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Assembly of Single-Walled Carbon Nanohorn Supported Liposome Particles

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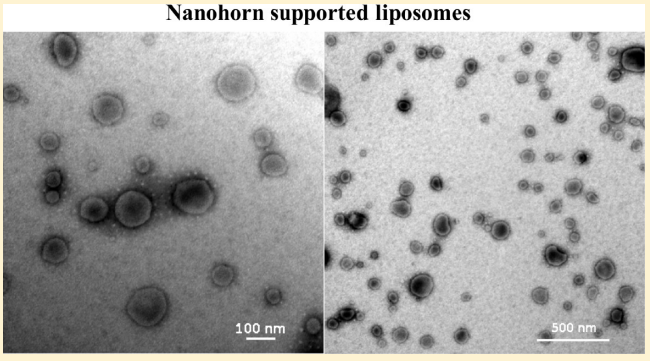
ABSTRACT: Nanoparticle-supported liposomes can be a promising platform for drug delivery, vaccine development, and biomedical imaging. Single-walled carbon nanohorns are a relatively new carbon nanomaterial, and they could be used as carriers of drug and imaging reagents. Assembling lipids around carbon nanohorns would confer this nanomaterial much broader applications such as vaccine development and targeted drug delivery by embedding a target protein or immunogenic protein into the lipid bilayer structure. Here, we show the assembly of functionalized single-walled carbon nanohorns ($-CH_2-CH_2-COOH)_x$, ~100 nm) with positively charged lipids through a freeze and thaw cycle. The assembled complex particles can be readily separated from individual nanohorns or liposomes under specific centrifugation conditions. The results from transmission electronic microscopy, flow cytometry through nitrobenzoxadiazole labeled lipids, and zeta potential analysis clearly show that the nanohorns are encapsulated by liposomes with a median size of ca. 120 nm.



Liposomes and many types of nanoparticles have been studied as potential drug delivery vehicles. Despite the similarities, i.e., they both can have a desirable loading capacity and release the contents at a controlled rate in a specific location when attached with targeting ligands, liposomes and nanoparticles are complementary delivery systems because (1) nanoparticles tend to have a longer half-life during the circulation in blood and the extravasations from plasma to the tumor cells;[1,2] (2) liposomes provide a better chance to deliver drugs across cell membrane at the targeting site; and (3) they have different drug preferences, loading, and releasing chemistry.[1,2] However, the combination of the two types of particles could possess unique advantages over any of the particles alone. Nanoparticle supported liposomes (NsLs) have recently been assembled and described.[3] Nanoparticles usually stabilize the liposomes through electrostatic interactions and may also increase the concentrations of poly(ethylene glycol) (PEG) that can be attached to the surface of liposomes, resulting in NsLs, when intravenously injected, with improved ability to evade clearance by the reticuloendothelial system (RES).[3] NsLs could have a large loading capacity and a desirable controlled release rate depending on the large adsorption area and special pore surface chemistry of the encapsulated nanoparticle.[4] Moreover, a targeting ligand attached liposome could transport nanoparticles into cells through receptor-mediated endocytosis. This is critical when large molecule drugs or therapeutic genes are to be released into the cytoplasm.[5]

In this report, functionalized (carboxylated) carbon single-walled nanohorns, SWNH $(-CH_2-CH_2-COOH)_x$, were prepared to provide SWNHs with hydrophilic surfaces for encapsulation into the liposomes. The SWNH$(-CH_2-CH_2-COOH)_x$ are nontoxic[6,7] nanoparticle aggregates of single graphene tubules and are spherical dahlia-like-shaped with a narrow diameter range reported as 80−100 nm.[8] The surface area of SWNHs range from 300 to 1000 m^2/g.[9] The functionalization process introduces mesoporous structures on the SWNHs, and consequently, drug molecules could bind not only to the surface, but also to the inner graphene wall. Research has also shown that molecules that have stacking aromatic rings once diffused into SWNHs will release in a slow and stable manner.[10]

Single-walled nanohorns were synthesized by Nd:YAG laser vaporization of graphite rods in an argon atmosphere at 1100 °C as described in detail elsewhere.[11,12] Functionalized SWNH-$(-CH_2-CH_2-COOH)_x$ with carboxyl groups was obtained by high-speed vibration milling as previously described.[13] Briefly, a mixture of nanohorns and succinic acid acyl peroxide (1:100 in mass) was vigorously shaken in a stainless steel capsule for 1.5 h. The ground ultrafine powder was washed three times with acetone and centrifuged to collect the sediment. Twenty minute

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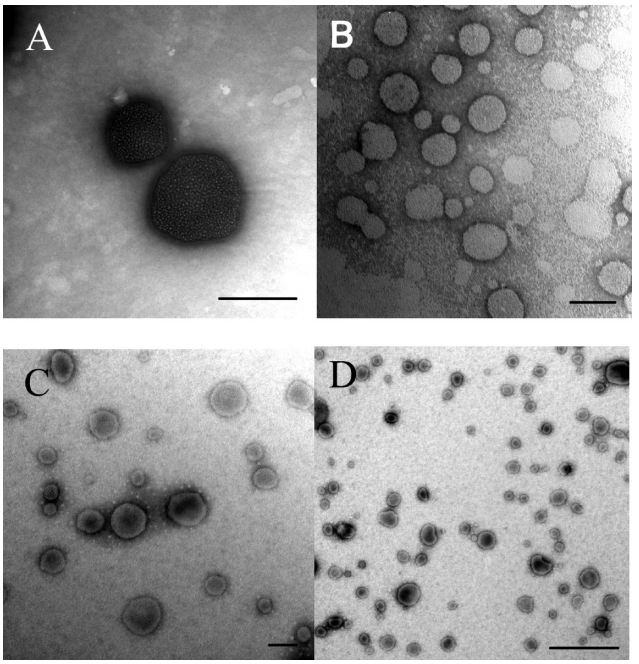

Figure 1. Representative TEM images of (A) SWNH($-CH_2-CH_2-COOH)_x$, (B) hollow DOTAP liposomes, and (C) and (D) SWNH($-CH_2-CH_2-COOH)_x$ supported liposomes. Scale bar in (A), (B), and (C) = 100 nm. Scale bar in (D) = 500 nm. Samples were deposited onto carbon coated copper grids. Negative staining was performed using 2% phosphotungstic acid.

sonication was performed to dissolve the sediment in ultrapure water yielding solutions of SWNH($-CH_2-CH_2-COOH)_x$. On the other hand, cationic liposomes were made by extruding 0.4 mg hydrated 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) lipid film through polycarbonate membranes with pore sizes of 100 nm. The hydration buffer contains 0.9 NaCl, 5% dextrose, and 10% sucrose in Tris-HCl buffer with a pH at 7.4. SWNH($-CH_2-CH_2-COOH)_x$ were briefly sonicated to break up aggregates and incubated with freshly made cationic liposomes. Particles of these functionalized SWNHs and liposome were quantified by flow cytometer (FCM), and the concentrations were both optimized to ~100 particles/μL. After gently homogenizing the optimized samples at a volume ratio of 1:10 SWNH($-CH_2-CH_2-COOH)_x$:liposomes, a ratio chosen to ensure there are excessive liposomes for functionalized SWNHs, three freeze and thaw cycles were applied using liquid nitrogen and a warm water bath. Samples were then centrifuged under 10 000 rcf for 10 min, and SWNH($-CH_2-CH_2-COOH)_x$ supported liposomes were collected in the top layer. It was also found that, in the presence of 10% sucrose, DOTAP liposomes could be collected in the top layer after 30 min of centrifugation under 16 000 rcf, and SWNH($-CH_2-CH_2-COOH)_x$ could be collected in the top liquid layer after 30 min of centrifugation under 10 000 rcf. Therefore, washing the SWNH($-CH_2-CH_2-COOH)_x$ supported liposomes with the hydration buffer and repeating the centrifugation (10 000 rcf, 10 min) can significantly increase the purity.

Transmission electronic microscopy (TEM) images for SWNH($-CH_2-CH_2-COOH)_x$, hollow liposomes, and SWNH-($-CH_2-CH_2-COOH)_x$ supported liposomes are shown in Figure 1. As can be seen, all particles are of spherical shape and

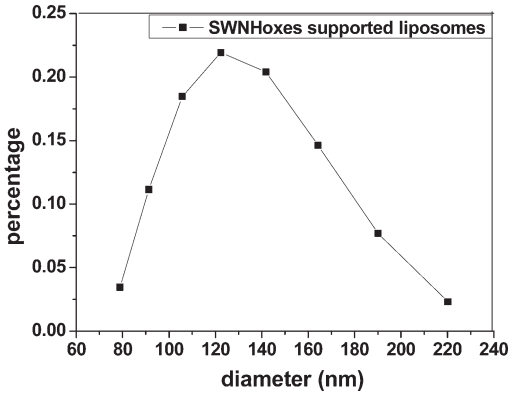

Figure 2. Size distribution of SWNH($-CH_2-CH_2-COOH)_x$ supported liposomes. Refractive index = 1.33. Viscosity = 0.8872 cP. Samples were measured at 25 °C.

have a narrow distribution centered at around 120 nm, which was confirmed by the dynamic light scattering test result shown in Figure 2. As shown in Figure 1C, a SWNH($-CH_2-CH_2-COOH)_x$ supported liposome is a combination of the particles shown in (A) and (B). A negatively charged SWNH($-CH_2-CH_2-COOH)_x$ is encapsulated in a cationic liposome and could stabilize and support the liposome from inside. The large-scale view of the particles shown in Figure 1D illustrates that a high purity of the assembled complex particles can be achieved by the centrifugation step described above. Centrifugation in the presence of 10% sucrose was performed only once in this case, but no hollow liposome or unencapsulated SWNH($-CH_2-CH_2-COOH)_x$ was observed in Figure 1D.

To further confirm the structure of the particles, nitrobenzoxadiazole (NBD) labeled 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine was added to the lipid mixture (up to 15% mass ratio) to produce NBD-DOTAP liposomes and SWNH($-CH_2-CH_2-COOH)_x$ supported NBD-DOTAP liposome assembly. Results of the fluorescence of different particles detected by FCM are shown in Figure 3. The overlay histogram shows a similar fluorescence intensity shared by NBD-DOTAP liposomes and SWNH($-CH_2-CH_2-COOH)_x$ supported NBD-DOTAP liposomes, and SWNH($-CH_2-CH_2-COOH)_x$ particles without liposomes assembled around show no fluorescence signal. These results indicate that the surface structure of SWNH($-CH_2-CH_2-COOH)_x$-supported liposome samples is most likely the same as that of hollow NBD-DOTAP liposomes, since the granularity and fluorescence intensity are almost identical (see FCM scatter plots in Figure 3). Zeta potential and electrophoresis mobility of the three particles shown in Table 1 further confirm the similar surface characteristics of DOTAP liposomes and SWNH($-CH_2-CH_2-COOH)_x$-supported DOTAP liposomes (N^-sL^+, negatively charged nanohorn supported positively charged liposomes), since their zeta potentials significantly differ from that of SWNH($-CH_2-CH_2-COOH)_x$. As expected, those N^-sL^+ particles and SWNH($-CH_2-CH_2-COOH)_x$ particles travel in opposite directions in an applied electric field. These results confirm the encapsulation of SWNH-($-CH_2-CH_2-COOH)_x$ in liposomes and that the gray circle surrounding SWNH($-CH_2-CH_2-COOH)_x$ in Figure 1C and D represents DOTAP lipid bilayers. The FCM results shown in Figure 3 also serve as a strong indication that a high purity of the N^-sL^+ particles can be achieved using the purification method described above.

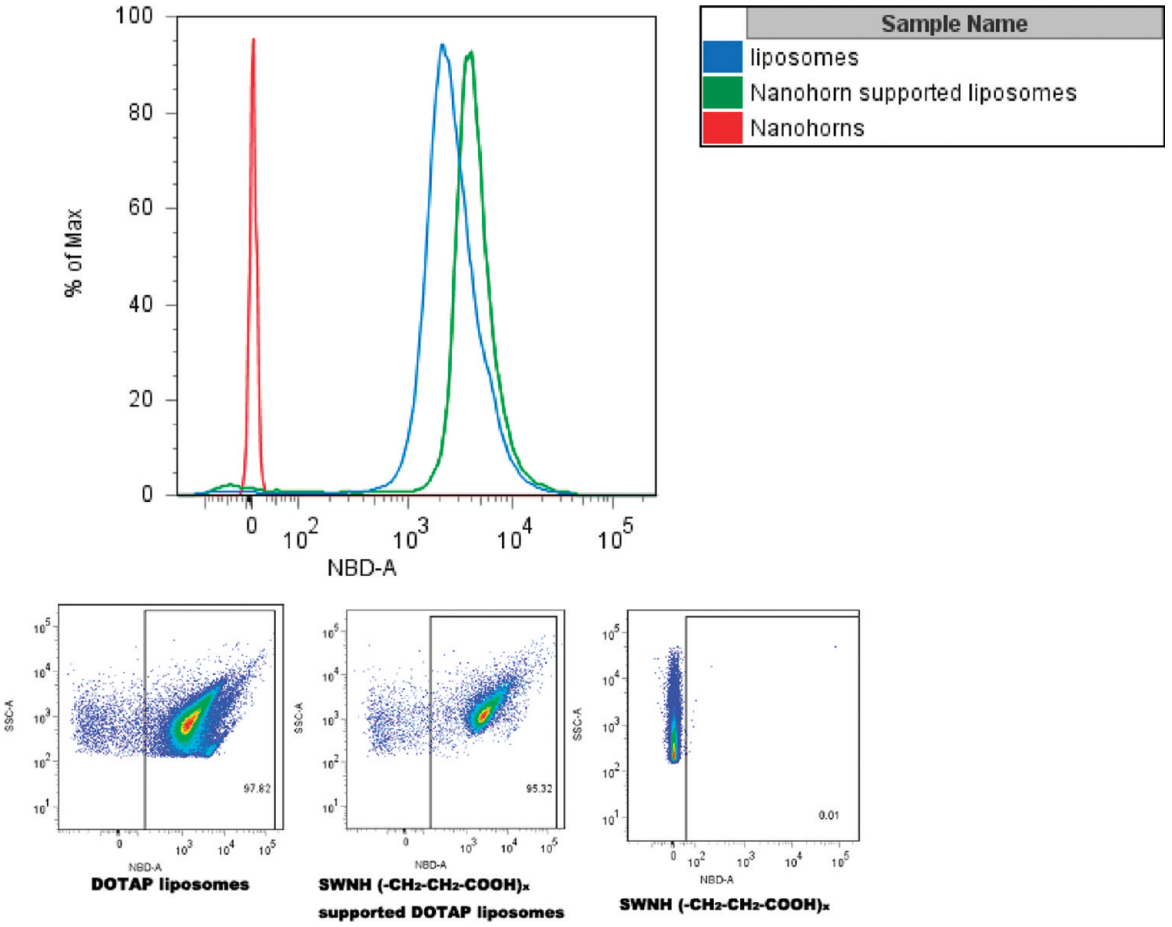

Figure 3. Fluorescent intensity of different particles. The histogram shows the fluorescent intensity distribution of NBD-DOTAP liposomes (blue), SWNH($-CH_2-CH_2-COOH)_x$ (red), and SWNH($-CH_2-CH_2-COOH)_x$ supported liposomes (green). The three corresponding FCM scatter plots show distribution of fluorescent intensity (horizontal axis, NBD-A) versus granularity (vertical axis, SSC-A).

Table 1. Zeta Potential and Electrophoresis Mobility of Particles[a]

sample	zeta potential (mv)	electrophoretic mobility (μm·cm/V·s)
hollow DOTAP liposome	24.6	1.928
nanohorn	34.4	−2.694
SWNH($-CH_2-CH_2-COOH)_x$ supported liposome	22.1	1.732

[a] All samples are in the hydration buffer (pH = 7.4), 25 °C.

Conceivably, functionalized nanohorn supported liposomes will not be limited just to N^-sL^+ types of particles. Positively charged nanohorns can be synthesized by the aryl diazonium functionalization as reported,[14] and therefore, it is possible to assemble the complex particles using negatively charged lipids (N^+sL^-), which are more commonly found in various biological membranes, for biomimetic purposes. The combination of positively or negatively charged nanohorns with anionic or cationic liposomes will significantly broaden the types of drugs that can be loaded, depending specifically on their charge or hydrophobicity. So far, many successful cases have shown that SWNH($-CH_2-CH_2-COOH)_x$ could be a very promising drug delivery vehicle in terms of the loading capacity and the controlled release. A good example is the anticancer drug cisplatin, which has recently become a commonly used molecule to test the two properties of certain functionalized nanohorns mentioned above.[15,16] However, the effects were mainly evaluated by directly providing cancer cell cultures with treatment of the drug carrying nanohorns. The targeting effect of nanohorns *in vivo* has not been well addressed. How many of the particles injected would nonspecifically bind to normal living cells and lead to their apoptosis is still unknown. On the other hand, liposomes conjugated with targeting ligands such as antibodies can navigate themselves to the cells that have corresponding receptors presented on the cell surface.[17] The particles assembled by the combination of ligand-attached liposomes and nanohorns could present a new and much improved drug delivery platform. For example, cisplatin could be loaded into SWNH($-CH_2-CH_2-COOH)_x$ particles,[15,16] which could subsequently be assembled with liposomes that contain lipids sensitive to the change of pH (such as dioleoyl-phosphatidyl-ethanolamine, DOPE). Targeting antibodies, such as antihuman epidermal growth factor receptor-2 (HER2) antibody (Herceptin),[18] could be attached to the assembled particles via poly(ethylene glycol) (PEG) on PEGylated phospholipids.[3] The presence of the lipid

bilayer will deter the escape of cisplatin from the particles through diffusion during circulation in blood. However, once the particles enter the targeted cells (such as breast cancer cells via Herceptin-HER interaction[18]), defects will appear on the lipid bilayer structure due to the presence of pH-sensitive lipids,[19] and cisplatin will be released from the particles via the lipid bilayer defects. Research on this novel drug delivery system is currently under investigation in our laboratories.

Functionalized single-walled nanohorn supported liposomes can have more novel applications beyond drug delivery systems. Magnetic resonance imaging (MRI) contrast agents containing Gd^{3+} ions have been loaded into SWNH$(-CH_2-CH_2-COOH)_x$ in the form of $Gd_3N@C_{80}$, trimetallic nitride template endohedral metallofullerenes (TNT-EMFs)[10] to lower the toxicity to the host because (1) lower amount is required due to the high ^1H magnetic resonance (MR) relaxivities of these TNT-EMF contrast agents[20] and (2) the encapsulation of $(Gd_3N)^{+6}$ clusters into fullerene cages limits the exposure to free Gd^{3+} ions.[12] Gadolinium containing SWNH$(-CH_2-CH_2-COOH)_x$ supported liposomes reveal an opportunity to make the drug carrier itself an MRI biosensor. This allows the in situ monitoring of the in vivo biodistributions of drugs in a macro or real-time manner.[21] For example, clearance of liposomes from the bloodstream by the RES, which has greatly limited the development and applications of liposomal models, can be further studied and understood in detail. Locating the pathogenic cells also becomes a possibility when using $Gd_3N@C_{80}$ TNT-EMFs encapsulated targeting nanohorn supported liposomes. The SWNH$(-CH_2-CH_2-COOH)_x$ along with the contrast agent will most likely be found inside the cytoplasm and remain there allowing a slow release of the therapeutic drugs, as the $(Gd_3N)^{+6}$ clusters remain detectable for several days post infusion.[12]

Liposomal vaccines are a new type of vaccine that are assembled by associating antigens with liposomes, and they have attracted much attention because of their high immunogenicity[22] and gene-free nature.[23] Size, exposure of epitopes, and stability of the liposomal vaccine particles are parameters most critical for their immunogenicity.[24,25] A commonly accepted measure to increase the stability is to incorporate PEGylated phospholipids into the liposome, especially when the vaccines are to be injected intravenously. However, the grafting density and polymer length of PEG strongly influence the exposure of the epitopes that need to be presented.[26] SWNH$(-CH_2-CH_2-COOH)_x$ stabilizes liposomes from inside and enables the entire liposome surface to be solely used for epitope presenting, and the size of the assembled particle (80−220 nm, see Figure 2) falls within the range that can be efficiently taken up by dendritic cells.[24]

In summary, we have shown the assembly and structure of SWNH$(-CH_2-CH_2-COOH)_x$ supported DOTAP liposomes. The combination of functionalized single-walled nanohorns with liposomes reveals a variety of opportunities for the complex particle not only as drug delivery vehicles in terms of high loading capacity, broad drug selectivity, cell targeting, and controlled release, but also as many more potential promising applications such as drug biodistribution real-time MRI, MRI contrast agent targeting, and liposomal vaccine assembly. It can be a useful platform to load with different types of molecules or a promotion for developing other similar nanoparticle complexes for various applications.

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