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Multilayered Polymer Coated Carbon Nanotubes to Deliver Dasatinib

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Abstract

Multilayered, multifunctional polymer coatings were grafted onto carbon nanotubes (CNT) using a one-pot, ring-opening polymerization in order to control the release kinetic and therapeutic efficacy of dasatinib. Biocompatible, biodegradable multilayered coatings composed of poly(glycolide) (PGA), and poly(lactide) (PLA) were polymerized directly onto hydroxyl-functionalized CNT surfaces. Sequential addition of monomers into the reaction vessel enabled multilayered coatings of PLA-PGA, or PGA-PLA. Poly(ethylene glycol) capped the polymer chain ends, resulting in a multifunctional amphiphilic coating. Multilayer polymer coatings on CNTs enabled control of anticancer dasatinib's release kinetics and enhanced the in vitro therapeutic efficacy against U-87 glioblastoma compared to monolayer polymer coatings.

Keywords

Multilayered polymer coatings; carbon nanotube; synthesis; encapsulation

Introduction

Nanoparticles have been the subject of significant research as a methodology for controlled drug delivery. Nanoparticles offer distinct advantages over conventional systemic drug delivery by localizing drug at the tumor site through passive tumor uptake or active molecular targeting, maintaining drug concentrations at the tumor via controlled release, and increasing intracellular delivery via cellular uptake. Furthermore, polymeric nanoparticles are in clinical trials as therapeutic vectors in cancer therapy.^{1, 2} However, monolayer nanoparticles generally exhibit an initial non-ideal “burst” release which limits the delivery of drug for prolonged periods.^{3, 4} Here we report multilayered polymer coated carbon nanotubes (CNT) facilitates drug loading, controls drug release, and improve therapeutic efficacy in vitro against U-87 glioblastoma. A one-pot room temperature ring-opening polymerization approach was used to functionalize carbon nanotubes (CNT) with multiple polymer layers composed of poly(glycolide) (PGA), poly(lactide) (PLA), and poly(ethylene glycol) (PEG).

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Multilayered particles offer a solution to impart better control over drug release characteristics when compared to single layer particles. Loo, Lee, and Widjaja reported multilayered microparticles composed of PGA, PLA, PLGA, poly(caprolactone) (PCL), poly(styrene) (PS), and poly(ethylene-co-vinyl acetate) (EVA) that controlled the release of the model drugs ibuprofen, metoclopramide HCl, and lidocaine.^{5–8} Multilayered particles were fabricated via an emulsion solvent evaporation method resulting in an EVA core, with PS, PLA, and PLGA shells. These particles not only altered the release kinetics, but the polymer layers exhibited spatial separation of drug loading within distinct layers. While single layered microparticles generally exhibited an initial burst release, closer to zero-order release kinetics were achieved with the use of multilayered particles. Multilayered microparticles have been previously fabricated using onestep solvent evaporation,⁷ biomimetic solution polymerization,⁹ spray drying,¹⁰ layer-by-layer assembly,¹¹ and liquid jets with acoustic disruption.^{12, 13} With CNTs as a nanoparticle template, we created a controlled drug delivery system for the anticancer drug dasatinib by polymerizing multiple polymer layers directly onto CNTs.

A variety of methods have been developed to functionalize CNTs as mechanisms for delivering drugs.^{14–17} Liu et al. functionalized CNT surfaces using a phospholipid-PEG molecule, whereby the phospholipid strongly adsorbs to the CNT surface and the PEG improves aqueous dispersion.^{15, 18, 19} Drug molecules could be further adsorbed to the CNT surface, or PEG could be modified to attach drugs. Another approach involves the direct conjugation of drug to the CNT surface.²⁰ While not yet in clinical trials, Ensyce Biosciences Inc. is investigating carbon nanotube-based systems for the delivery of siRNA.²¹ Previously, we have demonstrated that monolayer polymer coated CNTs can release paclitaxel for one week, improve therapeutic efficacy against U-87 glioblastoma compared to free drug, and reduced CNT-associated toxicity in vitro and in vivo.¹⁷ Here we investigated polymer coated CNT for the delivery of dasatinib (DAS). Nanoparticle formulations for the delivery of DAS have previously been investigated.^{22, 23} However DAS, an Src/Abl kinase inhibitor, is administered as an oral formulation.^{24–26} Solubility of DAS plays a significant role in its oral bioavailability, and the solubility of DAS is highly dependent on pH.²⁷ At an acidic pH of 2.6, DAS is soluble at approximately 18 mg/ml. This solubility drops steeply at a milder pH of 7 to <1 µg/ml. Parenteral administration of nanoparticle-drug formulations offer advantages such as avoidance of first-pass elimination, and improvement of drug bioavailability. Thus encapsulation of hydrophobic DAS into a NPDDS may enable different routes of administration, improve accumulation in tumor due to the EPR effect, and potentially improve therapeutic efficacy.

Here, we report a one-pot “grafting from” approach to sequentially add block copolymers, i.e. PGA-PLA, where the polymer coating thickness can also be controlled. Capping the ring opening polymerization with PEG improved the aqueous stability of these coated CNTs. The significance of this work is that (1) synthesis occurs via a one-pot reaction at room temperature using organocatalysts; (2) CNTs are coated with multiple layers to control drug release kinetics. To our knowledge this is the first report of polymer-grafted multilayered inorganic nanomaterials to deliver drugs to cancer cells. In Figure 1 we illustrate our approach for polymerizing multiple polymer layers onto carbon nanotubes.

Experimental

One-pot Room Temperature Ring-opening Polymerization

Hydroxyl-functionalized multi-walled carbon nanotubes (MWCNT-OH) were purchased from Sun Innovations, Inc. (Fremont, CA). D,L lactide was purchased from Purac Biomaterials (Lincolnshire, IL). Methoxy-poly(ethylene glycol)_{5k}-isocyanate (mPEG-isc) was purchased from Nanocs, Inc (New York, NY). Glycolide, phosphazene base 1-*tert*-butyl-2,2,4,4,4-pentakis(dimethylamino)-2 Λ ,⁵4 Λ ⁵-catenadi(phosphazene) (P₂-t-Bu), and all solvents were purchased from Sigma-Aldrich. MWCNT-OH was dried overnight in a vacuum oven at 32 in Hg with the first monomer, either d,l lactide or glycolide. Next, both CNT and monomer were dissolved in 4 mL of acetonitrile (ACN) by stirring for 30 min. Phosphazene base P₂-t-Bu was added as a catalyst and the reaction was continued overnight. The next day an aliquot of sample was removed, washed, and lyophilized for analysis. The second monomer was then dissolved in 2 ml of ACN and added directly into the reaction vessel. More P₂-t-Bu was added, the reaction vessel was purged with N₂, and the reaction was continued overnight. Next, mPEG-isc was added directly into the reaction vessel. This reaction was continued for 6 hours. The product was lyophilized and stored at -20 °C under N₂. TGA was performed on a TA Instruments Hi-Res TGA 2950 thermogravimetric analyzer under nitrogen from 25 °C to 600 °C at 20 °C/min. Transmission electron microscopy was performed on a Hitachi H7600T at 115kV.

Dasatinib Loading and Release

Dasatinib (DAS) was purchased from LC Laboratories (Woburn, MA). High performance liquid chromatography (HPLC) was used to determine the release kinetics of DAS from coated CNTs. DAS was encapsulated by dissolving coated CNTs at 5 mg/ml in a solution of DAS at 1 mg/ml in a 50/50 ACN/dimethylsulfoxide (DMSO) solution. This CNT/DAS solution was then dropped at a 1:2 ratio in HyPure water and stirred for 2 hours, and DAS was encapsulated via solvent evaporation. Samples were washed three times in HyPure water via centrifugation through a 100 kD MWCO centrifugal filter unit. To measure total drug loading content, CNTs were immediately lyophilized after washing. DAS-loaded CNTs were dissolved in a 50/50 ACN/DMSO solution to extract the drug from the CNTs, and CNTs were removed through a 0.1 μ m PTFE syringe filter. These solutions with free drug were then lyophilized and prepared for HPLC. To measure release kinetics, DAS-loaded CNTs were re-dispersed in HyPure water and added to the top of a 3.5 kD Slide-a-Lyzer MINI dialysis unit. Each dialysis unit was placed in a 1.5 ml microcentrifuge tube containing HyPure water. Five repeats per formulation were used and samples were stored at 37 °C. 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 (4.6 $\times$ 25 mm, 5 μ m) was used with mobile phase of 50/50 solution of ACN and 0.4 v/v% triethylamine (TEA) in water. The mobile phase flow rate was 0.8 ml/min and DAS was detected at a wavelength of 280 nm with an average elution time of 7.5 minutes.

Cell Culture and Controlled Release Therapeutic Efficacy

U-87 glioblastoma and Eagle's minimum essential medium (EMEM) were purchased from American Type Culture Collection (Manassas, VA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). U-87 glioblastoma cells were grown in EMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were subcultured at 35 °C and 5% CO₂. To test DAS efficacy in vitro, U-87 cells were seeded at 10,000 cells/well in a 96-well plate. To measure cell viability of polymer coated CNTs, CNT-PLA-PEG, CNT-PGA-PEG, and CNT-PGA-PLA-PEG were dispersed at 5 mg/ml in ACN. CNT solutions were then dropped at a 1:2 ratio in sterile HyPure water, and stirred for 2 hr. CNTs were then washed as previously described, twice in sterile HyPure water and once in sterile phosphate buffered saline. CNTs were re-dispersed at 10, 25, and 50 µg/ml. Cells were incubated with CNTs for 24 hr, and then cell viability was measured via a Presto Blue assay by comparing fluorescent intensity of each formulation to the average fluorescent intensity of cells grown in only media.

DAS was loaded as previously described, and CNTs were incubated with cells at 10 µg/ml. Free DAS was prepared by first dissolving at 0.1 mg/ml in DMSO and diluting 1:1000 in EMEM for a final concentration of 100 nM. Fresh media, free DAS, or DAS-loaded CNTs were added to cells and incubated for 6hr or 24 hr. There were five repeats per formulation. After the initial time point, media was removed and cells were washed gently with EMEM. Cells were incubated in fresh EMEM and incubated for a total of 72 hr after initial exposure to free drug or CNTs. Cell viability was measured with a Presto Blue assay.

Results and Discussion

Polymer coated CNT synthesis and characterization

Commercially available CNTs functionalized with surface hydroxyl groups were coated by initiating the ring-opening polymerization of glycolide directly on the CNT surface using P₂-t-Bu in anhydrous acetonitrile.^{28, 29} CNTs coated with a layer of PGA (CNT-PGA-OH) was the result. As PGA has a terminal hydroxyl group, PLA was directly polymerized from CNT-PGA-OH as a second layer by introducing d,l lactide into the reaction vessel. A multilayered coating of PGA-PLA on the CNT surface (CNT-PGA-PLA-OH) was the result. Finally, the polymer chain ends were capped by introducing methoxy-poly(ethylene glycol)-isocyanate (mPEG-isc) into the reaction vessel. Here the reactive isocyanate groups form a urethane linkage with terminal hydroxyl groups on the polymer chain ends, thereby coating CNTs with an external PEG layer (CNT-PGA-PLA-PEG). This capping step effectively terminated the polymerization, affording CNTs coated with multiple layers. Multilayer polymer coating was confirmed via thermogravimetric analysis (TGA). Single layer CNTs were synthesized by simply introducing mPEG-isc directly into the reaction after the first polymerization step. Figure 2 shows that single layered CNT-PLA-PEG, and CNT-PGA-PEG could be synthesized.

Polymer coating thickness can be controlled by varying the ratio of monomer (i.e. glycolide or d,l lactide) to initiator (i.e. hydroxyl-functionalized CNT), and tunable levels of PLA and PGA were polymerized onto CNTs. Moreover, the relative amounts of polymer in the

multilayered CNT were controlled in the same manner. Figure 3A shows multilayered CNTs coated with a thin base layer of PGA (12 wt%) and a thick outer layer of PLA (42 wt%). By changing the reaction conditions, however, a thick base layer of PGA (48 wt%) and a thin outer layer of PLA (12 wt%) can be polymerized on CNT surfaces (Figure 3B). The isocyanate capping was also validated via TGA; Figure 3C shows TGA data for a multilayered CNT composed of all three polymer layers: 25 wt% PGA, 25 wt% PLA, and 6 wt% PEG. The order of the monomer polymerization can also be changed. Figure 3D shows a multilayered CNT with 58 wt% PLA, 20 wt% PGA, and 10 wt% PEG. Transmission electron microscopy of single layer, and multilayer CNTs was shown in Figure 4. While the thin single layer coating is difficult to discern, the multilayer coating clearly shows polymeric coating on the carbon nanotubes compared to control. Furthermore, by holding the mass of CNT constant and varying molar ratio of glycolide or d,l lactide, the weight percent of polymer coating could be controlled (Figure 5). This effectively changes the monomer/initiator ratio and enables a means to increase polymer coating thickness. Thus, this synthesis approach allows control over not only the thickness of the multiple layers, but also the spatial organization of these layers.

Multilayered Nanoparticle Release Kinetics

DAS release kinetics from the CNT NPDDS were measured with high performance liquid chromatography (HPLC), and single layered CNTs were compared to multilayered formulations. Drug loading efficiency is shown in Table 1, and the drug loading efficiency of DAS is influenced by polymer composition of the coating. This data suggests that the PGA coating is critical for high drug loading. Each formulation also showed different release kinetics due to different polymer composition on the surface of the CNTs (Figure 6). Though DAS released immediately from PGA (instant), there was a lag time (delayed) in the release of DAS from PLA coatings. This variation in DAS release kinetics is possibly attributable to differences in drug loading, interactions between either the drug and the polymer matrix, or the polymer degradation kinetics. Figure 6 shows the amount of drug release (Figure 6A), and also the percentage of total drug loaded released (Figure 6B). While low DAS loading in CNT-PLA may result in slower release, CNT-PGA and CNT-PGA-PLA load similar amounts of DAS. Percent total drug release data from the multilayered CNT shows that the PLA coating effectively slows and linearizes the DAS release profile. These results suggest that PLA coating is not suitable to deliver high drug concentrations, and although PGA coating increases drug loading it is not suitable because it releases DAS very quickly. Thus, multilayered coatings with both PGA and PLA could utilize these characteristics by increasing the DAS loading, but slowing its release. Indeed the multilayered CNT-PGA-PLA released drugs in a near linear manner, suggesting that multilayered coatings offer a means of controlling both the loading and release profile to achieve significant cellular cytotoxicity.

In vitro efficacy of drug loaded multilayered nanoparticles

Controlling the release kinetics of DAS is expected to alter the therapeutic efficacy of these CNT drug delivery vehicles. Cell viability data using U-87 glioblastoma suggests that controlling release kinetics is critical for improving therapeutic efficacy of DAS, which is being investigated as a treatment against recurrent glioblastoma.^{24, 26} In Figure 7A, we show

the efficacy of different DAS-loaded polymer coated CNTs compared to free DAS. Cells were exposed to different CNT formulations at a CNT concentration of 10 $\mu\text{g/ml}$ for either 6 h or 24 h. Free DAS was at 100 nM. This concentration was chosen because preliminary in vitro toxicity studies with the free drug showed it to be the IC_{50} of DAS with U-87 cells. The different incubation times allowed for CNT uptake into cells. Cells were washed after each time point and cell viability was determined at 72 h. At these time points, the released concentration of DAS are estimated to be 14.7, 163.6, and 75.9 nM for CNT-PLA, CNT-PGA, and CNT-PGA-PLA, respectively. However, the in vitro release data is in water and only provides a simulation of trends for DAS release from CNT formulations. The therapeutic efficacy was measured by normalizing all formulations to cells treated with cell culture media only. Multilayered CNTs showed significant improvement (40% viability) over DAS loaded CNT-PLA-PEG (55% viability), and free DAS (75% viability). Results showed that at both time points the PGA, and multilayered formulations had significantly higher cytotoxicity than the free DAS and PLA coated CNTs. This is ostensibly due to the low therapeutic loading, and lag in DAS release from PLA. Thus it is apparent that altering the drug loading and release kinetics by controlling the multilayered coatings on CNTs is critical to improving therapeutic efficacy. After 24 h incubation, the results of the DAS-loaded multilayered CNT-PGA-PLA-PEG formulation suggest lower cell viability compared to the monolayer formulations. Differences in cell viability may be related to difference in NP uptake into cells, or in vitro release kinetics. By treating U-187 with a constant concentration of CNT (10 $\mu\text{g/ml}$), it is evident that total drug loading and release kinetics can directly influence treatment efficacy. Free DAS was shown to have an IC_{50} value at approximately 100 nM, and treatment with a low amount of polymer coated CNT (approximately 2 μg per well) resulted in significant improvement in efficacy. Furthermore, when comparing the efficacy of treatment with the estimated amount of DAS released, the multilayered CNT were more effective than both DAS-loaded CNT-PGA-PEG and free DAS. CNT-PGA-PEG was estimated to release approximately 164 nM, free DAS was given at 100 nM, and CNT-PGA-PLA-PEG was estimated to release approximately 75 nM (Figure 7B). The in vitro efficacy of polymer coated CNTs was compared to conventional spherical polymeric PLA-PEG nanoparticles (Figure 7C).

CNT drug delivery systems are controversial due concerns surrounding carbon nanomaterial-mediated toxicity. These concerns are largely due to the similarities between fibrillar CNT and asbestos, a known carcinogen.³⁰ However, studies have shown that carbon nanomaterial toxicity is closely associated with NP size, shape, route of administration, dose, and surface functionalization.^{17, 31–34} Studies show that well dispersed CNT can safely be administered intravenously, and CNT can in fact be safely metabolized via eosinophil peroxidase and myeloperoxidase.^{35, 36} Also, we have previously shown that PLA-PEG coatings on CNT can mitigated carbon nanomaterial associated toxicity.¹⁷ In order to demonstrate that controlled release of DAS was responsible for therapeutic efficacy, and decrease in U-87 viability was not due to CNT-associated toxicity, CNT formulations without drug were tested against U-87 cells (Figure 8). Increasing doses of CNT-PLA-PEG, CNT-PGA-PEG, and CNT-PGA-PLA-PEG were added to U-87 cells for 24 hr, and no significant toxicity was seen at doses up to 50 $\mu\text{g/ml}$. Toxicity was determined by normalizing viability of cells after exposure to CNTs versus exposure to only cell culture

media. This data suggests that multilayered CNTs kill U-87 cells with DAS more effectively than polymeric PLA-PEG nanoparticles, which showed 54% viability after 24 h incubation.

Conclusions

We have reported multilayered, polymer coated CNT that are able to sequester hydrophobic drug, tailor drug release kinetics of DAS, and improve therapeutic efficacy against U-87 glioblastoma in vitro. Multilayered coatings utilized the high encapsulation efficiency of PGA combined with the delayed release kinetics of PLA to improve therapeutic efficacy. CNTs were functionalized using a one-pot, room temperature synthesis to coat multiple polymer layers on CNTs for drug delivery applications. Multilayered coatings adopted more linear release kinetics compared to both homopolymers PGA and PLA. Moreover, multilayered coating of PLA-PGA-PEG significantly improved therapeutic efficacy in vitro against U-87 compared to monolayered coatings. Further work is required to investigate the efficacy of multilayered coatings in an in vivo tumor model. However, coating nanomaterials with multiple layers of polymers has broad impact for drug delivery applications.

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Figure 1.

Synthesis of multilayered CNTs is achieved with a simple one-pot reaction. At each step monomer is added sequentially polymerize a multilayered block copolymer coating, without washing in between. Isocyanate-terminated PEG reacts with terminal hydroxyl groups to cap the reaction.

Figure 2. Thermogravimetric analysis of (A) CNT-PLA-PEG, and (B) CNT-PGA-PEG shows the successful synthesis of single layered CNTs.

Figure 3.

Thermogravimetric analysis shows successful control of multilayered coatings on CNTs. (A) By decreasing the initial monomer to initiator ratio it is possible to coat a thin layer of PGA on CNTs, followed by a thick layer of PLA. (B) Conversely it is possible to coat a thick layer of PGA and then have a relatively thin layer of PLA. (C) PEG capping imparts a fully functional multilayered CNT with a PGA, PLA, and PEG coating. (D) By altering the order of monomers added into the reaction it is possible to get a CNT coated with PLA, then PGA, and finally PEG.

Figure 4. Transmission electron microscope images at 100k magnification of CNT, CNT-PGA, and CNT-PGA-PLA, and CNT-PGA-PLA-PEG coated carbon nanotubes. The polymer coating can be seen with the multilayered coating. Scale bars represent 100 nm.

Figure 5.

The thickness of polymer coated onto CNT surface can be controlled by varying the ratio of monomer (i.e. glycolide or lactide) to CNT. (A) Poly(glycolide) (PGA) coating thickness on CNT was shown to increase by increasing the molar amount of glycolide in the reaction. (B) Poly(lactide) (PLA) exhibited a similar control over the PLA thickness by varying the molar amount of d,l lactide in the reaction.

Figure 6.

Dasatinib release displayed as (A) μM , and (B) % of total loaded DAS. Release from polymer coated CNTs shows that a difference in the polymer coating exhibits control over the release kinetics. PGA coated CNTs show the quickest release. CNT-PLA had an initial lag period before release, and CNT-PLGA showed release similar to that of CNT-PGA. Multilayered CNT-PGA-PLA showed mostly linear release.

Figure 7.

Cell viability assays using U-87 glioblastoma indicated that polymer coating and dasatinib release kinetics result in different therapeutic efficacy. Cells were incubated with different formulations at 10 $\mu\text{g}/\text{ml}$ for 6hr or 24hr and viability was tested at 72hr after initial exposure to drug loaded CNT formulations. (A) Multilayered CNT-PGA-PLA-PEG drug loaded formulations had significantly higher cytotoxicity to U-87 compared to monolayer formulations at 24hr exposure. *Indicates significant difference (p -value < 0.05) from free DAS and CNT-PLA-PEG at each respective time point (B) CNT-PGA-PEG and CNT-PGA-PLA-PEG were plotted showing the estimated concentration of DAS delivered at the time of the toxicity study, and were compared to 100 nM free DAS. Even though multilayered CNT were predicted to release less DAS (75 nM) compared to both free DAS (100 nM) and CNT-PGA-PEG (164 nM), CNT-PGA-PLA-PEG was most effective in treating U-87. (C) Multilayered CNT formulations also improved efficacy compared to spherical PLA-PEG

nanoparticles. * Indicates significant difference (p-value < 0.05) compared to all other samples.

Figure 8.
Cell viability of U-87 after exposure to different polymer coated CNTs without drug.
Polymer coated CNTs did not exhibit significant cytotoxicity at doses up to 50 µg/ml.

Table 1

Dasatinib Encapsulation Efficiency

	Encapsulation Efficiency (wt%)
CNT-PLA	0.4
CNT-PGA	4.4
CNT-PGA-PLA	3.5