Bioconjugate Chemistry

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Single-Walled Carbon Nanotubes Noncovalently Functionalized with Lipid Modified Polyethylenimine for siRNA Delivery *in Vitro* and *in Vivo*

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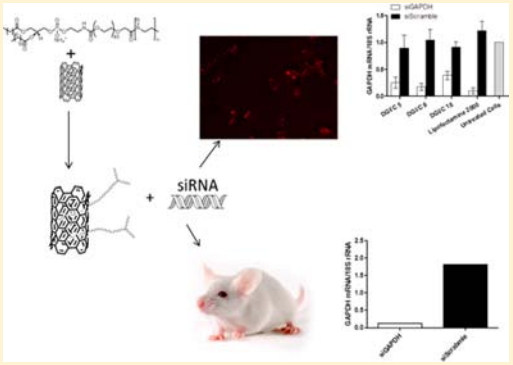
Supporting Information

ABSTRACT: siRNA can downregulate the expression of specific genes. However, delivery to specific cells and tissues *in vivo* presents significant challenges. Modified carbon nanotubes (CNTs) have been shown to protect siRNA and facilitate its entry into cells. However, simple and efficient methods to functionalize CNTs are needed. Here, noncovalent functionalization of CNTs is performed and shown to effectively deliver siRNA to target cells. Specifically, single-walled CNTs were functionalized by noncovalent association with a lipopolymer. The lipopolymer (DSPE-PEG) was composed of a phospholipid 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) and poly(ethylene glycol) (PEG). Three different ratios of polyethylenimine (PEI) to DSPE-PEG were synthesized and characterized and the products were used to disperse CNTs. The resulting materials were used for siRNA delivery *in vitro* and *in vivo*. The structural, biophysical, and biological properties of DGI/C and their complexes formed with siRNA were investigated. Cytotoxicity of the materials was low, and effective gene silencing in B16−F10 cells was demonstrated *in vitro*. In addition, significant uptake of siRNA as well as gene silencing in the liver was found following intravenous injection. This approach provides a new strategy for siRNA delivery and could provide insight for the development of noncovalently functionalized CNTs for siRNA therapy.



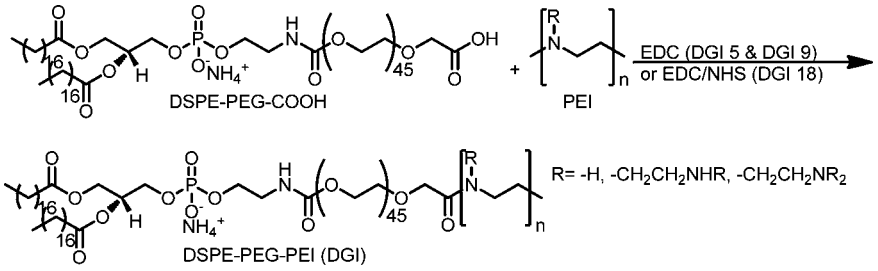
INTRODUCTION

Carbon nanotubes (CNTs) have been used for gene delivery,[1−11] and it has been reported that CNTs can enter cells as "nano-needles" by a diffusion-like mechanism.[12,13] The type and dimensions of CNTs, and the manner in which they are functionalized, determine the mechanism of cell entry.[14,15] However, pristine CNTs (p-CNTs) are not soluble in most solvents, so various modifications or functionalizations have been carried out to increase the solubility of CNTs in common solvents.[16] CNTs can be functionalized both covalently and noncovalently for better solubility.[17,18] Although CNTs can be functionalized to promote effective cell entry, they can carry only certain drugs or nucleic acids effectively. For example, CNTs can bind to single stranded DNA,[19] but the choice of sequence is limited, as the binding requires hydrophobic DNA bases to interact with the hydrophobic CNT surface. In order to use CNTs for drug or gene delivery, they must be functionalized specifically for the desired application.

Small interfering RNA (siRNA) is a powerful tool for research and is an attractive method to treat disease when the specific therapeutic target (RNA) is known. However, its application in the clinic remains limited, and efficient and specific delivery to target tissues and cells is a bottleneck.[20−23] It has been reported that siRNA can be covalently attached to CNTs, but both the CNT and siRNA must be modified before covalent attachment to the CNT, increasing the complexity of manufacture. In addition, covalent functionalization of CNTs by acidic oxidation can damage the CNT structure, and consequently only the side wall or defect sites in CNTs will bear the functional groups. If only functional groups on the side wall or defect sites are modified for siRNA delivery, the charge density of CNT will not be uniform, which can in turn lower the siRNA loading capacity or reduce colloid stability. In

Received: November 15, 2013
Revised: August 29, 2014
Published: September 12, 2014

Scheme 1. Synthesis of DGI



contrast, noncovalent functionalization methods are simple to perform and preserve the integrity of the CNT, making them particularly attractive for the development of CNTs for siRNA delivery.

One of the most efficient methods for the noncovalent functionalization of CNTs involves the use of surfactants.[24] By virtue of hydrophobic interactions involving the aliphatic tails of surfactants and the surface of carbon nanotubes, they can be dispersed in water for further applications. 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)-2000] (DSPE-PEG) is one of the most promising surfactants for noncovalent CNT functionalization. It has been reported that CNTs can be dispersed noncovalently through interactions with the lipid tail of DSPE. In addition, this approach has been used for siRNA delivery and the resulting materials were found to induce significant gene down-regulation.[10,25,26] However, this approach involved the covalent attachment of siRNAs to the lipid, and the release of siRNA is inefficient unless disulfides or other agents capable of inducing release are used for siRNA detachment. In addition, the amount of siRNA/DNA must be determined after noncovalent dispersion of the CNT, which makes its application complicated. Furthermore, siRNA needs to be modified for attachment to the lipid, which increases the cost of this approach. Therefore, an effective siRNA loading and unloading method with an easily controlled siRNA loading amount and universal siRNA attachment/complexation strategy is still particularly desirable.

Electrostatic interactions are an effective way to complex anionic nucleic acids. Moreover, the release of siRNA can be induced by the interaction of mRNA or anionic proteins in the cells. An additional advantage is that once the ratio of cationic species with siRNA are determined such that no free siRNA is present in the complexes, the amount of siRNA used in the preparation of the siRNA complex is the amount of siRNA in the resulting delivery vehicle. In preliminary work in our lab, DSPE-PEG was conjugated to poly(L-lysine) (PLL) to form DSPE-PEG-PLL and this was used to form DSPE-PEG-PLL/CNT for siRNA delivery. However, this complex could not deliver siRNA effectively (data not shown). Therefore, another polycation which could deliver siRNA better than PLL was sought. PEI is a polycation known to be effective in delivering pDNA.[27] It can also condense siRNA and facilitate endosomal escape.[28] It has been shown that CNTs covalently conjugated with PEI can provide increased transfection efficiency.[2,5] However, unmodified PEI is inefficient in delivering siRNA.[29,30] The covalent attachment of DSPE-PEG to PEI was proposed as an approach to enhance the delivery. In this study, CNTs were functionalized noncovalently with DSPE-PEG which was covalently conjugated to PEI. Three different ratios of DSPE-PEG to PEI were synthesized and characterized. The *in vitro* siRNA delivery capacities and cytotoxicities were determined. The *in vivo* distribution of the siRNA delivered by CNTs in five major organs was investigated and *in vivo* gene silencing in liver was demonstrated.

RESULTS AND DISCUSSION

Synthesis of DSPE-PEG-PEI (DGI) and Its Complexation with CNTs. It was anticipated that the degree of PEI modification with DSPE-PEG would require optimization. Therefore, the initial aim was to conjugate DSPE-PEG to PEI in varying ratios and then to study these different materials. As shown in Scheme 1, DSPE-PEG (2 kDa) conjugates with PEI (DGI) were synthesized by first activating the carboxylic acid group in DSPE-PEG-COOH using *N*-(3-(dimethylamino)-propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and then reacting this with varying ratios of PEI, either with or without *N*-hydroxysuccinimide (NHS), to prepare three different conjugates (DGI 5, DGI 9, and DGI 18) (Table 1). Uncoupled DSPE-PEG was removed by dialysis.

Table 1. Summary of Composition and Characterization of DGIs

	PEI: PEG feed ratio (wt:wt)	PEI:PEG ratio in product (^{1}H NMR, wt:wt)	M_n (SEC)	M_w (SEC)	PDI (SEC)
DGI 5	6.6:1	4.9:1	13400	21900	1.63
DGI 9	12.1:1	8.8:1	13200	21300	1.60
DGI 18	11.1:1	17.1:1	14600	24200	1.66

The resulting conjugates were characterized by ^{1}H NMR spectroscopy in D_2O. Peaks corresponding to the methylene protons of unconjugated PEI appear from 2.5 to 3.0 ppm. The formation of amide linkages through the conjugation reaction results in a shift of some of the peaks downfield, to 3.1 to 3.4 ppm, as these methylene protons become adjacent to amide bonds, consistent with successful conjugation. Peaks corresponding to methylene groups adjacent to some protonated amines on the PEI may also appear in these regions, along with the methylene protons adjacent to the carbamate linker of DSPE-PEG.[31] A representative spectrum is shown in Figure 1 for DGI 9. Additional spectra are included in the Supporting Information. It should be noted that the integral of the stearoyl tail was expected to be inaccurate under these conditions as the hydrophobic lipid was not well dispersed in D_2O. Other NMR solvents, including $CDCl_3$, DMF-d_6, DMSO-d_6, and CD_3OD were also investigated. DGI did not dissolve well in $CDCl_3$ or DMF-d_6. Residual solvent and water peaks of DMSO-d_6 and CD_3OD overlapped significantly with the peaks from the PEI, preventing accurate integration. Therefore, D_2O was used and the relative integrations of the peaks corresponding to PEI (2.5−3.4 ppm, after subtracting the integration for the

dx.doi.org/10.1021/bc500280q | *Bioconjugate Chem.* 2014, 25, 1744−1751

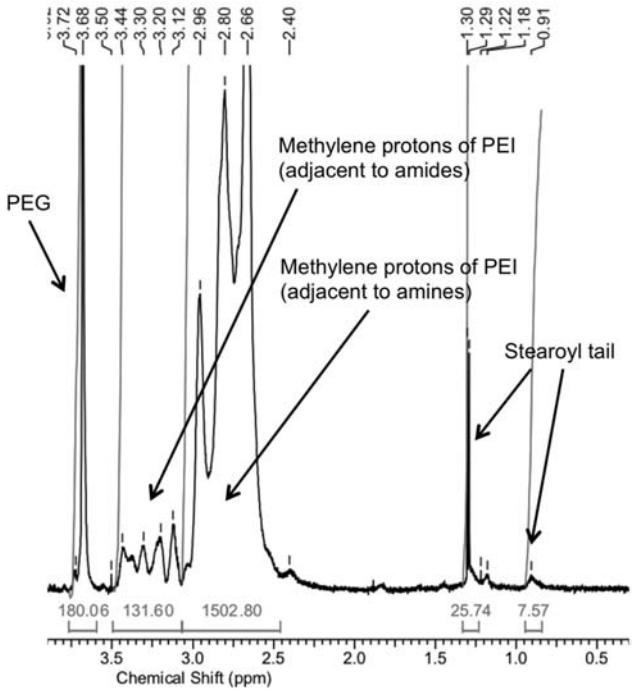


Figure 1. ^{1}H NMR spectrum of DGI 9 (600 MHz, D_2O).

methylenes adjacent to the carbamate in DSPE-PEG) and PEG (3.7 ppm) were used to calculate the weight ratio of the PEI:PEG, and these results are included in Table 1. These results were deemed to be accurate, as both of these components dissolve well in D_2O.

The DGIs were also characterized by size exclusion chromatography (SEC) in an aqueous buffer at pH 5.3, using a calibration curve based on PEG standards. The traces are shown in the Supporting Information. As shown in Table 1, the molar masses of DGI 5, DGI 9, and DGI 18 were similar, with number-average molecular weights (M_n) ranging from 13 kDa to 15 kDa and weight-average molecular weights ranging from 21 kDa to 24 kDa. Unreacted PEI or DSPE-PEG were not observed.

Noncovalent functionalization is an effective and simple way to obtain water-soluble CNTs. In order to obtain water-soluble CNTs which were able to condense siRNA by electrostatic interaction, DGI functionalized CNTs, DGI/C 5, 9, and 18, were prepared from DGIs 5, 9, and 18, respectively, using a previously reported method.[10] After dispersing CNTs using the three DGIs, the resulting DGI/Cs were imaged by transmission electron microscopy (TEM). As shown in Figure 2, the DGI/Cs were singly dispersed and had lengths of 600−800 nm. This is somewhat shorter than the length claimed by the manufacturer (800 to 1000 nm), suggesting that the CNTs may have been shortened during this process. This can likely be attributed to the sonication step, which has been previously reported to result in shorter CNTs.[32]

siRNA Binding of DGI/C. Next, the capacities of DGI/C 5, 9, and 18 to condense siRNA electrostatically were evaluated using a gel shift assay. In this assay, free siRNA migrates relatively unimpeded through the gel, while the migration of siRNA bound to DGI/C is slowed down or stopped. In addition, complexed siRNA cannot bind to ethidium bromide effectively and the fluorescence of siRNA in the presence of ethidium bromide is reduced. This assay showed that, for DGI/C 5 and DGI/C 9, the ratio of DGI/C:siRNA required to inhibit migration of siRNA was 1:1 (w/w) while for DGI/C 18 it was 2:1 (Figure 3). This was counterintuitive because the PEI content of DGI/C 18 is higher than that of DGI/C 5 or DGI/C 9, leading the expectation that it should bind more siRNA. However, as demonstrated by others,[33] the binding of siRNA to PEI alone is relatively weak whereas modified PEI can facilitate the binding of siRNA to PEI. Therefore, the relatively low degree of modification of PEI in DGI/C 18 resulted in lower siRNA binding capacity compared to DGI/C 9 and DGI/C 5.

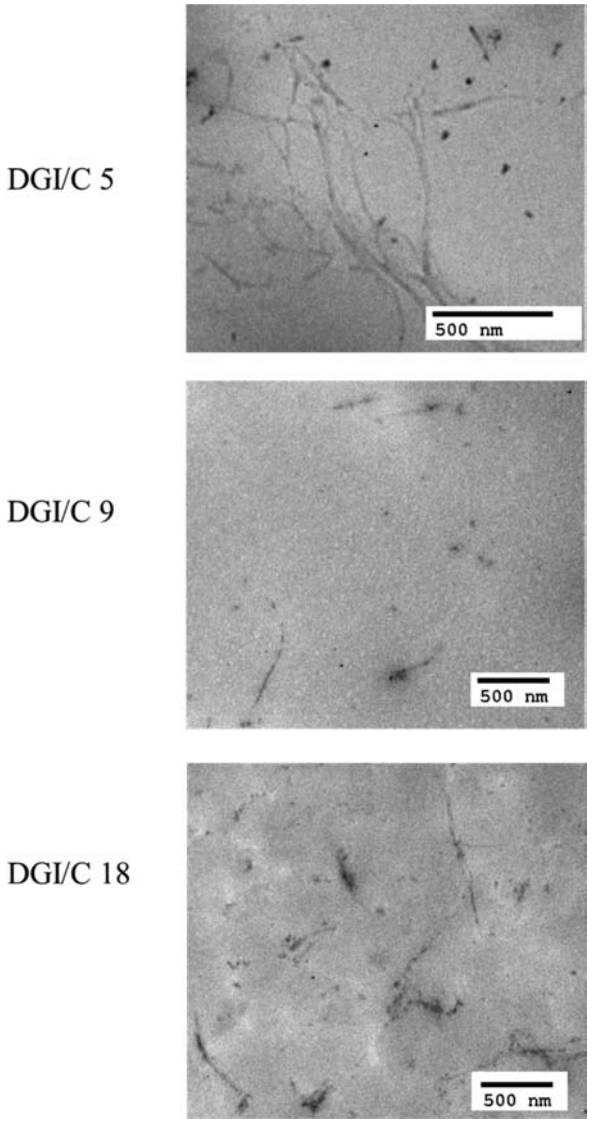


Figure 2. TEM images of DGI/C 5, 9, and 18.

Zeta Potential of DGI/C/siRNA. Colloid stability of DGI/C/siRNA complexes is important for effective siRNA delivery, and zeta potential is one of the indicators of colloidal stability. Electrostatic repulsion between complexes tends to increase stability, and the higher the zeta potential the more stable the complexes are expected to be. All of the DGI/C/siRNA complexes had positive zeta potentials (Figure 4) in the medium to high range (>30 mV). This can be attributed to the incorporation of PEI at CNT surfaces. The zeta potentials were similar for different CNT:siRNA ratios, and ranged from 47 to 42 mV for DGI/C 5; 54 to 46 mV for DGI/C 9; and 39 to 31 mV for DGI/C 18. It is interesting that there was a lack of

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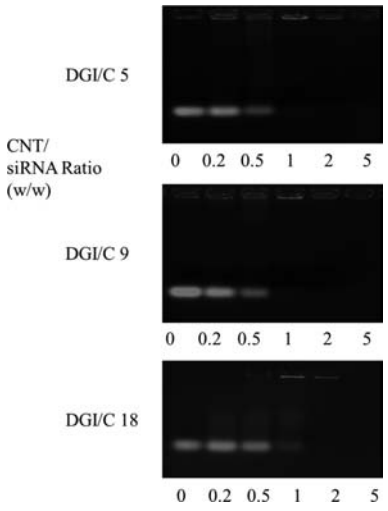

Figure 3. Gel shift assays of DGI/C/siRNA show that 1:1 (wt:wt, DGI/C:siRNA) was required for DGI/C 5 and 9 to fully bind siRNA and retard migration, while for DGI/C 18, 2:1 (wt:wt) DGI/C:siRNA was required.

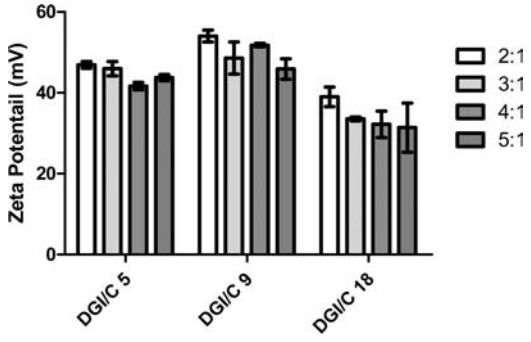

Figure 4. Zeta potential of DGI/C/siRNA at varying DGI/C:siRNA (wt:wt) ratios. Error bars represent the standard deviation from duplicate experiments with 10 runs each.

obvious trend in the zeta potential, even with increases in the CNT:siRNA ratio. Although the zeta potential is positive in the absence of serum, it is quite possibly different in the presence of adsorbed serum proteins.

Gene Silencing Mediated by DGI/C/siRNA *in Vitro*. It has been shown that PEI-conjugated CNTs could increase the transfection efficiency of PEI alone. To reveal the siRNA delivery capacities of three different DGI/C reagents, we set out to test the gene silencing in B16−F10 cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was the siRNA target. After treatment with DGI/C 5 and DGI/C 9 complexed with GAPDH siRNA, relative GAPDH mRNA levels were reduced by 80% compared to untreated cells. Treatment with DGI/C 18:siGAPDH reduced relative GAPDH mRNA levels by 60% compared to untreated cells (Figure 5a). All of the DGI/C/siGAPDH complexes were able to induce a substantial reduction in target mRNA levels.

The gene silencing capacity of DGI/C corresponds to the general trend of zeta potentials.[34,35] DGI/C 9 had the greatest gene silencing capacity, compared to treatment with non-targeting siRNA with a scrambled sequence (siScramble control), treatment with DGI/C 18, or to no treatment at all. This is possibly due to the strong interaction of the positively charged DGI/C 9/siRNA complexes with the negatively

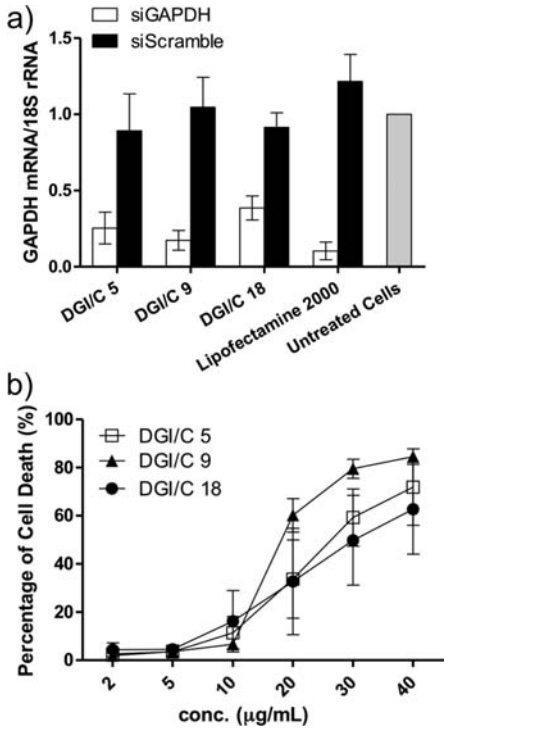

Figure 5. (a) *In vitro* gene silencing in B16−F10 cells by DGI/C and siGAPDH or siScramble 24 h after transfection. Error bars represent the standard deviation of 3 experiments. (b) *In vitro* cytotoxicity of DGI/C to B16−F10 cells based on 7AAD staining followed by flow cytometry analysis. Error bars represent the standard deviation of 3 experiments.

charged cell membrane. DGI/C 18 was less effective in inducing gene silencing, possibly due to inefficient protection of siRNA in media containing serum. When the PEI:PEG ratio was reduced, siRNA was protected more effectively and the efficiency of DGI/C 5 as an siRNA delivery reagent was similar to that of DGI/C 9, implying that siRNA delivery requires an optimal PEI:PEG ratio. Differences between the lipopolymer and the functionalized CNT were also compared to elucidate the role of the CNT in the siRNA delivery process. DGI/C 5 and DGI 5 were used for purposes of comparison of siRNA effectiveness (see the Supporting Information section). It was found that the reduction in mRNA target induced by DGI/C 5 was 2.1-fold higher than DGI 5. Therefore, in addition to capacity of the lipopolymer to enhance siRNA effectiveness, the CNT also played an important role in increasing the capacity of siRNA to reduce target mRNA.

It has been previously reported that CNTs functionalized with amine enter cells through a diffusion-like mechanism.[13] To explore the mechanism of entry of the DGI/C/siRNA complexes prepared in this study into cells, the cellular uptake of DGI/C 9 complexed with Cy-3 labeled siRNA in media containing increasing concentrations of sodium azide (0%, 0.005%, and 0.05%) was determined by flow cytometry (see Supporting Information). A PEI complex with Cy-3 labeled siRNA was used for purposes of comparison. Cell uptake of DGI/C 9/siRNA under the different conditions was similar to cell uptake of PEI/siRNA. In an energy-depleted environment, the cell uptake of both PEI/siRNA and DGI/C 9/siRNA was similar. These data suggest that DGI/C 9 enters cells by mechanism similar to that mediating PEI uptake, and that this mechanism requires energy-dependent endocytosis. It does not

dx.doi.org/10.1021/bc500280q | *Bioconjugate Chem.* 2014, 25, 1744−1751

enter cells by a diffusion-like mechanism. Taken together, the results suggest that incorporation of CNT is the primary reason for the enhanced siRNA delivery. The enhanced gene silencing is possibly induced by enhanced cellular uptake of DGI/C/siRNA complexes.

Cytotoxicity of DGI/C. To investigate the cytotoxicity of DGI/C, various amounts of DGI/C were used to treat B16−F10 cells for 24 h followed by staining of dead cells with 7AAD and analysis by flow cytometry (Figure 5b). All of the DGI/Cs have low cytotoxicity up to 5 μg/mL, which was higher than the concentration used in the *in vitro* gene silencing experiment described above. DGI/C 9 had negligible cytotoxicity up to 10 μg/mL. The percentage of cell death was 60% when the concentration was increased to 20 μg/mL and continued to increase to 85% at 40 μg/mL. The cytotoxicities of DGI/C 5 and DGI/C 18 were similar: cell death was approximately 10% at a concentration of 10 μg/mL and increased gradually from 30% to 70% at 20−40 μg/mL. The cytotoxicity of DGI/C 9 at concentrations below 20 μg/mL was low. The PEI content of DGI/C 18 was highest and, if PEI was primarily responsible for toxicity, would have been the most toxic of the DGI/C species. This was not the case. Again, this suggests that DGI/C 18 may ultimately have less surface PEI. To compare the cytotoxicity of the DGI/C and DGI at each PEI:PEG ratio, 10 μg/mL of each were tested in B16−F10 cells (Supporting Information). Compared to DGIs, DGI/Cs exhibited reduced cytotoxicities, indicating that CNTs actually reduce rather than increase cytotoxicity. This can perhaps be attributed to the reduction in any surfactant-induced toxicity as the lipids would be buried through binding to the CNT surface.

DGI/C 9 Delivers siRNA and Mediates Gene Silencing in Liver *in Vivo*. *In vivo* siRNA delivery capacity of DGI/C with Cy3-labeled siGAPDH was evaluated. DGI/C 9 had the best gene silencing capacity *in vitro* and was, therefore, selected for this study. DGI/C 9 complexed with Cy-3 labeled siRNA was injected intravenously into CD-1 mice. Most of the red fluorescence was found in the liver and spleen (Figure 6a). This is not surprising because the reticuloendothelial system (RES) usually takes up particles delivered into the body intravenously. There was also fluorescence found in lung and kidney. Quantitative real time polymerase chain reaction (qRT-PCR) results showed that DGI/C 9:siGAPDH mediated greater than 80% down-regulation of target mRNA in liver (Figure 6b), the organ which exhibited the most intense fluorescence.

CONCLUSION

A novel and efficient noncovalent functionalization strategy for complexing and delivering siRNAs using CNTs was developed. This strategy involved the preparation of conjugates (DGIs) incorporating DSPE-PEG, where the phospholipid complexes with the hydrophobic surface of the CNT and the PEG enhances both solubility and cargo protection, and PEI which mediates formation of complexes with siRNA. The most effective siRNA complex formation was found when using DGI/C with lower PEI content (DGI/C 5 and DGI/C 9). These materials also provided greater down-regulation of GAPDH mRNA *in vitro*. The cytotoxicity of the DGI/C was low at the concentration used in this *in vitro* transfection experiment. A complex between DGI/C 9 and siGAPDH mediated significant gene silencing *in vivo* in the liver. Overall, this research provides new insights for the further development of CNT-based siRNA delivery systems.

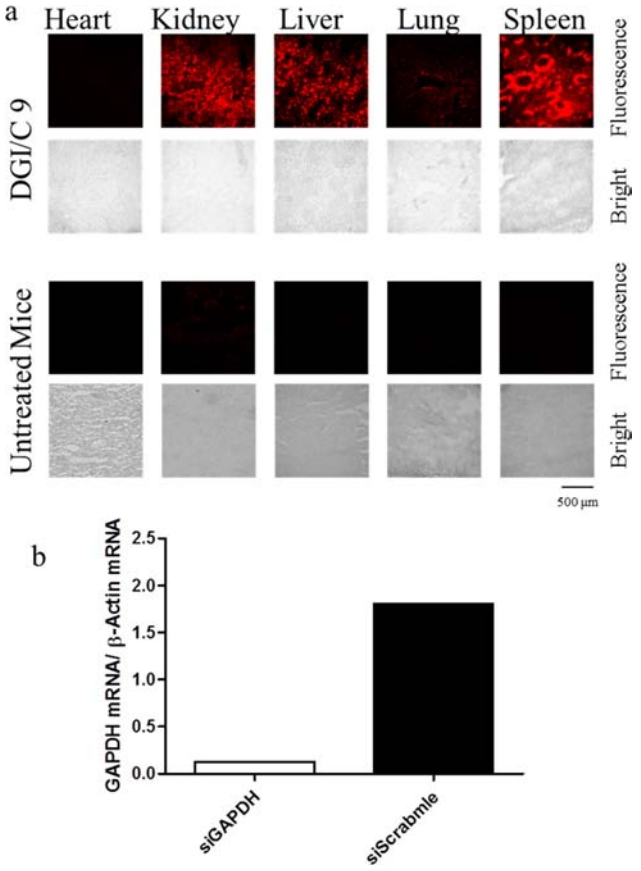

Figure 6. *In vivo* siRNA distribution and gene silencing in liver: (a) Fluorescence and bright field microscopy images of tissue cryosections taken from a CD-1 mouse injected with DGI/C 9:siGAPDH complexes, or an untreated control mouse, 24 h after the injection. Representative images are shown. (b) Gene silencing of GAPDH in the liver of CD-1 mice treated with DGI/C/siGAPDH or DGI/C/siScramble 24 h after injection (n = 2).

EXPERIMENTAL PROCEDURES

General Procedures and Materials. Single-walled carbon nanotubes were purchased from Nano-C (MA, USA). Polyethylenmine (PEI, M_w 25 000, M_n 10 000), *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), and *N*-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (St. Louis, USA). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[carboxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG-COOH) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Dimethyl sulfoxide (DMSO) was purchased from Caledon Laboratory Chemicals, Inc. (Georgetown, Canada). Double-stranded siRNA (Silencer Cy3 labeled GAPDH siRNA) (siGAPDH) was obtained from Invitrogen (Burlington, Canada). Luciferase GL2 Duplex was used as control, nontargeted scrambled siRNA (siScramble) which was obtained from Thermo Scientific (Ottawa, Canada). NMR spectroscopy was performed in D_2O (99%, Sigma) on an INOVA 600 spectrometer (Agilent, Santa Clara, USA) (600 MHz). Chemical shifts (δ) are given in parts per million and the residual HOD peak in the D_2O (4.7 ppm) was used as the reference. SEC was performed using a Waters 2695 Separations Module (Waters, Mississauga, Canada), a Waters 2414 Refractive Index Detector, and three PLaquagel-OH 40 8 μm (300 × 7.5 mm) columns (Polymer Laboratories/Varian, Santa Clara, CA) connected in series to a PLaquagel-

dx.doi.org/10.1021/bc500280q | *Bioconjugate Chem.* 2014, 25, 1744−1751

OH 8 μm guard column. The eluent was 0.2 M ammonium acetate/acetic acid (pH 5.3) at 1 mL/min at room temperature for 35 min/run. Samples were prepared at a concentration of 10 mg/mL in the eluent, filtered through 0.2 μm Supor membrane filters (Pall, Quebec, Canada) and injected with a 100 μL volume loop. The calibration curve was obtained from PEO/PEG standards (Polymer Laboratories/Varian, Santa Clara, CA).

Synthesis of DGI 5 and DGI 9. 200 μL (1.75 μmol) of DSPE-PEG-COOH (2.5 mg/mL in chloroform) was added to a round-bottom flask and the solvent was removed using a stream of compressed air. Next, 5 mL of DMF and 10 mg (52 μmol) of EDC were added. The mixture was stirred for 15 min and then the desired amount of PEI solution (23 mg, 2.3 μmol for DGI 5; and 42 mg 4.2 μmol for DGI 9) in MES buffer (0.1 M, pH 5.5) was added. The reaction was allowed to proceed overnight and then the reaction mixture was dialyzed overnight using a 50 kDa molecular weight cutoff (MWCO) regenerated cellulose membrane (Spectrum Laboratories, Rancho Domingo, USA) against deionized water for 48 h. The product was then lyophilized. **DGI 5**: Yield: 27 mg; 96%; ^{1}H NMR (D_2O, 600 MHz): δ 0.91 (m, 6H), 1.13−1.30 (m, 21H), 2.50−3.44 (m, 914H), 3.68 (s, 180H); SEC: M_n = 13400 Da, M_w = 21900 Da, PDI = 1.63. **DGI 9**: Yield: 53 mg; >99%; ^{1}H NMR (D_2O, 600 MHz): δ 0.91 (m, 8H), 1.13−1.30 (m, 26H), 2.50−3.44 (m, 1635H), 3.68 (s, 180H); SEC: M_n = 13200 Da, M_w = 21300 Da, PDI = 1.60.

The integration of the PEG peak was set to 180 (corresponding to 45 repeat units) and then the PEI:PEG weight ratio was calculated using the following equation:

$$\begin{aligned}&\text{Weight ratio of PEI:PEG}\\&= \{[(\text{Integral of PEI peak} - 2 \text{ for DSPE methylene})\\&\quad /4\text{H per PEI monomer}] \times 43 \text{ g/mol}\}\\&\quad /[(\text{Integral of PEG peak}/4\text{H per PEG monomer})\\&\quad \times 44 \text{ g/mol}]\end{aligned}$$

Synthesis of DGI 18. The same procedure as described above for DGI 5 and 9 was followed except for the following changes. Half the amount of DSPE-PEG-COOH (2.5 mg; 0.9 μmol) was used. Then, 5 mL of DMF, 27.0 mg (26.6 μmol) of EDC, and 2.80 mg (24.3 μmol) of NHS were added. The mixture was stirred for 15 min and then 19.3 mg (1.93 μmol) of PEI solution was added. The reaction was allowed to proceed overnight and then the reaction mixture was dialyzed overnight using a 50 kDa MWCO regenerated cellulose against deionized water for 48 h. The product was then lyophilized. Yield: 26 mg; >99%; ^{1}H NMR (D_2O, 600 MHz): δ 0.91 (m, 28H), 1.13−1.30 (m, 34H), 2.50−3.44 (m, 3150H), 3.68 (s, 180H); SEC: M_n = 14600 Da, M_w = 24200 Da, PDI = 1.66.

Noncovalent Functionalization of CNTs by DGIs. 5 mg of DGI was weighed in a tube and 20 mL of deionized water was added to dissolve the polymer. Then, 5 mg of CNT was added into the DGI solution. The solution was sonicated for 1 h at 60 °C. The undissolved CNTs were removed by repeated centrifugation. The unbound polymer was removed by using ultra-15 centrifugal filter units (100 kDa MWCO, Amicon, Billerica, MA). The concentrated DGI/CNT (DGI/C) was lyophilized providing a gray solid. The recovery of DGI/C 5 was 24%, DGI/C 9 was 25%, and DGI/C 18 was 23%, which was calculated by the following equation:

$$\text{Percentage of recovery} = \frac{\text{weight of DGI/C recovered}}{\text{weight of CNT} + \text{weight of DGI}} \times 100\%$$

Transmission Electronic Microscopy (TEM). DGI/C was dispersed in water and dropped onto a copper grid. The grid was incubated for 20 min and then dried under air. The sample was analyzed by Hitachi 7000 (Hitachi, Dallas, TX) operating at 100 kV with 40 μm aperture.

Gel Shift Assay. Equal volumes (10 μL) containing 0.5 μg of siRNA and the desired amount of DGI/C were mixed and incubated for 30 min. The complexes were then subjected to electrophoresis at 100 mV with 1.5% agarose gel and EtBr in TAE buffer. After 20 min the gel was removed, visualized, and the picture recorded with FluoroChem M (Protein simple, Santa Clara, USA).

Zeta Potential. DGI/C/siRNA complexes were prepared by mixing equal volumes (10 μL) of siScramble (5 μg) and the desired amount of DGI/C followed by incubation for 30 min. The solution was then transferred to a disposable capillary cell and analyzed using a Malvern Zetasizer Nano ZS (Malvern, Worcestershire, UK) equipped with a He−Ne laser (633 nm) with an energy output of 10 mW with automatic laser attenuation. The mean and standard deviation of zeta potential data were calculated based on 2 measurements of 10 runs each.

***In Vitro* Gene Silencing in B16−F10 Cells.** 24 h before transfection, B16−F10 cells (ATCC, Manassas, VA) were seeded in a 12-well plate at a density of 1.2×10^5 cells/well in 1 mL culture media. On the date of transfection, DGI/C/siRNA complexes were made by mixing equal volumes of siRNA and DGI/C (5-fold, wt:wt) followed by incubation for 30 min. Then DGI/C/siRNA solution was added to serum-containing media to generate a final siRNA concentration of 2 μg/mL. Treatment of cells with Lipofectamine 2000 (Invitrogen) was used as positive control according to manufacturer's protocol. 24 h after transfection, RNA was isolated using Trizol, according to the manufacturer's protocol (Life Technologies, Burlington, ON) and cDNA were synthesized using that RNA as a template. The amount of GAPDH mRNA in samples, relative to 18S rRNA, was measured by quantitative real time polymerase chain reaction (qRT-PCR). GAPDH primer, 18S primer, and the qRT-PCR TaqMan Assays buffer were obtained from Life Technologies Inc. (Burlington, ON) and the reactions were done in duplicate using the MX 3005p qRT-PCR systems (Stratagene, Mississauga, ON). The reaction condition was 10 min at 95 °C, then 40 cycles on 30 s at 95 °C, 1 min at 58 °C, and 1 min at 72 °C.

Cytotoxicity in B16−F10 Cells. 24 h before experiment, B16−F10 cells were seeded in a 24-well plate at a density of 5×10^4 cells/well in 500 μL of DMEM with 10% FBS. At the date of experiment, the media was then replaced with culture media with serum containing the desired amount of DGI/C. The cells were then incubated for 24 h. The media was removed and the cells were trypsinized, collected, and resuspended in phosphate-buffered saline (PBS) with 2% fetal bovine serum (FBS) and 5 μg/mL 7-Aminoactinomycin D (7AAD). The cells were then analyzed by flow cytometry. The percentage of cell death was calculated according to the number of 7AAD-positive cells in the treated population (cell death due to treatment plus background cell death) minus the 7AAD-positive cells in the untreated population (background cell death).

***In Vivo* siRNA Delivery and Gene Silencing.** Complexes of DGI/C 9 and Cy-3 labeled siGAPDH or siScramble were prepared as described above at a 3:1 (wt:wt) of DGI/C:siRNA. 6−8 week old CD-1 mice (Charles River, Canada) were injected with complexes containing 200 μg of siGAPDH or siScramble by tail vein injection and untreated mice were used as negative controls. 24 h after injection, the mice were sacrificed and the organs were taken and frozen in optimal cutting temperature compound (OCT) or for later use. Cryosectioning was done on the organs and they were observed by fluorescence microscopy (Olympus BX51, Canada). RNA was isolated using the Trizol method. cDNA was synthesized and qRT-PCR was used to quantify the level of GAPDH of the samples were analyzed and β-Actin was used as internal control. The reaction condition was 10 min at 95 °C, followed by 40 cycles with 30 s at 95 °C, 1 min at 62 °C, and 1 min at 72 °C. The primers for GAPDH and β-Actin were as follows: GAPDH: 5′-GGGGTGAGGCCGGTGCTGAGTAT-3′ (forward) and 5′-CATTGGGGTAGGAACACGGAAGG-3′ (reverse). β-Actin: 5′-AGGGAAATCGTGCGTGACAT-CAAA-3′ (forward) and 5′-ACTCATCGTACTCCTGC-TTGCTGA-3′ (reverse).

All animals were housed under pathogen-free conditions. All experiments were conducted in accord with the *Guide for the Care and Use on Animals Committee Guidelines*. The animal protocol was approved by the Animal Use Subcommittee (AUS) at Western University.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional NMR spectra, SEC traces, comparison of gene silencing capacities, relative cytotoxicities of polymer and polymer/nanotube composite, and the mechanism of cell uptake of polymer/nanotube composite. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was partially supported by grants from Natural Science Foundation of China (NSFC, No. 81160286, No. 91229119), grants from Canadian Institute of Health Research (CIHR), the London Regional Cancer Program (LRCP), and Canadian Breast Cancer Foundation (CBCF). Di Chen and King Sun Siu were supported by the CIHR Strategic Training Program in Cancer Research and Technology Transfer (CaRTT).

■ ABBREVIATIONS

RNAi, RNA interference; siRNA, small interfering RNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CNTs, carbon nanotubes; siGAPDH, siRNA specific to GAPDH; PEI, poly(ethylenimine); PEG, poly(ethylene glycol); DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; DGI, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-poly(ethylene glycol)-poly(ethylenimine); DGI/C, DGI on CNTs; 7AAD, 7-Aminoactinomycin D

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