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# Prevention of Carbon Nanohorn Agglomeration Using a Conjugate Composed of Comb-Shaped Polyethylene Glycol and a Peptide Aptamer

**Sachiko Matsumura,[†,‡] Shigeo Sato,[§] Masako Yudasaka,[‡,||,⊥] Akihiro Tomida,[§] Takashi Tsuruo,[§,#] Sumio Iijima,[‡,▽] and Kiyotaka Shiba*[,†]**

*Department of Protein Engineering, Cancer Institute, Japanese Foundation for Cancer Research, 3-10-6 Ariake, Koto-ku, Tokyo 135-8550, Japan, SORST-JST, c/o NEC Corporation, 34 Miyukigaoka, Tsukuba, Ibaraki 305-8501, Japan, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 3-10-6 Ariake, Koto-ku, Tokyo 135-8550, Japan, NEC Corporation, 34 Miyukigaoka, Tsukuba, Ibaraki 305-8501, Japan, and Department of Material Science and Engineering, Meijo University, 1-501 Shiogamaguchi, Tenpaku-ku, Nagoya 468-8502, Japan*

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**Abstract:** Assured dispersibility is a prerequisite for clinical application of nanomaterials. Carbon nanomaterials have hydrophobic surfaces and thus readily agglomerate under aqueous conditions. Various conjugates composed of a carbon surface-binding moiety and polyethylene glycol (PEG) have been examined as dispersants for carbon nanomaterials. Here we synthesized a conjugate composed of a comb-shaped PEG (cPEG) and carbon nanomaterial-binding peptide (NHBP-1). The resultant cPEG−$NHBP_3$ conjugate displayed multiple units (2.4 on average) of NHBP-1 on a single cPEG molecule whose average molecular weight was 15−20 kDa. cPEG−$NHBP_3$ endowed single-walled carbon nanohorns (SWNHs) with good dispersibility *in vitro*, which could not be achieved with 20PEG−NHBP, a conjugate composed of linear 20 kDa PEG and a single NHBP-1 peptide. Notably, cPEG−$NHBP_1$, which was similar to 20PEG−NHBP but had a comb-shaped PEG backbone, functioned better as a dispersant than 20PEG−NHBP, suggesting a graft-type PEG formula is better-suited for dispersing nanomaterials. Finally, cPEG−$NHBP_3$ treatment substantially suppressed formation of SWNH agglomerates in mouse lung, suggesting the potential utility of SWNHs as a carrier in drug delivery systems.

**Keywords:** Biocompatibility; carbon nanotubes; nanoparticles; peptides; polyethylene glycol

## Introduction

Nanosized materials have been attracting attention in medicinal areas because of their potential to serve as drug carriers and imaging agents.[1−4] In addition to organic polymer-based nanoparticles, some of which have already been clinically approved as anticancer drug delivery carriers,[5,6] inorganic material-based nanoparticles, including superparamagnetic ion oxide crystals, quantum dots and carbon nanomaterials, among others, are now arousing interest.[7−9] Carbon nanotubes are one such inorganic material-based nanocarrier. Over the past few years, they have been shown to have a strong potential for use in drug delivery,[10,11] imaging[12−14] and photodynamic therapy[15,16] applications. Among the various types of carbon nanotubes, we have been focusing on single-walled carbon nanohorns (SWNHs),

* Corresponding author: Department of Protein Engineering, Cancer Institute, Japanese Foundation for Cancer Research, 3-10-6 Ariake, Koto-ku, Tokyo 135-8550, Japan. Tel: +81-3-3570-0489. Fax: +81-3-3570-0461. E-mail: kshiba@jfcr.or.jp.

[†] Cancer Institute, Japanese Foundation for Cancer Research.

[‡] SORST-JST.

[§] Cancer Chemotherapy Center, Japanese Foundation for Cancer Research.

[||] NEC Corporation.

[⊥] Present address: Nanotube Research Center, National Institute of Advanced Industrial Science and Technology (AIST), Central 5, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, Japan.

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[▽] Meijo University.

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which form stable spherical aggregates 50−100 nm in diameter.[17] A SWNH particle consists of approximately 2,000 graphitic tubes that are similar to single-walled carbon nanotubes, but the diameters of the tubes are somewhat larger (2−5 nm in diameter), and the ends of the tubes are closed like a horn.[17] SWNHs inherently contain no metal contamination because they are prepared by laser ablation of a pure graphite target without a metallic catalyst, which reduces the risk of possible toxicity. Indeed, *in vivo* toxicology testing of SWNHs using animals showed no indication of undesirable effects on the animals' health.[18] The ability of SWNHs to load and release drugs such as dexamethasone,[19] doxorubicin[20] and cisplatin[21,22] has also been shown. In those experiments, an oxidized form of SWNHs (oxSWNHs), which have nanosized (∼2 nm) openings on the surfaces, was used.[23] It has also been proposed that oxSWNHs could serve as carriers for magnetic resonance imaging (MRI)[24] and photodynamic therapy applications.[25]

With respect to the clinical use of SWNHs, it is very important to endow the particle with sufficient dispersibility under aqueous conditions; otherwise, undesired agglomeration within capillaries could result in their occlusion and serious organ damage. The method most commonly employed to disperse nanoparticles is modification with polyethylene glycol (PEG). PEG ornamentation of the surfaces of nanoparticles not only prevents agglomeration but also inhibits the binding of plasma proteins, which would retard clearance of the nanoparticles from blood.[26,27] Murakami et al. have shown that a PEG−doxorubicin conjugate can endow oxSWNHs with excellent dispersibility,[20] and that


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the doxorubicin molecule binds to the surface of SWNHs. But because this formulation limits the spectrum of drugs that can be used, we developed the new formulation in which a peptide aptamer against SWNHs (NHBP-1)[28] was used as the binder instead of doxorubicin.[22] This conjugate (20PEG−NHBP) endows oxSWNHs with good dispersibility, even in the presence of salt, which is known to enhance agglomeration of nanoparticles.[22] Moreover, we were able to load the anticancer agent cisplatin into the inner space of the oxSWNHs. As shown below, however, 20PEG−NHBP did not sufficiently disperse SWNHs if the conjugate/SWNHs ratio within the complex was reduced from 3:1 to 1:1. Therefore, before SWNHs can be used clinically, it will be necessary to establish a robust new method to endow them with good dispersibility.

Our recent studies of peptide aptamers have highlighted the importance of the valency of the displayed peptide on the conjugate. Multivalent display of peptide aptamers dramatically increases the affinity of the conjugate for the target,[29] and in one case, multivalency increased the adsorption of the conjugate.[30] We therefore decided to synthesize a new PEG−NHBP conjugate in which NHBP-1 was multivalently coupled to a comb-shaped PEG (cPEG). We then compared the dispersibility of the newly synthesized cPEG−NHBP conjugate with 20PEG−NHBP using both *in vitro* and *in vivo* dispersion assessment tests.

## Experimental Section

**Preparation of oxSWNHs and PEG−NHBP.** The oxidized forms of SWNHs (oxSWNHs) were prepared as described previously.[23] Linear 20 kDa PEG with *N*-hydroxy succinimide ester at one end (SUNBRIGHT ME-200CS) and comb-shaped PEG (copolymer of polyoxyethylene monoallyl monomethyl ether and maleic anhydride, SUNBRIGHT AM-1510K, $M_n$ = 18,200, $M_w$ = 33,700) were purchased from NOF Corp. (Tokyo, Japan). The peptide NHBP-1 was purchased from Anygen Co., Ltd. (Gwangju, Korea). 20PEG−NHBP was synthesized as described previously.[22] To synthesize cPEG−$NHBP_3$ and cPEG−$NHBP_1$, comb-shaped PEG and NHBP-1 were first mixed at molecular ratios of 1:3 (for cPEG−$NHBP_3$) and 1:1 (for cPEG−$NHBP_1$) in acetonitrile/DMF (2/1) containing a trace amount of triethylamine, and then stirred for 3 days at room temperature. The resultant PEG−NHBP conjugates were purified by gel filtration on a Sephadex G-25 (eluate: 10% acetic acid/water), evaporated several times to remove acetic acid, and lyophilized. Amounts of NHBP-1 attached to the conjugate were estimated both by amino acid analysis and optical absorption (derived from the tyrosine residues). Both methods gave comparable values: peptide:PEG ratios were 2.4:1, 0.7:1 and 0.7:1 for cPEG−$NHBP_3$, cPEG−$NHBP_1$, and 20PEG−NHBP, respectively.

**Preparation of Dispersed Solutions of oxSWNHs.** Dispersed solutions of oxSWNHs were prepared by first suspending oxSWNHs in 50% ethanol/water by sonication (Bioruptor UCD-200TM, Cosmo Bio, Tokyo, Japan; power = 130 W, time = 30 s × 5 times),[22] after which a solution of PEG−NHBP conjugate was added so that the final concentrations of the molecules in the mixture were 0.1 mg/mL oxSWNHs, 0.05−0.3 mg/mL PEG−NHBP and 2.5% ethanol. The mixture was agitated for 30−60 min using a vortex mixer to obtain dispersed oxSWNHs.

**Spectrophotometric Evaluation of the Dispersed States of the Modified oxSWNHs.** oxSWNHs/PEG−NHBP complexes prepared as described above were diluted in PBS (10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, 137 mM NaCl, and 2.7 mM KCl, pH7.4). Final concentrations of oxSWNHs, PEG−NHBP and ethanol were 0.03 mg/mL, 0.015−0.09 mg/mL and 0.8%, respectively. Absorbance at 800 nm was then recorded using a Shimadzu UV-2550 spectrophotometer (Kyoto, Japan).

**Dynamic Light Scattering (DLS) Measurements.** The size distribution of the PEG−NHBP/oxSWNHs complexes in PBS was analyzed by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments Ltd. Worcestershire, U.K.).

**Fluorescent Observation of oxSWNHs Modified with cPEG−$NHBP_3$.** Fluorescein-labeled cPEG−$NHBP_3$ (FAM-cPEG−$NHBP_3$) was synthesized by modifying the cPEG−peptide conjugation reaction described above. First, Boc-ethylenediamine (approximately 3 equivalents to cPEG) was added to the reaction mixture containing NHBP-1 and cPEG to introduce an ethylenediamine group. After removing the Boc group using trifluoroacetic acid, the polymer was reacted with carboxyfluorescein (FAM) *N*-hydroxy succinimide ester and purified by gel-filtration chromatography. Amino acid analysis revealed that the NHBP-1 content in FAM−cPEG−$NHBP_3$ was similar (2.3 units) to that in cPEG−$NHBP_3$. The FAM−cPEG−$NHBP_3$ was also able to disperse oxSWNHs in PBS (see Supporting Information), indicating that the FAM moiety did not hinder the ability of cPEG−$NHBP_3$ to act as a dispersant.

To observe the fluorescent signal from the FAM−cPEG−$NHBP_3$, 50 μL of the complex was mixed with 200 μL of culture medium (RPMI1640 containing 5% FBS) in a Laboratory-Tek chambered cover-glass (Nunc, Thermo Fisher Scientific), after which it was incubated for 12 h (the concentration of oxSWNHs under these conditions was 20 μg/mL). After removal of the culture medium, the cover-glass was rinsed with PBS, and the attached complex was observed using a confocal laser scanning fluorescence microscope (FV1000, Olympus Corp. Tokyo, Japan). The

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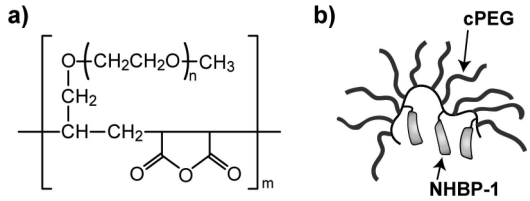


***Figure 1.*** (a) Chemical formula of cPEG. (b) Schematic structure of cPEG−$NHBP_3$.

excitation wavelength was 488 nm, and the detection range was 495−591 nm.

**Intravenous Injection of Dispersed oxSWNHs into Mice.** All animal care and experimentation was in compliance with the Guidelines for Animal Experiments at the Japanese Foundation for Cancer Research. Female 6-week-old BALB/c mice were purchased from Japan Charles River Co., Ltd. (Yokohama, Japan), and housed in our laboratory for 12 days (their weights were 20−22 g). For animal experiments, dispersed cPEG−$NHBP_3$/oxSWNHs or 20PEG−NHBP/oxSWNHs were prepared in 4% glucose solution using an oxSWNHs/conjugate ratio of 1:1. The mice were divided into three groups, and the two types of oxSWNHs/conjugate complexes or untreated oxSWNHs were injected via the tail vein at a dose of 3 mg/kg (~0.2 mL) ($n = 3$). The mice were then sacrificed 24 h later, and their organs were removed, fixed in 10% neutral buffered formalin, embedded in paraffin, thin sectioned, and observed using an optical microscope (DMLP microscope, Leica Microsystems, Wetzlar, Germany). For detection of oxSWNH agglomeration, we used unstained sections. For histological analysis, the samples were stained with hematoxylin and eosin.

## Results

**Preparation of cPEG−$NHBP_3$ and cPEG−$NHBP_1$.** NHBP-1 is a 12 amino acid peptide aptamer (DYFSSPYYEQLF) originally isolated as a specific SWNH binder using a peptide phage system.[28] We previously showed that the conjugate formed by NHBP-1 and 20 kDa PEG (20PEG−NHBP) was able to act as a good dispersant for oxSWNHs *in vitro*.[22] In that earlier study, 20PEG−NHBP/oxSWNHs complexes remained well dispersed in PBS or culture medium for up to 3 days. As shown below, however, 20PEG−NHBP lost its excellent ability to prevent agglomeration when a smaller amount of the molecule was used in the preparation of the dispersion solution for oxSWNHs.

cPEG is an alternative copolymer composed of PEG and maleic anhydride moieties (Figure 1a). Because the molecule can conjugate more than one peptide via its carboxyl groups, we expected that multivalent display of the NHBP-1 on each cPEG molecule would result in a stronger affinity of the conjugate for SWNHs (Figure 1b). In this study, we used AM-1510K, a cPEG containing ten 1.6 kDa PEG moieties, on average, resulting in an approximate $M_w$ of 15−20 kDa. We prepared two types of cPEG−NHBP conjugates: one displaying an average of 2.4 units of NHBP-1 per molecule (cPEG−$NHBP_3$) and another displaying 0.7 unit per molecule (cPEG−$NHBP_1$). These different conjugates were prepared by controlling the molar ratios of cPEG and NHBP-1 in the coupling reaction, as described in the Experimental Section. For a comparison, we also used the 20PEG−NHBP we synthesized in our earlier study, which displays an average of 0.7 unit of NHBP-1 on an unbranched 20 kDa PEG moiety.

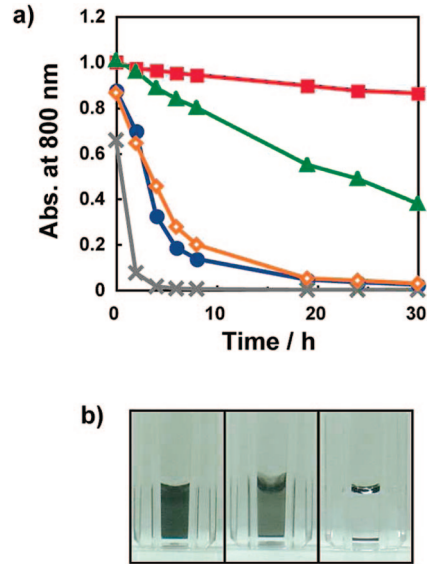


***Figure 2.*** Dispersibility in PBS of oxSWNHs treated with the dispersants. (a) Dispersibility was evaluated by measuring the absorbance at 800 nm at the indicated times. oxSWNHs were mixed at a ratio of 1:1 with cPEG−$NHBP_3$ (red squares), 20PEG−NHBP (blue circles), cPEG−$NHBP_1$ (green triangles), cPEG (orange diamonds), or nothing (crisscrosses). (b) Visual inspection of the oxSWNHs mixed with cPEG−$NHBP_3$ (left), 20PEG−NHBP (center) or nothing (right) after incubation for 24 h.

**Effects of Different PEG−NHBP Conjugates on Dispersion of oxSWNHs.** We first compared the abilities of cPEG−$NHBP_3$, cPEG−$NHBP_1$ and 20PEG−NHBP to mediate dispersion of oxSWNHs using an *in vitro* assessment method similar to the one we used in our earlier work.[22] In PBS, untreated oxSWNHs rapidly formed agglomerates and the absorbance at 800 nm, which corresponds to the scattering of the molecules within the sample, was lost within 5 h (Figure 2). By contrast, when the oxSWNHs were pretreated with a PEG−NHBP conjugate, agglomeration was inhibited to varying degrees, depending on the conjugate used. In particular, cPEG−$NHBP_3$ had a remarkable effect. The cPEG−$NHBP_3$/oxSWNHs complex remained dispersed for at least 1 day with only a slight reduction in absorbance (Figure 2). By contrast, 20PEG−NHBP showed only a modest ability to prevent agglomeration under the conditions used; indeed, its efficacy was little better than that of free cPEG without NHBP-1. Note that the conditions we used in this study were different from the ones used in our earlier study. In the present study, we employed a low PEG−NHBP:oxSWNHs ratio (1:1 by weight), which we found to highlight the difference in the ability of the conjugates to endow dispersibility. When we increased the conjugate:oxSWNHs

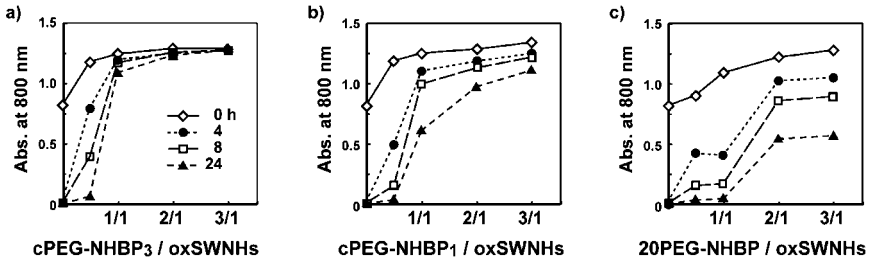


***Figure 3.*** Dispersion of oxSWNHs treated at the indicated weight ratios with cPEG−$NHBP_3$ (a), cPEG−$NHBP_1$ (b) or 20PEG−NHBP (c) after incubation for 0 (diamonds), 4 (circles), 8 (squares) or 24 (triangles) h.

ratios, the differences among the three conjugates gradually declined, and even 20PEG−NHBP could act as a reasonable dispersant, as we reported previously (Figure 3).

Interestingly, cPEG−$NHBP_1$ showed a greater ability to disperse oxSWNHs than 20PEG−NHBP, though they have similar molecular compositions; i.e., they both display an average of 0.7 unit of NHBP-1 on PEG having an approximate MW of 20 k. This may mean that the backbone cPEG moiety somehow contributes to the interaction between the conjugate and oxSWNHs, or that the graft-type structure of cPEG is somehow more suitable for endowing hydrophilicity. Although the backbone moiety is likely involved in the binding, the contribution of NHBP-1 was indispensable, which was evident when comparing the dispersant properties of cPEG−$NHBP_3$, cPEG−$NHBP_1$ and free cPEG. Overall, multivalency resulted in better dispersibility.

**DLS Analysis of the Sizes of Dispersed PEG−NHBP/oxSWNHs Complexes.** We next used dynamic light scattering (DLS) to determine the hydrodynamic diameters of the oxSWNHs dispersed using the three PEG−NHBP conjugates (Figure 4). When oxSWNHs were treated with cPEG−$NHBP_3$ or cPEG−$NHBP_1$ at a 1:1 ratio, the average diameter of the complexes was estimated to be approximately 210 nm (Figure 4a and Figure 4c). By contrast, when oxSWNHs were treated with 20PEG−NHBP at the same ratio, DLS analysis indicated the presence of populations of aggregates, in addition to a monodispersed population. The approximate average diameter was 1 $\mu$m (Figure 4b), reflecting the rapid agglomeration observed in Figure 2. A similar DLS profile was obtained with oxSWNHs treated with free cPEG (Figure 4d). When the 20PEG−NHBP:oxSWNHs ratio was increased to 3:1, the population with the 1 $\mu$m diameter was suppressed just after preparation. After incubation for 48 h, however, the size distribution had broadened, and populations having larger diameters were apparent (Figure 4f). By contrast, there were no changes in the distribution of diameters when oxSWNHs were treated with cPEG−$NHBP_3$ under the same conditions (Figure 4e). Thus, DLS analyses confirmed the superior ability of cPEG−$NHBP_3$ to disperse oxSWNHs, as compared to 20PEG−NHBP.

***In Vivo* Evaluation of the Dispersibility of oxSWNHs Treated with Various PEG−NHBP Conjugates.** To evaluate the dispersibility of conjugate/oxSWNHs complexes *in vivo*, oxSWNHs dispersed with cPEG−$NHBP_3$ or 20PEG−NHBP were injected into the tail veins of BALB/c mice at a dose of 3 mg/kg (approximately 0.06 mg per body).

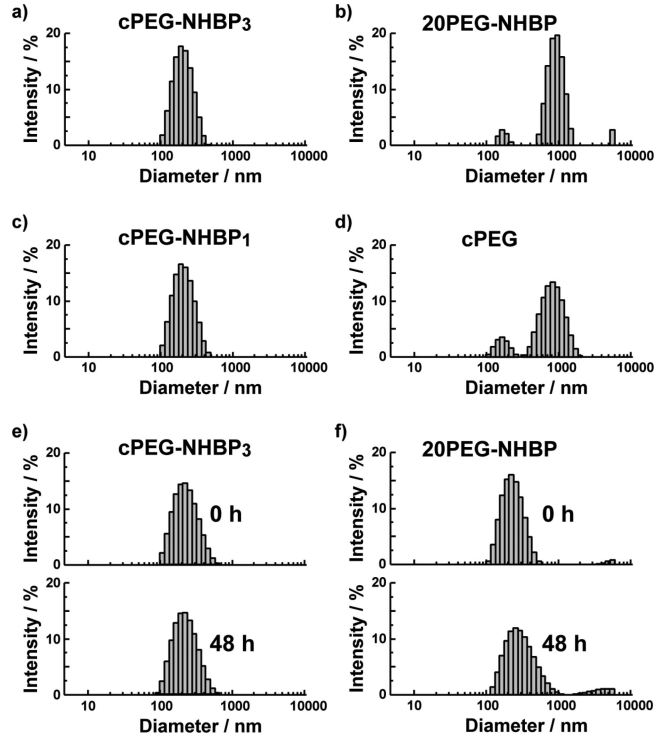


***Figure 4.*** DLS analysis of the size distributions of the dispersed oxSWNHs. (a−d) DLS measurements were performed immediately after the indicated dispersants were mixed with oxSWNHs at a ratio of 1:1. (e, f) Differences of size distributions for oxSWNHs treated with cPEG−$NHBP_3$ (e) or 20PEG−NHBP (f) after 0 and 48 h of incubation. The PEG−NHBP conjugate:oxSWNHs ratio was 3:1.

As a control, we also injected untreated oxSWNHs. The mice were then sacrificed 24 h later, after which their organs were retrieved, fixed and prepared for histological examination. In specimens from mice injected with untreated oxSWNHs, oxSWNH agglomerates were visible as black $\mu$m-sized particles predominantly in liver and spleen, and to a lesser degree in the lung (data not shown and Figure 5a and Figure 5b). Note that these depositions were not accompanied by inflammation detectable on pathological examination, and the mice showed no abnormal behavior (data not shown and Figure 5a). Under higher magnification ($\times$100, Figure 5c), we also observed many smaller agglomerates, whose diameter was less than

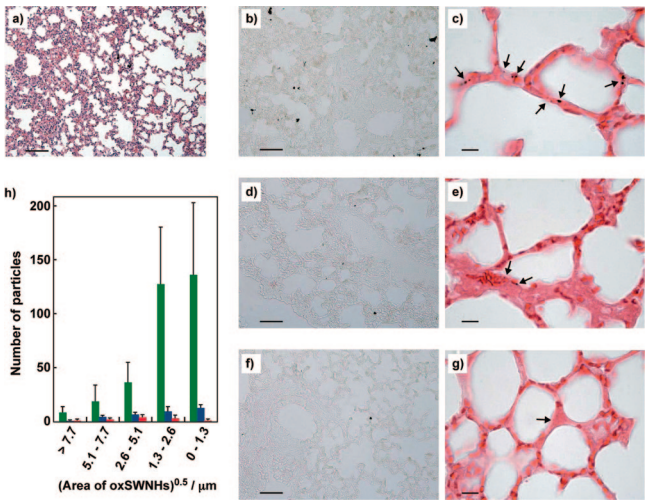


***Figure 5.*** Accumulation of agglomerated oxSWNHs in the lung after intravenous injection into mice. Shown are photomicrographs of lung sections from mice injected with unmodified oxSWNHs (a, b, c), 20PEG−NHBP/oxSWNHs (d, e) or cPEG−$NHBP_3$/oxSWNHs (f, g). The sections were stained with hematoxylin and eosin (a), unstained (b, d, f), or stained with eosin (c, e, g). Arrows indicate aggregated oxSWNHs observed as black dots in lung capillaries. In the unstained micrographs, the numbers of oxSWNHs agglomerates having the indicated sizes were counted using Image-J (h). The green, blue, and red bars represent the numbers of unmodified oxSWNHs, 20PEG−NHBP/oxSWNHs and cPEG−$NHBP_3$/oxSWNHs, respectively. Scale bars represent 50 $\mu$m (a, b, d, f) or 10 $\mu$m (c, e, g).

approximately 1 $\mu$m, in lung capillaries (Figure 5c). In contrast, the mice injected with 20PEG−NHBP/oxSWNHs or cPEG−$NHBP_3$/oxSWNHs formed few larger particles in lung (Figure 5d and Figure 5f, respectively). Similarly, the smaller aggregates were hardly observed from the capillaries of the mice (Figure 5e and Figure 5g). The semiquantitative analyses of various sized agglomerates in lung tissue were carried out using unstained images ($\times$20 magnification) (Figure 5h).

## Discussion

In this study, we synthesized a new PEG−NHBP conjugate (cPEG−$NHBP_3$) in which multiple units (2.4 on average) of oxSWNH-binding peptide (NHBP-1) were coupled to a comb-shaped PEG (cPEG). Comparison of cPEG−$NHBP_3$ to 20PEG−NHBP, in which a single oxSWNH-binding peptide (actually 0.7 on average) was conjugated to one linear PEG, revealed the superior ability of the multivalent molecule to disperse oxSWNHs. The ability of 20PEG−NHBP to act as a dispersant was dramatically diminished when the 20PEG−NHBP:oxSWNHs weight ratio was reduced to 1, whereas cPEG−$NHBP_3$ showed robust activity at ratios ranging from 1 to 3 (Figure 3), indicating that cPEG−$NHBP_3$ is relatively tolerant of concentration variation.

The fact that another graft-type PEG−NHBP, cPEG−$NHBP_1$, displayed only 0.7 unit of NHBP-1 on cPEG, but still showed better dispersant ability than 20PEG−NHBP, which also displays 0.7 unit of NHBP-1, suggests that the molecular structure of cPEG is better suited to endow nanoparticles with hydrophilicity than linear PEG.

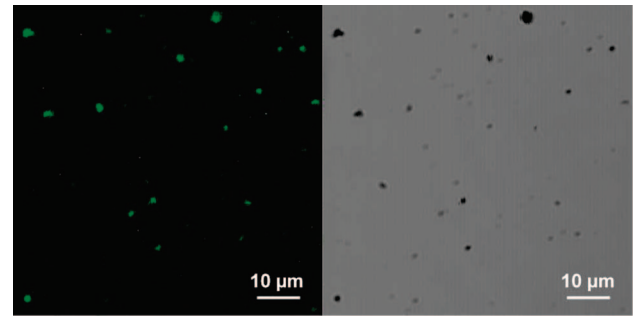


***Figure 6.*** Confocal laser scanning micrographs of oxSWNHs modified with FAM−cPEG−$NHBP_3$ (fluorescein-labeled cPEG−$NHBP_3$); shown are fluorescence (left) and differential interference contrast (right) images. No granular fluorescence was detected with oxSWNHs without modification or with FAM−cPEG−$NHBP_3$ alone (data not shown).

In addition, when oxSWNHs were treated with FAM-cPEG−$NHBP_3$ (see Experimental Section), the particles could be observed using fluorescence microscopy (Figure 6), which suggests that the new cPEG−NHBP conjugate could also serve as the basis for developing new imaging agents, or the free carboxyl groups of cPEG could serve as multifunctional carriers.

Injection of untreated oxSWNHs into the tail veins of mice led to formation of oxSWNH agglomerates in the lung and elsewhere. Although these mice showed no deleterious effects and no apparent occlusion was observed in lung capillaries, the formation of large agglomerates *in vivo* must be avoided for the development of new DDS system. Our observations indicated that lung capillaries contained only smaller particles of oxSWNHs (Figure 5c), suggesting that larger agglomerates were formed by phagocytosis by alveolar macrophages (Figure 5b). Because the unmodified oxSWNHs tended to form oligomers due to their poor dispersibility, they might be retarded in capillaries, which may stimulate phagocytosis by macrophages. Treating oxSWNHs with cPEG−$NHBP_3$ or 20PEG−NHBP substantially suppressed the oligomer formation (Figure 5e and Figure 5g) and the agglomerate formation (Figure 5d, Figure 5f and Figure 5h). It is noteworthy that 20PEG−NHBP, which was relatively ineffective *in vitro*, showed an ability to prevent agglomeration *in vivo* that was comparable to that of cPEG−$NHBP_3$. It may be that some plasma proteins also contribute to the dispersion state of SWNHs, as has been reported for carbon nanotubes.[13] That said, considering the variability of conditions within the living body and the versatility of its functionalization, we believe that the new cPEG−NHBP formulation is better suited for use as a dispersant than linear PEG−NHBP. We are now using labeled SWNHs to quantitatively assess the disposition of dispersed SWNHs.

## Conclusions

We have shown that cPEG−$NHBP_3$, a comb-shaped PEG, coupled with multiple units of carbon nanohorn binding peptide (NHBP-1), stably disperses the oxidized form of single-walled carbon nanohorns (oxSWNHs), both *in vitro* and *in vivo*. Assured dispersibility is a prerequisite for the clinical application of nanomaterials. In particular, agglomerate formation in pulmonary capillaries must be avoided. Our *in vivo* experiments showed that whereas untreated oxSWNHs form agglomerates in the lung, treating oxSWNHs with cPEG−$NHBP_3$ dramatically suppresses agglomerate formation.

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**Supporting Information Available:** Dispersibility in PBS of oxSWNHs treated with FAM-cPEG−$NHBP_3$. This material is available free of charge via the Internet at http://pubs.acs.org.

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