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Assessing the Role of Poly(ethylene glycol-*bl*-propylene sulfide) (PEG-PPS) Block Copolymers in the Preparation of Carbon Nanotube Biocompatible Dispersions

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ABSTRACT: Amphiphilic block copolymers based on hydrophobic polysulfides (poly(propylene sulfide), PPS) and hydrophilic polyethers (poly(ethylene glycol), PEG) have been used to solubilize and disperse single-walled carbon nanotubes (SWNTs). The obtained highly concentrated aqueous dispersions are stable for months. The factors that affect the dispersant activity of the studied block copolymers have been characterized, and comparisons with the much more investigated oxygen analogues (Pluronics) are reported. The biocompatibility and the stability after dilution of the most representative suspensions have been investigated as prospective drug carriers.

Introduction

The 1D structure of carbon nanotubes (CNTs)[1] endows them with outstanding electronic, thermal, and mechanical properties that are unrivaled by any other substance class. Potential applications of these unique nanoscale materials encompass a wide range of composite materials from electrically conducting polymers and nanoelectronics to biosensors[2] and biomaterials[3,4] that interface directly with biological systems and biomedical applications. Recently, CNTs have also received remarkable attention as delivery systems.[5] However, all proposed bioapplications have so far been limited by their virtual insolubility in aqueous solvents. In particular, single-walled carbon nanotubes (SWNTs)[6] pack into bundles or "ropes" that typically contains hundreds of tubes arranged in a close-packed triangular lattice held together by strong van der Waals interactions.[7]

Chemical functionalization can drastically enhance the solubility of SWNTs in various solvents. Nevertheless, it alters the intrinsic physical properties of the CNT because it modifies the sp^2 carbon framework. Noncovalent functionalization by surface adsorption by ionic and nonionic surfactants,[8,9] polymers,[10] and ionic liquids[11] may equally allow the formation of stable dispersions of individual nanotubes while preserving the intrinsic electronic and mechanical properties of SWNTs.[12] Therefore, depending on the elected application, a tailored surfactant or polymer can be chosen to suspend properly SWNTs. For example, in the biomedical field, poly(ethylene oxide) is the preferred solubilizing polymer.[13] Moore et al.[10] had recently investigated the dispersing activity of a large number of amphiphilic poly(ethylene glycol-*bl*-propylene oxide-*bl*-ethylene glycol) (PEG-PPO-PEG) block copolymers, known as Pluronics or poloxamers (Chart 1a). Their good suspendability of SWNTs was attributed to the steric stabilization brought about by the PEG chains that flank the adsorbing hydrophobic central block.

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Numerous papers have already been published on the preparation,[14] bioapplications,[15,16] and self-aggregation[17,18] of a new class of biocompatible block copolymers, structurally related to Pluronics, the triblock poly(ethylene glycol-*bl*-propylene sulfide-*bl*-ethylene glycol) and the diblock poly(ethylene glycol-*bl*-propylene sulfide) copolymers, referred to as PEG-PPS diblock and triblock copolymers, respectively (Chart 1b,c respectively). The PEG-PPS triblock system is analogous to the well-characterized Pluronics; however, the replacement of the oxygen atom in the chain backbone of poly(propylene glycol) with the sulfur atom of PPS renders the central block considerably more hydrophobic[19] and presumably more prone to interact with the hydrophobic surface of the nanotubes. As a matter of fact, it has been demonstrated that the adsorption of the SWNTs by water-soluble polymers is a general phenomenon, thermodynamically driven to eliminate the hydrophobic interface between the tubes and their aqueous medium.[20]

In this study, we investigate the affinity of PEG-PPS di- and triblock copolymers for SWNTs and compare their behavior with that of PEG-PPO analogues. The dispersing efficiency of the copolymer has been evaluated in terms of concentration of dispersed CNTs versus concentration of the dispersing agent. Different features of the PEG-PPS block copolymers have been considered: (i) the hydrophilic/hydrophobic balance; (ii) the presence of two or three blocks; and (iii) the concentration with reference to their CAC (critical aggregation concentration).

We have pointed out[17] that the PEG-PPS block copolymers self-assemble in aqueous solution into aggregates whose morphology and thermodynamics depend on the sample preparation protocol. Preparation by dilution from organic solvent only yielded structures that were close to equilibrium distributions, whereas preparation by direct hydration of polymer films yielded an extraordinary variety of morphologies far from equilibrium. For this reason, in the present study, we also compare the dispersing ability of PEG-PPS block copolymer suspensions

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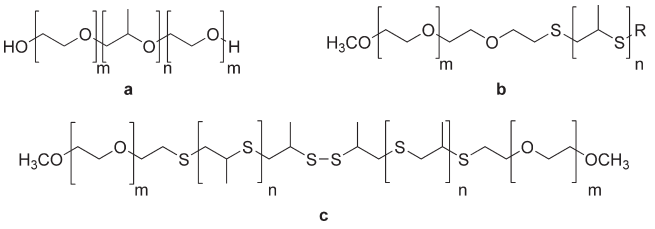
Chart 1. General Structure of (a) Pluronics, (b) Diblock Poly(ethylene glycol-*bl*-propylene sulfide) Copolymers, and (c) Triblock Poly(ethylene glycol-*bl*-propylene sulfide-*bl*-ethylene glycol) Copolymers[a]



[a] R = allyl, acetamido.

obtained by either dilution from THF or direct hydration of polymer films.

Water-soluble functionalized CNTs and Pluronic dispersed MWNTs exhibit a high propensity to cross cell membranes while preserving a high cell viability.[21,22] Moreover, several of the investigated PEG-PPS block copolymers are extensively used in the pharmaceutical field[23] as biocompatible gels, emulsions, nanoparticles, and micellar drug carriers.[24] The final aim of this study is to verify if PEG-PPS block copolymers may favor the entry of individual CNTs into the cell in view of their potential use as carriers of drug or of biomolecules into targeted cells. An ideal noncovalent coating of CNTs for biological applications should have the following features: (a) the dispersant molecules should be biocompatible and nontoxic; (b) the affinity of the dispersant molecules should be sufficiently stable to resist detachment from the nanotube surface in biological solutions, especially in the protein-rich serum; and (c) the amphiphilic coating molecules should have a low CAC so as to confer maximum stability under conditions of extreme dilution (i.e., "sink" conditions) as those met by the drug carrier following physiological administration.[25,26] Therefore, we also estimate the cytotoxicity of PEG-PPS-dispersed SWNTs, and, for the sake of comparison, that of PEG-PPO-dispersed SWNTs, by systematic in vitro studies in media containing 10% fetal bovine serum.

Experimental Section

Materials. Pristine HiPCO SWNTs were provided by Carbon Nanotechnologies (Houston, Texas).

Poly(ethylene glycol)−poly(propylene sulfide) copolymers were synthesized as reported by Napoli et al.[14,24] Purified polymers were analyzed via gel permeation chromatography (GPC) using Waters Styragel THF columns (HR 2, 3, and 4), and analyzed using both refractive index and UV/vis detectors using THF as the mobile phase at 1 mL/min and at 40 °C.

The water-soluble Pluronics were received as a gift from BASF Corporation. Pyrene and all organic solvents were purchased from Aldrich and were used without further purification. Ultra pure Milli-Q water (Millipore model Direct-Q 3) with a resistivity of >18.2 MΩ·cm was used to prepare all solutions.

Preparation of Copolymeric Micellar Solutions. Block copolymer aggregates were prepared in aqueous suspension following two different protocols. In the first protocol, a concentrated stock solution of the copolymer in THF, a good solvent for both of the blocks, was diluted in a large amount of aqueous solution or phosphate-buffered saline (pH 7.4, 578 mOsm), depending on the experiment. In the second preparation protocol, a CH_2Cl_2 copolymer solution was evaporated to dryness by rotary evaporation, followed by further drying under vacuum for at least 30 min. The obtained thin copolymeric film was rehydrated by addition of water (or phosphate-buffered saline).

Determination of the Critical Aggregation Concentration of Block Copolymers. The aim of this study is to investigate the dispersant activity of these block copolymers for SWNTs, and because of their wide variety of self-assembly, we choose to investigate only micelle-forming PEG-PPS block copolymers (with $f_{PEG} \geq 0.5$, as recently evidenced)[19] and to measure CACs for the aggregates obtained by dilution in aqueous solution from copolymeric THF solution. CACs were spectrofluorimetrically determined by using the acknowledged pyrene method.[19] Pyrene is a hydrophobic fluorescent molecule that exhibits[27] different intensity of its vibrational fine structured fluorescence emission spectrum depending upon the polarity of the solubilizing medium and can be used to characterize the hydrophobic cores of micelles. The solvent polarity dependence of pyrene's emission is expressed[28] in terms of the intensity ratio, I_1/I_3, which is the intensities of bands I and III of the pyrene emission spectrum [$\lambda_{em}(I_1) = 373$ nm and $\lambda_{em}(I_3) = 383$ nm]; the larger the ratio, the more polar the medium. The CAC can be obtained as the amphiphile concentration at which I_1/I_3 sharply decreases, reflecting the preferential solubilization of pyrene in the hydrophobic environment of PPS core. Steady-state fluorescence spectra were recorded at 25 ± 0.1 °C by using a Jasco FP-6500 spectrofluorimeter with an excitation band-pass of 3 nm and an emission band-pass of 1 nm. A few microliters of a stock solution of the polymer in THF were diluted into phosphate-buffered saline (pH 7.4, 578 mOsm) or in ultrapure water so as to obtain a final polymer concentration in the range 1×10^{-7} to 2×10^{-3} M. A given volume of a pyrene stock solution in ethanol was added to the copolymer suspension (as to reach a final [pyrene] $= 2 \times 10^{-6}$ M) under stirring and left to equilibrate for at least 10 min before the measurement was performed. The excitation wavelength was $\lambda_{ex} = 335$ nm.

Preparation of SWNT-Stabilized Dispersions. We prepared SWNT aqueous dispersions by adding 4 mL of aqueous solution of the copolymer to 1 mg of SWNTs. The sample was then sonicated with an ultrasonic bath (Bandelin Sonorex, 35 kHz) and centrifuged for 10 min at 4000 rpm by using an Universal 32 (Hettich) centrifuge to separate the supernatant from the precipitate. The supernatant was decanted and used for the following measurements. The final concentration of SWNTs in the aqueous dispersions was spectrophotometrically measured[10,11,29,30] by using a Cary 100-Varian instrument. Because the examined copolymers do not absorb at 377 nm, we take advantage of the calibration curve constructed by Di Crescenzo et al. ($\varepsilon_{377\ nm} = 106.0$ mL/mg cm) in aqueous solution of SDBS (sodium dodecylbenzenesulphonate).[11]

Cryo-TEM Measurements. An EM grid with holey carbon film was held in tweezers and 4 to 5 μL of sample solution was applied on the grid. The tweezers are mounted in a plunge freezing apparatus (guillotine), and after blotting, the grid was immediately immersed in a small metal container with liquid ethane that is cooled from the outside by liquid nitrogen. The speed of cooling is such that ice crystals do not have time to form. Observation was made at −170 °C in a Philips CM12 EM (Eindhoven, The Netherlands) operating at 100 kV and equipped with a cryo-specimen holder Gatan 626 (Warrendale, PA). Digital images were recorded with a Gatan MultiScan charge-coupled device (CCD) camera 1024 × 1024. The image processing software was a Gatan Digital Micrograph.

Cell Culture and Cytotoxicity Assay. Both HeLa cells and 3T3 mouse fibroblasts were employed for cell viability test. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All chemicals were purchased from Gibco Invitrogen Corporation. Cells were maintained in culture at 37 °C in a 5% CO_2 humidified incubator. Cells were plated in 96-well culture plates (0.32 cm^2) at ∼10 000 cells per well and cultured up to 70% confluency. Cells were then exposed to different concentrations of SWNT-copolymer complex for 36 h under humid conditions of 5% CO_2 at 37 °C. The Alamar blue (AB) assay was used to determine the variation in cellular metabolic activity. After removal of the residual media, SWNT-treated cultures were incubated in 10% v/v Alamar blue dye solution in DMEM for 1 h at 37 °C. Following the incubation, AB fluorescence, directly proportional to cell viability, was

Table 1. Characterization of the Investigated Copolymers

copolymer[a,b]	M_w (PDI)	f_{PEG}[c]	CAC at 25 °C in PBS (10^{-5} M; mg/mL)	CAC at 25 °C in H_2O (10^{-5} M; mg/mL)
$E_{46}S_{13}E_{46}$	5092 (1.22)	0.81	11.6(±1.5); 0.59	
$E_{46}S_{28}E_{46}$	6101 (1.31)	0.66	3.92(±0.39); 0.23	4.92(±0.03); 0.30
$E_{45}S_{56}E_{45}$	8152 (1.21)	0.49	0.91; 0.07[d]	
$E_{110}S_{18}$	6330 (1.14)	0.78	4.74(±0.40); 0.30	4.86(±0.86); 0.31
$E_{49}S_{13}$	3081 (1.17)	0.69	6.72(±0.04); 0.28	
$E_{44}S_{20}$	4350 (1.14)	0.57	4.27(±0.18); 0.19	6.29(±2.69); 0.27
$E_{110}S_{54}$	9000 (1.17)	0.55	0.47(±0.07); 0.042	0.78(±0.10); 0.07
$E_{54}S_{32}$	4708 (1.20)	0.50	6.62(±0.24); 0.31	6.99; 0.33
$E_{54}S_{36}$	5064 (1.22)	0.47	1.09(±0.02); 0.055	1.34(±0.16); 0.068
$E_{122}O_{44}E_{122}$	9427 (1.11)	0.81	200; 19.0[d]	
$E_{128}O_{74}E_{128}$	12961 (1.15)	0.72	120; 15.0[d]	
$E_{37}O_{56}E_{37}$ (Pluronic P105)[e]	6500 (1.11)	0.50	28.2; 1.83	54.0; 3.51

[a] Molecular weight distribution was determined by size exclusion chromatography (SEC) and confirmed from ^{1}H NMR measurements. [b] Number of units calculated using the average molecular weight. [c] Average molecular weight of hydrophilic fraction to total average molecular weight ratio ($M_{w(PEG)}/M_w$). [d] Ref 19. [e] Average molecular weight provided by the manufacturer.

quantified at the respective excitation (550 nm) and emission (600 nm) wavelength using a plate reader (TECAN).

Statistical Analysis. Experiments were performed in quadruplicate. Control values were set at 100%. Cytotoxicity data were expressed as mean percentage relative to the unexposed control ± standard deviation (SD). One-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test, was used for the evaluation of statistical significance ($p \leq 0.001$). A star (*) indicates that the value is statistically different from the control.

Results and Discussion

Determination of the Critical Aggregation Concentration of Block Copolymers. The ability of amphiphilic block copolymers to undergo self-assembly into a variety of ordered structures above a certain CAC, when dispersed in solvents that selectively solubilize only one of their domains, has been widely studied in the past decades.[31−33] The driving force for the aggregation is the tendency of the hydrophobic domains to minimize their contact with water and of the hydrophilic domains to orient themselves toward the aqueous phase and become solvated.

Compared with low-molecular-weight surfactant, amphiphilic block copolymers are generally characterized[34,35] by relatively low CAC and consequent high stability during extreme dilution, as specifically requested in the case of physiological administration. Table 1 reports the CAC values obtained in PBS and in pure water for the investigated PEG-PPS block copolymers and some oxygen analogues. The copolymers are indicated as E_xS_y or $E_xS_yE_x$, where E represents the PEG domain, S is the PPS domain, and x,y are the repeating units in the respective polymer chains. The two series of values, those determined in pure water and those determined in PBS, are very similar, highlighting that the presence of ions and the pH control (in the range of 5 to 7.4) do not significantly affect these measurements. The close superimposition of the two series is confirmed by the fact that the decrease in the I_1/I_3 ratio covers a large interval of block copolymer concentrations. (See, as an example, Figure 1). As a matter of fact, the critical transition interval from monomers to mature micelles with the proper aggregation number involves different steps and different species (i.e., monomolecular micelles and preaggregates with different aggregation numbers).[19]

Because the aggregation process is driven by the hydrophobic effect, the CAC value decreases with increasing length of the hydrophobic PPS block. However, "the increase in the CAC with increasing relative hydrophilic composition (f_{PEG}) is mainly due to the decrease in the relative number of PPS units, which is the primary factor governing both stability and self-assembly".[19]

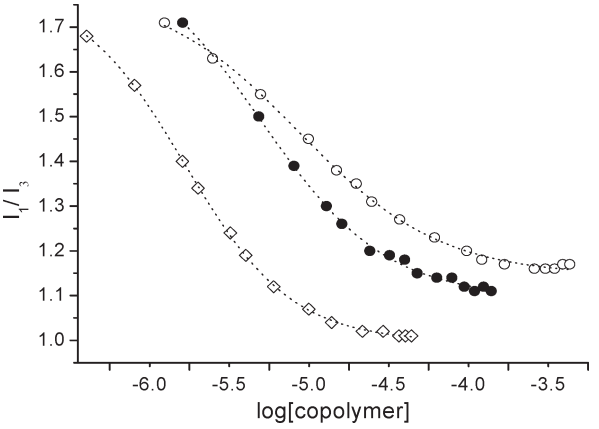


Figure 1. Plots of the I_1/I_3 ratio as a function of copolymer concentration for the three investigated triblock copolymers $E_xS_yE_x$, essentially characterized by an increase in the length of the central hydrophobic block and an almost constant weight of the two hydrophilic domains. ○, $E_{46}S_{13}E_{46}$ (f_{PEG} = 0.8); ●, $E_{46}S_{28}E_{46}$ (f_{PEG} = 0.7); ◇, $E_{45}S_{56}E_{45}$ (f_{PEG} = 0.5).

SWNT Dispersions. Images of the ink-like dispersions obtained by adding 4 mL of a differently concentrated copolymeric solution to 1 mg of SWNTs are presented in Figure 2. Visual inspection allows discrimination between well-dispersed (sample A) and partially dispersed SWNTs (samples B and C). The effect of the dispersing polymers on the electronic state of SWNTs was investigated using UV−vis spectroscopy. In Figure 3, we report, as an example, the UV−vis spectra of SWNTs dispersed in aqueous solutions of $E_{46}S_{13}E_{46}$ block copolymer. The distinctive subband transition peaks characterizing the absorption spectra of SWNTs in this and in all investigated copolymers demonstrate the ability of these PEG-PPS block copolymers to form well-dispersed SWNT solution.[8] As a matter of fact, the observation of pronounced spectral features suggests the presence of individual SWNTs in solution because bundling would lead to a broadening of the absorption spectra.[8,36] Nevertheless, the dispersant activity depends on many factors.

Effect of the Copolymer Concentration. As evidenced in Figures 2 and 3, the copolymer concentration is a crucial factor in determining the dispersant activity of PEG-PPS block copolymers. The most efficient dispersant is the triblock $E_{45}S_{56}E_{45}$ with a dispersing ratio of 0.58 (CNT to polymer). The best diblock copolymers appear to be $E_{110}S_{18}$

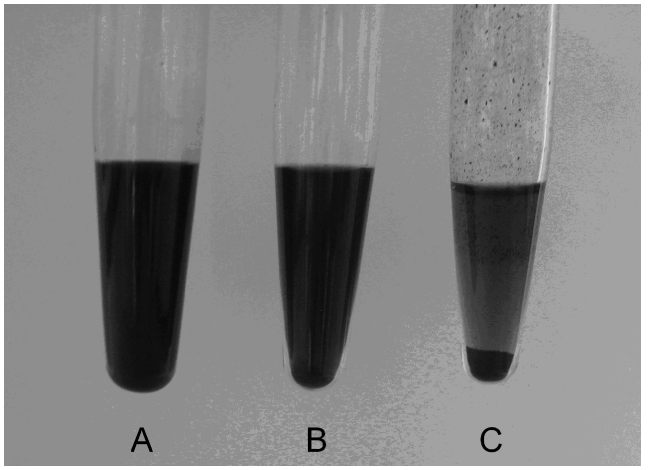


Figure 2. Dispersions of SWNTs (0.025 wt %) in polymeric solutions obtained by film rehydration after 7 h of sonication: (A) 0.3 mg/mL $E_{45}S_{56}E_{45}$ in water, (B) 2 mg/mL $E_{45}S_{56}E_{45}$ in water, and (C) 5.4 mg/mL $E_{45}S_{56}E_{45}$ in water. Images were taken more than 10 months after preparation.

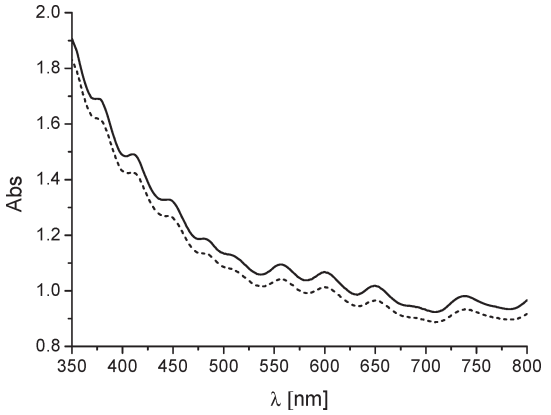


Figure 3. Absorption spectra of debundled SWNTs in the presence of 0.56 (---) and 2.81 mg/mL (—) of $E_{46}S_{13}E_{46}$ after 3 h of sonication. The copolymer samples are obtained by film hydration.

and $E_{44}S_{20}$ with a dispersing ratio of 0.13 and 0.12, respectively, whereas Pluronics show a dispersing ratio (< 0.32) lower than those of PEG-PPS triblock analogues. A general trend can be outlined from Table 2. The best dispersant activity of $E_xS_yE_x$ block copolymer is obtained at a concentration between 1 and 5 times the CAC for triblock copolymers, whereas a higher concentration is necessary for diblock copolymers. Despite their lower dispersant power, Pluronics are able to disperse individual nanotubes in aqueous solutions well below their CAC (see Table 3); therefore, a steric stabilization via single-chain adsorption of the latter copolymers onto SWNTs had been previously proposed.[37]

The typical scenario for steric stabilization of SWNTs via $E_xO_yE_x$ block copolymers (where O indicates the PPO block) involves[38] the adsorption of the hydrophobic block to the nanotube surface, the other hydrophilic blocks dangling into the solvent. In this way the interaction of the copolymer with the SWNTs depends only on the affinity of the hydrophobic block for the nanotube surface, whereas the hydrophilic blocks, dangling from the CNT surface, repel the other polymer-coated SWNTs and are responsible for the stabilization of SWNT dispersions. Apparently, from inspection of data reported in Table 2, copolymeric micelles are instead necessary when the SWNTs solubilization is performed by using PEG-PPS block copolymers. Nevertheless, Figure 4 highlights that when the best dispersed samples are considered for each polymer, the amounts of adsorbed solvophobic blocks in SWNT dispersion of PEG-PPS block copolymers or Pluronics are comparable (compare violet, magenta, and yellow bars). Therefore, we propose a similar steric solubilization model also for the present PEG-PPS block copolymers, the only difference with Pluronics being that the CACs of the $E_xS_yE_x$ block copolymers in the absence of SWNTs are generally much lower than those of the oxygen analogues. Indeed, an increase in PEG-PPS block copolymer concentration with respect to the optimal threshold value does not necessarily favor the dissolution of a higher number of SWNTs (Table 2), thus confirming that the SWNT dissolution is independent of micelle concentration. Actually, micelles had been previously considered to be the main cause of nanotube aggregation at sodium dodecyl sulfate (SDS) concentration well above its CAC.[39]

Moreover, the weight ratio between the solubilized SWNTs and the copolymer is always in favor of the copolymers (i.e., dispersing ratio lower than 1; Tables 2 and 3), but among the investigated samples, only $E_{45}S_{56}E_{45}$ allows dispersion of half as much SWNTs as its weight. The other $E_xS_yE_x$ triblocks, the diblocks $E_{110}S_{18}$ and $E_{44}S_{20}$, and the Pluronic $E_{128}O_{74}E_{128}$ solubilize SWNTs at a concentration from 3 to 11 times higher than that of SWNTs. All other polymers solubilize SWNTs at a concentration from 20 to 60 time higher than that of SWNTs. This is not surprising if one considers that the best suspension obtained with sodium dodecyl benzenesulfonate, the surfactant generally considered the best dispersant of SWNTs, requires a concentration of surfactant that is 7 times higher than that of SWNTs under analogous experimental conditions (i.e., dispersing ratio = 0.15).[11] This points to the good dispersant ability of PEG-PPS block copolymers with respect to other amphiphilic molecules and also with respect to Pluronics.

Effect of the Copolymer Hydrophilic/Hydrophobic Balance. The presence of SWNTs modifies[40] the self-aggregation of Pluronics. As a matter of fact the presence of micelles complicates the adsorption process by introducing an equilibrium system where there is competition between the adsorption of unimers onto the surface and micelle formation.[41] It has been demonstrated that micelles with a small central hydrophobic block are less stable than micelles formed from longer hydrophobic blocks, thus pushing the equilibrium toward the breakup of micelles and the adsorption of unimers.[19] This explanation has been already used for explaining the enhanced adsorption of Pluronics with short PPO chains on a model hydrophobic surface.[42] A similar model can be suggested also for PEG-PPS block copolymers, for which copolymers with a smaller PPS block appear to be able to solubilize a higher number of SWNTs. (See Figure 4). Figure 4 also points out a higher affinity of the PPS block for the nanotube surface with respect to the oxygen analogous PPO block because at similar concentration of hydrophobic block, PEG-PPS block copolymers are able to solubilize a higher number of SWNTs. Nevertheless, the role of the hydrophilic dangling PEG units cannot be neglected. Figure 5 reports the dispersing ratio (CNT to polymer) against the hydrophilic/hydrophobic balance of the polymer. With the exception of the low dispersant $E_{122}O_{44}E_{122}$ and the high dispersant $E_{45}S_{56}E_{45}$ block copolymers, a quite good linear correlation can be drawn for either triblocks (circles) and diblocks (squares). A higher hydrophilic/hydrophobic balance favors the dispersant activity of these block copolymers, with the diblocks being less sensitive to the hydrophilic/hydrophobic balance change. This lower sensitivity of diblocks toward the hydrophilic/hydrophobic balance and the

Table 2. Characterization of SWNT Solutions Obtained by Using PEG-PPS Block Copolymer Suspensions Obtained by Either Dilution from THF or Direct Hydration of Polymer Films

copolymer	copolymer concn (mg/mL)	copolymer/ CAC	hours of sonication	concn of dispersed SWNTs (mg/mL; %)[a]	dispersed SWNTs/ copolymer
$E_{46}S_{13}E_{46}$			Film Hydration		
	0.56	1	7	0.17; 76	0.304
	2.81	5	5	0.17; 67	0.061
			Dilution THF → H_2O		
	0.56	1	5	0.12; 57	0.217
	8.91	15	5	0.12; 53	0.013
	10.7[b]	18	4	0.14; 58	0.013
$E_{46}S_{28}E_{46}$			Film Hydration		
	0.23	1	1	0.02; 11	0.109
	0.50	2	3	0.14; 55	0.278
	1.12	5	4	0.17; 71	0.153
			Dilution THF → H_2O		
	0.15	0.6	4	No dispersion	No dispersion
	0.27[b]	1	8	0.09; 43	0.326
	3.67	15	5	0.07; 37	0.018
$E_{45}S_{56}E_{45}$			Film Hydration		
	0.12	1.6	4	No dispersion	No dispersion
	0.31[b]	4	7	0.18; 67	0.583
	2.00	27	3	0.05; 23	0.027
	5.37	73	4	0.05; 7	0.003
			Dilution THF → H_2O		
	0.31	4	8	0.18; 57	0.591
$E_{110}S_{18}$			Film Hydration		
	0.30	1	4	No dispersion	No dispersion
	1.34	4.5	3	0.12; 49	0.091
	1.48[b]	5	11	0.19; 75	0.130
$E_{49}S_{13}$			Film Hydration		
	1.36	6	12	0.07; 28	0.049
	2.58[b]	9	4	0.05; 19	0.020
	5.05	18	4	0.04; 17	0.008
$E_{44}S_{20}$			Film Hydration		
	0.22	1.2	5	No dispersion	No dispersion
	1.60	8.4	5	0.88; 75	0.118
	2.24[b]	12	5	0.19; 77	0.086
$E_{110}S_{54}$			Film Hydration		
	0.044	1	3	No dispersion	No dispersion
	0.23	5.5	3	0.001; 1	0.006
	1.60	38	2	0.06; 24	0.037
			Dilution THF → H_2O		
	1.74[b]	41	5	0.07; 29	0.041
$E_{54}S_{32}$			Film Hydration		
	2.62	8	2	0.06; 24	0.024
$E_{54}S_{36}$			Film Hydration		
	0.57	10	4	0.02; 6	0.031
	1.28	23	3	0.006; 2	0.005
	2.53	46	2	0.006; 2	0.002

[a] Calculated on the initial concentration of SWNTs (~0.25 mg/mL). [b] Dispersion subjected to cytotoxicity assay.

necessity of a higher diblock concentration with respect to those necessary for triblocks could be ascribed to the fact that a certain density of PEG chains should be ensured[37,38] to obtain a good SWNT dispersion. The good dispersant activity of $E_{44}S_{20}$ is highlighted by Cryo-TEM images reported in Figure 6.

Sonication Time. In general, the number of dispersed SWNTs increases with the time of sonication until a constant

Table 3. Characterization of SWNT solutions obtained by using Pluronics suspensions obtained by either dilution from THF or direct hydration of polymer films

copolymer	copolymer concentration (mg/mL)	copolymer/ CAC	hours of sonication	concn of dispersed SWNTs (mg/mL)	% of dispersed SWNTs[a]	dispersed SWNTs/ copolymer
$E_{122}O_{44}E_{122}$				Film Hydration		
	0.34	0.02	1	0.005	1	0.015
	8.15[b]	0.4	8	0.13	50	0.016
$E_{128}O_{74}E_{128}$				Film Hydration		
	0.34	0.02	1	0.08	38	0.233
	2.50	0.2	6	0.003	12	0.001
$E_{37}O_{56}E_{37}$ (Pluronic P105)				Film Hydration		
	0.10	1	4	0.02	7	0.172
	0.22[b]	1.5	2	0.06	23	0.264
	0.34	2	6	0.11	49	0.317
	2.21	15	3	0.18	69	0.079
	22.5[b]	157	8	0.13	64	0.006
				Dilution THF → H_2O		
	0.22	1.5	1	0.06	23	0.282
	2.20	15	4	0.22	74	0.100

[a] Calculated on the initial concentration of SWNTs (~0.25 mg/mL). [b] Dispersion subjected to cytotoxicity assay.

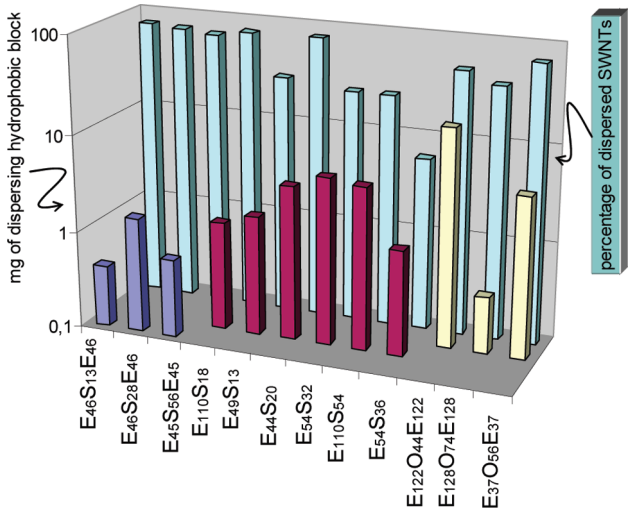


Figure 4. Mass of dispersing hydrophobic blocks (violet, magenta, and yellow bars) and percentage of dispersed SWNTs (clear blue bars). The concentrations of polymers reported in the graph are those that ensured the highest dispersion of SWNTs among the concentrations investigated. Violet data refer to $E_xS_yE_x$ triblocks, magenta data refer to E_xS_y diblocks, and yellow data refer to Pluronics.

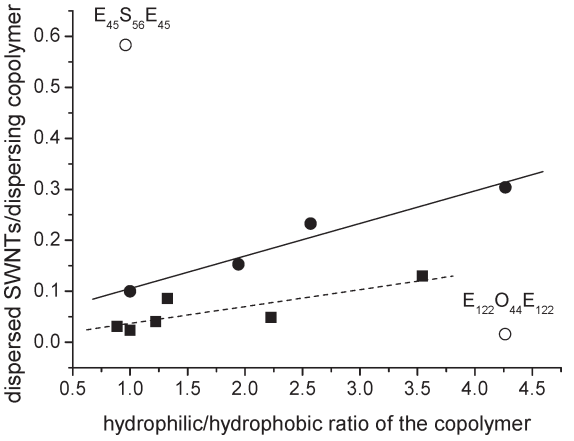


Figure 5. Dispersed SWNTs/dispersing polymer ratio against the hydrophilic/hydrophobic balance of the same polymers for the samples that assured the highest dispersion of SWNTs among the investigated polymer concentrations. ■, diblock PEG-PPS block copolymers; ●, triblock copolymers.

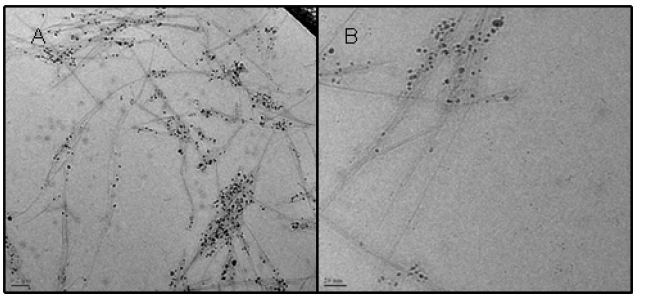


Figure 6. Cryo-TEM of small bundles and individual SWNTs (0.188 mg/mL) dispersed using $E_{44}S_{20}$ at a concentration of 1.6 mg/mL and 5 h of sonication (scale bar equal to 0.2 μm and 20 nm in A and B, respectively). Black dots are due to catalyst particles from the HiPCO SWNTs. Some spherical micelles of block copolymer can be observed in sample A.

value is reached. Nevertheless, a time of sonication above this maximum often reduces the SWNTs dispersion. We ascertained that the reduction of the number of SWNTs dispersed was not a consequence of the sonication-induced break of the investigated polymers. As a matter of fact, GPC of freeze-dried micelle suspension of $E_{44}S_{20}$ or $E_{46}S_{13}E_{46}$ copolymers redissolved in THF did not evidence any variation before or after 2, 4, and 6 h of sonication. An optimum sonication time for the investigated suspension was 5−7 h (as an example, Figure S1 in the Supporting Information reports the effect of sonication in dispersions of SWNTs with $E_{46}S_{13}E_{46}$), as also obtained in previous papers on SWNTs solubilization.[11] Figure 6 confirms that the mild sonication employed, although prolonged for 5 h, does not damage the dispersed SWNTs.

Effect of the Preparation Procedure of Copolymer Solution. The protocol of preparation of the copolymeric solution seems to have little effect on the dispersing efficiency of the investigated copolymer. For this reason, we tested thoroughly only triblock copolymers. It appears that the more

thermodynamically stable aggregates obtained through dilution[17] are less indicated for the preparation of highly concentrated SWNT dispersions. This is in agreement with the above-mentioned tendency of unimers from less-stable micelles to adsorb to the nanotube surface.

Stability of the Dispersions. The stability of SWNT dispersions was monitored over months through UV−vis measurements (corresponding ζ-potential measurements and related discussion are reported in detail in the Supporting Information). As reported in Table S1 of the Supporting Information, almost all PEG-PPS block-copolymer-coated SWNT dispersions are stable over time, with an average variation of 10% in the dispersed concentration of SWNTs within 1 month. In a few cases, the effect of the solvent evaporation seems to affect the concentration of SWNTs more than the eventual reaggregation and the consequent precipitation from the suspension of SWNTs. On the contrary, 80% of SWNTs dispersed with Pluronic P105 precipitate in the solution within 3 days. Samples obtained by dilution from a THF solution or by film hydration behave similarly.

In view of a potential administration in a biological environment, the stability of SWNT suspensions after a 10-fold dilution was also monitored over time. It appears that the diluted samples are also relatively stable. (See Table S1 of the Supporting Information). It is worthwhile to underline that whereas the as-prepared 1.2 mg/mL $E_{44}S_{20}$ does not seem to disperse SWNTs, the 10-fold diluted sample from 12 mg/mL $E_{44}S_{20}$ is very stable with an apparent increase in dispersed SWNTs over 1 month presumably because of solvent evaporation. This latter evidence confirms once more that micelle concentration is not related to the formation of stable SWNT dispersion, the key factor being a proper coverage of SWNT surface by the PPS blocks.

Biocompatibility Assessments. CNTs interfere with a number of dyes used for cytotoxicity assessment,[43] making toxicological investigations quite difficult. For example, the widely used and sensitive MTT test is particularly inappropriate because of the interaction of SWNTs with the water-insoluble MTT-formazan crystals.[44] Indeed, several MTT-based determinations resulted in false positive and mismatched findings if compared with other viability tests run on the same samples.

Because the AB assay had recently been found[43] to be the most sensitive and reproducible test when dealing with CNTs, we chose this assay to make a preliminary in vitro screening of the investigated polymer-SWNT suspensions.

This cell viability indicator uses the natural reducing power of living cells to convert resazurin to the fluorescent molecule resorufin. Cellular reducing conditions are well-known indicators of cell viability and cell death. In the present assay, the higher the obtained fluorescence, the greater the cell viability.

To compare the different copolymeric dispersions, we exposed the cells to comparable amounts of SWNTs (in the range of 0.625−62.5 μg/mL). The choice of this concentration interval was made on the basis of previously performed similar toxicity tests,[43−45] and the estimation of the number of nanotubes required for biomedical applications.

As displayed in Figure 7, we did not observe any significant loss of cell viability (the percentage of living cells remains well above 80%) upon incubation for 36 h of the 3T3 cells with a concentration of SWNTs from 0.625 to 12.5 μg/mL dispersed with $E_{44}S_{20}$, as compared with untreated cells. $E_{44}S_{20}$ alone, as expected from previously studies highlighting the nontoxicity of $E_xS_yE_x$ block copolymers,[16,46] turned out to be completely nontoxic at all investigated concentrations. Similar results were obtained

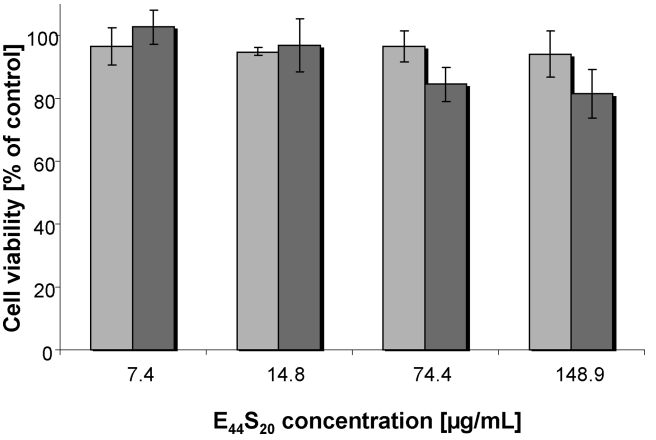


Figure 7. Cytotoxicity of $E_{44}S_{20}$ aqueous solution in the absence (light gray bars) and in the presence (dark gray bars) of SWNTs on 3T3 cells. SWNT concentrations in the samples are, respectively, 0.625 (7.4 μg/mL $E_{44}S_{20}$), 1.25 (14.8 μg/mL $E_{44}S_{20}$), 6.25 (74.4 μg/mL $E_{44}S_{20}$), and 12.5 μg/mL (148.9 μg/mL $E_{44}S_{20}$).

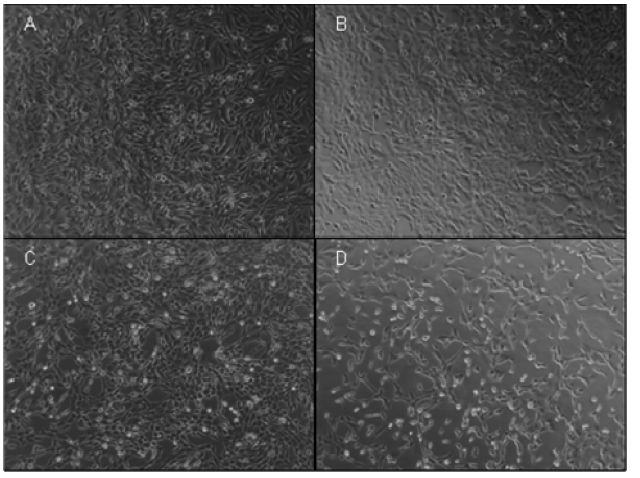


Figure 8. Optical microscopy images (performed with a Zeiss microscope (Feldbach, Switzerland)) of HeLa cells incubated for 36 h with the dispersions of SWNT-$E_{44}S_{20}$ with a final SWNT concentration of (B) 36.9, (C) 62.5, and (D) 125 μg/mL. (A) Control sample.

when SWNT-$E_{44}S_{20}$ dispersions were tested on the HeLa cell line (Figure S2 of the Supporting Information). HeLa cells are widely used for scientific research, and, in the present case, compatibility of the investigated dispersions for these immortalized human tumor cells would be a further step toward the use of nanotubes in the biopharmaceutical field. HeLa cells were exposed to even higher nanotube concentrations. Significant cytotoxicity ($p \leq 0.001$) was recorded only above SWNTs concentration of 60 μg/mL. These same conclusions can be drawn by comparing the morphological images reported in Figure 8, which show typical examples of HeLa cells under nontoxic (Figure 8B) and toxic dispersion conditions (Figure 8C,D). Cell cytostaticity and loss in viability (>20%) can be observed only at the highest investigated concentrations of SWNTs (Figure 8C,D).

An analogous behavior, and therefore a significant toxicity only above a nanotube concentration of 60 μg/mL, was displayed by exposure of 3T3 cells to SWNTs dispersed with two other diblock copolymers ($E_{110}S_{18}$ and $E_{110}S_{54}$; Figure S3 of the Supporting Information) or with several triblock copolymers (Figure S4 of the Supporting Information).

The dispersion with $E_{110}S_{54}$ appears to be particularly toxic (above a SWNTs concentration of 60 μg/mL) probably

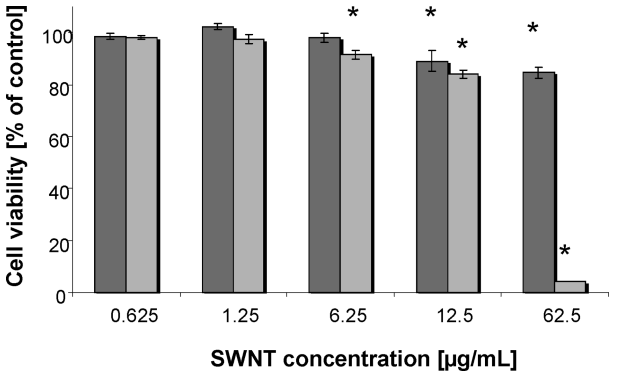


Figure 9. Cytotoxicity of SWNT dispersions prepared with two Pluronics, $E_{122}O_{44}E_{122}$ (dark gray bars), and $E_{37}O_{56}E_{37}$ or Pluronic P105 (light gray bars) on 3T3 cells at various SWNT concentrations. Copolymer concentrations in the samples are, respectively, 39.2 and 2.29 μg/mL (at 0.625 μg/mL SWNTs); 78.4 and 4.58 μg/mL (at 1.25 μg/mL SWNTs), 392 and 22.9 μg/mL (at 6.25 μg/mL SWNTs); 784 and 45.8 μg/mL (at 12.5 μg/mL SWNTs); and 3920 and 229 μg/mL (at 62.5 μg/mL SWNTs).

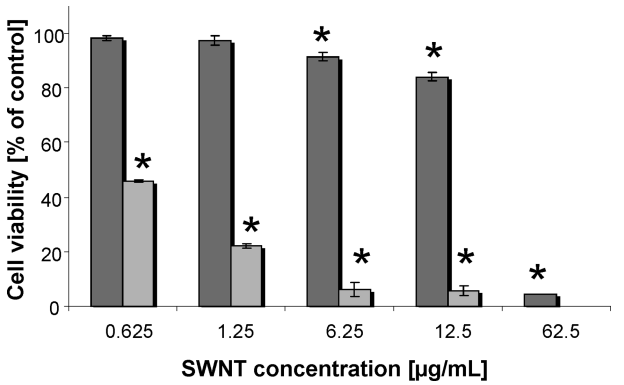


Figure 10. Comparison of the 3T3 cell cytotoxicity of SWNT-$E_{37}O_{56}E_{37}$ (Pluronic P105) dispersion prepared with low concentration of copolymer (dark gray bars) and high concentration of copolymer (light gray bars). Copolymer concentrations in the samples are, respectively, 2.29 and 108 μg/mL (at 0.625 μg/mL SWNTs); 4.58 and 216 μg/mL (at 1.25 μg/mL SWNTs), 22.9 and 1082 μg/mL (at 6.25 μg/mL SWNTs); 45.8 and 2163 μg/mL (at 12.5 μg/mL SWNTs); and 229 and 10800 μg/mL (at 62.5 μg/mL SWNTs).

because of the lower dispersant capacity of this copolymer with respect to the other ones. (See Table 2). As a matter of fact, it is well known[47,48] that the main causes of toxicity of CNTs are the presence of residual metal catalyst and the insolubility of the material.

In summary, we can conclude that we have obtained PEGylated and exfoliated SWNTs whose dispersions are stable and biocompatible at least up to a nanotube concentration of 50 μg/mL, as reported in the literature for properly functionalized[49,50] or differently dispersed SWNTs,[51] thus allowing an eventual use in the biomedical field.

Pluronics were widely used to disperse CNTs; in particular, many attempts were made with Pluronic F68 (poloxamer 188), Pluronic F87 (poloxamer 237), Pluronic F108 (poloxamer 338), and Pluronic F127 (poloxamer 407), approved by the US FDA as biocompatible materials. It was found that the toxicity of Pluronics is fairly low and is correlated to their hydrophobicity as it decreases with increasing their molecular weight and PEG content.[52]

Toxicological investigations were carried out to assess the influence of such copolymers on the toxicity of nanotube dispersions. Monteiro-Riviere et al. studied the effect of some Pluronics on the disaggregation of MWNT bundles in water and the cytotoxicity of the resulting dispersions on human epidermal keratinocytes.[53] They concluded that Pluronic F127 (at a concentration of 1% w/v) does not increase the MWNT cytotoxicity, although it reduces MWNT aggregation. Pluronic F127 alone starts to reduce cell viability at a concentration of 10%.[54] Sayes et al. observed that SWNTs (2 μg/mL) in 1% Pluronic F108 produces 65% of cell mortality on human dermal fibroblasts.[50]

We studied SWNT dispersions obtained with Pluronic $E_{122}O_{44}E_{122}$ and Pluronic P105. At the same concentration of SWNTs (62.5 μg/mL), the dispersion prepared with Pluronic $E_{122}O_{44}E_{122}$ presents no toxicity, whereas that prepared with P105 is very toxic. (See Figure 9).

This result is not surprising because the stability experiments showed that SWNTs dispersing ability being equal, the suspensions obtained with P105 are not stable over the time. The reaggregation of SWNTs, probably due to a scarce ability of P105 to adsorb properly to the nanotube surface, results in a higher toxicity of the dispersion. Indeed, it has been previously shown[54] that the ability to simply disperse nanotubes in aqueous solutions was not a sufficient criterion for predicting cell interactions, although several studies had hypothesized[4,49] that the dominant factor leading to 3T3 fibroblasts rupture is the SWNT hydrophobicity, and Lacerda et al. demonstrated[55] that only "if the injected SWNTs nanomaterial is in aggregates or bundles, the latter will not be able to cross the glomerular filter and will accumulate in the liver, spleen, or lungs".

We also found (Figure 10) that the toxicity of SWNTs dispersion with Pluronic P105 depends on the copolymer concentration, although the Pluronic alone has been demonstrated to be nontoxic at all the investigated concentrations. Cytotoxicity therefore depends on specific aggregates formed on increasing copolymer concentration, that is, coated bundles of CNTs, as previously suggested,[56] or the preference of this Pluronic, above its CAC, to associate with other Pluronic molecules rather than with the nanotube surface.[57]

It is important to highlight that metal content (Co, Fe, Ni, Mo) could influence the toxicity of CNT dispersions.[58,59] Nonpurified SWNTs can contain a certain percentage of metal catalysts used for their synthesis. Nevertheless, these impurities are partially, although not completely, removed in our dispersions through sonication and the subsequent centrifugation,[4] which are two essential steps of the preparation protocol of SWNT dispersions. (See the Experimental Section).

Conclusions

We have succeeded in producing well-dispersed and stable aqueous solutions of SWNTs with the aid of PEG-PPS block copolymers. These block copolymers can assist the exfoliation process of SWNT bundles essentially through steric stabilization and appear to behave better than the well-known and widely used Pluronics. In particular, the hydrophobic block strongly anchors onto the nanotube surface with the hydrophilic PEG chain extending to the aqueous phase and imparting water solubility and biocompatibility to the nanotubes. Unlike nanotubes suspended by typical surfactants, the PEGylated SWNTs prepared by the present method are highly stable in aqueous solutions including serum. The high affinity of the PPS block for the nanotube surface has also been demonstrated by the low toxicity of SWNT-PEG-PPS block copolymer suspensions toward 3T3 and HeLa cells (at concentration of SWNTs below 50 mg/mL) because only properly functionalized and water-soluble CNTs have been demonstrated[49,56] to be nontoxic.

In particular, the dispersing efficiency of the investigated copolymers and the stability of the obtained dispersions depend on many factors such as the length of the hydrophobic block and the hydrophilic/hydrophobic balance of the copolymer, the concentration of the PEG-PPS block copolymer, and the sonication time.

Finally, the PEG-PPS block copolymers demonstrated to be a better dispersant of SWNTs with respect to Pluronics.

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Supporting Information Available: ζ-Potential measurements; absorption spectra of SWNTs in aqueous solution of $E_{46}S_{13}E_{46}$ 0.56 mg/mL; cytotoxicity of SWNT-$E_{44}S_{20}$ dispersion on HeLa cells at various SWNT concentrations; and cytotoxicity of SWNT dispersions prepared with $E_{110}S_{18}$, $E_{110}S_{54}$, $E_{46}S_{13}E_{46}$, $E_{46}S_{28}E_{46}$, or $E_{45}S_{56}E_{45}$ on 3T3 cells at various SWNT concentrations. This material is available free of charge via the Internet at http://pubs.acs.org.

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