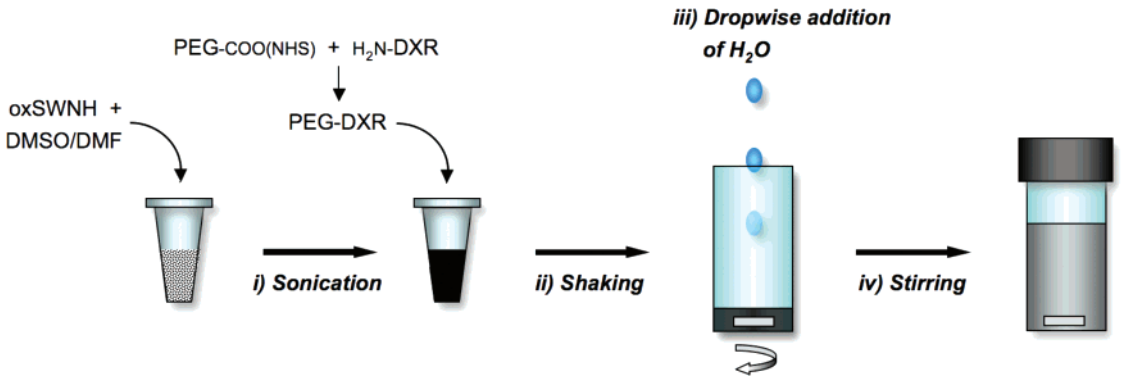
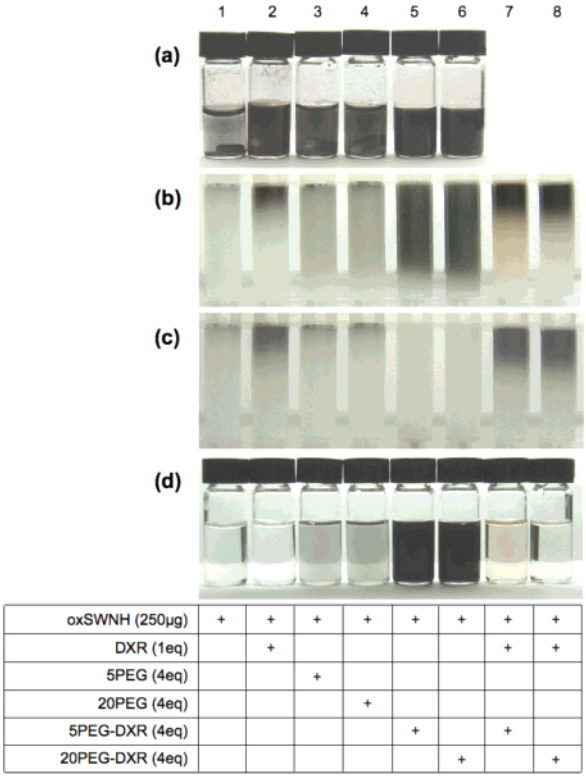


Figure 2. Schematic drawing depicting the synthesis of PEG−DXR−oxSWNHs. See the text for details.



oxSWNH (250μg)	+	+	+	+	+	+	+	+
DXR (1eq)		+					+	+
5PEG (4eq)			+					
20PEG (4eq)				+				
5PEG-DXR (4eq)					+		+	
20PEG-DXR (4eq)						+		+

Figure 3. Evaluation of the dispersibility of PEG−DXR-treated oxSWNHs using PD-10 gel filtration chromatography. (a) Photographs of vials that contained the oxSWNH suspension (0.1 mg/mL). Each suspension has been prepared as descried in Materials and Methods by using materials shown in the bottom table. (b) Photographs of gel filtration columns after loading with the oxSWNH suspensions. (c) Photographs of gel filtration columns after eluting with 3.5 mL of water. Note that only PEG−DXR-treated oxSWNHs (lanes 5 and 6) were eluted. (d) Photographs of vials containing the eluates.

oxSWNHs, and the PEG moieties protruded into the aqueous solution. Once the DMSO or DMF content was reduced to 10%, the mixture was stirred at 4 °C overnight to produce PEG−DXR−oxSWNHs. Figure 3a shows oxSWNH suspensions prepared according to our method under various conditions. Treating oxSWNHs with two kinds of PEG−DXR, in which the PEG moieties had average molecular weights of either 5000 (5PEG) or 20 000 (20PEG), provided well-dispersed oxSWNHs (lanes 5 and 6), whereas untreated oxSWNHs (lane 1) and oxSWNHs treated with either DXR (lane 2) or PEG (lanes 3 and 4) were precipitated. Increased dispersion of oxSWNHs has been also achieved using PEG-fluorescein (data not shown). Fluorescein has multiple aromatic rings like DXR does, suggesting that other molecules having similar molecular structures could be used in the conjugate. Further, pyrene has been used as an anchor molecule for noncovalent surface functionalization of carbon nanotubes,[23,27,28] expanding the repertoire of applicable molecules

Characterization of PEG−DXR−OxSWNHs. Water-dispersed nanoparticles with a diameter of 100−200 nm have been reported to pass through size exchange chromatography columns.[38] To evaluate the dispersibility of the PEG−DXR−oxSWNHs, we assessed the ability of the complexes to pass through a size exchange chromatography column. The column we used was a PD-10 gel filtration column composed of dextran-based beads ranging from 38 to 235 μm in diameter. Figure 3b shows a picture taken after the PD-10 columns were loaded with 2.5 mL of a suspension containing 250 μg of oxSWNHs treated with the indicated molecules. When oxSWNHs treated as described above but in the absence of PEG−DXR were loaded onto a column pre-equilibrated with H_2O, they stacked at the top of the column (lane 1), as did oxSWNHs treated with either DXR (lane 2) or PEG (lanes 3 and 4), indicating that they had formed agglomerates. By contrast, the PEG−DXR-treated oxSWNH suspension penetrated into the column (lanes 5 and 6 in Figure 3b) and was completely eluted with H_2O (lanes 5 and 6 in Figure 3c,d). A small amount of precipitate formed in the collected eluate after a few hours. Thereafter, however, the complexes remained dispersed in the solution, even when left for several days (data not shown). It is noteworthy that by the co-addition of DXR and PEG−DXR (lanes 7 and 8) the penetration and elution of oxSWNHs were inhibited, which implies that PEG−DXR interacts with oxSWNHs via its DXR moiety.

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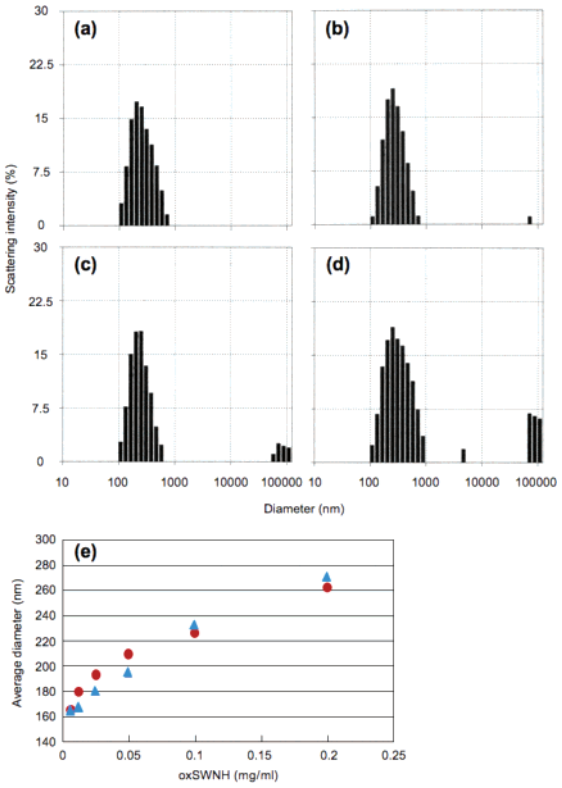

Figure 4. DLS analysis of PEG−DXR−oxSWNHs: (a) 5PEG−DXR−oxSWNHs in deionized water, (b) 20PEG−DXR−oxSWNHs in deionized water, (c) 5PEG−DXR−oxSWNHs in physiological saline (0.9% NaCl), and (d) 20PEG−DXR−oxSWNHs in physiological saline. (e) Concentration dependency of the apparent diameters of PEG−DXR−oxSWNHs in deionized water. Red circles represent data for 20PEG−DXR−oxSWNHs and cyan triangles data for 5PEG−DXR−oxSWNHs. All data were obtained at 25 °C.

We next estimated the amount of bound PEG−DXR within the complexes. After PD-10 separation, we further purified the complexes by passing them through an ultrafiltration membrane to remove residual unbound PEG−DXR and organic solvent (see Materials and Methods). The purified PEG−DXR−oxSWNHs were then boiled in 2% sodium dodecyl sulfate (SDS) for 5 min to liberate PEG−DXR from the complexes. After electrophoresis in a SDS−polyacrylamide gel, the PEG moieties were stained with I_2 and quantified by densitometry (Supporting Information).[39] From these analyses, we estimated that the complexes contained 260 mg of 5PEG−DXR or 280 mg of 20PEG−DXR per gram of oxSWNH, which corresponds to approximately 12 and 3 PEG−DXR molecules, respectively, adsorbed on each nanohorn sheath.

DLS analysis was then used to determine the average hydrated diameter of the PEG−DXR−oxSWNHs (Figure 4a−d). Data were initially obtained at a concentration of 0.2 mg of oxSWNHs/mL of deionized water (a and b) or physiological saline (c and d). The mean diameters indicated by the major peaks obtained for 5PEG−DXR−oxSWNHs and 20PEG−DXR−oxSWNHs were 250 and 255 nm in distilled water and 230 and 310 nm in physiological saline, respectively. In these experiments, we noticed that greater numbers of micrometer-sized particles were present in the physiological saline (Figure 4c,d), indicating that additional effort will be needed to improve the solubility of PEG−DXR−oxSWNHs in ionic solutions.

When apparent hydrated diameters are determined on the basis of DLS measurements, higher concentrations of sample can lead to overestimation due to suppression of diffusive motion.[40] We therefore measured the diameters of PEG−DXR−oxSWNHs in deionized water using various concentrations of sample. Figure 4e shows how the size of the apparent hydrated diameter varied as a function of concentration. As expected, estimated diameters declined as the concentration of PEG−DXR−oxSWNHs was reduced, and by extrapolating these data to zero concentration, we were able to estimate the diameters in the absence of particle interactions. We calculated that both the 5PEG− and 20PEG−DXR−oxSWNH complexes have hydrated diameters of approximately 160 nm. It is noteworthy that these diameters are within the range of 120−200 nm because, at that size, they would not be rapidly trapped in liver and spleen, enabling them to persist in the blood circulation for long periods.[6,7] Moreover, long-circulating nanoparticles of this size have been known to accumulate within solid tumors due to the EPR effect.

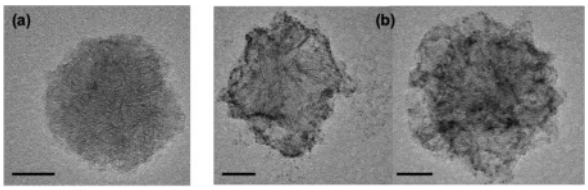

Figure 5. TEM images of oxSWNHs (a) and 5PEG−DXR−oxSWNHs (b) negatively stained on a collodion-carbon-coated grid support using a 2% (w/v) solution of PTA (pH 7.2). The bars are 20 nm.

TEM Imaging. For TEM analysis, purified PEG−DXR−oxSWNHs in H_2O were freeze-dried and then negatively stained with 2% PTA (pH 7.2), which facilitates electron optical imaging of synthetic polymers, like PEG.[41] As shown in Figure 5, the surfaces of 5PEG−DXR−oxSWNHs were stained with PTA, whereas untreated oxSWNHs were not. This further confirms that PEG−DXR occupied the outer surfaces of oxSWNHs.

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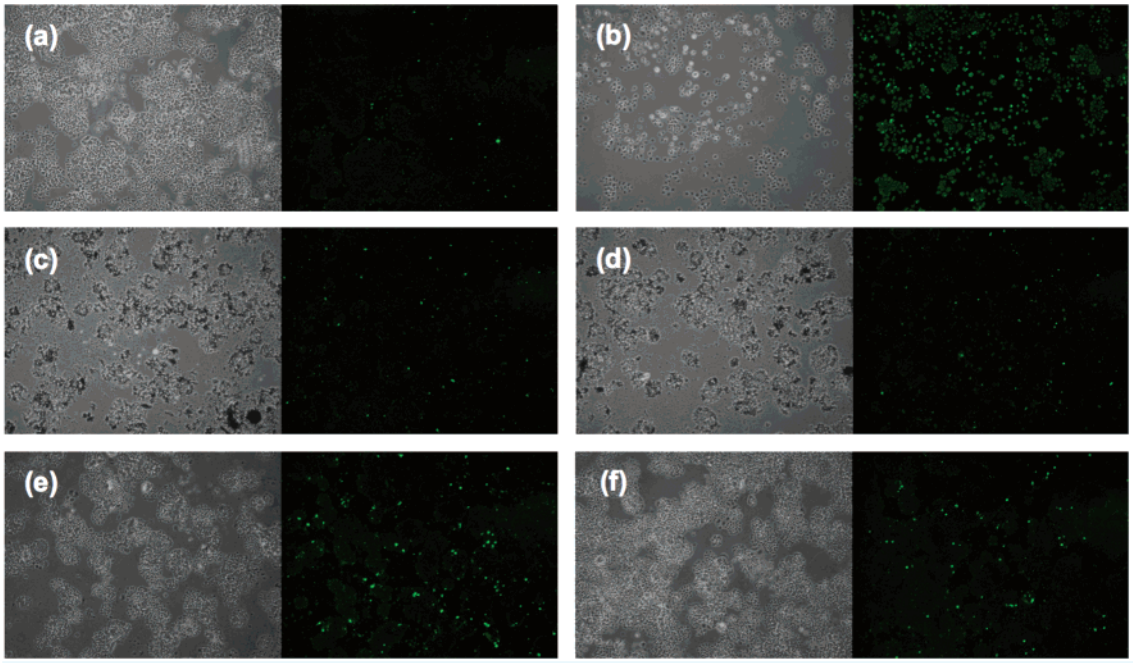

Figure 6. Detection of apoptotic cells by TUNEL staining in (left) phase-contrast micrographs and (right) FITC fluorescence images: (a) control, (b) DXR (10 ng/mL), (c) 5PEG−oxSWNHs, (d) 20PEG−oxSWNHs, (e) 5PEG−DXR−oxSWNHs, and (f) 20PEG−DXR−oxSWNHs. After incubation for 72 h under various conditions, the cells were fixed in 4% paraformaldehyde, stained, and observed under light (left) and fluorescence microscopes (right). In panels c−f, the cell culture medium contained 0.2 mg/mL oxSWNHs.

In Vitro Cytotoxicity of PEG−DXR−OxSWNHs. DXR is a clinically approved anticancer agent that induces apoptosis in a variety of cancer cells. Because the interaction between PEG−DXR and oxSWNHs is noncovalent, released PEG−DXR might be expected to exert an apoptotic effect. We therefore evaluated the cytotoxicity of PEG−DXR−oxSWNHs by subjecting NCI-H460 human non-small cell lung cancer cells to TUNEL staining. Figure 6 shows phase-contrast micrographs (left panels) as well as FITC fluorescence images (right panels) in which apoptotic cells are green in color (see Materials and Methods). We found that exposing NCI-H460 cells to 10 ng/mL DXR for 3 days induced apoptosis in almost all cells (Figure 6b), whereas few apoptotic cells were detected among the controls (Figure 6a). The addition of 5PEG− or 20PEG−DXR−oxSWNHs at amounts corresponding to 0.2 mg/mL oxSWNH also increased the incidence of apoptosis among NCI-H460 cells (Figure 6e,f). That an apoptotic effect was not seen upon addition of 5PEG− or 20PEG−oxSWNHs (Figure 6c,d) confirmed the DXR dependency of the cytotoxic effect of PEG−DXR−oxSWNHs.

We estimate that if all the DXR molecules were released from 0.2 mg/mL 5PEG−DXR−oxSWNH, it would correspond to 5.4 μg/mL DXR. Obviously, however, the incidence of apoptosis obtained with 0.2 mg/mL 5PEG−DXR−oxSWNHs was lower than that obtained with 10 ng/mL DXR. This is thought to be because most of the PEG−DXR was retained on the surfaces of oxSWNHs under our experimental conditions. In addition, the DXR moiety likely needs to be released by hydrolysis from the PEG−DXR conjugate before it can exert an apoptotic effect, and one also should consider the physical interaction between the released DXR and the surfaces of the oxSWNHs. Apparently, the cleavage at the ester linkage between PEG and succinate is relatively slow under our experimental conditions. Perhaps more fragile or environment-responsive linkers (e.g., acid-sensitive,[42] β-lactamase-sensitive,[43] or lysosomally degradable linkers[44]) would increase the bioactivity of PEG−DXR conjugates.

At present, we do not know whether PEG−DXR−oxSWNHs are taken up into cells. Given that water-soluble carbon nanotubes are reportedly taken up via endocytosis,[12] it is possible that PEG−DXR−oxSWNHs also are taken up into cells, where PEG−DXR is hydrolyzed and the free DXR induces apoptosis. Alternatively, PEG−DXR may be released into the extracellular milieu. We also cannot yet rule out the possibility that a trace amount of free DXR that was not eliminated from the preparation was present in the PEG−DXR conjugate and responsible for the observed apoptotic

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activity. We are now studying in detail the mechanism by which the PEG−DXR−oxSWNHs induce apoptosis.

In our assay system, a significant increase in the number of apoptotic cells was not observed in the treatment with DXR-free oxSWNHs at a concentration of 200 μg/mL (Figure 6c,d). On the contrary, some of the other drug carrier candidates, silica (10 μg/mL)[45] and carbon nanotubes[46] (150 μg/mL), reportedly induce apoptosis in a large fraction (>50%) of mammalian cells. Recently, there has been concern about the safety and biocompatibility of nanomaterials. Although there is room for further investigation in in vivo assay systems, our results lead us to hypothesize that oxSWNHs are a biocompatible drug carrier.

Conclusion

We have described a method for using an amphipathic polymer−drug complex (PEG−DXR) to endow hydrophobic carbon nanohorns with dispersibility in aqueous solution. PEGylation of oxSWNHs was achieved via physical adsorption of the DXR moiety of PEG−DXR onto oxSWNHs. On the basis of DLS analysis, the average diameter of PEG−DXR−oxSWNHs was estimated to be 160 nm, which is advantageous because, at that size, the complexes would not be rapidly cleared from the blood by the liver and spleen and would exert an EPR effect in tumors showing neovascularization.[6,7] TEM images of negatively stained PEG−DXR−oxSWNHs further confirmed coverage of surfaces of oxSWNHs by PEG−DXR, and the complexes exhibited DXR-dependent apoptotic activity against cancer cells. We believe that our method that uses the conjugate between the biocompatible PEG and drug should pave a new path for preparing carbon nanomaterial-based drug carriers for cancer chemotherapy.

Notably, the method described here made use of only the outer surfaces of oxSWNHs: the PEG−DXR−oxSWNH complexes carried an anticancer drug on their outer surfaces, but their interior space was not used. In that regard, Ajima et al.[16] showed that the interior space of oxSWNHs can serve as a carrier for the anticancer drug cisplatin. These successes imply that oxSWNHs have the potential to serve as double reservoirs capable of simultaneously carrying two different drugs, inside and outside of the molecule. Constructing such unparalleled multifunctional nanocarriers is one of the main goals of nanomedicine.

Abbreviations Used

C_{60}, fullerene; DLS, dynamic light scattering; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; DXR, doxorubicin; NHS, *N*-hydroxysuccinimide; EDTA, ethylenediaminetetraacetic acid; EPR, enhanced permeability and retention; FBS, fetal bovine serum; HPLC, high-performance liquid chromatography; MWCO, molecular weight cutoff; SWNH, single-wall carbon nanohorn; oxSWNH, oxidized SWNH; PBS, phosphate-buffered saline; PEG, polyethylene glycol; PTA, phosphotungstic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TEA, triethylamine; TEM, transmission electron microscopy; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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Supporting Information Available: SDS−PAGE analysis of PEG−DXR absorbed onto oxSWNHs. This material is available free of charge via the Internet at http://pubs.acs.org.

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