Electronic Supplementary Material (ESI) for Chemical Communications.
This journal is © The Royal Society of Chemistry 2014

Membrane analysis with amphiphilic carbon dots

Sukhendu Nandi,[a] Ravit Malishev,[a] Kaviya Parambath Kootery,[a]

Yelena Mirsky,[b] Sofiya Kolusheva[b] and Raz Jelinek[*a,b]

[a]*Department of Chemistry, Ben Gurion University of the Negev, Beer Sheva 84105, Israel*

[b]*Ilse Katz Institute for Nanotechnology, Ben Gurion University of the Negev, Beer Sheva 84105, Israel.*

**Author to whom correspondence should be addressed: razj@bgu.ac.il*

Experimental section

Materials

All chemicals used for carbon dot synthesis were purchased from the Sigma-Aldrich Company and have been used without further purification. All solvents were HPLC grade. *L*-α-phosphatidylcholine (egg PC), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-*N*-(7-nitro-2–1,3-benzoxadiazol-4-yl) (*N*-NBD-PE) were obtained from Avanti Polar Lipids (AL). Amyloid beta 1-40 (Aβ40) was purchased from Peptron (South Korea) in a lyophilized form at >90% purity (HPLC). 1,1,1,3,3,3-hexafluoro-2-propanol and sodium phosphate monobasic were purchased from Sigma- Aldrich (Rehovot, Israel). BODIPY-PH was provided by Professor M. Meijler, Ben Gurion University. The dye was synthesized according to a published protocol.[1]

Syntheses and spectroscopic characterisation of compound 1-3

Syntheses and spectroscopic characterisation of (3*R*,4*R*)-2,5-Dioxotetrahydrofuran-3,4-diyl didodecanoate (compound 1)

166 mL of lauroyl chloride (0.7 mol) was added to 30 g of finely powdered *L*-tartaric acid (0.2 mol) in a 500 mL round bottom flask equipped with a magnetic stirrer bar and an air bubbler. The reaction mixture was heated at 90 °C for 24 h and then cooled to room temperature. In order to remove lauric acid and excess lauroyl chloride, the crude mixture was dissolved in a minimum amount of *n*-hexane and kept at room temperature for 12 h. The product was precipitated in *n*-hexane and it was filtered, washed thoroughly with *n*-hexane, and dried under vacuum. 89 g of **1** (90%) was obtained as white powder.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 5.67 (s, 2H), 2.48 (t, *J* = 7.5 Hz, 4H), 1.68 (m, 4H), 1.49 – 1.09 (m, 32H), 0.90 (t, *J* = 7.1 Hz, 6H) ppm.

^{13}C NMR (101 MHz, $CDCl_3$) δ = 172.60, 163.48, 72.05, 33.30, 31.87, 29.55, 29.54, 29.36, 29.29, 29.12, 28.85, 24.51, 22.64, 14.04 ppm.

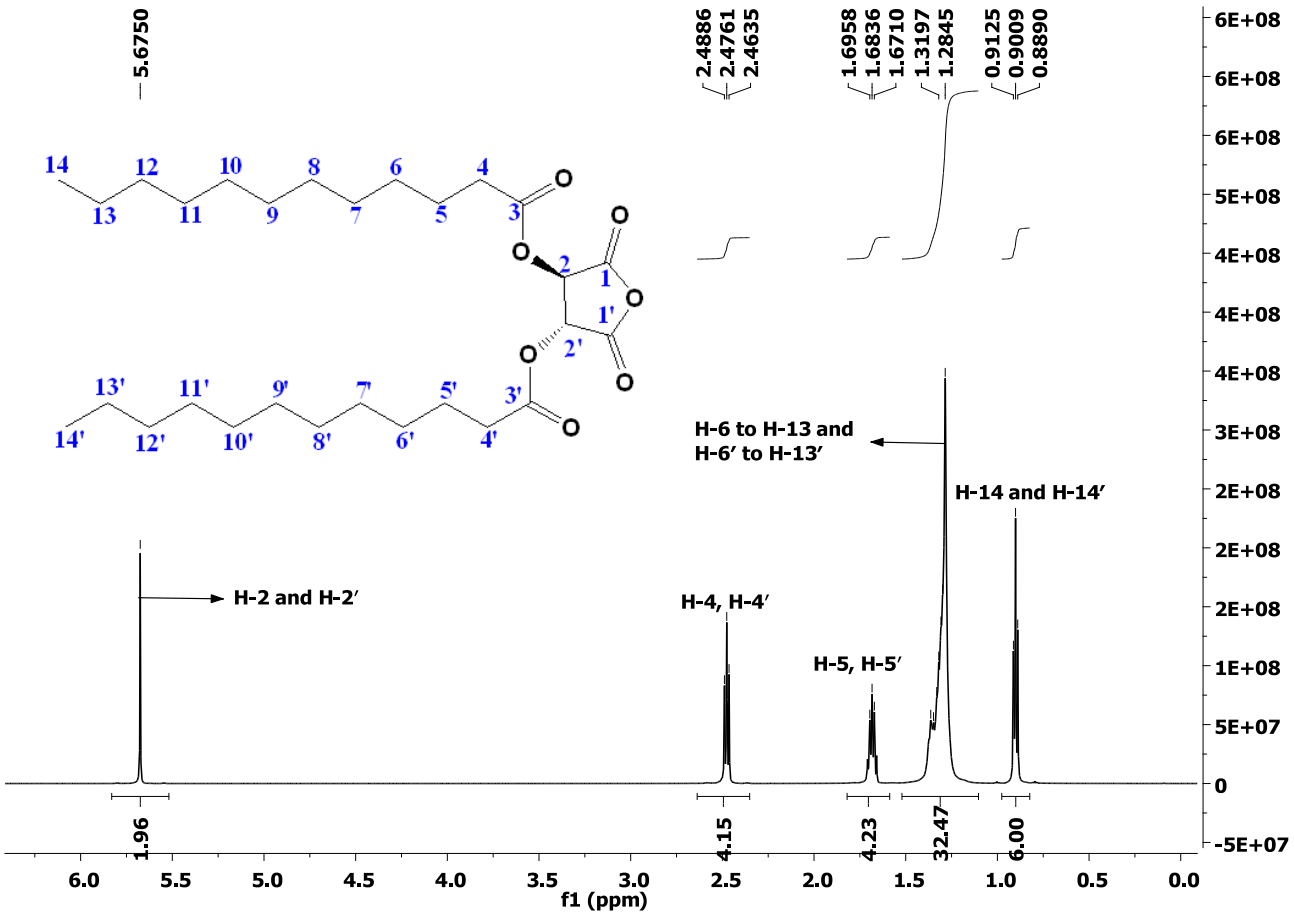

Figure 1, SI: ^{1}H NMR spectrum of 1 in $CDCl_3$ recorded at 298 ºK.

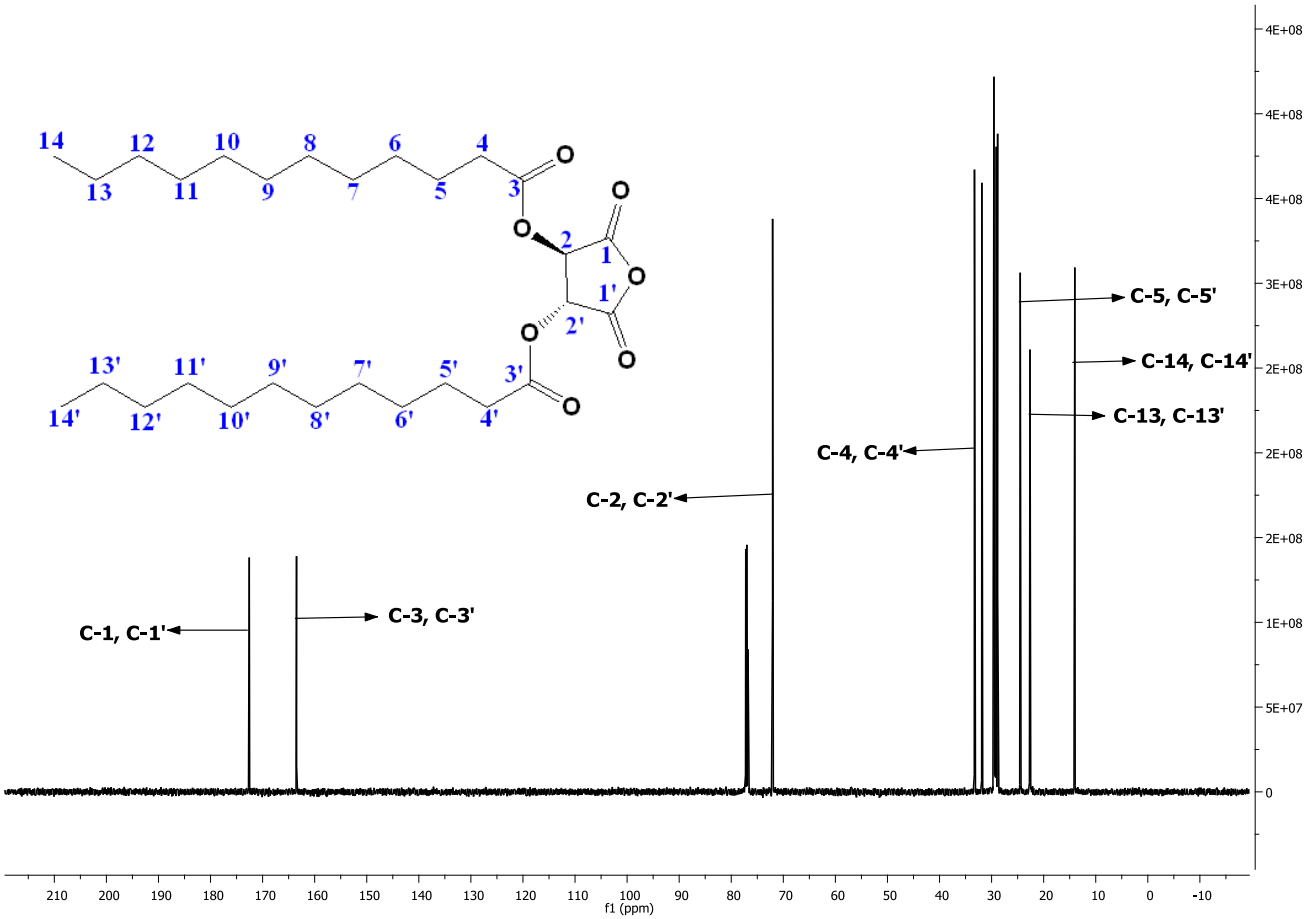

Figure 2, SI: ^{13}C NMR spectrum of 1 in $CDCl_3$ recorded at 298 °K.

Synthesis and characterisation of (2*R*,3*R*)-2,3-Bis(dodecanoyloxy)-4-oxo-4-(((2*R*,3*S*,4*S*,5*R*)-3,4,5,6-tetrahydroxytetrahydro-2H-pyran-2-yl)methoxy)butanoic acid (compound 2)

To a solution of *D*-glucose (20 g, 0.11 mol) in anhydrous DMF (150 mL) 1 (11 g, 0.022 mol) was added with stirring under argon and the reaction mixture was allowed to cool down to 0 °C, followed by addition of dry pyridine (1.8 mL, 0.022 mol). The reaction was continued under an argon atmosphere at 0 °C for 2-3 h, followed by an additional 3 days at room temperature. After completion of the reaction, the mixture was poured into ice-water mixture and then 2 N HCl was added at 0 °C vigorous stirring. The product was extracted with ethyl acetate, washed four times with brine solution, dried over sodium sulphate and the organic solvent was removed under reduced pressure to obtain the crude product (12.5 g, 83%). The crude product contains a mixture of regioisomers (monoesters) of *D*-glucose (as confirmed by TLC and HPLC-MS and ^{13}C NMR). The 6-substituted monoester 2 (~ 80%) was precipitated from the mixture in the following manner: the crude mixture was dissolved in a minimum amount of *n*-hexane under reflux and a half volume of acetone was added. The solution was cooled to 0°C in an ice-water bath and then kept 12 hrs at room temperature. **2** was precipitated from the mixture, filtered and dried to obtain 3.1 g (21%, α/β = 1.8/1) of **2** as a mixture of anomers.

^{1}H NMR (400 MHz, acetone-d_6) δ = 5.88 (d, *J* = 2.8 Hz, 1H), 5.85 (d, *J* = 2.6 Hz, 1H), 5.24 (d, *J* = 3.6 Hz, 1H), 4.64 (dd, *J* = 8.1, 4.9 Hz, 1H), 4.46 – 4.32 (m, 1H), 4.09 (ddd, *J* = 9.8, 4.7, 1.9 Hz, 1H), 3.84 (t, *J* = 9.2 Hz, 1H), 3.57 – 3.47 (m, 2H), 2.55 (m, 4H), 1.78 (d, *J* = 7.2 Hz, 4H), 1.66 – 1.27 (m, 32H), 1.03 (t, *J* = 6.7 Hz, 6H) ppm.

^{13}C NMR (101 MHz, acetone-d_6) δ = 173.61, 173.38, 168.12, 167.33, 98.95, 94.49, 78.54, 76.92, 75.64, 75.37, 74.28, 72.31, 72.08, 72.05, 72.01, 70.96, 66.59, 66.41, 34.97, 34.92, 33.29, 30.88, 30.67, 30.35, 30.33, 29.86, 26.21, 26.19, 23.95, 14.94 ppm.

HRMS (ESI$^-$, m/z, [M−H]$^-$) calculated for $C_{34}H_{59}O_{13}$: 675.3956 Found: 675.3951

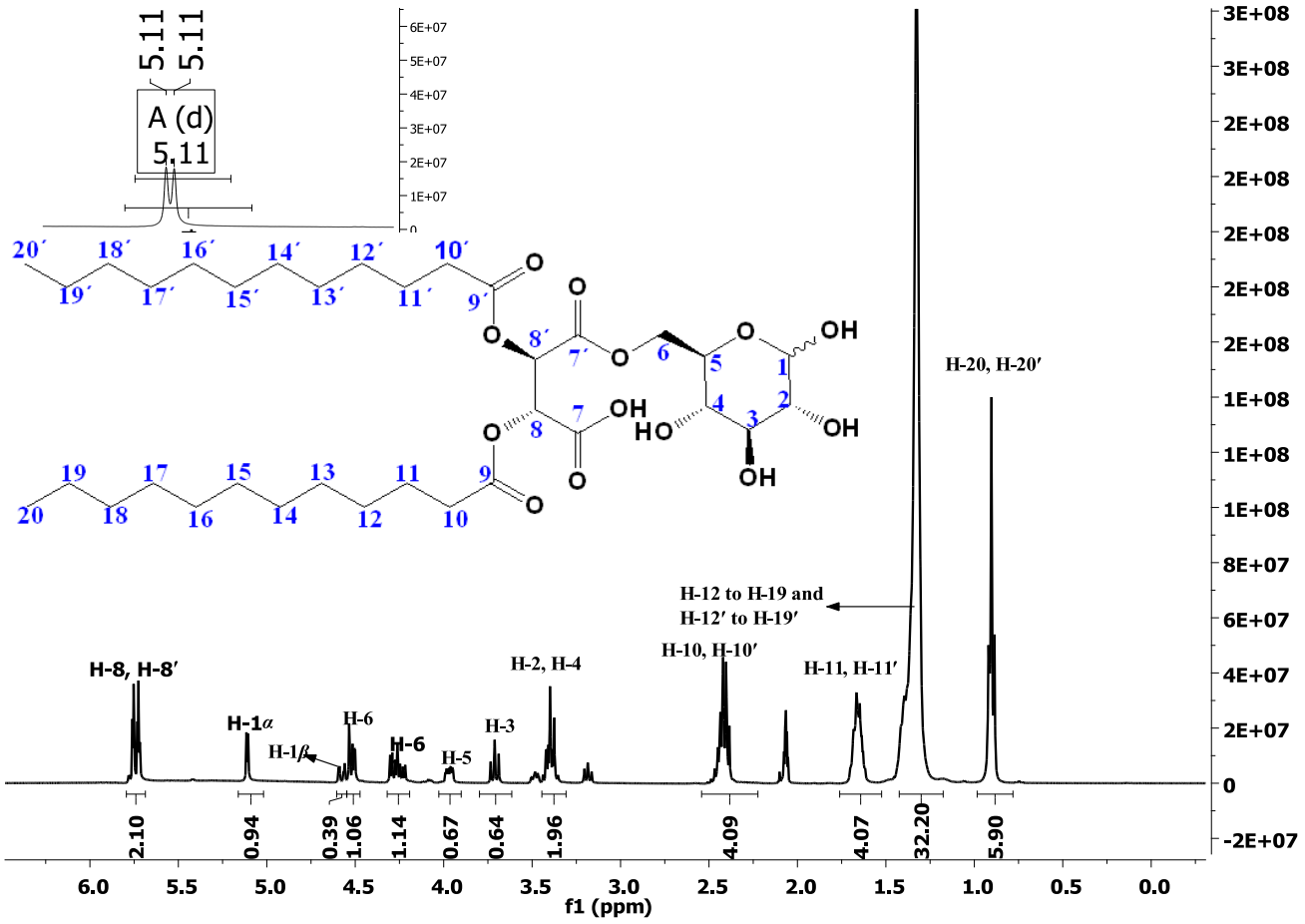

Figure 3, SI: ^{1}H NMR spectrum of 2 in acetone-d_6 recorded at 298 °K.

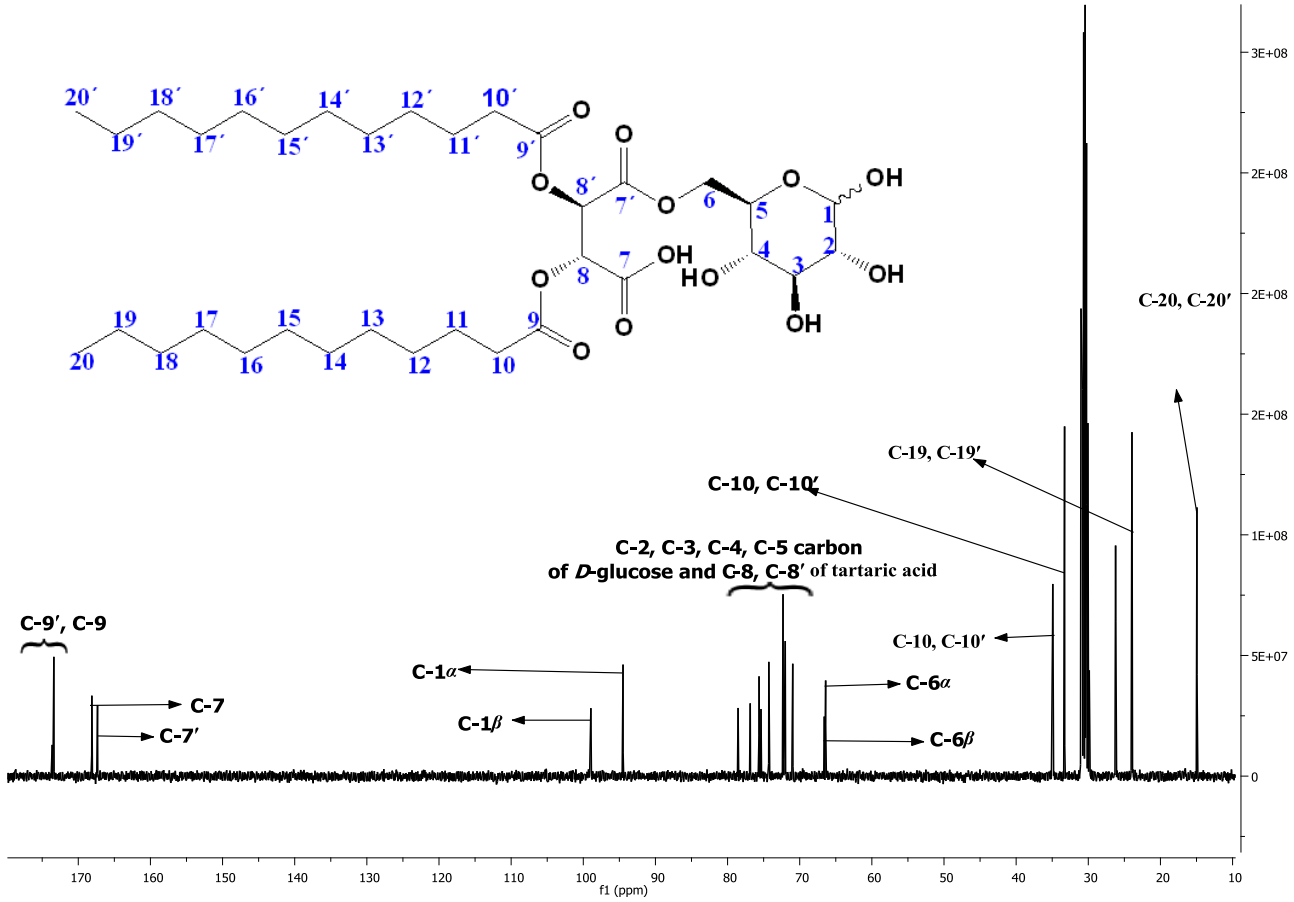

Figure 4, SI: ^{13}C NMR spectrum of 2 in acetone-d_6 recorded at 298 °K.

Synthesis and characterisation of amphiphilic carbon dots (carbon dots, compound 3)

100 mg of compound **2** and 330 µL DI water were placed in a teflon film tightened, septum-capped test tube and then heated in an oven to 125 °C for 2.5 h. Upon completion of the hydrothermal reaction, the resultant mixture was allowed to cool to room temperature yielding a brown precipitate implying the formation of carbon dots. The brown precipitate was then re-dispersed in 5 mL of chloroform through vortexing and centrifuged at 14,000 rpm for 30 min to remove high-weight precipitate and agglomerated particles. Chloroform was gently evaporated under reduced pressure to obtain a brown solid. The same procedure was repeated with 5 mL acetone, followed by solvent removal under reduced pressure to obtain monodisperse carbon dots (**3**) with a yield of 63 mg.

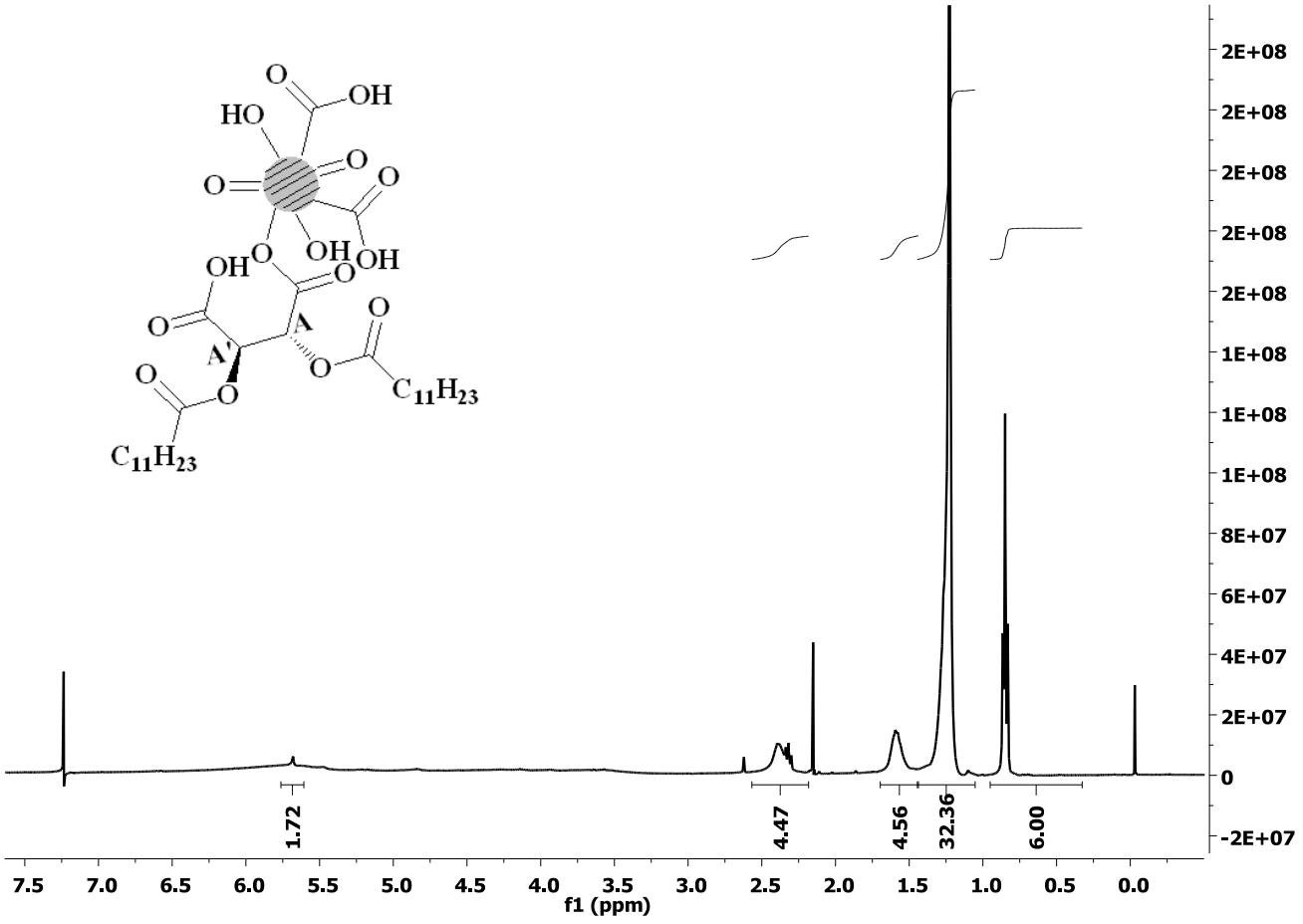

Figure 5, SI: ^{1}H NMR spectrum of carbon dots (3) in $CDCl_3$ recorded at 298 °K. The ^{1}H NMR spectrum of CQDS shows the proton with chemical shifts δ in the range of 2.36 (m, , 4H), 1.59 (m, 4H), 1.23 (m, 32H), 0.85 (t, J = 6.8 Hz, 6H) ppm are the characteristic signal of the lauroyl residue clearly suggesting carbon dots is coated with hydrocarbon chains (C_{12}). Protons with the chemical shifts of δ = 5.69 – 5.66 (m, 2H) ppm are the H-A and H-A′ proton of the tartaric acid unit.

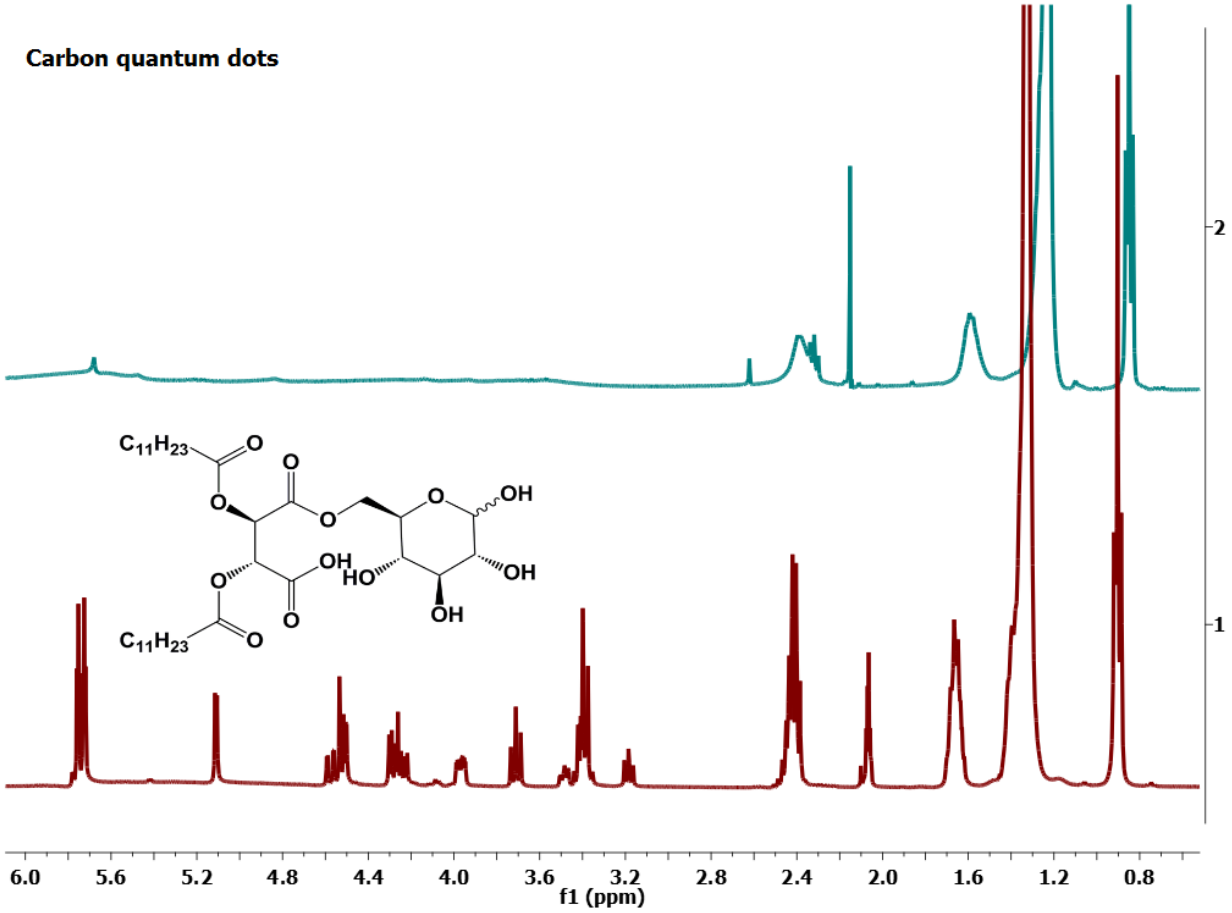

Figure 6, SI: Carbonisation of compound 2 in which glucose constitutes the carbon source. ^{1}H NMR spectra of compound **2** (bottom spectrum) and the final product **3** (carbon dots, top spectrum). In compound **2,** peaks in the range of 5.24–3.57 ppm are ascribed to protons in the carbohydrate ring whereas as in case of the CQDS, these signals disappear confirming that carbonisation occurred.

High resolution transmission electron microscopy (HRTEM)

HRTEM images were recorded on a 200 kV JEOL JEM-2100F. For the HRTEM measurement 0.5 mg of as-synthesised carbon dots were dissolved in 500 µL chloroform and 10 µL of the solution was placed upon an ultrathin carbon film coated-copper grid, dried at room temperature for 2 h and imaged.

Preparation of giant vesicles (GVs) comprising phospholipids and amphiphilic carbon dots

27.78 µL of prepared stock solution of 90 mM egg PC was added to a 4 mL glass vial, to which we further added through vortexing (for about 15 min) and sonication (for 30 min) 1 mg of carbon dots dissolved in 500 µL chloroform. The mixture was then transferred to a 250 mL round bottom flask and the aqueous phase (2.5 mL of 0.1 M sucrose, 0.1 mM KCl, 50 mM Tris solution, pH 7.4) was then carefully added with a pipette and gently stirred for about 5 min. The organic solvent was removed in a rotary evaporator under reduced pressure (final pressure 20 mbar) at room temperature. After evaporation for

4–5 min, an opalescent fluid was obtained with a volume of approximately 2.5 mL. 1.5 mL of the fluid was transferred to a quartz cuvette and used for the photoluminescence study.

Determination of quantum yield

Quantum yield of the as-synthesised carbon dots in a particular solvent was determined by comparing integrated photoluminescence intensity (in the measured range between 400-700 nm) upon excitation at 375 nm and absorbance value of carbon dots at 375 nm, with the respective values recorded for *quinine sulfate* in 0.1M H_2SO_4 (refractive index (η) of 1.33) according to the following equation:

$$\Phi = \Phi_R \times \frac{I}{I_R} \times \frac{A_R}{A}$$

ϕ=quantum yield

I=Integrated fluorescence intensity

A=Absorbance

n=refractive index

The index R indicates the standard.

Peptide Sample Preparation

Aβ40 was dissolved in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) at a concentration of 1 mg/mL and stored in at −20 °C until use to prevent fibril formation. For each experiment, the solution was thawed, and the required amount was dried by evaporation for 6−7 h to remove the HFIP. The dried peptide samples were dissolved in buffer consisting of 10 mM NaH_2PO_4, pH = 7.4, at room temperature.

In the confocal fluorescence microscopy experiment the same procedure was carried out using 55.56 µL stock solution of 90 mM egg PC. For the polymexin-B (PMB) experiment 1 mM PMB solution was prepared in deionized water, and 75 µL of this solution was then added to the carbon dot/GVs vesicles and for Aβ40 experiment 0.3 mM of Aβ40 was prepared in NaH_2PO_4 buffer and then 10 µL of such prepared solution was then added to the carbon dot/GVs vesicles.

Fluorescence spectroscopy

Steady-state fluorescence spectra were recorded using a Fluorolog 3 (Jobin-Yvon) steady-state Spectrometer.

Forster resonance energy transfer (FRET) experiments

FRET experiment using CQDS and NBD-PE as donor-acceptor pair embedded in giant vesicles (GVs) comprising of egg PC

For the FRET experiment, GVs comprising 1 mM egg PC and NBD-PE (NBD-PE: egg-PC 1:100 mole ratio) were prepared by the rapid evaporation technique.[2] Specifically, 55.5 μL stock solution of 90 mM egg PC and 27.78 μL of 1.8 mM of NBD-PE in chloroform/ethanol mixture (1:1, v/v) was transferred into a 250 mL round bottom flask. 1 mL chloroform was then added and homogeneously mixed through vortexing and sonication. The aqueous phase (5 mL of 0.1 M sucrose, 0.1 mM KCl, 50 mM Tris solution, pH 7.4) was then carefully added with a pipette immersed to the bottom of the flask wall and stirred gently for about 5 min. The organic solvent was removed in a rotary evaporator under reduced pressure (final pressure 20 mbar) at room temperature. After evaporation for about 4-5 min 1 mM GVs comprising of egg-PC/NBD-PE was obtained with a volume of approximately 5 mL. For the FRET analysis, we transferred 500 μL, 750 μL, and 1000 μL of the egg-PC/NBD-PE into three quartz cuvettes, respectively, and 500 μL of carbon dots solution (C = 10 mg/mL) dissolved in NaH_2PO_4buffer (10 mM, pH = 7.4) were added to each cuvette. The final volume of the solution in each cuvette was adjusted to 1.5 mL through addition of aqueous solution (5 mL of 0.1 M sucrose, 0.1 mM KCl, 50 mM Tris solution, pH 7.4).

FRET experiment using carbon dots and BODIPY-PH as donor−acceptor pair embedded in giant vesicles (GVs) comprising of egg PC

For the FRET experiment GVs comprising 1 mM egg PC and BODIPY-PH (BODIPY-PH: egg-PC 1:1000 mole ratio) were prepared by the rapid evaporation technique.[2] Specifically, 55.5 μL stock solution of 90 mM egg PC and 128.87 μL of 0.0194 mM BODIPY-PH in chloroform/ethanol mixture (1:1, v/v) was transferred into a 250 mL round bottom flask. 1 mL chloroform was then added and homogeneously mixed through vortexing and sonication. The aqueous phase (5 mL of 0.1 M sucrose, 0.1 mM KCl, 50 mM Tris solution, pH 7.4) was then carefully added with a pipette immersed to the bottom of the flask wall and stirred gently for about 5 min. The organic solvent was removed in a rotary evaporator under reduced pressure (final pressure 20 mbar) at room temperature. After evaporation for about 4-5 min 1 mM GVs comprising of egg-PC/BODIPY-PH was obtained with a volume of approximately 5 mL. For the FRET analysis, we transferred 50 μL, 100 μL and 200 μL of the egg-PC/BODIPY-PH GVs into three quartz cuvettes, respectively, and 100 μL of carbon dots solution (C = 1 mg/mL) dissolved in NaH_2PO_4 buffer (10 mM, pH = 7.4) were added to each cuvette. The final volume of the solution in each cuvette was adjusted to 1 mL through addition of aqueous solution (5 mL of 0.1 M sucrose, 0.1 mM KCl, 50 mM Tris solution, pH = 7.4).

Cell growth and labeling with carbon dots

T-REx-CHO-K1 cells (Chinese Hamster Ovary cells supporting the T-REx inducible system, Invitrogen) were grown in Alpha MEM Earle's Salts (Biological industries) supplemented with 10% FBS (Biological industries), 100 U/mL penicillin - 100 μg/mL streptomycin – 0.292 mg/mL *L*-glutamine (Gibco), and 10 μg/mL Blasticidin (InvivoGen) at 37ºC in the presence of 5% CO_2 under a humidified atmosphere.

Cell imaging

Small unilamellar vesicles (SUVs) comprising carbon dots embedded within egg PC were prepared according to the following procedure: 44.6 μL of stock solution of 90 mM egg PC was added to a 4 mL sample vial and then 5 mg of carbon dots dissolved in 1 mL chloroform/ethanol mixture (1:1, v/v) were added through vortexing (for about 15 min) and sonication (for 30 min). The mixture was then dried *in vacuuo*, followed by dissolution in 2 mL aqueous phase (2.5 mL of 0.1 M sucrose, 0.1 mM KCl, 50 mM Tris solution, pH = 7.4) by probe-sonication at room temperature for 10 min. The carbon dots-containing SUV solution was then cooled to room temperature for an hour and then added to the cells.

After culturing, the cell growth medium was removed and the cells were incubated for one hour with 500 μL of freshly prepared carbon dot/PC SUV solution. Prior to imaging, the multiwell plate wells were washed twice with fresh medium, and then substituted with low fluorescence medium αMEM (without Phenol red, riboflavin, folic acid, and vitamin B12; Biological Industries Inc., custom made) and 5% FBS.

Confocal fluorescence microscopy for cell imaging

Confocal microscopy images of carbon dot/PC giant vesicles were acquired on a PerkinElmer Ultra VIEW system (PerkinElmer Life Sciences Inc., MA, USA) equipped with Axiovert-200 M (Zeiss, Germany) microscope and a Plan-Neofluar 63×/1.4 oil objective. The excitation wavelengths at 440, 488, 514, 568 nm were produced by an argon/krypton laser. Cell images were acquired using a revolution XD spinning disk confocal microscope (Andor), with 60x Oil objective.

Cell viability

Chinese Hamster Ovary (CHO K1) cell line were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% heat inactivated foetal bovine serum (FBS), 2mM *L*- glutamine and 50U/mL penicillin and 50μg/mL streptomycin in humidified 5% CO_2 atmosphere at 37 °C. The cell lines were maintained at a density of 0.7-1×10^6 cells/mL before harvesting and were sub cultured three times a week.

Cell viability was estimated by the trypan blue dye exclusion method. Small unilamellar vesicles (SUVs) comprising of carbon dots embedded egg PC were prepared using the above mentioned method and 500 μL of such carbon dots embedded SUVs were incubated with the cells at 37 °C for 30 minutes, followed by incubation with 0.4% trypan blue stain for 10 minutes. Percentage of dead cells (blue stained) and live cells (unstained) were determined using a haemocytometer in a transmission microscope.

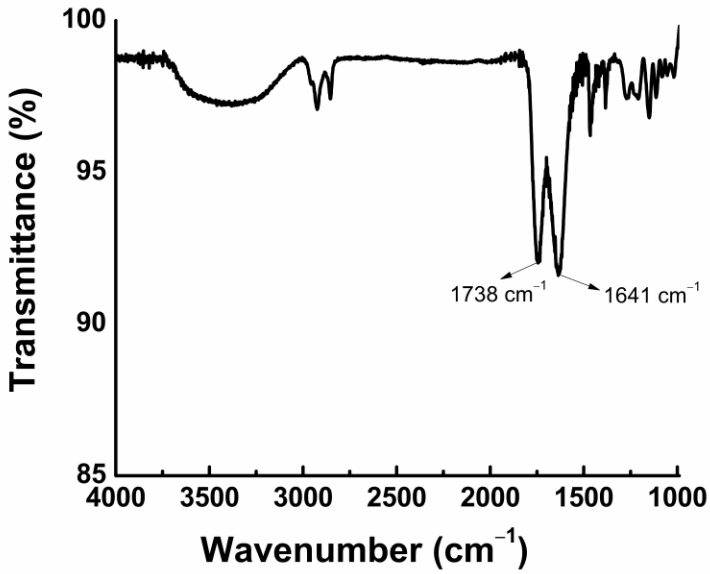

Figure 7, SI: FT-IR spectrum of the as-synthesized carbon dots (3). The two peaks indicated correspond to the carbonyl (1738 cm^{-1}) and alkyne (1641 cm^{-1}) residues, The absorption band observed within the range 3000–3600 cm^{-1} is associated with the stretching modes of the hydroxy (–OH) group. The bands at 2850–2925cm^{-1} are attributed to C−H asymmetric and symmetric stretching vibration, indicating the existence of hydrocarbon chains coated on the carbon dots surface. The FT-IR spectrum was recorded using a Nicolet-380 FT-IR spectroscopy. FT-IR experiments were carried out by placing a drop of chloroform solution of the sample onto a KBr palette and drying it prior to FT-IR analysis.

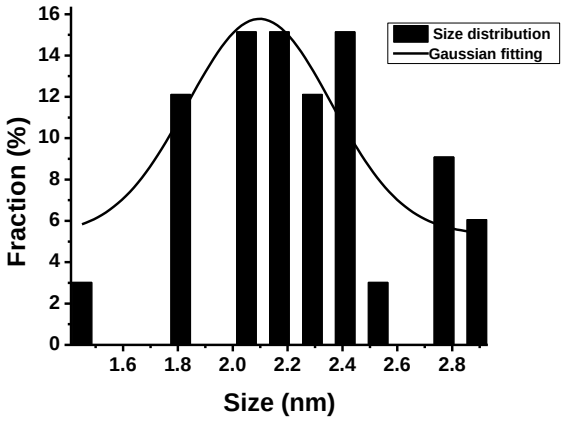

Figure 8, SI: Size distribution of amphiphilic carbon dots. The histogram of size was based upon image analysis of the TEM data (Figure 1B in the manuscript). Diameters of particles were measured using Image J software and the size distribution was plotted based on this statistics.

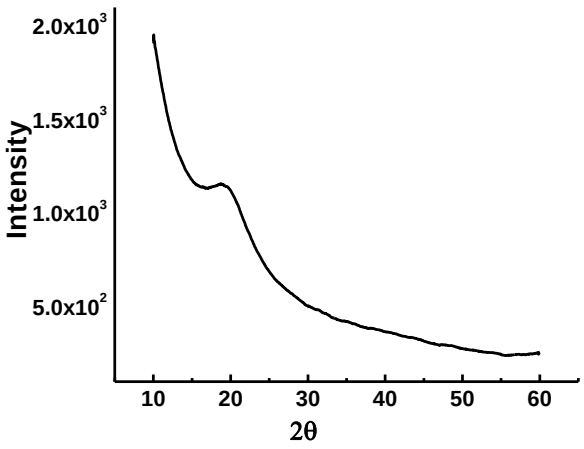

Figure 9, SI: carbon dot crystallinity. **X-ray diffraction (XRD) pattern of the as-synthesized amphiphilic carbon dots.** The peak indicates an interlayer spacing of 0.46 nm. Powder x-ray diffraction (XRD) XRD patterns were obtained using Panalytical Empyrean Powder Diffractometer equipped with a parabolic mirror on incident beam providing quasi-monochromatic Cu Kα radiation (λ=1.54059 Å) and X'Celerator linear detector. XRD profile depicted in Figure 4 exhibits a weak broad reflection peak centered at around 19.3 °, suggesting the interlayer spacing of (002) diffraction peak is 0.46 nm. The larger interlayer spacing than that of bulk graphite (0.34 nm) is ascribed to increase in amorphous nature, the existence of abundant oxygen containing functional groups and coated hydrocarbon chains on the surface.[3-5]

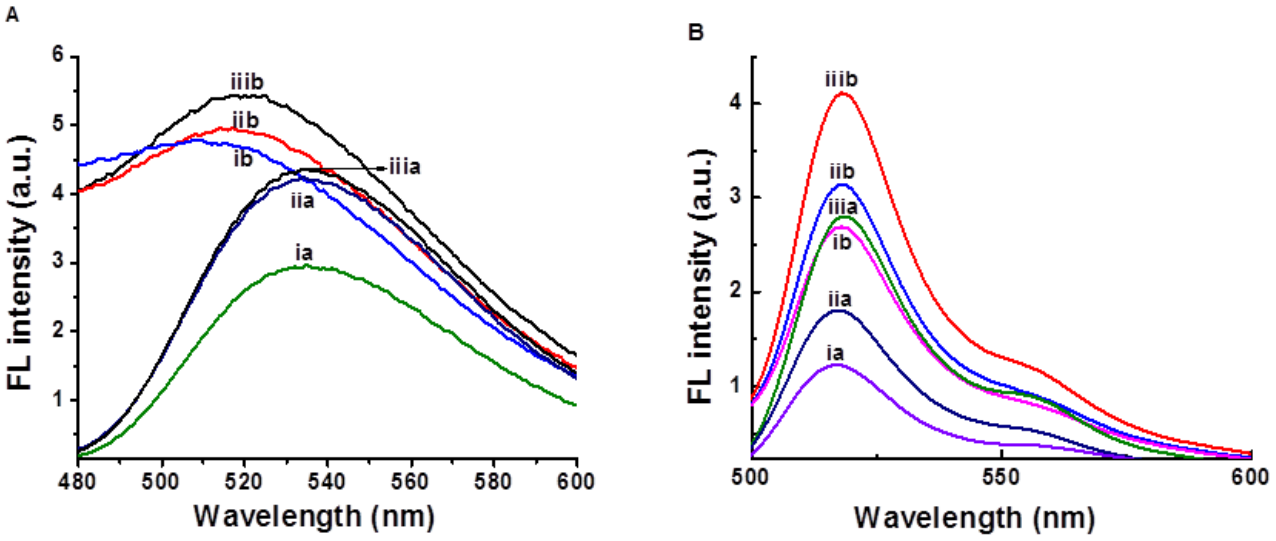

Figure 10, **SI: Fluorescence emission of dyes with and without the presence of carbon dots:** (**A**): Fluorescence spectrum of NBD-PE embedded in GVs comprising egg PC *without* carbon dots (**a**), and in the presence of carbon dots (b). The numerals i-iii correspond to different concentrations of NBD-PE: i. 3.3 µM NBD-PE; ii. 5 µM NBD-PE; iii. 6.6 µM NBD-PE. Excitation was at 370 nm; (**B**) Fluorescence spectrum of BODIPY-PH embedded in GVs comprising egg PC *without* carbon dots (**a**) and in the presence of carbon dots

(**b**). The numerals i-iii correspond to different concentrations of the fluorescent acceptor dye: i. 0.05 μM BODIPY-PH; ii. 0.1 μM BODIPY-PH; iii. 0.2 μM BODIPY-PH. Excitation was at 390 nm.

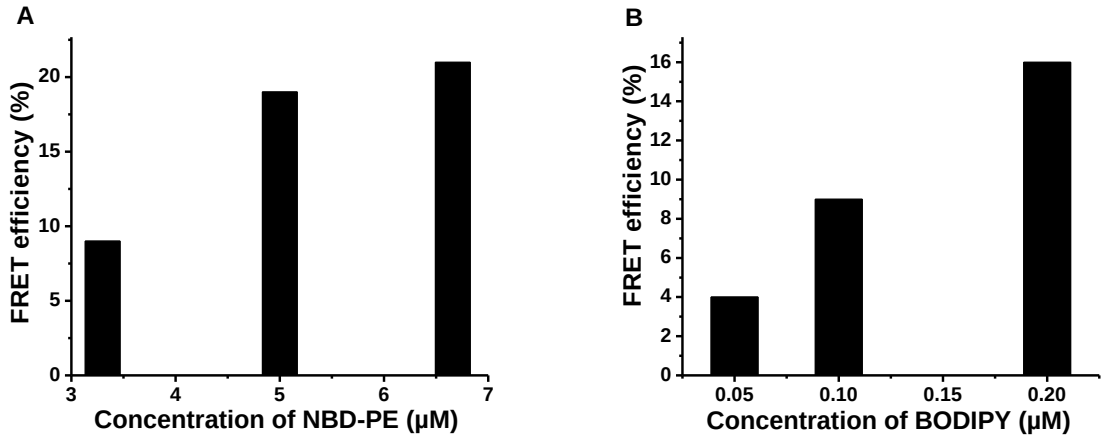

Figure 11, SI: FRET efficiency. FRET efficiency calculated for carbon dots / NBD-PE (**A**) and carbon dots / BODIPY-PH (**B**). The FRET efficiency was calculated according to the equation[6]:

$$\mathbf{E = 1 - \frac{F_{DA}}{F_D}}$$

Where F_{DA} represents fluorescence intensity of donor in the presence of acceptor, and F_D represents fluorescent intensity of donor without the acceptor.

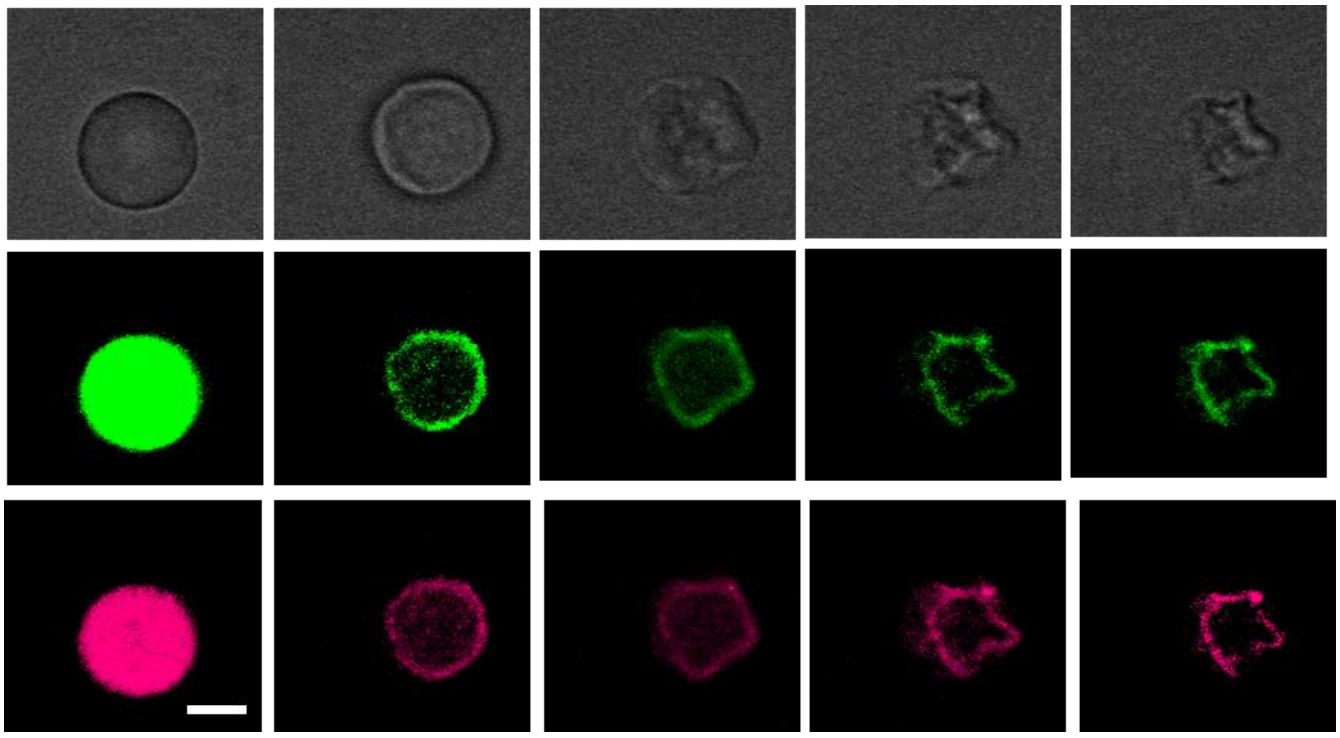

Figure 12, SI: Imaging membrane interactions of Aβ40. Bright field microscopy (top), and confocal fluorescence microscopy images recorded upon excitation at 440 nm emission filter EM 477/45 (green); excitation at 488 nm emission filter EM 525/50 (magenta) of giant vesicles labeled with the carbon dots following addition of Aβ40. From left: before addition; 1 minute after addition; 10 minutes after addition and 20 minutes after addition and 1 h after addittion. Scale bar is 5 μm.

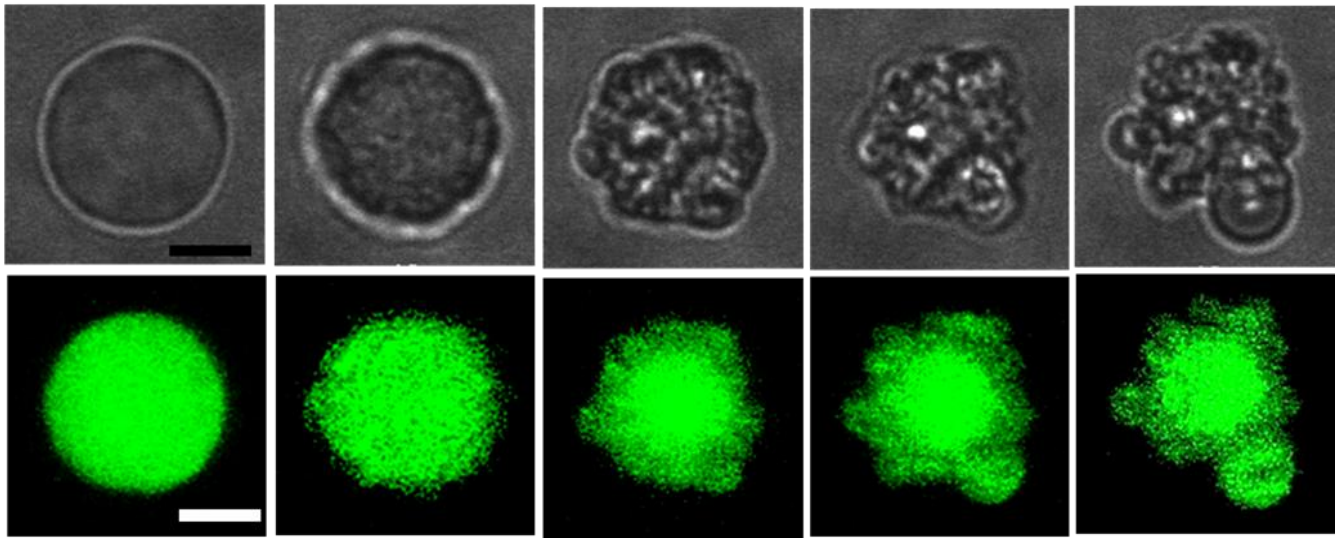

Figure 13, SI: Imaging membrane interactions of polymyxin-B (PMB). Bright field images (**top)** and fluorescence microscopy images (exc. 440 nm, **bottom**) recorded at different times after addition of PMB to giant vesicles labeled with the carbon dots (from left): 0 sec (control); 5 sec; 20 sec; 30 sec; 40 sec. Scale bar is 5 μm.

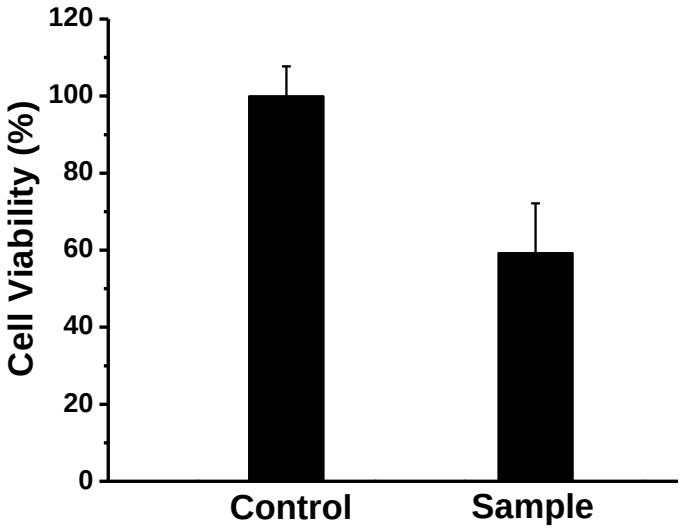

Figure 14, SI: Viability of Chinese Hamster Ovary (CHO K1) cell incubated with CQDS embedded SUVs for 30 minutes at 37 °C, evaluated using the trypan blue assay.

References

(1) J. Rayo, N. Amara, P. Krief and M. M. Meijler, *J. Am. Chem. Soc.* 2011, **133**, 7469-7475.

(2) A. Moscho, O. Orwar, D. T. Chiu, B. P. Modi and R. N. Zare, *Proc. Natl. Acad. Sci. U. S. A.* 1996, **93**, 11443-11447.

(3) H. Li, Z. Kang, Y. Liu, S.-T. Lee, *J. Mater. Chem.* 2012, **22**, 24230-24253.

(4) A. Salinas-Castillo, M. Ariza-Avidad, C. Pritz, M. Camprubí-Robles, B. Fernández, M. J. Ruedas-Rama, A. Megia-Fernández, A. Lapresta-Fernández, F. Santoyo-Gonzalez, A. Schrott-Fischer and L. F. Capitan-Vallvey, *Chem. Commun.* 2013, **49**, 1103-1105.

(5) L. Tang, R. Ji, X. Cao, J. Lin, H. Jiang, X. Li, K. S. Teng, C. M. Luk, S. Zeng, J. Hao and S. P. Lau, *ACS Nano* 2012, **6**, 5102-5110.

(6) H. Lu, O. Schops, U. Woggon and C. M. Niemeyer, *J. Am. Chem. Soc.* 2008, **130**, 4815-4827.