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Multifunctional Polymer-Coated Carbon Nanotubes for Safe Drug Delivery

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Abstract

Though progress in the use carbon nanotubes in medicine has been most encouraging for therapeutic and diagnostic applications, any translational success must involve overcoming the toxicological and surface functionalization challenges inherent in the use of such nanotubes.

Ideally, a carbon nanotube-based drug delivery system would exhibit low toxicity, sustained drug release, and persist in circulation without aggregation. We report a carbon nanotube (CNT) coated with a biocompatible block-co-polymer composed of poly(lactide)-poly(ethylene glycol) (PLA-PEG) to reduce short-term and long-term toxicity, sustain drug release of paclitaxel (PTX), and prevent aggregation. The copolymer coating on the surface of CNTs significantly reduces *in vitro* toxicity in human umbilical vein endothelial cells (HUVEC) and U-87 glioblastoma cells. Moreover, coating reduces *in vitro* inflammatory response in rat lung epithelial cells. Compared to non-coated CNTs, *in vivo* studies show no long-term inflammatory response with CNT coated with PLA-PEG (CLP) and the surface coating significantly decreases acute toxicity by doubling the maximum tolerated dose in mice. Using polymer coatings, we can encapsulate PTX and release over one week to increase the therapeutic efficacy compared to free drugs. *In vivo* biodistribution and histology studies suggests a lower degree of aggregation in tissues in that CLP accumulate more in the brain and less in the spleen than the CNT-PLA (CL) formulation.

Graphical Abstract

Mult-walled carbon nanotubes are coated with poly(lactide)-poly(ethylene glycol). This coating imparts aqueous solubility thereby improving the toxicological profile of carbon nanotubes. Moreover, the amphiphilic coating enables the ability to load hydrophobic anticancer drugs into the inner PLA layer, making this nanostructure suitable for drug delivery.

Carbon nanotubes coated with PLA-PEG for the controlled delivery of anticancer drugs

Keywords

carbon nanotubes; controlled release; drug delivery; nanomedicine; polymer coating

1. Introduction

Current challenges in chemotherapy include the non-ideal biodistribution of drugs, the limited dose due to systemic toxicity, and excessive dosing regimen required because of rapid drug clearance. These challenges can be overcome with nano-sized drug delivery vehicles capable of prolonged drug release, improved circulation time, and localized delivery at the site of interest. Recent research indicates the efficacy of carbon nanotubes as drug delivery vehicles.^[1–9] In cancer nanomedicine, carbon nanotubes are currently investigated for their ability to act as drug delivery vehicles, mediators for hyperthermia, or imaging agents because they can be easily internalized into cells.^[7–14] Though the large specific surface area of CNTs provides the potential for delivering high drug doses, pristine

CNTs are limited in their applications as drug delivery vehicles in that their high hydrophobicity causes aggregation and toxicity.^[15–21]

Most approaches for nanotube drug loading utilize adsorption or covalent conjugation of drugs on the surface of CNTs.^[9, 22] Hongjie Dai's group has developed a drug delivery system whereby a phospholipid-PEG molecule is adsorbed onto the CNT surface.^[8–10, 22] Here, tumor targeting moieties or modified drugs may be either chemically conjugated to the PEG chain ends, or drugs may be adsorbed (doxorubicin) onto the nanotube surface due to π -stacking of benzene rings. Previously, Pastorin et al.^[23] directly functionalized the sidewall of CNTs with methotrexate, an anti-cancer drug, via 1,3-dipolar cycloaddition. Surface adsorption or conjugation is controlled by the available CNT surface functional groups and reactivity, however this reduces the availability of functional groups for further attachment of PEG or targeting ligands. Moreover, the surface properties and pharmacokinetics of the CNT is expected to change with surface adsorbed drugs, drugs conjugated to the surface, or drugs conjugated to PEG chain ends.^[8, 24] In a study of CNTs functionalized with PEG-PTX, the conjugation of PTX to the PEG molecule decreased the circulation half-life of CNT-PEG from 3.3 hr to 1.1 hr for CNT-PEG-PTX. Here we describe an amphiphilic copolymer, PLA-PEG, that functionalizes the CNT surface. In this system, hydrophobic PLA acts as a reservoir to sequester hydrophobic drugs and mediate their release, while hydrophilic PEG stabilizes CNTs to reduce aggregation in an aqueous solution. While current CNT-based therapies for drug delivery and imaging have shown encouraging results, the translational success of this system is expected to be facilitated by preventing aggregation due to surface properties, and improving drug loading to increase therapeutic efficacy.

2. Results and Discussion

2.1. Preparation and Characterization of CNT-PLA-PEG

The CLP drug delivery system was synthesized by using non-toxic organocatalyst phosphazene base P_2 -t-Bu. We eliminated the need for high temperature polymerization methods and potential toxicity from tin-based catalysts.^[27–29] PLA was grafted on the surface of hydroxyl-functionalized MWCNT with an inner diameter of 5–15 nm, outer diameter of 30–50 nm, and lengths from 10–20 μ m. Carbon nanotubes were used as provided and were dissolved in the presence of d,l lactide in anhydrous acetonitrile. CL particles were PEGylated by adding methoxy-PEG_{5k}-isocyanate directly into the reaction. Our rationale for this approach was to utilize the high reactivity of isocyanate groups to chemically cap PLA with the PEG molecule on the surface of CNTs without using a purification step.^[30–32] The CLP particles were washed through centrifugation to remove residual monomer, PEG, and catalyst, and the amount of polymeric coating was quantified by thermogravimetric analysis under N_2 flow (Figure 1a). Pristine CNT showed no decomposition over the entire temperature range, from 30 °C to 600 °C, whereas CL exhibited a single stage of decomposition around 280 °C, revealing that approximately 60 wt.% of CL was the polymer PLA. CLP samples showed a two-stage decomposition, with PLA decomposing at approximately 280 °C and PEG decomposing at approximately 430 °C. This two-stage decomposition indicates the presence of two polymers, with

approximately 60 wt.% PLA and 20 wt.% PEG, respectively. The chemical bonds in the CNT composites were characterized via Fourier transform infrared spectroscopy (Figure 1b). The carbonyl stretch peak at 1745 cm^{-1} , present in both the CL and CLP samples, corresponds to the ester bond present in PLA. In the CLP sample there is a peak at 2881 cm^{-1} , which corresponds to the ether bond in PEG (Figure 1b, inset). ^1H NMR was taken on a Bruker Avance 300 NMR (300 MHz, CDCl_3) for CL (Figure 1c) and CLP (Figure 1d). Peaks for PLA were observed at 1.59 and 5.17 ppm. PEG had a single peak at 3.65 ppm. This data indicates the successful coating of CNT with an inner layer of PLA and an outer layer of PEG. Transmission electron microscope images of CNT (Figure 1e) and CLP (Figure 1f) show that the polymer coating was approximately 10–15 nm.

2.2. *In Vitro* Uptake, Toxicity, Inflammatory Response, and Controlled Release

To decrease the toxicity associated with CNTs, we coated them with PLA and PEG, two FDA-approved polymers. The rationale is based on controlling the surface properties of CNTs, allowing them to disperse in an aqueous solution and allowing the polymer to mediate the interactions of carbon nanomaterials with cells. By improving aqueous dispersion, the interaction of CNT with cells is reduced and aggregation of the nanoparticles in blood is subsequently reduced. Nanoparticles loaded with a hydrophobic fluorescent dye (Alexa Fluor 647) were used to study the uptake of CLP in two different cell lines: U-87 human glioblastoma cells and human umbilical vein endothelial cells (HUVEC) as a control. Cells were incubated with dye-loaded CLP for 24 hr in a chambered glass slide. Cells were washed, fixed, and stained for actin filaments and nuclei. Figure 2a and Figure 2b show the confocal images of the uptake of CLP in HUVEC and U-87 cells, respectively.

Toxicity in both HUVEC and U-87 cell lines was measured with a Presto Blue cell viability assay in which metabolically active cells reduced the non-fluorescent resazurin in the Presto Blue working solution into a red-fluorescent resorufin.^[33] Cells were treated with varying concentrations of pristine CNT or CLP, and percent cell viability was determined by normalizing the fluorescent intensity of each test group with the average intensity of cells receiving only media. CNT showed a dose dependent toxicity in both HUVEC (Figure 2c) and U-87 (Figure 2d) cell lines. A Student's t-test ($p\text{-value} < 0.05$) was performed to compare toxicity between CNT and CLP at each concentration. CNTs were significantly more toxic than CLP in HUVEC cells at all concentrations, up to $700\text{ }\mu\text{g cm}^{-2}$. U-87 cells exhibited similar results where CNTs were significantly more toxic than CLP, from $140\text{ }\mu\text{g cm}^{-2}$ to $700\text{ }\mu\text{g cm}^{-2}$. Moreover, Tukey's Honestly Significant Difference (HSD) test determined a demonstrable significance between each concentration of CNT and the 0 nM concentration (cell blank). Though pristine CNT was significantly toxic in both HUVEC and U-87 cell lines in concentrations of approximately $70\text{ }\mu\text{g cm}^{-2}$, no significant toxicity was evident in CLP using concentrations 10 times higher than pristine. Thus, coating our samples with the biocompatible PLA-PEG polymer reduces the toxicity associated with CNTs *in vitro*, which is critical for medical applications.

Inflammatory responses to CNT and CLP were tested in rat lung epithelial cells *in vitro*. Expression of interleukin-6 (IL-6), a cytokine involved in the inflammatory response, and intercellular adhesion molecule-1 (ICAM-1), a cell surface molecule involved in leukocyte

extravasation, was measured following exposure to CNT and CLP at concentrations of $1 \mu\text{g cm}^{-2}$ and $10 \mu\text{g cm}^{-2}$.^[34–36] The fold difference in expression of IL-6 between CNT and CLP when normalized to the $1 \mu\text{g cm}^{-2}$ CNT group is illustrated in Figure 2e. At $10 \mu\text{g cm}^{-2}$ the expression of IL-6 for CNT was nearly three fold higher than for CLP. Moreover, ICAM-1 expression following CNT exposure at $10 \mu\text{g cm}^{-2}$ was approximately 50% higher than the CLP (Figure 2f), indicating that coating CNT with PLA-PEG significantly decreased both toxicity and inflammatory response compared to non-coated CNTs.

In a controlled release study performed to quantify the amount of PTX released from CLP (Figure 3a), we determined that CNT-PLA-PEG could load 1.65 wt.% PTX and release the drug in a controlled manner for at least one week. To carry out *in vitro* efficacy experiments, U-87 cells were incubated for 6 hours and at 24 hours with drug loaded CLP to allow for nanotube uptake and intracellular release. Three different amounts of NP were tested containing the equivalent concentrations of 70, 140, and 280 nM of PTX (Figure 3b) compared to free drug. At the end of the time point, cells were washed with sterile PBS and allowed to grow for a total of 72 hours following initial exposure to the nanoparticles. This data emphasizes the importance of rapid nanoparticle uptake and controlled release due to short circulation time of free drugs. Thus, this figure illustrates that the nanotubes are uptaken into the cells in as little as 6 hours and can mediate drug release over an extended period of time. Free paclitaxel efficacy is significantly reduced when the cells are exposed to drug for a short period of time (i.e. 6 hours). Free PTX at 140 nM incubated for 6 hours was only able to kill 12% of cells, compared to the 50% viability after 24 hours of exposure to free drug. However with the 6 hour incubation, CLP at a concentration equivalent to 70 nM of PTX was as effective as free PTX at twice the concentration (140 nM). This demonstrates that, with a shorter incubation time, CLP loaded with PXT was twice as effective as free PTX. A Tukey's HSD test showed that at 6 hr incubation with free drug or drug-loaded CLP, only CLP loaded with the equivalent of 140 nM and 280 nM were significantly different than the 0 nM control (cell blank). CLP loaded with an equivalent of 280 nM, however was as efficient after 6 hours of incubation as it was at 24 hours, perhaps due to the rapid uptake of the drug delivery vehicle saturation and the intracellular controlled release of PTX. To achieve a PTX concentration of 280 nM, a concentration of only $10 \mu\text{g cm}^{-2}$ CLP was required. Therefore, only $3 \mu\text{g}$ of CLP loaded with PTX was able to eliminate 50% of U-87 cells in a 96-well plate. These results illustrate the potential of CLP as a safe, potent mediator of drug delivery and cancer therapy using low doses of CNTs.

2.3. *In Vivo* Biodistribution in Mice

Biodistribution studies in Balb/c mice showed clearance of fluorescently loaded CLP through the kidneys and bladder, as well as through the reticuloendothelial system, i.e. the liver and spleen (Figure 4). NPs loaded with a fluorescent dye Alexa Fluor 647 were administered via tail vein injection at a dose of 10 mg kg^{-1} to enable live animal imaging with an IVIS Lumina XR small animal imaging system. Animals were imaged at 5 minutes, 30 minutes, and 24 hours post injection (Figure 4a), and fluorescent images show fluorescent signal from CLP in the bladder. Kostarelos et al.^[20, 37–40] determined that dispersed single-walled CNTs are cleared through the kidneys due to the alignment of thin diameter tubes with the pores of the glomerulus, approximately 5–20 nm in diameter.^[37, 38]

Our results suggest that some of the CLP nanotubes may be cleared via this mechanism while the majority of tubes are cleared via the liver and spleen similar to other nanomaterial drug delivery systems. Mice were euthanized and organs were explanted for fluorescent imaging after 24 hours. Figure 4b shows representative images of the fluorescent signal in the spleen (iii) and liver (iv) from three repetitions. In addition to fluorescent biodistribution studies, mice were injected with saline, pristine CNT, CL, and CLP to analyze tissues using histology after 24 hours. Figure 5 shows the representative haematoxylin and eosin (H&E) staining of the liver, spleen, lung, and brain. Bright field microscope images show the accumulation of CNT aggregates in the lungs, liver, and spleen, with CL aggregates also in the liver, lung, and brain. In contrast, though CLP particles were found in the lungs and brain, the images suggest no CLP aggregation.

2.4. *In Vivo* Inflammatory Response and Toxicity

Inflammatory response in C57BL/6 mice was monitored following the pulmonary instillation of both CNT and CLP. Lung tissue was collected 30 days after pulmonary instillation and stained with Masson's trichrome (Figure 6a–c). Figure 6b shows that mice instilled with CNT clearly had aggregates throughout the lung tissue, with some inflammation, slight granuloma formation around the CNT aggregates, and a small amount of collagen deposition. No CNTs were visible with the CLP group when the entire lung was analyzed with histology (Figure 6c). Thus, the CLP particles effectively reduced the inflammation following instillation in the lungs.

The inflammatory response was further investigated following pulmonary instillation in mice via bronchoalveolar lavage (BAL). Though macrophages collected from BAL were shown to uptake pristine CNT (Figure 6e), none containing CLP were found following pulmonary instillation. Figure 6g shows the percent of alveolar macrophages containing CNTs, comparing pristine CNT to PLA-PEG coated CNT. The results demonstrate that coating CNT in PLA-PEG can mitigate the inflammatory response associated with CNTs, possibly due to decreased NP aggregation and by imparting a hydrophilic superficial coating. The polymer coating also increased the CNTs maximum tolerated dose. In a dose escalation study, Balb/c mice were injected intravenously via the tail vein with increasing amounts of either pristine CNT or CLP. Five mice were injected at each concentration. At 25 mg kg⁻¹, 85% of mice injected with pristine CNT expired in less than two minutes. However, all CLP mice injected at 25 mg kg⁻¹ (data not shown) and 40 mg kg⁻¹ survived. Importantly, 60% of mice injected with CLP at 50 mg kg⁻¹ survived. Figure 6h shows a Kaplan-Meier survival curve of mice injected with pristine CNT or CLP. Coating CNT with PLA-PEG reduces acute toxicity associated with intravenous administration of CNTs, possibly due to decreasing particle aggregation and thus decreasing capillary blockage and NP accumulation in the lungs as shown with histology analysis.^[21, 41–43] Moreover the maximum tolerated dose of coated CNT was twice greater compared to the pristine CNT.

3. Conclusions

In this work we showed the critical aspects of both hydrophobic and hydrophilic polymer coating on the surface of CNTs to reduce toxicity and increase therapeutic efficacy. Here, the

PLA layer serves to encapsulate and mediate the controlled release of hydrophobic drugs, while the PEG layer serves to improve aqueous solubility and improve pharmacokinetic properties. This CLP drug delivery system was significantly less toxic in both *in vitro* and *in vivo* when compared to pristine CNTs with a maximum tolerated dose of approximately 50 mg kg⁻¹ and 25 mg kg⁻¹, respectively. Moreover, PTX-loaded CLP at equivalent concentrations of 70 nM PTX was as effective as free PTX incubated for 6 hr at 140 nM. Though our approach now facilitates novel methods for safely using a CNT-based controlled release drug delivery system, more advanced studies for cancer therapy efficacy and pharmacokinetics studies are required to fully understand the potential of CNT drug delivery systems.

4. Experimental

Synthesis of CNT-PLA-PEG

Carbon nanotubes were covalently coated with PLA using a room temperature, ring-opening polymerization. Prior to each reaction, reagents were dried under vacuum at 32 in Hg. In a typical reaction, 40 mg of MWCNT-OH (Sun Innovations, Fremont, CA, USA) and varying amounts of D,L lactide (Purac Biomaterials, Lincolnshire, IL, USA) were dissolved in 4 ml of anhydrous acetonitrile for 30 minutes under N₂. Phosphazene base P₂-t-Bu (2M in tetrahydrofuran) (Sigma-Aldrich) was added as a catalyst. The reaction was then continued under nitrogen gas. Next, methoxy-PEG-isocyanate (Nanocs Inc, New York, NY, USA) was dissolved in 2 ml of solvent and added directly to the reaction mixture. The reaction then proceeded under nitrogen and was washed via centrifugation. The product was lyophilized and stored at -20 °C under nitrogen. NMR was performed with a Bruker Avance 300 and ATR FT-IR was performed with a Thermo-Nicolet Magna 550 equipped with a Thermo-SpectraTech Foundation series Endurance Diamond ATR. ¹H-NMR (300 MHz, CDCl₃, δ): 7.26 (s, CDCl₃), 5.17 (q, -C(=O)-CH(CH₃)-), 3.65 (s, -CH₂CH₂-O-), 1.59 (d, -CH(CH₃)-); IR 2881 cm⁻¹ (-CH₂-CH₂-O-), 1745 cm⁻¹ (C=O). Thermogravimetric analysis was performed on a TA Instruments Hi-Res TGA 2950 thermogravimetric analyzer under nitrogen from 25 °C to 600 °C at 20 °C min⁻¹. Transmission electron microscope images were taken on a Hitachi HD2000 at 200 kV.

Cell Culture

Human umbilical vein endothelial cells (Lonza, Walkersville, MD, USA) were grown on collagen-1 coated flasks (Thermo Scientific, Rockford, IL, USA) in endothelial growth media (Lonza, Walkersville, MD, USA). U-87 glioblastoma cells (American Type Culture Collection, Manassas, VA, USA) were grown on CellBind-treated flasks (Corning, Tewksbury, MA, USA) in EMEM (American Type Culture Collection) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA). Cells were subcultured at 37°C and 5% CO₂. Rat lung epithelial cells RLE-6TN (American Type Culture Collection) were grown in Ham's F12 medium with 2 mM L-glutamine supplemented with 0.01 mg ml⁻¹ bovine pituitary extract, 0.005 mg ml⁻¹ insulin, 2.5 ng ml⁻¹ insulin-like growth factor, 0.00125 mg ml⁻¹ transferrin, 2.5 ng ml⁻¹ epidermal growth factor, 1000 U ml⁻¹ penicillin-G, 100 µg ml⁻¹ streptomycin, and 10% fetal bovine serum.

CNT-PLA-PEG Uptake In Vitro

CLP uptake in HUVEC and U-87 cells was determined via confocal microscopy. Cells were seeded at 1,200 cells per chamber in a Lab-Tek 8-chambered slide (Thermo Scientific). Nanoparticles were loaded with Alexa Fluor 647 cadaverine (Life Technologies, Grand Island, NY, USA), a fluorescent dye, by dissolving Alexa Fluor 647 (AF647) in a 50/50 dimethylformamide/acetonitrile (DMF/ACN) solution. CLP was dissolved in the AF647 solution on a rotisserie. Next, fluorescent dye-loaded CLP solution was prepared by solvent evaporation in HyPure water (Thermo Scientific). Samples were washed via centrifugation twice in HyPure water and once in sterile phosphate buffered saline (Sigma-Aldrich). Samples were re-suspended at 0.25 mg ml^{-1} in cell culture media and added to cells. After 24 hours, media was removed and cells were washed twice with sterile phosphate buffered saline (PBS). Cells were fixed with a 4% formaldehyde solution in PBS for 30 minutes, and then washed with PBS. Cells were incubated with a 0.1% Triton-X solution for 2 minutes, washed with PBS, and stained with Alexa Fluor 488 phalloidin. Finally, cells were mounted with VectaShield fluorescent mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). Slide chambers were removed and the slide was cover slipped. Confocal microscope images were taken with a Nikon Eclipse Ti confocal microscope.

Paclitaxel Release from CNT-PLA-PEG

Release of PTX from CLP was measured using high performance liquid chromatography (HPLC). Paclitaxel (LC Laboratories, Woburn, MA, USA) was encapsulated by dissolving CLP and PTX in ACN and rotating on a rotisserie protected from light. The CLP/PTX solution was then dropped in HyPure deionized water and PTX was encapsulated via solvent evaporation. Samples were washed three times in HyPure water via centrifugation. CLP/PTX was re-dispersed in HyPure water and added to the top of a 3.5 kD Slide-a-Lyzer MINI dialysis unit (Thermo Scientific). Each dialysis unit was placed in a vial with HyPure deionized water. Five repeats were used in this release study and samples were placed in an incubator at 37°C and 5% CO_2 for the duration of the study. At each time point dialysate was removed and frozen, and fresh HyPure water was replaced. Lyophilized aliquots were analyzed via HPLC on a Waters 1525 Binary HPLC pump with a 2998 photodiode array detector. An Alltima C18 column (Grace, Deerfield, IL, USA) was used that was $4.6 \times 25 \text{ mm}$ with $5 \mu\text{m}$ pores. Samples and standards were dissolved in acetonitrile. The mobile phase used was 60% acetonitrile and 40% water. The mobile phase flow rate was 1 ml min^{-1} and PTX was detected at a wavelength of 227 nm with an average elution time of 11.5 minutes.

In Vitro Inflammation and Toxicity Studies

RLE-6TN cells were plated at 100,000 cells per well in a 12-well plate (Corning) and incubated at 37°C and 5% CO_2 for 24 hours. Media was exchanged and returned to temperature before being dosed with NP at 1 or $10 \mu\text{g cm}^{-2}$ for 6 hours. Media was then removed and cells were lysed by Trizol (Life Technologies) and stored at -80°C . RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA, USA) and quantitated on a Nanodrop (Thermo Scientific). Reverse transcription of $1 \mu\text{g}$ of RNA was performed using a QuantiTect reverse transcription kit (Qiagen). QuantiTect primer assays (Qiagen) and SYBR

green master mix (Qiagen) were utilized for Quantitative real-time PCR to examine mRNA expression levels of IL1 β , IL-6, ICAM1 in RLE cells. Cycle threshold (Ct) values and internal reference cDNA levels for the target genes were determined by an Applied Biosystems StepOnePlus Real-Time PCR System (ABI). The cDNA levels of each treatment were then normalized to GAPDH, used as an internal reference, by the equation $Ct_{\text{target}} - Ct_{\text{gapdh}}$. The relative expression of each target was then calculated by $2^{[-Ct]}$, where Ct is defined as $Ct_{\text{treatment}} - Ct_{\text{control}}$.

HUVEC and U-87 cells were seeded at 10,000 cells per well in a 96-well plate (Corning) with five repeats per test concentration. CNT and CLP were prepared by solvent evaporation in HyPure water. Solutions were stirred for 2 hours and washed by centrifugation, and nanoparticles were then re-dispersed in media and added to cells at different concentrations. Cells were incubated with the NP for 24 hours at 37°C and 5% CO₂. Next, NP media was removed and cells were gently washed twice with PBS. A Presto Blue cell viability assay (Life Technologies) was performed by mixing Presto Blue reagent at a 1:9 ratio with cell culture media. 100 μ l of working solution was added to the cells and incubated protected from light for 45 minutes at 37°C and 5% CO₂. Fluorescent intensity was measured with a BioTek Synergy 4 plate reader at an excitation wavelength of 560 nm and emission wavelength of 590 nm. Cell viability was determined by normalizing fluorescent intensity to the average intensity of five wells of cells only treated with media.

Efficacy studies of PTX-loaded CLP were performed using U-87 cells, in which cells were seeded at 10,000 cells per well in a 96-well plate. CLP was loaded with PTX as previously described and CLP/PTX was added to cells at 2.5, 5, and 10 μ g cm⁻². Estimated PTX concentrations, based upon the release data were 70, 140, and 280 nM, respectively. Cells were incubated for 6 hours or 24 hours, and washed twice with sterile PBS. Fresh media was added to cells and they were grown for a total of 72 hours after initial exposure to NP. Cell viability was measured with a Presto Blue assay.

In Vivo Biodistribution Studies

Animals were housed at Clemson University's Godley-Snell Research Center or within the Department of Comparative Medicine at East Carolina University. All studies were done in accordance with Clemson and East Carolina University Institutional Animal Care and Use Committee (IACUC) approved protocols. CL or CLP was loaded with AF 647 as previously described. The final wash in the centrifugal filter unit was done in sterile PBS. Samples were re-dispersed at 1 mg ml⁻¹ in sterile PBS, and 200 μ l of NP was administered via a tail vein injection. Live animal imaging was done with an IVIS Lumina XR small animal imaging system. At the final time point of 24 hours, animals were euthanized and kidney, liver, spleen, muscle, fat, heart, lung, and brain tissue was retrieved. Tissues were fixed in 4% formaldehyde and stored protected from light at 4 °C. For tissue staining, organs were incubated overnight in a 30% sucrose in PBS solution. Next, samples were embedded in optimal cutting temperature (O.C.T.) compound (Sakura, Torrance, CA, USA) and sectioned at 10 μ m on a Microm HM 505N cryostat microtome. Sections were then stained with haematoxylin and eosin. Histology images were taken on a Nikon Labopho-2 microscope with a Sony HDR-HC9 camera.

In Vivo Inflammatory Response and Maximum Tolerated Dose

C57BL/6 mice were obtained from Jackson Labs at 4 weeks of age. Mice were instilled with 4 mg kg⁻¹ of CNT or CLP by oropharyngeal aspiration, and euthanized 30 days following aspiration of CNT or CLP. Mouse lungs underwent *in situ* bronchoalveolar lavage (BAL). The right lung was lavaged 4 times (26.25 ml kg⁻¹ body weight) with ice-cold Hank's balanced salt solution (HBSS). Bronchoalveolar lavage fluid (BALF) was collected and centrifuged at 1000 g for 10 min at 4 °C. Cell pellets were re-suspended in 1 ml HBSS and counted. In order to obtain differential cell counts, 20,000 cells from each BALF sample were centrifuged using a Cytospin IV (Shandon Scientific Ltd, Cheshire, UK) and stained with a three-step hematology stain (Richard Allan Scientific, Kalamazoo, MI). Cell differential counts were determined and imaged for presence of CNTs or CLPs. Left lungs from CNT or CLP treated mice were perfused with 10% neutral buffered formalin fixative and stored for 24 h, then processed and embedded in paraffin. Samples were then sectioned at a thickness of 5 µm and were mounted on slides for staining with Masson's trichrome to detect morphological changes and collagen deposition.

For the maximum tolerated dose studies, NPs were prepared as previously described. After washing, NPs were re-dispersed in sterile PBS at concentrations determined by mouse body mass. NP solutions were sonicated just prior to injection due to rapid aggregation of pristine CNTs. Following injection mice were monitored for 6 hours for signs of distress or deteriorating conditions. Mouse body mass and food consumption was monitored every day for one week following injection. Subjects whose body mass went below 20% of initial body mass or who were non-responsive were euthanized immediately. All subjects were euthanized at the final time point per IACUC protocol requirements.

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Figure 1.

Chemical characterization of coated CNTs. (a) Thermogravimetric analysis (TGA) of CNT, CNT-PLA (CL), and CNT-PLA-PEG (CLP) showing the percent of polymer coating. Inorganic materials, i.e. CNT, did not vaporize below 600 °C and therefore displayed no mass change. PLA degraded at 280 °C and TGA is able to measure the percent of mass remaining, or the amount of carbon nanotube remaining, across the tested temperature range. CLP showed degradation of PLA at 280 °C and then a second degradation of PEG at 300 °C. (b) Fourier transform infrared spectroscopy of coated particles with expanded view to show the corresponding peaks for PEG at 2881 cm⁻¹ (inset). (c, d) ¹H nuclear magnetic resonance (NMR) spectra for CL and CLP, with an inset of the polymer structure of PLA-PEG. Letters a, b, and c in red indicate which hydrogen protons correspond with the peaks in the NMR

spectra. (e, f) Transmission electron microscope image of CNT (e) and CNT-PLA-PEG (f) showing a 10–15 nm thick polymer coating. Scale bars represent 150 nm.

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Figure 2.

In vitro studies illustrated the uptake, toxicity, and inflammatory response of CNT-PLA-PEG (CLP). (a, b) Confocal microscopy images show the uptake of fluorescently-loaded CLP particles (red) in HUVEC and U-87 cells stained for actin filaments (green) and cell nuclei (blue). Scale bars represent 50 μm . (c, d) Toxicity studies of CNT and CLP in HUVEC and U-87 cells showed that polymer coating decreased NP toxicity. * indicates significant difference between each sample and the 0 nM concentration, determined by Tukey's HSD test (e, f) Polymer coating on CNT also decreased expression of inflammatory cytokines IL-6 and ICAM-1.

Figure 3.

(a) Controlled release of paclitaxel (PTX) from CNT-PLA-PEG (CLP) was monitored by high performance liquid chromatography with the release sustained over a 1-week period. (b) PTX toxicity with free drug compared to drug-loaded CLP shows that NP are more effective with shorter incubation time, apparently due to NP uptake and controlled intracellular release. * indicates significant difference between each sample and the 0 nM concentration, determined by Tukey's HSD test.

Figure 4.

(a) Representative images from three repetitions of live Balb/c mice injected intravenously with saline, fluorescently-loaded CNT-PLA (CL), or fluorescently-loaded CNT-PLA-PEG (CLP) show clearance of coated CNT through the bladder. (b) Fluorescent imaging of explanted organs after 24 hours shows accumulation of fluorescent signal in the spleen liver and brain. Organs shown are i) muscle, ii) fat, iii) spleen, iv) liver, v) kidneys, vi) heart, vii) lung, viii) brain.

Figure 5. H&E staining of mouse organs following intravenous administration of saline, CNT, CL, and CLP showed some deposits of NP in tissue. NPs are indicated with black arrows and white scale bars indicate 50 μm.

Figure 6.

C57BL/6 mice underwent either a pulmonary aspiration of saline or 2 mg kg⁻¹ CNT or CNT-PLA-PEG (CLP) (a–g). Masson's trichrome staining of lung tissue of (a) saline, (b) CNT, and (c) CLP (not easily detected in lung tissue) showed that CNT caused mild inflammation. Alveolar macrophages were collected via bronchoalveolar lavage following pulmonary instillation of NP (d–g). Arrows in (e) indicate macrophages containing CNT. No CLP particles were detected in macrophages (f, g). CNT or CLP particles were counted in macrophages retrieved through bronchoalveolar lavage, and no CLP was detected (g). Kaplan-Meier survival curves for Balb/c mice receiving intravenous tail vein injections of CNT or CLP (h) show that the polymer coating increases the maximum tolerated dose of CNT.