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Role of Polyethylene Glycol Integrity in Specific Receptor Targeting of Carbon Nanotubes to Cancer Cells

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ABSTRACT

We demonstrate that dispersion of single walled carbon nanotubes (SWNTs) by ultrasonication with phospholipid-polyethylene glycol (PL-PEG) fragments it, thus interfering with its ability to block nonspecific uptake by cells. However, unfragmented PL-PEG promoted specific cellular uptake of targeted SWNTs to two distinct classes of receptors expressed by cancer cells. Since fragmentation is a likely consequence of ultrasonication, a technique commonly used to disperse SWNTs, this maybe a concern for certain applications such as drug delivery.

Carbon nanotubes (CNTs), whether single- or multiwalled (SWNTs and MWNTs, respectively) have been evaluated as emerging drug delivery vehicles that can be efficiently functionalized to transport therapeutics. For example, functionalized CNTs with lengths ≤ 1 μm have been used to deliver into cells a variety of molecules including proteins,[1−4] nucleic acids,[5−9] or drugs.[10−16]

In general nanoparticles that are used for drug delivery are usually functionalized by polyethylene glycol (PEG) with molecular weight (MW) between 1 and 40 kDa.[17] This is a desirable coating for nanoparticles because it reduces their immunogenicity and reduces their nonspecific uptake by cells within the reticuloendothelial system, which diminishes their phagocytosis, thus leading to prolonged circulation time of nanoparticles.[17] In the case of SWNTs, it has been found that adsorbing phospholipid-PEG (PL-PEG) prevents nonspecific binding of proteins to the surface of SWNTs.[18−20] In that case, the PL part strongly and stably adsorbs to the sidewalls of SWNTs while the PEG moiety extends into the aqueous solution.[21,22] On the other hand, evaluation of cellular uptake of SWNTs functionalized by adsorbed PEG led to unexpected findings. It was demonstrated that adsorbing PL-PEG2000 (i.e., the MW of PEG is ~2000) to SWNTs did not prevent uptake of SWNTs *in vitro*[21] nor *in vivo*[22] while functionalizing with longer chain PEG, PL-PEG5000 (i.e., the MW of PEG is ~5000) reduced their nonspecific uptake by cells *in vivo*.[22,23] However, *in vitro* studies of SWNTs adsorbed to PEG5000 gave contradictory results. Two separate studies reported that adsorption to PL-PEG5000 imparted a block on nonspecific binding and uptake of SWNTs by cells,[21,23] whereas another study reported the uptake of SWNTs functionalized with fluorescein-PEG5000 by cells *in vitro*, where adsorption to SWNTs in this case was through interactions of the fluorescein moiety with SWNTs.[24] Thus, the findings for employing PEG to block nonspecific binding and uptake of SWNTs by cells have been inconsistent for the longer chain PEG5000 and contrary to what is expected for the shorter chain PEG2000.

Functionalization of SWNTs with PEG serves another purpose besides blocking nonspecific protein binding, which is enhancing SWNTs dispersion in liquids. SWNTs are insoluble in many liquids including water, and generally their dispersion in liquids involves introducing functional groups onto SWNTs' surfaces via physical adsorption by ultrasonication or by chemical modifications.[25−27] The usual method for functionalizing SWNTs with PL-PEG involves ultrasonicating SWNTs in a solution of PL-PEG for 1 h.[21−24] Ultrasonication for 1−2 h has been used to functionalize SWNTs with different biomolecules or polymers; however the effect of this on such molecules has not been evaluated yet.

In this paper, we (1) evaluate the effect of ultrasonication on PL-PEG that is used to functionalize SWNTs and define the role of PEG in blocking nonspecific uptake thereby resolving existing contradictory findings, and (2) employ PEG and specific ligands to exploit high affinity targets that are expressed on cancer cells for targeted uptake of SWNTs.

In this study, we evaluated the effect of ultrasonication on PL-PEG that is adsorbed to SWNTs. SWNTs (with length 300−1000 nm) were functionalized with PL-PEG2000, which has a MW of ~2790 Da, by ultrasonication for 1 h followed by removal of excess PL-PEG2000 as described in Supporting Information. We used matrix-assisted laser desorption ionization (MALDI) mass spectrometry to confirm

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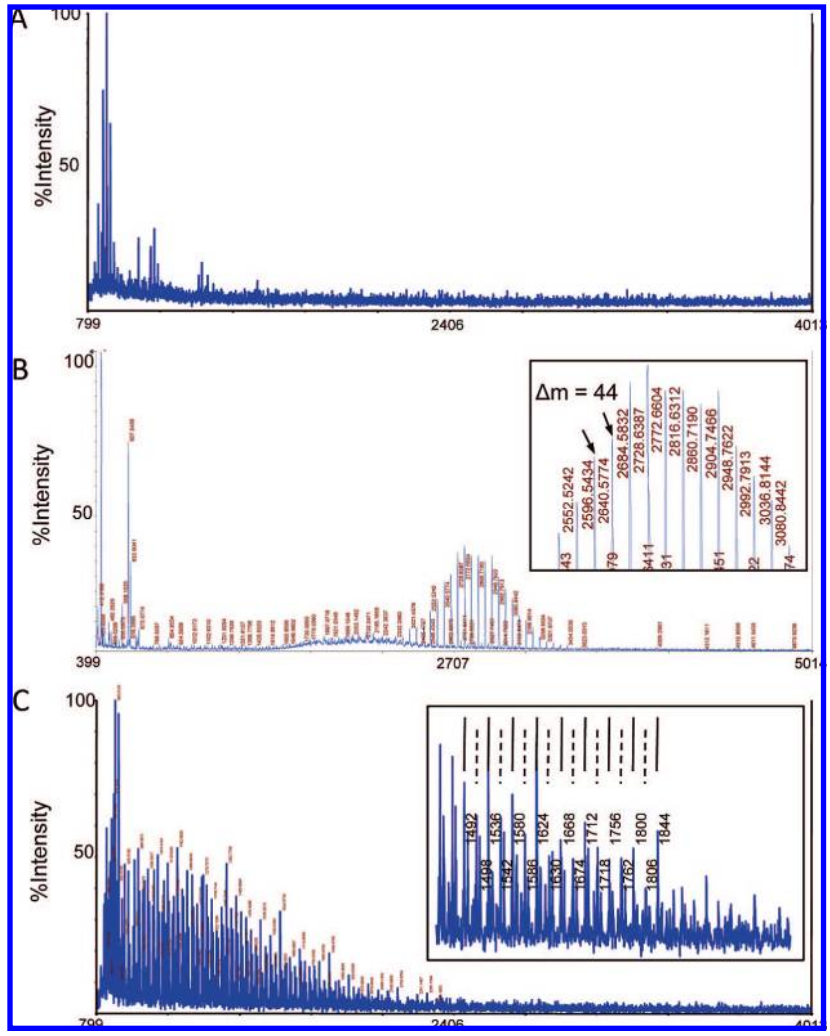


Figure 1. Confirmation of functionalizations of SWNTs with PL-PEG2000 using MALDI mass spectrometry. Mass spectrum for (A) SWNTs, (B) PL-PEG2000, and (C) SWNTs functionalized with PL-PEG2000 by ultrasonication for 1 h. For clarity, we deleted from the spectrum the peaks with *m*/*z* below 800. The insets represent magnifications of parts of the spectra.

functionalizations of SWNTs by PL-PEG2000 using an Applied Biosystems 4700 Proteomics Analyzer (Framingham, MA). The mass spectrum for SWNTs alone had peaks with mass-charge ratio (*m*/*z*) at the lower end of the spectrum, which is distinctive of CNTs[28] (Figure 1A). The mass spectrum of PL-PEG2000 also had the expected symmetric spectrum that is distinctive of a polydisperse polymer with peaks in the *m*/*z* range of 2335 to 3301 (Figure 1B). The difference between adjacent peaks (Δm) of 44 Da corresponds to the mass unit difference between adjacent oligomers (Figure 1B). In contrast, the mass spectrum for SWNTs functionalized with PL-PEG2000 displayed several peaks with *m*/*z* ranging from 800 to 1888, which is not what we expected (Figure 1C). Closer examination revealed at least two overlapping sets of peaks; each set has a Δm of 44 Da as indicated in Figure 1C by solid lines for one set and broken lines for the second set. This indicated that the SWNTs actually were functionalized with PL-PEG; however the lower *m*/*z* peaks indicated the possibility of fragmentation of PL-PEG2000 by ultrasonication. To evaluate this possibility, we ultrasonicated PL-PEG2000 in PBS for 10 and 30 min then examined its mass spectrum, which revealed that 10 min ultrasonication of PL-PEG2000 did not change its mass spectrum (Figure 2A compared to Figure 1B), whereas 30 min ultrasonication caused a shift of peaks to the left with *m*/*z* ranging from 766 to 2642 (Figure 2B).

On the basis of these findings, we hypothesized that functionalization of SWNTs with PL-PEG2000 by ultrasonication causes fragmentation of PL-PEG in a time-dependent manner, which interferes with their ability to block nonspecific uptake of SWNTs by cells. To evaluate this, we tested the cellular uptake of functionalized SWNTs via ultrasonication with fluorescently labeled PL-PEG2000 for either 10 min or 1 h by an ovarian cancer cell line, SKOV-3. PL-PEG2000 (150 μL) labeled with carboxyfluorescein (PL-PEG2000-fluor) in chloroform was dried by nitrogen gas, followed by 10 min vacuum, and was resuspended by adding 500 μL of 1 mg/mL unlabeled PL-PEG2000. The amount of fluorescein on PL-PEG2000 was 0.8:1 (mole/mole), as quantitated by optical absorbance measurement at 488 nm. The fluorescein moiety on PL-PEG2000-fluor is at the terminus that extends into the aqueous solution, and thus fragmentation of PL-PEG2000-fluor could lead to loss of fluorescein and reduction of fluorescence on functionalized SWNTs. However, examination of the absorbance spectrum

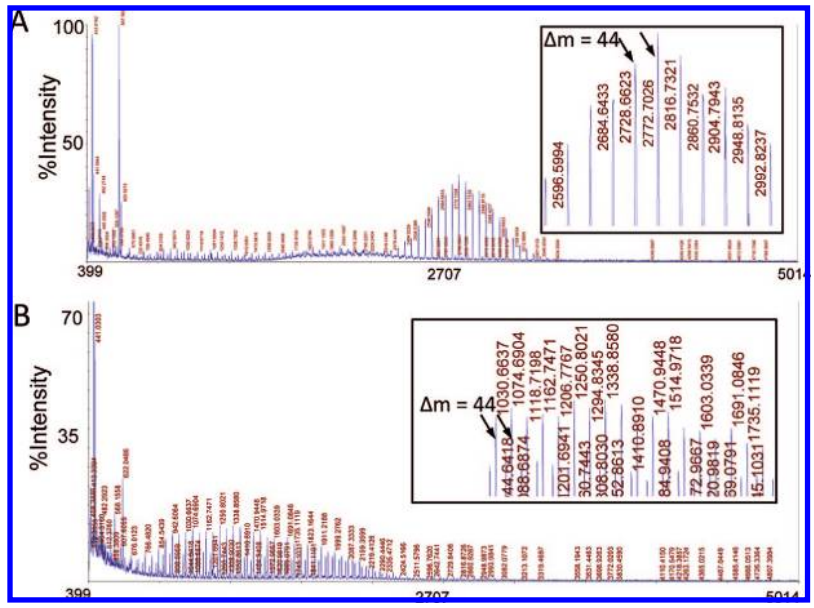


Figure 2. Examination of fragmentation of PL-PEG2000 by ultrasonication using MALDI mass spectrometry. Mass spectrum for (A) PL-PEG2000 ultrasonicated for 10 min and (B) PL-PEG2000 ultrasonicated for 30 min. The insets represent magnifications of parts of the spectra.

revealed that SWNTs functionalized with PL-PEG2000-fluor by ultrasonication for 10 min or 1 h, after removing excess PL-PEG2000 as described in Supporting Information, displayed comparable peaks for fluorescein absorbance at ~488 nm (Figure 3A). Thus, although ultrasonication fragmented the adsorbed PL-PEG, a fluorescein peak was still detected on SWNTs. This can be explained by either that some PL-PEG2000-fluor remained intact, or some of the fragments of PL-PEG2000-fluor that had the fluorescein moiety got readsorbed to SWNTs during the ultrasonication and incubation periods with PL-PEG2000-fluor. The latter case is possible because adsorption of PL-PEG was reported to cause incomplete coverage of SWNTs surface,[14] which allows for further molecular interactions with SWNTs' surfaces. Furthermore, since we demonstrated that the chains of PL-PEG got fragmented by ultrasonication, then this interferes with their ability to block nonspecific binding of molecules with SWNTs' sidewalls. In this case, the adsorption of fragments carrying fluorescein is probably through $\pi-\pi$ stacking as a result of interactions of fluorescein with SWNTs as previously reported.[24]

Evaluation of cellular uptake of functionalized SWNTs was carried out by confocal microscopy using a Zeiss LSM 510-META confocal microscope (Carl Zeiss Micro Imaging Inc., Thornwood, NY), which revealed that PL-PEG2000 ultrasonicated with SWNTs for 10 min blocked the uptake of SWNTs by SKOV-3 cancer cells after 24 h incubation (Figure 3B), whereas the PL-PEG ultrasonicated with SWNTs for 1 h did not block the uptake of SWNTs (Figure 3C) as fluorescent SWNTs were detected inside cells. Similar results were obtained with another ovarian cancer cell line, OVCA 433 (data not shown). This confirmed our hypothesis that the fragmentation by long time (1 h) ultrasonication of PL-PEG2000 prevented its ability to block nonspecific uptake of SWNTs by cells. On the basis of these findings, we continued functionalizing SWNTs with PL-PEG2000 by ultrasonication for only 10 min followed by overnight incubation.

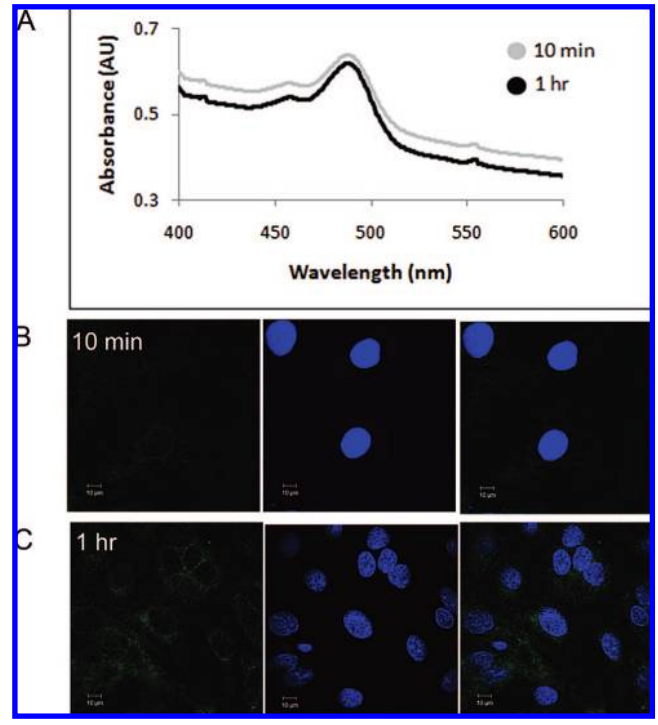


Figure 3. Fragmentation of PL-PEG2000 by ultrasonication prevents its ability to block nonspecific uptake of SWNTs by SKOV-3 cancer cells. (A) Absorbance spectrum of SWNTs functionalized with PL-PEG2000-fluor by ultrasonication for 10 min (gray trace) or 1 h (black trace) after removing excess PL-PEG. (B,C) Confocal microscopy images for SKOV-3 cancer cells after incubating for 24 h with SWNTs that were functionalized by PL-PEG2000-fluor via ultrasonication for either 10 min (B) or 1 h (C). Blue represents DAPI (4′,6-diamidino-2-phenylindole) staining for nucleus. Green is fluorescein on PL-PEG-fluorescein-functionalized SWNTs. The scale bar = 10 μm.

Since we successfully blocked the uptake of SWNTs with unfragmented PL-PEG2000, we aimed at evaluating specific targeting of different types of high affinity receptors on cancer cells. For that reason, we studied two receptors that belong to two different classes, as one is GPI-anchored to the cell membrane, and the second one has one transmembrane domain. The first receptor was folate receptor α (FRα), a 38 kDa GPI-anchored membrane glycoprotein that is detected at high frequency in ovarian cancer (90%).[29] FRα has a limited distribution to the kidneys, lungs, choroid plexus, and placenta[30] where the receptors in these tissues, except the placenta, are localized in the apical membrane facing away from the blood (toward urine and airway). This makes FRα inaccessible to folate conjugates administered intravenously and intraperitoneally. However, cancer cells lose this polarity, so FRα is expressed all over the cell surface and at high levels, which make FRα accessible intravenously and intraperitoneally. Thus, FRα is a good target for selective cancer therapy because it is a common tumor marker expressed at high levels within cancers of epithelial origins, such as ovarian cancer and by activated macrophages that are tumor associated and secrete cytokines and angiogenic factors that promote tumor growth.[31] FRα has a high affinity for folic acid (Kd ∼ 1 nM).[30] Other folate derivatives are transported into cells via another receptor, that is the reduced folate carrier (RFC), which is ubiquitously present in all cells; nevertheless this carrier has a low affinity for folic acid (Km ∼ 100−400 μM).[30] The proof of the principal of targeting cancer cells expressing FRα was demonstrated with SWNTs coated by folate-conjugated PL-PEG, where only the cancer cells that expressed FRα internalized the SWNTs, but not other cells.[32,33]

To evaluate the role of PL-PEG in specific targeting of FRα, we used folic acid that is conjugated to PL-PEG2000 (PL-PEG2000-folate) mixed with PL-PEG2000 and PL-PEG2000-fluor to functionalize SWNTs. The amount of folic acid on PL-PEG2000 was 1:1 (mole/mole), as quantitated by optical absorbance and extinction coefficient at 270 nm. As mentioned above, FRα is a high affinity receptor for folic acid, and there is a lower affinity receptor for folic acid, which is RFC. We examined the levels of these two receptors in seven ovarian cancer cell lines, but we are presenting results for two of them, OVCA 433 and SKOV-3. We found that the level of FRα was higher in SKOV-3 cells than other cells as detected by flow cytometry using a FACScan flow cytometer (Becton-Dickinson, Sunnyvale, CA) (Figure 4A). On the other hand, immunoblot analysis revealed that the level of RFC in both cell lines is similar (Figure 4B). On the basis of that, we chose SKOV-3 cells as FRα(+) cells for testing the targeting of FRα with folic acid- and PL-PEG-functionalized SWNTs. In addition, we evaluated SWNTs that were functionalized with PL-PEG alone or fluorescein alone. For that PL-PEG2000-fluor was dried as described above, then resuspended in a mixture of 167 μL of 1 mg/mL of PL-PEG2000-folate in PBS and 333 μL of 1 mg/mL of PL-PEG2000 in PBS. SWNTs were functionalized by ultrasonication for 10 min with the PL-PEG2000 mixture as described in Supporting Information. In addition, SWNTs

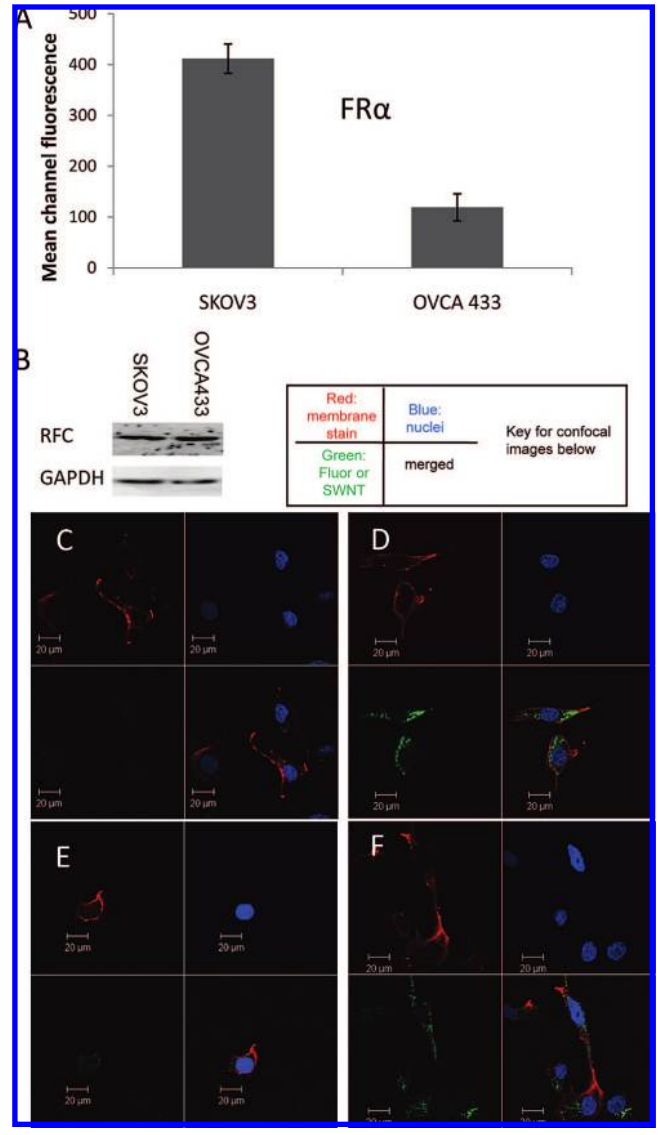


Figure 4. Specific uptake of folate-functionalized SWNTs by FRα(+) cells. (A) Expression level of FRα in ovarian cancer cell lines OVCA 433 and SKOV-3 as detected by flow cytometry. The mean channel fluorescence represents values obtained for three independent experiments ± SD. (B) Immunoblot analysis for RFC in both cell lines with GAPDH as an internal loading control. (C−F) Confocal microscopy images of SKOV-3 cells that express FRα serum-deprived for 24 h, and folic acid-deprived for 4 h followed by incubation for 24 h with (C) no treatment of cells, (D) SWNT-Fluorescein without PEG, (E) SWNT-PL-PEG2000-Fluor, and (F) SWNT-PL-PEG2000-folate. Blue represents DAPI staining for nucleus. Green is fluorescein on SWNTs. Red is a membrane stain CM-DiI. The scale bar = 20 μm.

were functionalized with fluorescein without any PL-PEG by ultrasonication for 1 h, which causes adsorption of fluorescein to SWNTs' surfaces through $\pi-\pi$ stacking.[24]

The FRα-expressing (FRα(+)), SKOV-3 cells were incubated with 20 μL of 6 μg/mL of SWNTs functionalized with either fluorescein alone without PL-PEG2000, PL-PEG2000 (with PL-PEG2000-fluor), or PL-PEG2000-folate (with PL-PEG2000-fluor and PL-PEG2000). The cells were stained with a lipophilic carbocyanine membrane probe, CM-DiI to visualize the cell membrane. The SWNTs that were functionalized with fluorescein alone were taken up by cells (Figure 4D), whereas coating SWNTs with PL-

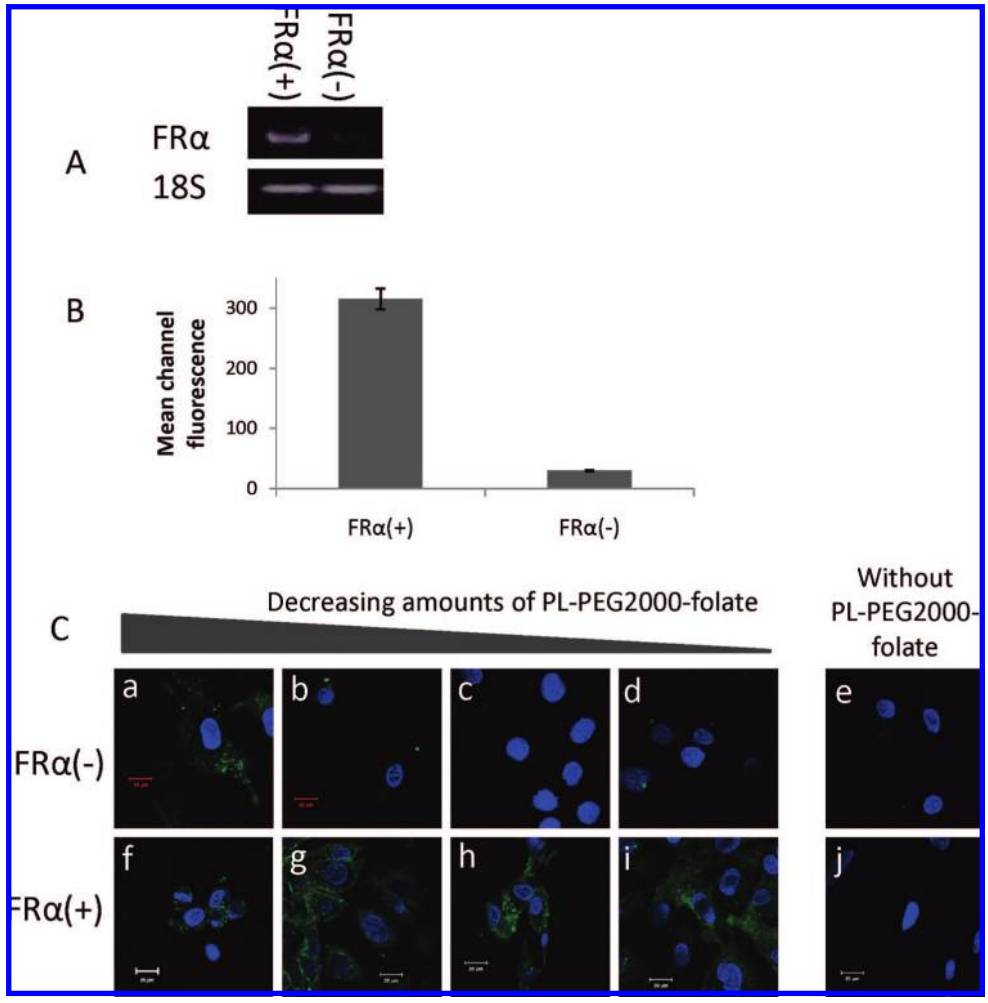


Figure 5. Specific uptake of folic acid-functionalized SWNTs by FRα(+) cells but not FRα(−) cells. (A) RT-PCR for FRα in parental cell line SKOV-3 (FRα(+)) and cells with knocked-down expression of FRα (FRα(−)) with 18S as internal control. (B) Flow cytometry confirming knock-down of FRα. The mean channel fluorescence represents values obtained for three independent experiments ± SD. (C) Confocal microscopy images for FRα(+) cells and FRα(−) cells to test uptake of SWNTs functionalized with decreasing amounts of folic acid (by a factor of one third) as panels advance from left to right and with PL-PEG2000 (right panels). Cells were serum-deprived for 18 h and folic acid-deprived for 4 h before treatments. The scale bar = 20 μm.

PEG2000 blocked internalization by cells (Figure 4E). On the other hand, coating SWNTs with PL-PEG2000-folate promoted their uptake by the FRα(+) cells (Figure 4F). This indicated that PL-PEG2000 blocked uptake of SWNTs by cells; on the other hand, internalization was promoted by targeting the FRα through folate- and PL-PEG2000-functionalized SWNTs. Our finding that SWNT-fluor is taken up by cells agrees with previous reports of uptake of SWNTs functionalized by a variety of molecules, including fluorescein, in absence of PEG.[1,3,6,7,12,34] Functionalization by adsorbing PL-PEG2000 for the first time is reported to block nonspecific cellular uptake, again because it is not fragmented. On the other hand, once the nonspecificity is blocked by PL-PEG2000 ligand mediated targeting of FRα is achieved, in this case through presenting folic acid on PL-PEG2000 that is adsorbed to SWNTs.

In order to confirm the specificity of the uptake of ligand-functionalized SWNTs through FRα, we suppressed the expression of FRα in SKOV-3 cells to generate FRα-negative cells (FRα(−)). We did that by stably transfecting a predesigned commercially available FRα gene specific short hairpin RNA into SKOV-3 cells according to manufacturer's instructions (Origene, Rockville, MD). Confirmation of gene knock-down was carried out by RT-PCR to examine the level of RNA for FRα and by flow cytometry to examine the protein level of FRα. Figure 5A shows that the level of RNA expression was significantly reduced in FRα(−) cells and that the protein level of FRα in FRα(−) cells was reduced to ∼one tenth that of the parental cell line, FRα(+) (Figure 5B).

We aimed at evaluating whether suppressing FRα expression successfully eliminated the uptake of folate-functionalized SWNTs, thus confirming the specificity of the receptor-mediated uptake. However, these cells express a low affinity receptor for folic acid, that is, RFC. We expect that at high concentration of folic acid on SWNTs, folic acid will facilitate the uptake of SWNTs through both the high affinity receptor (FRα) and the low affinity receptor (RFC). However, as the concentration of folic acid is reduced, the uptake of SWNTs will be only through FRα. In order to test this, we functionalized SWNTs with different amounts of PL-PEG2000-folate in order to examine the possibility of

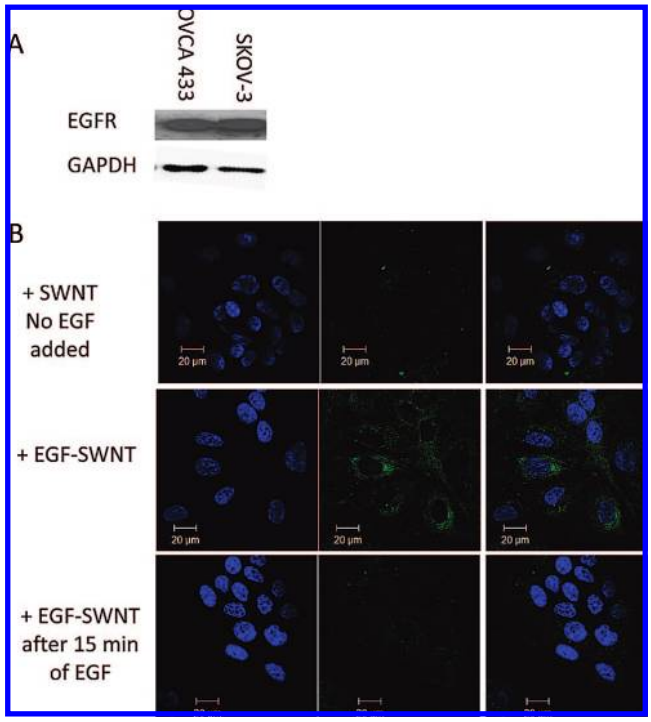
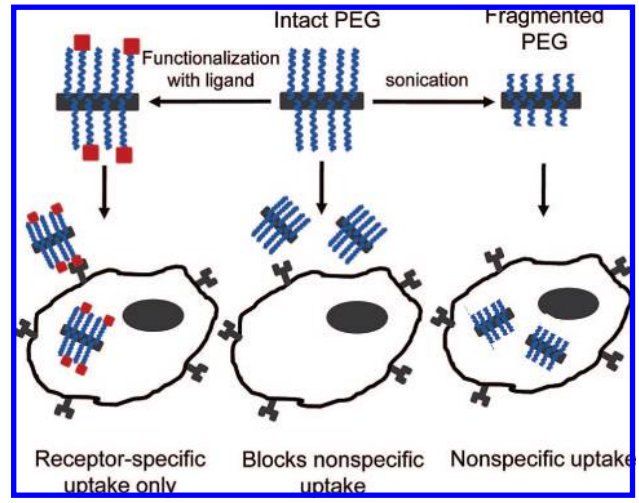


Figure 6. Specific uptake of EGF-functionalized SWNTs by EGFR-expressing cells. (A) Immunoblot analysis for EGFR protein level in OVCA 433 and SKOV-3 cells with detecting GAPDH as an internal loading control. (B) Confocal microscopy images of OVCA433 cells that express EGFR. OVCA 433 cells were serum deprived for 24 h then incubated with one of the following: top panels, SWNTs functionalized with PL-PEG2000 only and in absence of EGF; middle panels, SWNTs functionalized with EGF and PL-PEG2000; and bottom panels, SWNTs functionalized with EGF and PL-PEG2000 after incubating cells with 20 nM EGF for 15 min. The scale bar = 20 μm.

nonspecific uptake due to RFC expression by these cells. We decreased the amounts of PL-PEG2000-folate by a factor of one third by diluting it with unconjugated PL-PEG2000. Thus, the total volume of 500 μL of PL-PEG2000 was reduced by an equal volume for added PL-PEG2000-folate of 500, 167, 56, or 19 μL. We found that only the highest amount of folic acid on SWNTs caused nonspecific uptake of nanotubes (Figure 5C-a), whereas the lower amounts of folate-functionalized SWNTs (Figure 5C-b−d) did not promote their uptake by FRα(−) cells as compared with the blocked uptake of SWNTs functionalized with PL-PEG2000-fluor without folate (Figure 5C-e). In contrast, all the amounts tested promoted uptake of folate-functionalized SWNTs by FRα(+) cells, which is expected because these cells express FRα (Figure 5C-f−i versus blocked uptake Figure 5C-j). Our findings indicate that the expression of the low affinity receptor RFC by cells promoted uptake of folate-functionalized SWNTs when higher amounts of folic acid were used. However, when lower amounts of folic acid were used, we established specific uptake through FRα, and suppressing its expression blocked the uptake of SWNTs functionalized by folic acid. On the basis of our findings, it is desirable to titrate down the amount of folic acid on SWNTs so as to promote specific uptake through the tumor marker FRα.

Scheme 1. Scheme on the Role of PEG in Promoting Specific Cellular Uptake[a]



[a] The middle shows SWNTs functionalized by unfragmented PL-PEG, which blocks nonspecific uptake of SWNTs by cells. The right shows SWNTs functionalized by fragmented PL-PEG as a result of 1 hr ultrasonication, which does not block cellular uptake of SWNTs. The left shows SWNTs functionalized by a ligand in addition to unfragmented PL-PEG, which promotes specific uptake by receptor-expressing cells.

To confirm our ability of specifically targeting high affinity receptors on cancer cells, we evaluated a different type of receptor that has one transmembrane domain and is often overexpressed or activated in cancers. That receptor is epidermal growth factor receptor (EGFR), and its ligand that we used here is EGF, which has a MW $\sim$ 6 kDa.

SWNTs were functionalized via adsorption by incubating with a mixture of 170 μL of 200 μg/mL EGF in PBS and 330 μL of PL-PEG2000 (containing PL-PEG2000-fluor). Evaluation of the specific EGFR targeting by EGF on SWNTs revealed that coating SWNTs with EGF combined with PL-PEG2000 promoted their specific uptake by OVCA 433 cells that express EGFR (Figure 6A,B middle panels), while coating with PL-PEG2000 blocked the nonspecific uptake of SWNTs (Figure 6B top panels). Adding EGF to cells 15 min before adding ligand-functionalized SWNTs inhibited the uptake of EGF-functionalized SWNTs (Figure 6B bottom panels). This is due to down-regulation of EGFR by its ligand, EGF, as it leads to directing EGFR to intracellular endosomes.[35] Thus, when EGF-functionalized SWNTs were added next, the level of EGFR on the surface was already significantly reduced so that no uptake of SWNTs was detectable. This was a second example of successful specific targeting of a high affinity receptor on cancer cells by employing its ligand while blocking nonspecific cellular uptake with unfragmented PL-PEG2000.

Our study demonstrated that although dispersion of SWNTs in various solvents/solutions involves ultrasonication of SWNTs, in the case of PL-PEG this is not advisable because longer-time ultrasonication fragments PEG. A similar finding was recently reported for high MW PEG ($\sim$6000 Da).[36] This outcome of ultrasonication, that is, fragmentation of PEG, explains the controversial findings about the ability of PL-PEG2000 and PL-PEG5000 to block nonspecific cellular uptake.[21−24] Similar implications may be

drawn to other molecules such as nucleic acids and other polymers that are used to functionalize carbon nanotubes by the same method.

Scheme 1 summarizes our findings, where SWNTs functionalized by ultrasonication for 1 h with PL-PEG2000 were taken up by cells due to fragmentation of PL-PEG caused by the 1 h ultrasonication (right side of Scheme 1). On the contrary, 10 min ultrasonication was not sufficient to fragment PL-PEG2000, so it blocked internalization of functionalized SWNTs (middle of Scheme 1). We also demonstrated that functionalizing SWNTs with a ligand in addition to PL-PEG led to specific targeting of high affinity receptors that are expressed on cancer cells such as FRα and EGFR (left side of Scheme 1). On the basis of our findings, ultrasonication of SWNTs with PL-PEG should be avoided as that fragments PL-PEG. Instead PL-PEG should be either ultrasonicated with SWNTs for a short time or chemically conjugated to SWNT without ultrasonication.

In summary, the integrity of PEG is important to promoting specific cellular uptake of ligand-functionalized SWNTs. Blocking nonspecific internalization is achieved only if PEG is not fragmented, which is important for effective targeting of disease cells by SWNTs while being used for drug delivery.

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Supporting Information Available: Description of the material. This material is available free of charge via the Internet at http://pubs.acs.org.

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