Supporting Information

Nanodiamond Decorated Liposomes as Highly Biocompatible Delivery Vehicles and a Comparison with Carbon Nanotube and Graphene Oxide

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1. Additional methods.

The names and structures of the phospholipids used in this work are shown in Figure S1, DOPC = 1,2-dioleoyl-sn-glycero-3-phosphocholine; DPPC = 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DOTAP = 1,2-dioleoyl-3-trimethyl ammonium-propane; DOPG = 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt); DOPE = 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; Rh-PE = 2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-((lissaminerhodamine B sulfonyl) (ammonium salt).

Figure S1. Names and structures of the lipids used in this work.

UV-vis spectra of nano-carbons. The UV-vis absorption spectra of ND (250 μg mL⁻¹), CNT (20 μg mL⁻¹) and NGO (50 μg mL⁻¹) were obtained in a quartz cuvette using an Agilent 8453A

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spectrophotometer. All the measurements were carried out in water.

**Liposome/nano-carbon complex studied by DLS.** To study the size of the complexes formed between liposome and nano-carbons, dynamic light scattering (DLS) was carried out. In a typical experiment, DOPC, DOPG or DOPC/DOTAP liposome with a concentration of 0.025 mg mL\(^{-1}\) was respectively mixed with various concentrations of ND (30 nm), CNT or NGO (weight ratios of carbon to liposome were 0.02, 0.1, 0.2, 1, 2, 4, 8). After 20 min incubation, the sample size was determined using DLS (Zetasizer Nano 90, Malvern) at 25 °C.

**TEM and cryo-TEM.** TEM measurements were performed on a Philips CM10 transmission electron microscope. The sample was prepared by pipetting a drop of the aqueous solution of ND (0.02 mg mL\(^{-1}\)) onto a 230 mesh copper grid and the sample was allowed to dry in air before measurement. Cryo-TEM experiment was performed by spotting the ND/DOPC, CNT/DOPC or NGO/DOPC mixture (5 μL) on a carbon-coated copper TEM grid (treated with plasma to ensure surface was hydrophilic) in a humidity controlled chamber (FEI Vitrobot). The humidity was set to be 95 to 100% during this operation. The grid was blotted with two filter papers for 2 sec and quickly plunged into liquid ethane. The sample was then loaded to a liquid N\(_2\) cooled cold stage and loaded into a 200 kV field emission TEM (FEI Tecnai G2 F20). The samples were imaged when the temperature was stabilized at -175 °C.

**Calcein-loaded DOPC liposome leakage test.** To test whether adsorption of nano-carbons can induce pore formation or leakage in DOPC liposomes, 10 μL Pd-10 column purified calcein-loaded DOPC liposome was mixed with 10 μL ND (1 mg mL\(^{-1}\), CNT (50 μg mL\(^{-1}\)) or NGO (250 μg mL\(^{-1}\)) and the mixture was quickly transferred to 600 μL of HEPES (10 mM, pH 7.6) in a quartz cuvette. The fluorescence intensity was monitored for ~15 min before 10 μL 5% Triton X-100 was added. Calcein was excited at 483 nm and the emission fluorescence was monitored at 515 nm using a Varian Eclipse fluorometer.

**Liposome centrifugation conditions.** In Figure 2B, nano-carbons were mixed with Rh-DOPC liposomes and the samples were centrifuged at 70,000 rpm. The free Rh-DOPC liposomes were also tested as a control group. Due to the adsorption of nano-carbons, the nano-carbon/DOPC conjugate was precipitated and the fluorescence of Rh-DOPC liposomes was quenched. However, at 70,000 rpm, free Rh-DOPC liposomes could not be spun down, and had strong red fluorescence in the solution.

**Temperature-dependent DPPC leakage.** 1 μL freshly prepared calcein-loaded DPPC liposomes were incubated with 50 μL different concentrations of ND, SWNT and nano-GO in HEPES buffer (10 mM, pH 7.6). The highest concentration for ND, CNT and NGO were 2 mg mL\(^{-1}\), 12.5 μg mL\(^{-1}\) and 250 μg mL\(^{-1}\) respectively. With such high concentrations of nano-carbons, the fluorescence intensity was already approaching zero and therefore, no higher concentrations were tested. Then 20 μL of the samples were loaded into real-time PCR tubes (replicate of 4) and the temperature was increased by 1 °C from 4 °C to 95 °C using a real-time PCR thermocycler (CFX 96, Bio-Rad). The incubation time at each temperature was 20 sec and fluorescence in the FAM channel was read at each temperature.

**Laurdan-loaded liposome assays.** 10 μL as prepared Laurdan-loaded DOPC liposome was mixed with different volumes of ND (4 mg mL\(^{-1}\), CNT (50 μg mL\(^{-1}\)) or NGO (500 μg mL\(^{-1}\)). The added nano-carbon volumes were 1, 2, 4, 10, 20, 50, 100, and 200 μL. Then HEPES buffer (10 mM, pH 7.6) was added to make the total volume 1 mL for each sample. The Laurdan fluorescence as a function of ND concentration was monitored by a Varian Eclipse fluorometer with an excitation wavelength at 340 nm. Note that the Laurdan dye was loaded in the hydrophobic bilayer region, while calcein was loaded in the internal aqueous compartment of the liposomes. As a control experiment, Laurdan-loaded DPPC was also tested and its fluorescence was measured at two temperatures (25 °C and 41 °C) since it has a phase transition temperature at 41 °C. The phase transition temperature of DOPC is -20 °C.

**Cell culture.** HeLa cell line was obtained from the American Type Culture Collection (ATCC, MD, USA). The cells were cultured in DMEM/F12 medium, supplemented with 10% FBS and 100 U mL\(^{-1}\) penicillin, 100 μg mL\(^{-1}\) streptomycin at 37 °C using a humidified 5% CO\(_2\) incubator.
Laser scanning confocal fluorescence microscopy. Hela cells were seeded onto 14 mm coverslips in 24-well plates with 50,000 cells per well and allowed to grow to ~60% confluency. For the low temperature incubation experiment at 4 °C, the cells were first kept at 4 °C for 30 min, and then washed twice with ice-cold PBS and incubated with Rh-DOPC/30 nm ND (weight ratio 1:10) at a Rh-DOPC concentration of 5 μg mL⁻¹ in the cell medium for another 1 h at 4 °C instead of the regular 37 °C condition. For the endocytosis experiment, the cells were washed twice with ice-cold PBS, and then incubated with Rh-DOPC, Rh-DOPC/30 nm ND, Rh-DOPC/50 nm ND, Rh-DOPC/100 nm ND or Rh-DOPC/CNT with same concentrations of Rh-DOPC and ND (or CNT) as mentioned above for the 4 °C incubation condition in the cell medium for 1 or 4 h at 37 °C. After the designated incubation time, the cells were washed twice with ice-cold PBS and fixed with fresh 4% paraformaldehyde for 15 min at room temperature. The cells were counterstained with DAPI for the cell nucleus, Alexa Fluor 488 phallloidin for the cell actin or Lysotracker Green for acidic organelles following the manufacturer’s instructions. The coverslips were mounted on glass microscope slides with a drop of antifade mounting media (Sigma-Aldrich Co., USA) to reduce fluorescence photobleaching. The intracellular localization was visualized under a laser scanning confocal fluorescence microscope (LSM510Meta, CarlZeiss Inc., Thornwood, NY).

To image co-delivery of calcein and doxorubicin, 1 μL of purified calcein-loaded DOPC liposomes were mixed with 99 μL of ND (500 μg mL⁻¹) and the sample was centrifuged at 70,000 rpm for 12 min. The supernatant was removed to get rid of free calcein and the precipitant containing the ND/DOPC complex was dispersed in 95 μL buffer (5 mM HEPES, pH 7.6). Doxorubicin (2.5 μL, 1 mg mL⁻¹) was then added to the solution. HeLa cells were seeded onto 14 mm coverslips in 24-well plates with ~50,000 cells per well and allowed to grow until ~60% confluent. Cells were washed twice with ice-cold PBS, and then incubated with doxorubicin/calcein-DOPC/ND or free calcein-DOPC with an equivalent calcein concentration of 45 μM and doxorubicin 5 μg mL⁻¹ in cell medium for 4 h at 37 °C. After incubation, the cells were washed twice with ice-cold PBS and fixed with fresh 4% paraformaldehyde for 15 min at room temperature. The cells were then counterstained with DAPI for the cell nucleus following the manufacturer’s instructions. The coverslips were mounted and visualized under the laser scanning confocal microscope.

Cell Growth Inhibition Assay. HeLa cells were seeded in 96-well plates at 5,000 cells per well in 100 μL of cell medium, and incubated at 37 °C in 5% CO₂ humidified atmosphere for 24 h. The culture medium was then replaced with 100 μL of freshly prepared culture medium containing ND, CNT, NGO, doxorubicin or doxorubicin/DOPC/ND at different carbon or doxorubicin concentrations (weight ratio of doxorubicin to ND is 1:10). The cells were further incubated for 72 h, and then 25 μL of MTT stock solution (5 mg mL⁻¹ in PBS) was added to each well to achieve a final concentration of 1 mg mL⁻¹, with the exception of the wells as blank to which 25 μL of PBS was added. After incubation for another 2 h, 100 μL of extraction buffer (20% SDS in 50% DMF, pH 4.7, prepared at 37 °C) was added to the wells and incubated for another 4 h at 37 °C. The absorbance was measured at 570 nm using a SpectraMax M3 microplate reader. Cell viability was normalized to that of HeLa cells cultured in the cell media.

2. IR spectroscopy. To characterize the surface chemistry of our ND sample, attenuated total reflection (ATR) FT-IR was used. The sample was prepared by placing the ND sample at the ATR crystal surface. As shown in Figure S2, the signal for C=O bonds of carboxylic acid usually appears at 1775 cm⁻¹. The broad band at 3000-3600 cm⁻¹ can be attributed to the hydrogen-bonded-OH of physisorbed water on the surface.1,2 The bands in the range 700-1450 cm⁻¹ also (in particular, 1275 cm⁻¹) have been ascribed to ether-like groups (O-C-O) on the diamond powders. Therefore, the surface of the ND contains carboxyl and other oxygenated species.
Figure S2. FT-IR spectra of our ND sample after acid treatment.

3. TEM. The TEM micrographs of ND sample is shown in Figure S3. The size distribution is relatively large and the shapes of the ND particles are irregular.

Figure S3. TEM of 30 nm ND samples.

4. Adsorption kinetics. To measure the adsorption kinetics of DOPC liposome to ND, the concentration of Rh-DOPC (50 μg mL⁻¹) was dispersed in HEPES buffer (10 mM, pH 7.6). Then 30 nm ND was added to the Rh-DOPC at different weight ratios. The Rh-DOPC fluorescence was monitored for 60 min on a fluorescence microplate reader by exciting at 532 nm (Infinite F200 Pro, Tecan). As shown in Figure S4, the adsorption kinetics was quite fast and most of the fluorescence was quenched in the first minute. Note that fluorescence quenching is used to monitor the adsorption reaction.
Figure S4. Adsorption kinetics of ND by DOPC.

5. Additional cryo-TEM micrographs. In addition to the cryo-TEM micrographs in the main paper, we also present small clusters of these hybrid materials. Figure S5A and S5B show the DOPC/ND complexes and the ND particles are decorated around the liposome surface. Figure S5C and S5D show the complex between individual CNTs and DOPC liposomes. Figure S5E and S5F show the complex formed between NGO and DOPC. Since the size, geometry, and surface property of these nano-carbons are all different, they display different binding interactions with the DOPC liposomes.

Figure S5. Cryo-TEM of DOPC with ND (A, B), CNT (C, D), and with NGO (E, F). Scale bars = 100 nm.

6. Laurdan fluorescence spectra in DPPC. In the main paper, we presented the Laurdan fluorescence spectra in DOPC liposome when titrated with ND. As a control experiment, we also embedded the Laurdan dye in the DPPC liposome, whose lipid phase transition temperature ($T_c$) is 41 °C. As shown
in Figure S6, when DPPC is close to the fluid phase (41 °C), Laurdan emission is red shifted to a maximum emission wavelength at 481 nm with respect to the emission observed in the gel phase (25 °C) with a maximum at 445 nm. This red spectral shift, observed in the gel phase and the liquid crystalline phase can be attributed to dipolar relaxation phenomena, originating from the sensitivity of the probe to the polarity of its environment. Therefore this result serves as a positive control to show that the Laurdan dye was successfully incorporated into our bilayer systems.\textsuperscript{3-5} The lack of fluorescence shift in our DOPC sample thus confirms that nano-carbon adsorption does not affect the bilayer structure.

![Normalized and emission spectra of Laurdan in DPPC liposome at 25 °C and at 41 °C. The emission spectra were measured at an excitation wavelength of 340 nm.](image)

**Figure S6.** Normalized and emission spectra of Laurdan in DPPC liposome at 25 °C and at 41 °C. The emission spectra were measured at an excitation wavelength of 340 nm.

Here, we also present the fluorescence spectra of Laurdan loaded DOPC titrated with NGO and CNT. In all these cases, we only observed fluorescence intensity drop without spectral shifting, suggesting that the fluidity of the DOPC bilayer was maintained when binding to these nano-carbons.

![Laurdan dye fluorescence spectra with CNT (A) and with NGO (B). The tested CNT and NGO concentrations are 0.05, 0.1, 0.2, 0.5, 1, 2.5, 5, 10, 20 μg mL\(^{-1}\) and 0.5, 1, 2, 5, 10, 25, 50, 100, 200 μg mL\(^{-1}\) respectively. The arrow heads point to the increase of nano-carbon concentration.](image)

**Figure S7.** Laurdan dye fluorescence spectra with CNT (A) and with NGO (B). The tested CNT and NGO concentrations are 0.05, 0.1, 0.2, 0.5, 1, 2.5, 5, 10, 20 μg mL\(^{-1}\) and 0.5, 1, 2, 5, 10, 25, 50, 100, 200 μg mL\(^{-1}\) respectively. The arrow heads point to the increase of nano-carbon concentration.
7. Nano-carbon assisted internalization of DOPC. In the main paper, we presented the delivery of DOPC complexed with CNT, ND of 30 nm and 100 nm, where DN was much more efficient. Here we also show that 50 nm ND and NGO can also assist the delivery similar to the other sized ND samples.

![Confocal fluorescence micrographs of HeLa cells incubated with Rh-DOPC/50 nm ND complex and DOPC/NGO at 37 °C for 4 hr. The cells were counterstained with DAPI (blue) for the cell nucleus, Alexa Fluor 488 phalloidin (green) for the cell actin, and the red fluorescence was from Rh-DOPC.](image)

**Figure S8.** Confocal fluorescence micrographs of HeLa cells incubated with Rh-DOPC/50 nm ND complex and DOPC/NGO at 37 °C for 4 hr. The cells were counterstained with DAPI (blue) for the cell nucleus, Alexa Fluor 488 phalloidin (green) for the cell actin, and the red fluorescence was from Rh-DOPC.

8. Cellular uptake mechanism. We also investigated the entry mechanism of Rh-DOPC/ND into Hela cells. Endocytosis is known as one of the important entry mechanisms for various extracellular materials which is energy dependent and can be hindered when incubation is performed at low temperatures (e.g., 4 °C instead of 37 °C). As shown in Figure S9A, incubation of Rh-DOPC/ND at 4 °C for 1 h resulted in significant depressed internalization of Rh-DOPC/ND compared to that incubation at 37 °C (Figure S9B). The confocal laser scanning microscopy image of Hela cells shown in Figure S10 also shows the red fluorescence from Rh-DOPC/ND was mainly localized in the acidic organelles labeled with Lysotracker Green (green) after 4 h of incubation.
Figure S9. Confocal fluorescence micrographs of HeLa cells incubated with ND/Rh-DOPC complex at 4 °C (A) or 37 °C (B) for 1 hr. The cells were counterstained with DAPI (blue) for the cell nucleus, Alexa Fluor 488 phalloidin (green) for the cell actin, and the red fluorescence was from Rh-DOPC.

Figure S10. Confocal images showing cellular uptake of nanoparticles (red) by HeLa cells after 4 h of incubation with ND/Rh-DOPC complex at 37 °C. The acidic organelles were stained with Lysotracker Green (green), and the cell nuclei were counterstained with DAPI (blue).

9. Doxorubicin adsorption capacity. Doxorubicin is a commonly used anti-cancer drug and we herein compare its loading capacity on various nano-carbons. 200 μL ND (100 μg mL⁻¹), CNT (10 μg mL⁻¹) or NGO (2 μg mL⁻¹) was mixed with 4 μL doxorubicin (1 mg mL⁻¹), respectively and the samples were centrifuged at 120,000 rpm using ultracentrifugation. The supernatant fluorescence containing non-adsorbed doxorubicin was measured. In another experiment, 200 μL ND (100 μg mL⁻¹), 1 μL DOPC (5 mg mL⁻¹) and 4 μL doxorubicin (1 mg mL⁻¹) was mixed and the sample was centrifuged at 70,000 rpm. The supernatant fluorescence was measured. The fluorescence emission spectra were recorded using a fluorometer with an excitation wavelength at 485 nm.
The loading capacities are plotted in Figure S11 normalized to 100 μg of each nano-carbon. NGO has the highest capacity, adsorbing about 3 times of the mass of the carrier. CNT has the second highest capacity, adsorbing around the same mass of doxorubicin as its own mass. ND has the lowest capacity and each 100 μg of ND loads ~20 μg of doxorubicin. This is understandable from the geometry of these materials. All the carbon atoms are on the surface for CNT and NGO, while ND has a solid carbon core. For NGO, both sides can adsorb the drug, while for CNT the drug cannot diffuse into the inside of the tube. Another note is that for ND, the loading capacity does not change even when ND is first mixed with DOPC liposome. This suggests that DOPC and doxorubicin occupies different sites on the ND surface. Considering its low toxicity, 20% loading capacity is still useful for drug delivery applications. This doxorubicin loading capacity agrees with that reported in the literature by ND.²

**Figure S11.** Doxorubicin loading capacity by 100 μg of ND, ND/DOPC complex, NGO, and CNT.

**Figure S12.** Quantification of Rh-labeled liposomes with various lipid composition adsorption by ND.
Additional references.


