

Noncovalent Pyrene-Polyethylene Glycol Coatings of Carbon Nanotubes Achieve in Vitro Biocompatibility

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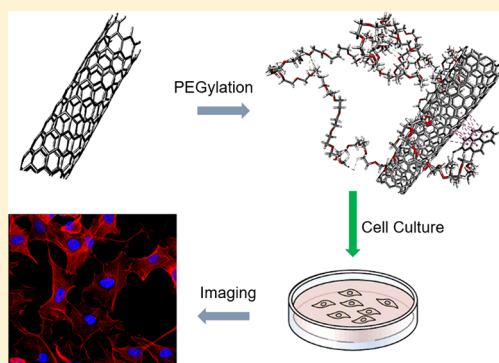
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Supporting Information

ABSTRACT: Single-walled carbon nanotubes (SWNTs) have become increasingly exploited in biological applications, such as imaging and drug delivery. The application of SWNTs in biological settings requires the surface chemistry to remain through the low solubility in aqueous media. In this research, a facile approach for the preparation of a polyethylene glycol (PEG)-coated SWNT-based nanocarrier was reported. We focused on the effect of PEG chain length and SWNT size on the cytotoxicity of PEG-coated SWNTs as a superior drug delivery nanovector. First, all-atom molecular dynamics (MD) simulations were employed to explore the stability and behavior of SWNT/pyrene-PEG (SWNT/Pyr-PEG) structures at a molecular level that is not attainable with experiments. The MD studies revealed that (i) π - π stacking interactions between the pyrene bearing PEG molecules and SWNTs are maintained in bulky situations, regardless of PEG molecular weight or SWNT size; (ii) pyrene molecules diffuse over the SWNT surface without detaching; and (iii) both short and long dynamic Pyr-PEG chains have the capability of effectively coating the SWNT surface. In light of the simulations, noncovalent (π - π stacking) assemblies of SWNT/Pyr-PEG with different molecular weights of PEG (M_w = 2000, 5000, and 12000) were successfully fabricated and characterized. For longer PEG chains, more effective coating of SWNTs was obtained, resulting in more biocompatible SWNT/Pyr-PEG nanomaterials. The number of SWNTs coated by Pyr-PEG was highly dependent on the length of pyrene bearing PEG polymers. Moreover, the short SWNTs showed a higher amount of PEG coating with respect to the long SWNTs. Cell viability results demonstrated a dose-dependent cytotoxicity of coated SWNTs. Short SWNTs coated with longer PEG chains have low cytotoxicity to be used in in vivo studies.



INTRODUCTION

Because of their intrinsic chemical, physical, and biological properties, single-walled carbon nanotubes (SWNTs) have been in the spotlight as one of the most promising nanomaterials for biomedical applications, such as cellular imaging,^{1,2} bone scaffolding,³ MRI contrast agents,⁴ tumor destruction agents,^{5,6} and intracellular protein transporters.⁷ Although pristine SWNTs have been reported to have the capability of transporting their cargo without metabolism in the circulatory system and directly entering into the intracellular medium,^{8–11} several studies revealed that carbon nanotubes have cytotoxic effects on cultured cells and adverse health outcomes on animals. Inhibition of cell proliferation,^{12,13} induction of oxidative stress,^{14,15} and apoptosis/necrosis¹⁶ are some of the negative impacts of carbon nanotubes. The toughest task in the employment of pristine SWNTs is their limited solubility in any biological environment due to their hydrophobic structure, which is prone to

form bundles. Covalent modification and noncovalent attachment of biocompatible macromolecules to SWNTs are two common approaches that trigger the required dispersion into the biological media. Compared to the covalent functionalization, the noncovalent attachment is preferred to retain the desired properties of SWNTs for cellular media, especially for NIR-II fluorescence imaging applications.⁹ Moreover, SWNTs wrapped by polymers, DNA, or proteins prevent the disruption of the π -network of pristine carbon nanotubes.¹⁷

Poly(ethylene glycol) (PEG) is an FDA-approved polymer and one of the most effective polymers for surface coating of SWNTs.¹⁸ Because of the hydrophilic, flexible nature and surface passivation of SWNTs, PEG chains provide the required resistance against immune system macrophages,

Received: March 25, 2018

Revised: September 15, 2018

Published: September 19, 2018

which results in PEG-modified SWNTs being recognized as biomaterials followed by prolonged blood circulation time and delayed clearance from the body.¹⁹ Several strategies have been developed to synthesize PEG-coated SWNTs.^{2,4,20} One of the versatile synthesis methods is the adsorption of aromatic moieties functionalized with PEG chains onto pristine SWNTs. For instance, pyrene conjugated PEG chains can strongly bind to the SWNTs surface via π - π interactions leaving the PEG chains extruding out from the sidewall.^{21,22} In addition, the SWNTs' size and the degree of PEGylation of SWNTs are clearly critical in affording the required biodistribution to the fabricated nanoplateforms for potential clinical applications. However, thus far there have been a few studies investigating these parameters from this aspect. PEG chains with at least 2 kDa molecular weight can provide sufficient biodistribution and protection against immune system macrophages.^{23,24} The Dai group explored the uptake of SWNTs noncovalently functionalized by PEGylated phospholipids in the reticuloendothelial system of mice.²⁵ They reported that PEG chains with branched structures (7 kDa) had the desired blood circulation behavior and biodistribution that are excellent for in vivo applications. Low solubility of SWNTs in the biological environment is another issue that is a strong size-dependent characteristic. As the size of SWNTs increases, because of strong van der Waals attractions between them, SWNTs tend to aggregate and form bundle structures, leading to highly cytotoxic activity against cell lines and animal species.^{26,27} In this study, an integrative approach using computational and experimental methods was applied to investigate the effect of PEG chain length (PEG₂₀₀₀, PEG₅₀₀₀, and PEG₁₂₀₀₀) and SWNT size on cell viability of human umbilical vein endothelial cells (HUVEC).

Effective coating of SWNTs is imperative for the integrity of the nanovectors in the bloodstream and is governed by inter- and intramolecular interactions. Therefore, we first used full-atom molecular dynamics (MD) simulations to observe the effect of PEG chain length, SWNT size, and PEG-to-SWNT ratio on the coating of SWNTs at the atomic level. For the past decade, molecular simulations have provided useful molecular level observations on the interactions between small molecules, polymer chains, and proteins and SWNTs to be used in different applications.^{28–32} Numerous computational studies confirmed that polymers with aromatic units make stable π - π interactions with SWNTs^{33,34} and similarly with graphene.³⁵ Conformational behaviors of interacting polymer chains were also investigated, and various simulations indicated that long polymeric chains wrap SWNTs with either stacking or van der Waals interactions.^{28,29,36,37} Besides other polymeric coatings, PEGylation of SWNTs has special importance in biomedical applications. In this line, simulations of covalently or noncovalently PEGylated SWNT systems give valuable insights on different conformations PEG chains can adopt, such as mushroom and brush conformations,^{34,38,39} and on their stability and applicability in biomedicine.^{39–42} A recent MD study on Pyr-PEG (pyrene-PEG) and SWNT systems³⁴ reported that the pyrene-SWNT stacking interactions are the main parameter in pyrene adsorption in various solvents. Although all these computational studies give useful insights into our system, they still lack clear answers for the synergetic effect of parameters on our design during the coating step, namely PEG chain size, SWNT size, and the stoichiometric ratio of Pyr-PEG to SWNT. Moreover, to mimic the experimental conditions, simulations were conducted in a

crowded environment that is effective in Pyr-PEG and SWNT and SWNT-SWNT interactions. Guided by the MD simulations, we designed and interpreted our experimental results and synthesized effective nanovectors for theranostic purposes.

■ COMPUTATIONAL AND EXPERIMENTAL METHODS

Molecular Dynamics Simulations. Three different SWNTs were modeled to reveal the effect of SWNT size on the interactions between Pyr-PEG chains and SWNT. We used the *Build* module of Materials Studio to generate a long SWNT having (9,13) chirality with a diameter of 14.0 Å and a length of 80.0 Å (Table 1). Another

Table 1. Diameter, Length, and Other Characteristics of the Simulated SWNT Systems

model	SWNT		Pyr-PEG chains	
	diameter (Å) × length (Å)	amount	molecular weight	amount
M ₁	7.0 × 40.0	1	2000	2
M ₂	14.0 × 80.0	1	2000	2
M ₃	7.0 × 40.0	1	5000	2
M ₄	7.0 × 40.0	1	2000	20
M ₅	7.0 × 120.0	1	2000	20
M ₆	7.0 × 40.0	1	5000	10

long SWNT with (6,5) chirality having a diameter of 7.0 Å and length of 120.0 Å was also constructed. The shorter SWNT had (6,5) chirality with 7.0 Å diameter and 40.0 Å length. Model SWNT dimensions agree with the synthesized nanotubes. Using the same module, we built PEG chains of molecular weight 2000 (PEG₂₀₀₀) and 5000 (PEG₅₀₀₀); we then linked these with the pyrene molecules. Generated structures were subjected to energy minimization satisfying an energy threshold of 0.001 kcal/mol using a combination of steepest descent, Newton-Raphson, and quasi-Newton methods.

Two different PEG chain lengths, 2000 and 5000, and three different Pyr-PEG-to-nanotube ratios, 2:1, 10:1, and 20:1, were studied. Using the *Amorphous Cell* module for systems M₁, M₂, and M₃ (Table 1), two Pyr-PEG complex and one SWNT were randomly packed together to the simulation box of an aqueous medium, fixing the density to 1.2 g/cm³. In models M₄ and M₅, 20 Pyr-PEG₂₀₀₀ chains and either a short SWNT (M₄) or a long SWNT (M₅) were randomly packed inside the simulation box. Model M₆ contained 10 Pyr-PEG₅₀₀₀ chains and one SWNT. Water was selected as the solvent in order to mimic both the polar solvent in the coating experiments and the hydrophilic property of body fluids. Simulation boxes were large enough to accommodate the SWNT, at the same time allowing the interaction of molecules from symmetric boxes providing the crowded medium of experimental conditions. Long-range interactions with their images from the neighboring symmetric boxes were attentively controlled.

After the energy minimization of the simulation cell using the same conditions as explained previously, MD simulations were performed with the *Forcite* module using the COMPASS force field for molecular interactions, which is suitable for polymer composites.^{43–45} Periodic boundary conditions were employed for the NVT ensemble at 300 K, which were maintained by applying the Berendsen thermostat with a decay constant of 0.1 ps.⁴⁶ Electrostatic interactions were computed using the Ewald summation technique.^{47,48} For the long-range van der Waals interactions, the cutoff distance was taken as 12.5 Å. All molecules were allowed to freely diffuse in the simulation box. Each system was run for 1 ns, which was sufficient to monitor the rapid coating process of SWNTs with Pyr-PEG chains. Each simulation was replicated at least two times leading to a total 2 ns of simulation time. Thanks to the energy minimization prior to MD simulations, systems were equilibrated within ~30 ps, as total energy and temperature profiles indicated (not shown).

From the MD simulations, we selected different configurations of Pyr-PEG chains adsorbed on SWNT and performed quench MD simulations. Quench MD simulations in Materials Studio is a classical MD simulation run between geometric optimizations, called *quench*, applied with a desired frequency. By this approach, an effective sampling of configurational space of an adsorbed ligand, i.e. Pyr-PEG, over a surface, i.e. SWNT, can be performed while significantly reducing simulation times. This analysis was used to reveal the effect of PEG chain length on the binding strength of pyrenes on SWNTs. Quench simulations were performed with the *Forcite* module and carried out at NVE ensemble for 500 ps, which was found adequate for the Pyr-PEG on SWNT system. The temperature was set as 350 K, which is a trade-off value providing enough kinetic energy for a trapped pyrene molecule to move on the surface as well as to prevent its desorption from the nanotube. For the sampling, quenching frequency was set to 2 ps. Geometric optimizations were performed with the same settings as explained before. After obtaining minimum energy conformations, we calculated binding energies E_{BE} (kcal/mol) between the Pyr-PEG and SWNT molecules by

$$E_{BE} = E_{\text{complex}} - (E_{\text{SWNT}} + E_{\text{Pyr-PEG}}) \quad (1)$$

Here, E_{complex} is the total energy of the Pyr-PEG chain adsorbed on SWNT, E_{SWNT} the total energy of the SWNT without PEG, and $E_{\text{Pyr-PEG}}$ the total energy of the polymeric chain without the nanotube.

■ EXPERIMENTAL SECTION

Materials. PEG monomethyl ether with 2000, 5000, and 12000 g/mol molecular weight were obtained from Sigma-Aldrich and were used as received. Dichloromethane (DCM) was purchased from Aldrich and distilled over P_2O_5 before usage. Tetrahydrofuran (THF; 99.8%, J.T. Baker) was dried and distilled over benzophenone–Na. Other solvents were purified using standard procedures. Succinic anhydride, dimethylaminopyridine (DMAP), trimethylamine (TEA), dimethyl sulfoxide (DMSO), N,N' -dicyclohexylcarbodiimide (DCC), and 1-pyrenemethanol (98%) were all purchased from Aldrich and used as received without further purification. Human umbilical vein endothelial cells were obtained from American Type Culture Collection (Manassas, VA). Nitric acid (78%) and phosphate-buffered saline powder were purchased from Sigma-Aldrich.

SWNTs Synthesis. SWNTs were produced by the fluidized-bed chemical vapor deposition^{49,50} (CVD) of acetylene (C_2H_2) on a magnesium oxide (MgO) powder impregnated with an iron nitrate ($Fe(NO_3)_3 \cdot 9H_2O$) solution. The CVD apparatus consisted of a vertical furnace and a quartz glass tube with a diameter of 3 cm in the middle of a quartz filter. A magnesium oxide ($100 \text{ m}^2 \cdot \text{g}^{-1}$) supported iron oxide powder produced by impregnation in an iron nitrate-ethanol solution was used as a precursor powder. To obtain a precursor with a MgO-to-Fe weight ratio of 5%, MgO that was suspended in ethanol and iron nitrate that was dissolved in 100 mL of ethanol were stirred together and sonicated for 20 min in order to homogenize the mixture. Afterward, the precursor was dried and ground into a fine powder.

For one deposition, typically 0.6 g of precursor powder was filled in the quartz tube, and the atmosphere was purged with argon for 5 min. Then, the furnace was heated to the synthesis temperature (800 °C). By heating the precursor powder, iron oxide clusters were formed because of the thermal decomposition of the iron nitrate at 125 °C. The synthesis was started with the introduction of acetylene mixed with argon for 30 min. After the synthesis, the SWNT material was purified using 6 M HNO_3 . To obtain short SWNTs, highly concentrated HNO_3 treatment was used for 7 h at a temperature of 120 °C. Then, the nanotubes were filtered, thoroughly washed with distilled water until pH reached ~ 7 , and dried in air.

Preparation of PEG–COOH. Succinic anhydride (1.46 g, 14.6 mmol) and DMAP (209 mg, 1.71 mmol) were completely dissolved in 15 mL of anhydrous THF and kept at 0 °C for 30 min. PEG₂₀₀₀ (4.28 g, 4.28 mmol) and trimethylamine (TEA) (1.8 mL, 12.8 mmol) were mixed in 15 mL of THF and transferred slowly into the succinic anhydride solution using a syringe. The solution was stirred at 0 °C

for 2 h, and the reaction continued at room temperature for 24 h under the nitrogen atmosphere. Then, PEG₂₀₀₀COOH was precipitated in cold diethyl ether. The precipitates were dissolved in dichloromethane again and then precipitated in cold diethyl ether. The precipitates were dried under vacuum at room temperature overnight. The same procedure was applied to synthesize PEG₅₀₀₀COOH and PEG₁₂₀₀₀COOH as reported in the literature.^{51,52}

Preparation of Pyrene Bearing Polymers (Pyr-PEG). A relatively straightforward method was used to synthesize Pyr-PEG.^{22,53,54} Initially, 1-pyrenylmethanol (1.0 g, 4.3 mmol), PEG₂₀₀₀COOH (3.5 g, 1.1 mmol), and 25 mL of dry THF were added to a round-bottomed flask. Then, to the reaction mixture were added DCC (1.33 g, 9.47 mmol) and DMAP (0.21 g, 1.71 mmol) in 15 mL of dry THF dropwise over a period of 30 min at 0 °C under nitrogen; the mixture was then stirred at room temperature overnight. At the end of the reaction time, the reaction solution was concentrated and the product was dissolved in diethyl ether, washed with the dilute $NaHCO_3$ aqueous solution two times, and dried over anhydrous Na_2SO_4 .

Functionalization of SWNTs with Pyr-PEG. First, 50 mg SWNTs, 0.25 g Pyr-PEG₂₀₀₀ (polymer to nanotubes ratio: 5, w/w), and 150 mL of dry THF were added to a round-bottomed flask. The obtained mixture was sonicated for 30 min in a low-power laboratory ultrasonic bath and stirred for an additional 72 h at room temperature. The mixture was filtered through a filter (Sartorius, PTFE; pore size, 0.2 μm) to remove nonattached Pyr-PEGs. Then, the remaining solid on the filter was sonicated well using an excessive amount of fresh THF and filtered again. This process was repeated until a free filtrate was acquired. The obtained black powder was then dried under vacuum at 35 °C for 24 h. The same procedure was applied to obtain SWNT_Pyr-PEG₅₀₀₀ and SWNT_Pyr-PEG₁₂₀₀₀.

Characterization of Materials. Thermal gravimetric analysis (TGA) was performed on Perkin–Elmer Diamond TA/TGA in the temperature range of 30–800 °C, with a heating rate of 10 °C/min under nitrogen.

UV–vis spectra were recorded on an Agilent Cary 100 spectrophotometer in CH_2Cl_2 .

High-resolution transmission electron microscopy (HR-TEM) images were collected using a JEOL 2100 instrument operating at 200 kV. HR-TEM samples were prepared by drop coating a dilute solution of the sample (in THF) on a holey carbon-coated copper grid.

The ^1H spectra were recorded in $CDCl_3$ with $Si(CH_3)_4$ as internal standard on an Agilent VNMRS 500 instrument (500 MHz for ^1H).

All FTIR spectra for methoxy PEG, PEG–COOH, and Pyr-PEG were collected by PerkinElmer (Waltham, MA) by using an ATR system.

Raman spectra were recorded using a Raman Microscopy System (U.K.) with excitation at 514 nm and 10 $\times$ objective, 10% laser power.

Cell Culture and Cell Viability Assays. Human umbilical vein endothelial cells were cultured in Dulbecco's Modified Eagle Medium F-12 (DMEM/F12, Gibco, UK) supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin at 37 °C in 5% CO_2 . Cells were seeded into a 6-well plate (10^6 cells/well) and were incubated for 48 h prior to treatment with increasing concentrations (5, 10, 50, and 100 $\mu\text{g/mL}$) of Pyr-PEG coated SWNTs. The cytotoxic effect of Pyr-PEG coated SWNTs was tested with two different assays: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, United States) and water-soluble tetrazolium salt (WST-1, Sigma, United States). After incubation of HUVECs with Pyr-PEG coated SWNTs for 48 h, MTT (final concentration 0.1 mg/mL) was added, and the mixture was incubated for 4 h at 37 °C. Following the decantation of supernatants, DMSO was added, and the mixture was incubated at room temperature in the dark for 30 min. Absorbance was measured at 570 nm with a plate reader (Biotek, United States). WST-1 cell proliferation/viability assay was conducted according to the method reported in the literature.⁵⁵

Laser Scanning Confocal Microscopy (LSCM). HUVECs were seeded on round coverslip with 10^6 cells in each well. The day after,

cells were incubated with Pyr-PEG coated SWNTs for 6 h. Cells were washed with PBS and incubated for 30 min for increasing the membrane permeability with 0.1% Triton X-100-PBS. This was followed by 3 washes with PBS, which was repeated at the end of each step. Paraformaldehyde-PBS (2%) was used for the fixation for 1 h at 4 °C, and then Phalloidin-TRITC (1:1000, Sigma) was used to determine the intracellular actin distribution.⁵⁶ For labeling the nuclei, 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) was implemented, and the preparations were imaged with a confocal laser scanning microscope (Leica, TCS-SPE) equipped with lasers providing 532 and 405 nm laser lines by Leica Application Suit Advanced Fluorescence 3.3 (LAS AF) software.

Statistics. All experimental studies were performed at least three times, and each concentration was assayed in triplicate to achieve independent experiments for the viability assays. Results were expressed as mean $\pm$ standard deviation. Statistical analysis was carried out using one-way analysis of variance (ANOVA) and Student's *t* test by GraphPad Prism 6.01. Differences were considered significant from $p \leq 0.05$ and $p \leq 0.01$.

RESULTS AND DISCUSSION

Computational Results for PEG-Functionalized SWNTs. *Interactions between Pyr-PEG Chains and SWNTs.* Molecular interactions between the pyrene molecules and nanotubes are clearly important for an effective coating and later for maintaining the biocompatibility of SWNTs for therapeutic purposes. Therefore, we focused on the interactions between pyrene molecules and SWNTs and conformations of PEG chains to understand the effect of the size of nanotubes and PEG chains on the coating in a crowded environment.

Mimicking the coating experiments, Pyr-PEG chains and SWNTs were randomly packed into the simulation box. As a result, for all cases, they were either distant or close to the SWNT at the beginning of the simulations. For each simulation, we monitored the distances between the center of mass of pyrene molecules and the SWNT rotation axis. Figure 1 displays trajectories for some pyrene molecules adsorbed on SWNTs in each simulation. When the aromatic Pyr-PEG chains reached the SWNT, they were immediately adsorbed via stable π - π stacking interactions and remained on the SWNT wall. This observation agrees with previous experimental and computational studies on the Pyr-PEG and SWNT, multiwalled nanotubes, and graphene interactions.^{34,35,57} The stability of pyrene-nanotube interactions seemed to be affected neither by the size of SWNT nor molecular weight of PEG chains. Entanglements of PEG chains, even in a more crowded environment as in M_4 , M_5 , and M_6 , were not effective in detaching strongly adsorbed pyrene molecules. The reason behind this observation is the high pyrene-SWNT binding energy, $\sim$ -16.7 kcal/mol, previously estimated by a density functional theory approach in the gas phase.⁵⁸

Interestingly, pyrene molecules adopted different orientations on all SWNTs during their π - π interactions without leaving the nanotube surface. Figure 1 displays orientations of PEG attached pyrene molecules in a cartoon representation. The pyrene molecule built optimal π - π interactions with the SWNT wall in a parallel orientation (denoted as *ll*) at a distance of 3.5 Å, agreeing with the literature.⁵⁹ In this conformation, the minimum distance between the pyrene molecule and axis of SWNTs with diameter of 7.0 Å fluctuated around 7.0 Å (Figure 1a). This distance became approximately 10.5 Å for SWNTs of diameter 14.0 Å (Figure 1b). In addition, we observed a tilted, perpendicular orientation (denoted as *l*)

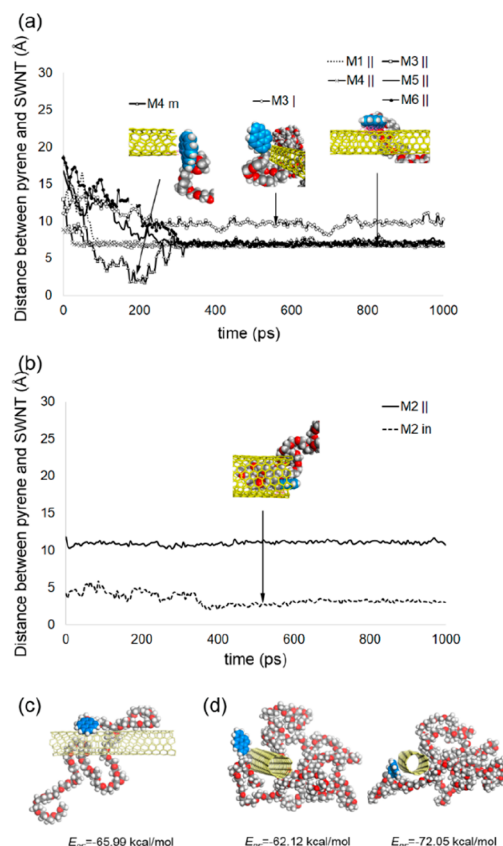


Figure 1. Distances between adsorbed pyrene molecules (blue) and SWNT (yellow) rotation axis. (a) Pyrene orientations are perpendicular (*l*), in the mouth (*m*), and parallel (*ll*). (b) Pyrene molecule is inside the SWNT (M_2). Binding energies between SWNT and different conformations of (c) Pyr-PEG₂₀₀₀ and (d) Pyr-PEG₅₀₀₀ chains.

of the pyrene molecule on the wall, where the minimum distance fluctuated around an average of 9.7 Å. A previous force-probe MD analysis⁵⁹ also detected this tilted orientation on a graphene surface and labeled it as an intermediate state of binding. Even though optimal interactions were lost in this orientation, pyrene molecules still remained adsorbed until the unbound state at a distance of around 8.0 Å above the graphene surface. According to our observations, the tilted position of pyrene on the nanotube seemed to be far from detachment.

Another interesting positioning of pyrene was at the mouth of SWNT (denoted as *m*). In M_1 and M_4 , pyrene molecules attached to long dynamic PEG chains kept a close distance to nanotubes at around 3.5 Å without desorbing. Interestingly, in M_1 , the pyrene molecule adsorbed to the mouth of SWNT and repositioned itself to make an optimal interaction with the nanotube. When the diameter of SWNT was large enough to accommodate a pyrene molecule, as in M_2 , pyrene diffused into the nanotube, pulling the long PEG chain (Figure 1b denoted as *in*).

From MD simulations, we randomly selected one Pyr-PEG₂₀₀₀ and one Pyr-PEG₅₀₀₀ chain adsorbed on the SWNT. Pyrene molecules were in parallel or tilted orientations on the nanotube surface. We performed configurational sampling using quench MD to investigate the effect of PEG chain length on binding energy between the pyrene and the nanotube. Results are displayed in panels c and d of Figure 1 for PEG₂₀₀₀

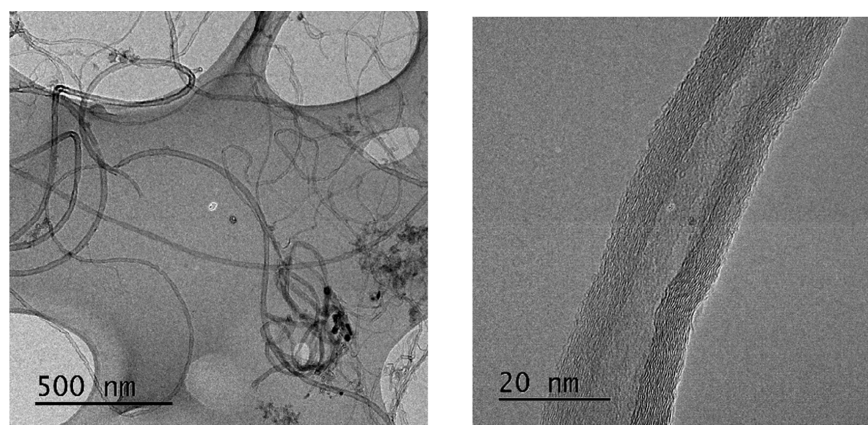


Figure 2. TEM images of SWNTs synthesized at 800 °C

and PEG₅₀₀₀, respectively. Energy calculations were performed using molecular mechanics; therefore, they do not represent absolute values. Nonetheless, they enabled the comparison between longer and shorter PEG chains. The starting conformation of Pyr-PEG₅₀₀₀ was in a tilted position with a binding energy of -62.17 kcal/mol. Then, pyrene reoriented itself in a more stable parallel position. One of the lowest-energy conformations is shown in Figure 1d with a binding energy of -72.05 kcal/mol. The shorter Pyr-PEG₂₀₀₀ chain adopted a more extended conformation in water while keeping its contact with SWNT through the pyrene molecule and few monomers on the chain. In these low-energy conformations, the binding energies were calculated to be in the range of -65.99 to -61.13 kcal/mol. The relatively small binding energy difference between the longer and shorter chains stems from the conformations of the polymers. These findings indicated that the size of the PEG chains and SWNTs have no influence on the interaction stability of pyrene molecules with the nanotubes. However, longer PEG₅₀₀₀ provides more efficient coating than PEG₂₀₀₀, which is important to achieve biocompatibility of the nanocarrier for longer periods of time.

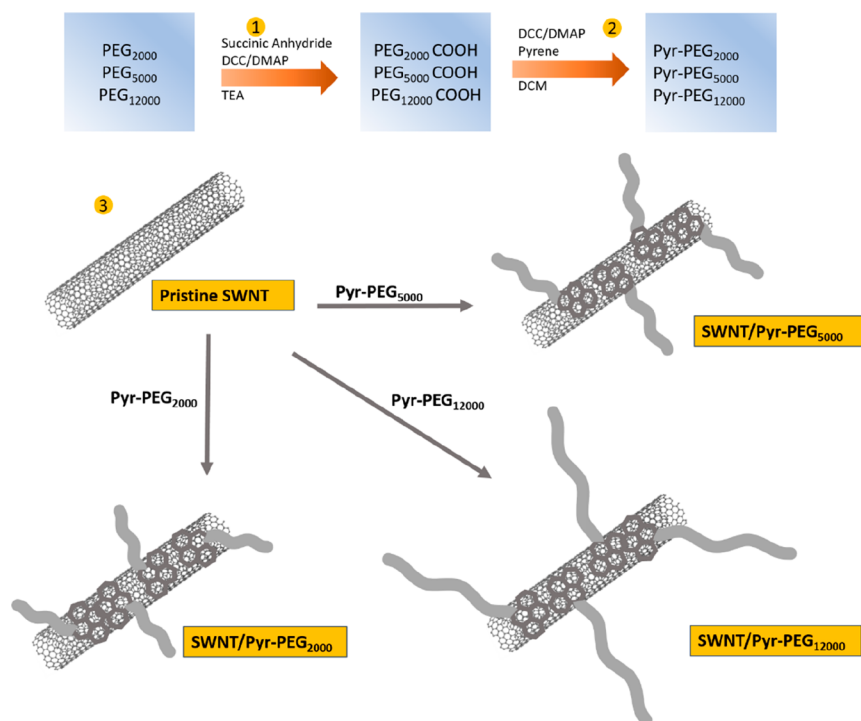
Dynamics of Pyr-PEG Chains. Previous MD studies indicated that long PEG chains can coat nanotubes or proteins by undergoing different conformations in water.^{34,39,60} For covalently attached PEG chains, with increasing grafting density on SWNTs, PEG conformations were shown to switch from mushroom to brush-like, which is important in preventing protein adsorption.³⁸ Even though we focused on the polymer length and SWCNT size, we also analyzed conformations of noncovalently attached PEG chains on the SWNTs during the fast coating process in a crowded environment. MD simulations showed that PEG chains were translocated on the SWNTs because of pyrene molecules that can laterally diffuse on the nanotube surface. Moreover, hydrophilic PEGs were interacting with water molecules as well as with other neighboring PEG chains leading to a highly dynamic behavior of the macromolecule. This behavior also provided a mixture of PEG conformations on the SWNT surface from wrapping to coiled chains, as shown in Figure S1. These conformational changes of the PEG chains on the nanotube in a crowded environment also reflect the dynamics of the nanovector in body fluids. As the molecular weight of PEG increased, the PEG layer on the SWNT thickened, improving the biocompatibility of the nanotube. This variety of conformations constantly changing on the SWNT can offer an important advantage for therapeutic purposes, besides

preventing macrophage or protein association by acting as an invisibility cloak. The nanovector can be designed to contain a reactive site to recognize a specific region, such as a receptor on a tumor cell. This reactive site can be either annealed on the PEG chain or on the SWNT. Because of the highly dynamic behavior of PEG chains, the reactive site will not stay hidden under PEG chains because of steric crowding and be quickly revealed to anchor the target that will promote fast accumulation of targeted nanovectors. Key points in designing a PEGylated nanovector discussed in this section are also reviewed in ref 61.

Interactions of SWNTs. For models M_1 , M_2 , and M_3 , the stoichiometric ratio of pyrene molecules to SWNT were set as 2:1. In some cases, PEG₂₀₀₀ and PEG₅₀₀₀ chains noncovalently coating a SWNT entangled with other SWNTs by short-range interactions in the neighboring periodic boxes. This situation set an obstacle to obtain single entities of PEG-coated SWNTs and led to bundles of SWNTs linked by PEG chains. Moreover, SWNTs can interact through stable π - π interactions with the symmetric nanotubes because of periodic boundary conditions, leading to partial aggregation of nanotubes (Figure S2a,b). This observation was more pronounced for SWNTs with larger diameter as in M_2 , where diffusion and adsorption of pyrene molecules to the surface were prevented (Figure S2a). When the ratio was increased to higher values, i.e. to 10:1 as in M_6 and 20:1 as in M_4 - M_5 , we detected that long PEG chains separated SWNTs by the steric blockage in this crowded environment still allowing pyrene adsorption (Figure S2c). These findings show that a high mole percent of PEG chains with a lower mole percent of SWNTs should be used in the synthesis. Here, the polymer-to-SWNT ratio of 10:1 was found to be adequate for experimental conditions.

Briefly, MD simulation studies provided the following information: (1) Adsorption of pyrene molecules with SWNT is strong enough to carry pyrene bearing PEG polymers on the surface of SWNTs. (2) As the length of the PEG chain increases, sufficient wrapping of SWNT increases by undergoing different conformations. (3) A high ratio of Pyr-PEG to SWNT should be used to prevent nanotube bundles during synthesis. In the light of obtained results from MD simulations, we decided to devise a biocompatible, optimized PEG-coated SWNT nanocarrier by utilizing SWNTs with different sizes and PEG polymers with 2000, 5000, and 12000 molecular weights to investigate the effect of length and size of

Scheme 1. Schematic Illustration of the Reactions Involved in the Preparation of the PEGylated SWNT



SWNT and PEG chains on the cell viability of the HUVEC cell line.

Experimental Results. SWNT Characterization. TEM images of the fabricated SWNTs are shown in Figure 2. TEM is significantly the highest-resolution technique that enables the capture of fine detail of carbon nanotube samples. It is evident that the structures synthesized by chemical vapor deposition method are carbon nanotubes.

Raman spectroscopy is a powerful technique for the characterization of the structure of carbon nanotubes. Figure S3 shows Raman spectra for a synthesized carbon nanotube deposit excited by 532 nm laser. Two different spectra of carbon nanotubes were observed at the G band (around 1580 cm^{-1}) and D band (around 1350 cm^{-1}). It can be seen that the intensity of the G band is considerably higher than that of the D band. The intensity ratio of D to G band (I_D/I_G) expresses the quality of SWNTs. The higher ratio indicates a higher amorphous carbon content and defect formation. The intensity ratio of G to D band of the synthesized SWNT sample was found to be 0.69. This observation led to a conclusion: SWNTs had low amorphous carbon content and defects. As seen from Figure S3, the spectrum in the RBM band, which is a characteristic of SWNTs, was observed for the sample. The reason for this spectrum is that the tube diameter was below 2 nm, and this result is consistent with other studies found in the literature.⁶² If nanotube diameter is greater than 2 nm, the RBM spectrum becomes difficult to be observed.

The RBM band can be used to calculate the mean diameter of SWNTs by eq 2

$$\omega \text{ (cm}^{-1}\text{)} = A/d \text{ (nm)} + B \text{ (cm}^{-1}\text{)} \quad (2)$$

where A and B are constants ($A = 223 \text{ cm}^{-1}\cdot\text{nm}^{-1}$ and $B = 10 \text{ cm}^{-1}$) and d is the diameter of the SWNT. The calculated mean diameter of the synthesized SWNT samples was found to be approximately 1.4 nm.

Synthesis and Characterization of PEG-Coated SWNTs.

The synthetic routes used for the preparation of Pyr-PEG functionalized short SWNTs (sSWNTs) and long SWNTs (lSWNTs) are illustrated in Scheme 1. PEG monomethyl ethers with molecular weights of 2000, 5000, and 12000 designated as PEG₂₀₀₀, PEG₅₀₀₀, and PEG₁₂₀₀₀, respectively, were selected to synthesize Pyr-PEGs. As shown in Scheme 1, the hydroxyl end group of the PEG₂₀₀₀, PEG₅₀₀₀, and PEG₁₂₀₀₀ chains were first transformed into the carboxylic acid group via reaction of PEG with succinic anhydride. Reactions for all PEG-COOH were carried out under mild conditions at room temperature through the catalysis of TEA and DMAP.

FTIR and ¹HNMR analysis were used to investigate the conversion of the hydroxyl group of methoxy PEG to PEG-COOH. Figure 3 shows the characteristic FTIR spectra of PEG₅₀₀₀ and PEG₅₀₀₀COOH. According to FTIR analysis, methoxy PEG exhibited its characteristic peak at 2882 cm^{-1} because of $-\text{OCH}_2\text{CH}_2$ units of the PEG backbone.⁶³ The

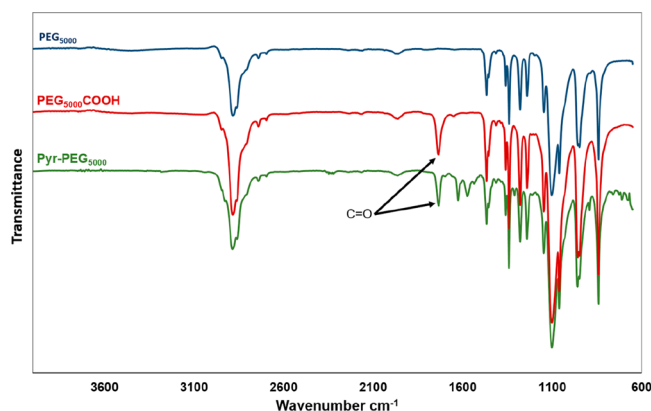


Figure 3. FTIR characterization of PEG₅₀₀₀ and PEG₅₀₀₀ COOH.

characteristic peaks for carboxyl group of the PEG₅₀₀₀COOH appeared at 1734 cm⁻¹, confirming the transformation of hydroxyl to the carboxyl groups. Figure 4a shows the ¹HNMR characteristic peaks of CH₂ group (δ = 2.5 ppm) of succinic anhydride, which proved the formation of PEG₅₀₀₀COOH.

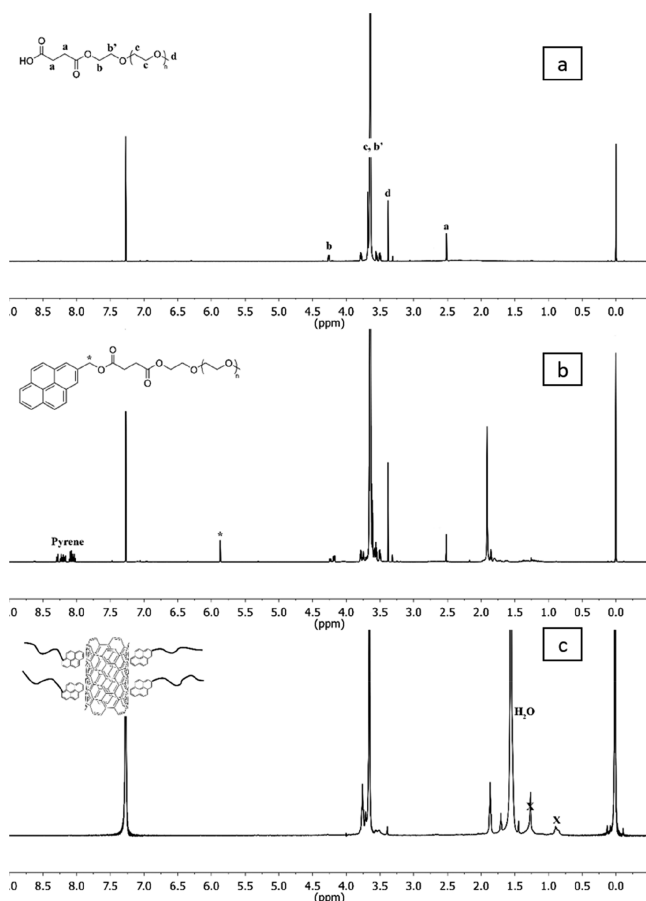


Figure 4. ¹HNMR characterization of (a) PEG₅₀₀₀COOH, (b) Pyr-PEG₅₀₀₀, and (c) Pyr-PEG functionalized SWNTs.

The formation of Pyr-PEG was confirmed by the FTIR and ¹HNMR measurements. As shown in Figure 4, the FTIR characteristic peaks of aromatic double bond for Pyr-PEG were obtained at 1574 and 1625 cm⁻¹.^{64,65} The formation of Pyr-PEG via esterification reaction was also corroborated by ¹HNMR. In Figure 4b, all expected signals attributed to the pyrene (δ = 8–8.2 ppm, ArH) and PEG (δ = 3.62 ppm, OCH₂CH₂O) segments can be seen for the synthesized Pyr-PEG₅₀₀₀. While Pyr-PEG was introduced to SWNTs, the ¹HNMR characteristic peaks of pyrene (δ = 8–8.2 ppm, ArH) disappeared because of the formation of π – π stacking interactions between the pyrene bearing PEG molecules and SWNTs (Figure 4c).

In the final step, the synthesized Pyr-PEG was noncovalently (π – π stacking) linked to SWNTs. The anchor molecule pyrene can attach irreversibly onto the graphitic surface of SWNTs because of van der Waals interactions.⁶⁶ This binding characteristic between pyrene molecule and SWNTs was used by Dekker and co-workers⁶⁷ to study the effect of attached enzymes on the electrical conductance of SWNT. The noncovalent functionalization of SWNTs based on a pyrene moiety was also studied by Chen et al.²¹ The group used 1-

pyrenebutanoic acid with anchored succinimidyl ester to form amide bonds for protein immobilization. They observed that the pyrenyl group irreversibly bound onto the sidewalls of SWNTs in an organic solvent. A similar modification strategy involves the attachment of pyrene-modified miktoarm star polymer to the sidewalls of the nanotubes, which was reported by Durmaz et al.⁶⁸ In this case, the group introduced the amphiphilic miktoarm star polymer, polystyrene-poly(ethylene glycol)-poly(methyl methacrylate), bearing a pyrene group at the end of polystyrene arm (pyrene-PS-PEG-PMMA) onto the multiwalled carbon nanotubes by noncovalent binding. In light of this approach as well as the MD simulations results, the synthesized Pyr-PEGs were noncovalently linked to SWNTs.

Fluorescence spectra of Pyr-PEG₅₀₀₀ and sSWNT_Pyr-PEG₅₀₀₀ at 375 nm excitation wavelength are shown in Figure 5. The Pyr-PEG₅₀₀₀ displayed the characteristic fluorescence

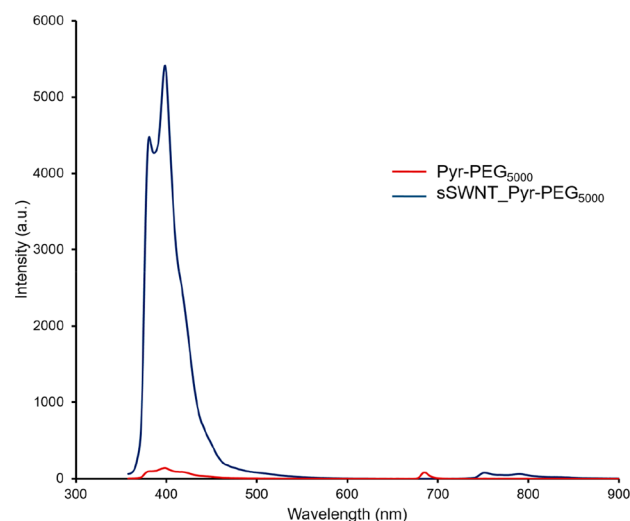


Figure 5. Fluorescence emission spectra of Pyr-PEG₅₀₀₀ and sSWNT_Pyr-PEG₅₀₀₀.

peak of pyrene unit; however, after the incorporation of Pyr-PEG₅₀₀₀ to SWNTs, this characteristic peak was completely quenched. This result is consistent with the literature²¹ and verifies that the pyrene side chain directly interacted with the nanotube surface as expected. Accordingly, both fluorescence spectroscopy and ¹HNMR measurements proved successful formation of PEGylated SWNT.

To gain a quantitative insight into the relative amount of Pyr-PEG incorporated with the SWNTs, TGA was carried out as described in the Experimental Section. According to TGA results, while short and long SWNTs were thermally stable up to 800 °C, Pyr-PEG coated SWNTs experienced significant weight losses. The thermal degradation of SWNT coated with Pyr-PEG is a multistage process (Figure S4). At temperatures up to 150 °C, a slight weight loss was detected regarding the evaporation of the adsorbed water. The degradation between 250 and 450 °C was attributed to the decomposition of PEG chains. Additional weight loss observed from 500 to 600 °C can be ascribed to the decomposition of pyrene attached to the PEG chains.

Molecular simulations suggested that the length of PEG chains did not affect the binding energy of pyrene to SWNT. On the other hand, PEG chain length may play a significant role in the amount of Pyr-PEG attachment on the SWNTs. At this point, solvents with different polarities such as THF and

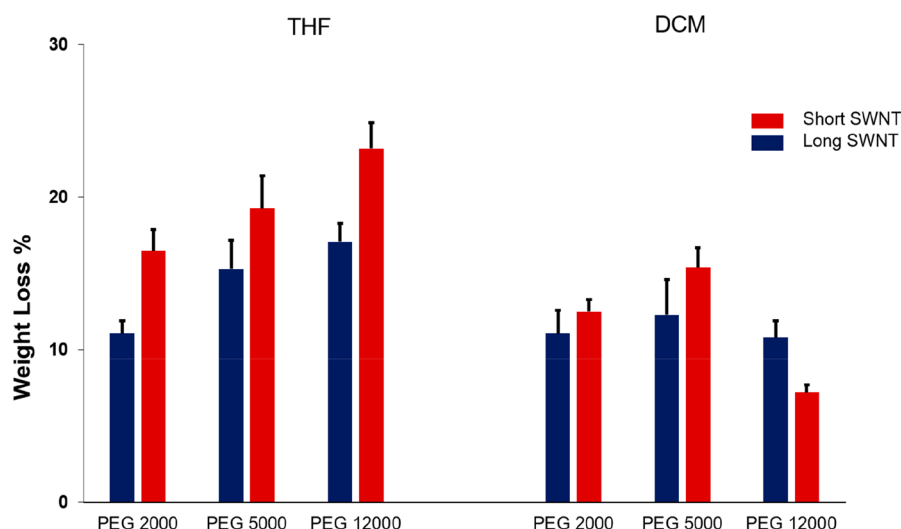


Figure 6. Thermogravimetric results of short SWNTs and long SWNTs coated with Pyr-PEG₂₀₀₀, Pyr-PEG₅₀₀₀, and Pyr-PEG₁₂₀₀₀ in THF and DCM solvents.

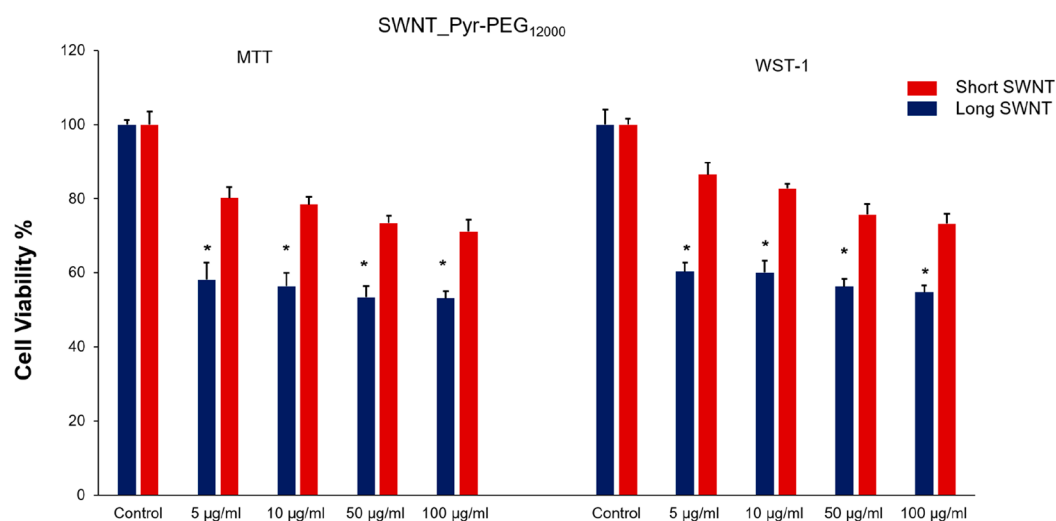


Figure 7. Cell viabilities of short and long SWNTs coated with Pyr-PEG₁₂₀₀₀. The asterisk (*) denotes a statistically significant difference for Student's *t* test between lSWNTs and sSWNTs coated with Pyr-PEG₁₂₀₀₀ treatment groups ($p \leq 0.05$).

DCM can provide an insight into this phenomenon. As it can be seen from Figure 6, the amount of linkage of Pyr-PEG to SWNTs (both sSWNTs and lSWNTs) in DCM exhibited completely different trends compared to the THF results. As the molecular weight of PEG increased for the samples that were prepared in THF solvent, the amount of weight loss increased. However, in the case of DCM, this amount experienced an increase up to Pyr-PEG₅₀₀₀ samples, with a subsequent sharp decrease observed for Pyr-PEG₁₂₀₀₀ samples. This can be attributed to the lower polarity index of DCM with respect to THF, which hinders the movement and therefore the diffusivity of Pyr-PEG chains in the solvent. This effect of the solvent was more pronounced once Pyr-PEG₁₂₀₀₀ was used for the coating. Therefore, to achieve a successful coating of SWNTs, we used THF as solvent for further studies. TGA analysis also revealed that the amount of noncovalent linkage for the Pyr-PEG coated SWNT samples with sSWNT was higher than that of lSWNT. This can be attributed to the higher number of attachments of Pyr-PEG to sSWNTs because of its lower length with respect to lSWNTs. The number of nanotubes per unit volume for sSWNTs in the solvent was

higher than that of lSWNTs; this may increase the number of successful collisions between Pyr-PEG chains and sSWNTs. Indeed, the noncovalent linkage quantity for Pyr-PEG₁₂₀₀₀ was higher than that for the Pyr-PEG₂₀₀₀ and Pyr-PEG₅₀₀₀ samples obtained with both lSWNTs and sSWNTs.

In Vitro Cell Viability and Imaging. MTT and WST-1 assays were used to evaluate the effect of PEGylated SWNT on HUVEC viability. HUVEC cell lines were utilized and treated with the short and long SWNTs coated with Pyr-PEG₂₀₀₀, Pyr-PEG₅₀₀₀, and Pyr-PEG₁₂₀₀₀ at concentrations of 5, 10, 50, and 100 µg/mL. The MTT viability results for pristine SWNTs showed that the fabricated carbon nanotubes were cytotoxic at the applied concentrations (Figure S5). After 12 h of incubation, lSWNTs coated with Pyr-PEG₂₀₀₀ exhibited more cytotoxic effect on the cell viability than sSWNTs coated with Pyr-PEG₂₀₀₀ in all concentrations (Figure S6). Similarly, after an exposure time of 12 h, lSWNTs coated with Pyr-PEG₅₀₀₀ and Pyr-PEG₁₂₀₀₀ were observed with higher cytotoxic behavior relative to their sSWNT counterparts (Figures S7 and 7). Analysis of MTT data with Student's *t* test revealed that the cell viability was decreased significantly after treatment

with PEGylated ISWNT compared to the sSWNT samples. MTT assay demonstrated that sSWNTs had lower cell proliferation inhibition behavior when compared to ISWNTs.

Furthermore, WST-1 in vitro cell viability assay was performed to verify the reproducibility of the results. Likewise, ISWNTs coated with different Pyr-PEGs reflected the same inhibitory trend of cell viability compared to their sSWNT counterparts. (Figures S6, S7, and 7). The enhanced cell growth inhibition with decreasing PEG chain length shows that the molecular weight of PEG polymer plays a critical role in camouflaging the SWNT side walls. Briefly, WST-1 confirmed the viability pattern for PEGylated SWNT cytotoxicity obtained from the MTT assays. These results indicated that the sSWNTs coated with longer PEG chains have excellent safety and biocompatibility over a wide range of concentrations among the nanocarriers fabricated in this study.

The actin cytoskeleton plays a major role in the physiology of endothelial cells, including motility, adhesion, and permeability.⁶⁹ To visualize the effect of PEGylated carbon nanotubes on the actin cytoskeleton, actin filaments were fluorescently labeled with Phalloidin-TRITC in HUVECs (Figure 8).

HUVECs cultured in control conditions displayed both cortical (peripheral) and stress fibers. In this study, PEGylated SWNT-treated and control HUVECs showed differences and similarities in the filament distribution information. Actin reorganization and cell shape changes were observed in cells treated with the short and long SWNTs at all concentrations, especially for the ISWNTs coated with Pyr-PEG₂₀₀₀ (Figure 8c). Interestingly, the actin filaments were more comparable to the control cells once treated with both short and long SWNT_Pyr-PEG₁₂₀₀₀ (Figure 8f,g). The breakdown of actin filament occurred for the cells treated with the short and long SWNT_Pyr-PEG₅₀₀₀ (Figure 8d,e). The impaired membrane integrity decreased as the molecular weight of PEG decreased for both short and long SWNTs.

In recent years, SWNT-based nanoparticles have offered promising perspectives in the field of regenerative medicine, including in situ cell engineering, building in vitro disease models, and in vitro instruction of therapeutic cells such as artificial extracellular matrixes.⁷⁰ The functionality of endothelial cells is essential for the vascular system.⁷¹ SWNTs can easily enter the bloodstream; consequently, endothelial cells would be the primary cells exposed to these nanoparticles.⁷² Currently, a complete understanding of how SWNTs interact with endothelial cells at the molecular level remains unclear. The cytoskeleton plays a fundamental role in many cellular processes, including maintaining cellular shape, endosomal transport, cell polarity, cell division, and formation of motility structures especially in signal pathways with many interacting proteins.⁷³ In this study, HUVECs were considered to approximately characterize the endothelial cells in blood vessels. Actin filament rearrangement was correlated to direct contact of PEGylated SWNTs with the HUVEC cells. We observed changes in actin organization and similar changes in cell proliferation. PEGylated SWNT effects were dose-dependent and most likely associated with mechanical damage to the cell membrane by actin reorganization. This study demonstrated that HUVEC cells experienced actin cytoskeleton disruption and induced reticulated fibrils formation and a concomitant diminished viability as a result of exposure to long SWNTs coated with the low molecular weight of PEGs. This resulted in disruption of the actin cytoskeleton and the

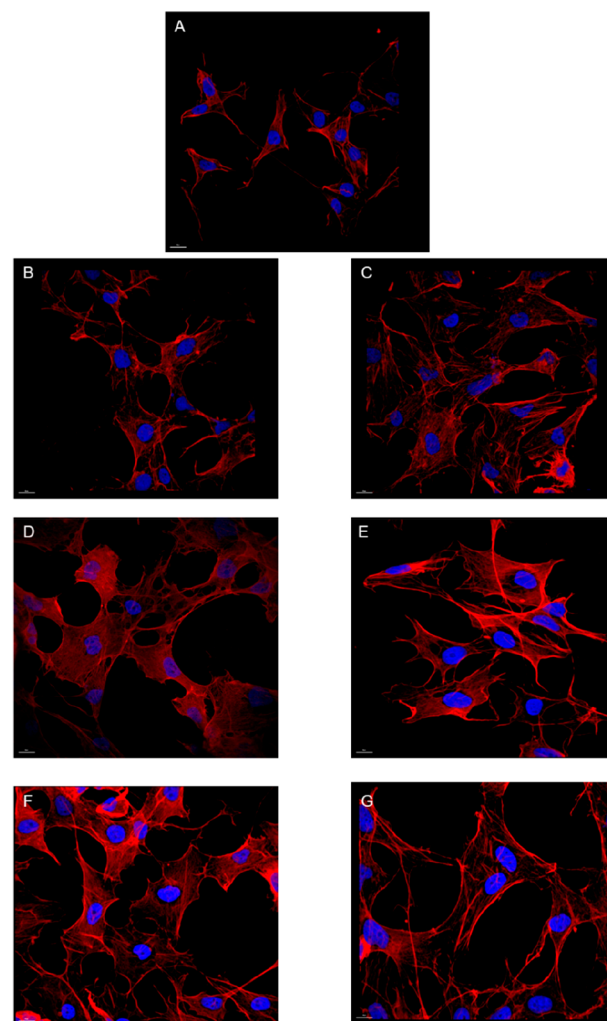


Figure 8. Confocal fluorescence microscopy images of the actin cytoskeleton in HUVECs treated with PEGylated SWNT: (a) control, (b) short SWNT-PEG₂₀₀₀, (c) long SWNT-PEG₂₀₀₀, (d) short SWNT-PEG₅₀₀₀, (e) long SWNT-PEG₅₀₀₀, (f) short SWNT-PEG₁₂₀₀₀, and (g) long SWNT-PEG₁₂₀₀₀. The actin cytoskeleton is stained in red with Phalloidin-TRITC, and nuclei are counterstained in blue with DAPI.

intercellular interactions, which may have multiple effects on endothelium function. However, the endothelial cells extremely tolerated the high concentrations of short SWNTs coated with PEG₁₂₀₀₀ without changes in viability, cytoskeleton, and function.

CONCLUSIONS

An integrative approach was utilized to devise a biocompatible PEG-coated SWNT nanocarrier for further drug delivery studies. We first explored the stability and behavior of Pyr-PEG coated SWNTs using MD simulations. MD studies revealed that pyrene bearing PEG chains and SWNTs have the required structural strength to maintain the nanocarrier intact in crowded environments. This strength and stability are both independent of PEG molecular weight or SWNT size. High stoichiometric ratios of Pyr-PEG chains to SWNTs should be used for effective coating. Based on the insights from MD simulations, noncovalent assemblies of SWNTs bearing PEG were synthesized by using pyrene as an anchor. Two types of SWNTs with different lengths were functionalized by

incorporation of different Pyr-PEGs (PEG₂₀₀₀, PEG₅₀₀₀, and PEG₁₂₀₀₀). Cell viability results revealed that short SWNTs supported cell proliferation; however, long SWNTs had an inhibitive behavior. Longer PEG chains provided lower cytotoxicity and higher biocompatibility to SWNTs in the endothelial cells lines (HUVEC). The present study indicated that effectively PEGylated short SWNT systems provide a promising platform not only for cellular imaging but also for therapeutic purposes in the treatment of different types of cancerogenic tissues.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.langmuir.8b00971](https://doi.org/10.1021/acs.langmuir.8b00971).

Molecular dynamics results, SWNT synthesis, thermogravimetric analysis of SWNTs coated with Pyr-PEG₅₀₀₀ and cell viability results (PDF)

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Notes

The authors declare no competing financial interest.

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