Electronic Supplementary Information

Preclinical evaluation of modified carbon nanohorns and complexation with insulin

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Experimental and instrumentation

Dipalmitoyl phosphatidylcholine (DPPC) and Dipalmitoyl phosphatidylglycerol (DPPG) were purchased from Avanti Polar Lipids Inc., (Albaster, AL, USA). Chloroform and methanol were purchased from LabScan (Dublin, Ireland). Insulin with a molecular weight of 5800 g/mol was purchased from Sigma–Aldrich and used without any further purification. The rest of reagents, and solvents were purchased from Sigma-Aldrich and used without further purification.

Infrared (IR) spectra were obtained on a Fourier Transform IR spectrometer (Equinox 55 from Bruker Optics) equipped with a single reflection diamond ATR accessory (DuraSamp1IR II by SensIR Technologies). Raman measurements were recorded with a Renishaw confocal spectrometer at 514 nm. The data were obtained and analysed with Renishaw Wire and Origin software. Thermogravimetric analysis was acquired using a TGA Q500 V20.2 Build 27 instrument by TA in a nitrogen (purity >99.999%) inert atmosphere. Microwave synthesis was performed with a CEM Discover microwave reactor equipped with infrared pyrometer and pressure control system.

TEM imaging was performed on a JEM-ARM200F (JEOL, Tokyo, Japan) operating at 200 kV.

Preparation of CNH-based material 3

In a microwave tube, pristine CNHs **1** (50 mg), aniline derivative *tert*-butyl 2-(2(2-(4-aminobenzamido)ethoxy)ethoxy) ethyl carbamate (602 mg, 1.64 mmol) and *o*-DCB (4.5 mL) were added under nitrogen and sonicated for 15 minutes. Then, isoamyl nitrite (0.440 mL, 3.3 mmol) was added to the suspension and the vial was sealed with a septum cap. The reaction mixture was heated at 150 ^{o}C and irradiated with 10 Watt for 90 minutes. After cooling, DMF was added to the reaction mixture and the dispersion was filtered on a PTFE membrane filter (pore size 0.2 μm). The solid residue was repeatedly bath-sonicated and washed with DMF, methanol, and CH_2Cl_2 to remove any organic species. The collected black solid was dried under high vacuum affording 55 mg of CNH-based material **3**.

Preparation of CNH-based material 4

In a round bottom flask, material **3** (52 mg) and CH_2Cl_2 (20 mL) were added and the resulting suspension was sonicated for 15 min. Then, TFA (12 mL) was added and the reaction mixture was stirred at room temperature overnight. Afterwards, the mixture

was distilled and the solid residue was bath-sonicated with MeOH and filtered on PTFE membrane filter (pore size 0.2 μm). The solid on the filter was washed with MeOH, CH_2Cl_2 and petroleum ether. The collected black solid was dried under high vacuum affording 51 mg of CNH-based material **4**.

Preparation of CNH-based material 5

In a round bottom flask, material **4** (48 mg), dry methanol (30 mL) and *N*-ethyldiisopropylamine (4 mL) were added and the resulting mixture was sonicated for 5 min. Then, methyl acrylate (6 mL) was added and the reaction mixture was stirred at 80 °C for 3 days under nitrogen atmosphere. After cooling, the reaction mixture was filtered on a PTFE membrane filter (pore size 0.2 μm) and the solid on the filter was washed by cycles of sonication and filtration with MeOH and CH_2Cl_2. The collected black solid was dried under high vacuum affording 47 mg of CNH-based material **5**.

Preparation of CNH-based material 6

In a round bottom flask, material **5** (43 mg) and dry methanol (20 mL) were added and the resulting mixture was sonicated for 5 min. Then, ethylenediamine (20 mL) was added. The reaction mixture was stirred at 80 °C for 3 days under nitrogen atmosphere and then filtered on a PTFE membrane filter (pore size 0.2 μm). The black solid on the filter was washed by cycles of sonication and filtration with MeOH and CH_2Cl_2. The collected black solid was dried under high vacuum affording 43 mg of CNH-based material **6**.

Preparation of CNH-based material 7

In a round bottom flask, material **6** (40 mg) and dry methanol (25 mL) were added and the resulting mixture was sonicated for 5 min. Then, methyl acrylate (5 mL) was added and the reaction mixture was stirred at 80 °C for 3 days under nitrogen atmosphere. After cooling, the reaction mixture was filtered on a PTFE membrane filter (pore size 0.2 μm) and the solid on the filter was washed by cycles of sonication and filtration with MeOH and CH_2Cl_2. The collected black solid was dried under high vacuum affording 40 mg of CNH-based material **7**.

Preparation of CNH-based material 8

In a round bottom flask, material **7** (37 mg) and dry methanol (25 mL) were added and the resulting mixture was sonicated for 5 min. Afterwards, ethylenediamine (25 mL) was added and the reaction mixture was stirred at 80 °C for 3 days under nitrogen

atmosphere and after cooling filtered on a PTFE membrane filter (pore size 0.2 μm). The solid on the filter was washed by cycles of sonication and filtration with MeOH and CH_2Cl_2. The collected black solid was dried under high vacuum affording 37 mg of CNH-based material **8**.

Preparation of CNH-based material 9

To a suspension of material **8** (32 mg) in dry methanol (25 mL) methyl acrylate (10 mL) was added. The reaction mixture was stirred at 80 °C for 3 days under nitrogen atmosphere and then filtered on a PTFE membrane filter (pore size 0.2 μm). The solid on the filter was washed by cycles of sonication and filtration with H_2O and MeOH. The collected black solid was dried under high vacuum affording 30 mg of CNH-based material **9**.

Preparation of f-CNHs

To a suspension of material **9** (25 mg) in dry methanol (15 mL) ethylenediamine (15 mL) was added. The reaction mixture was stirred at 80 °C for 3 days under nitrogen atmosphere and then filtered on a PTFE membrane filter (pore size 0.2 μm). The solid on the filter was washed by cycles of sonication and filtration with H_2O, MeOH and CH_2Cl_2. The collected black solid was dried under high vacuum affording 25 mg of **f-CNHs**.

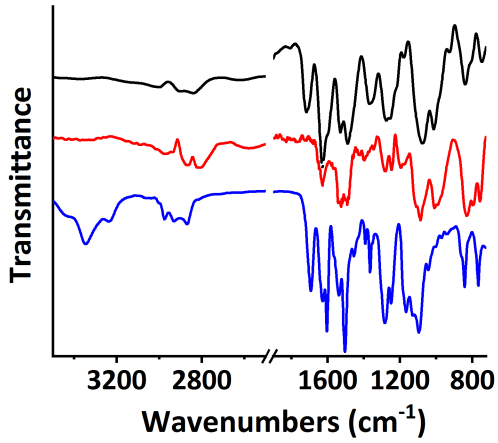

Figure S1. ATR-IR spectra for materials **2** (blue), **3** (black) and **4** (red).

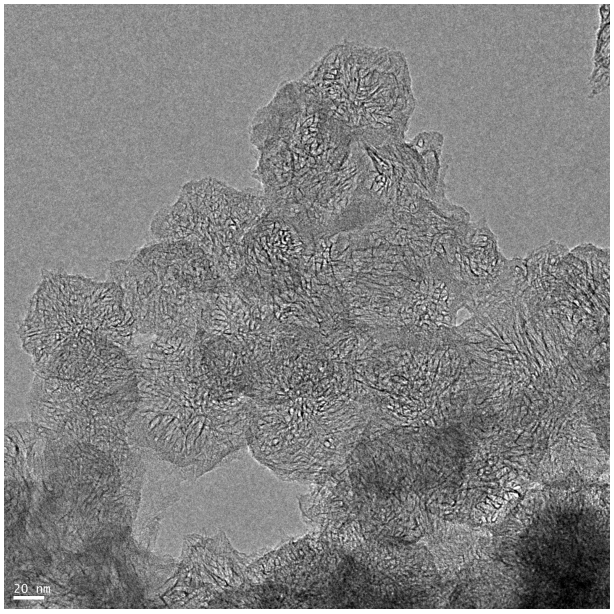

Figure S2. Representative low magnification TEM image of PAMAM-functionalized f-CNHs.

Table S1: Calorimetric values of lipid bilayers with **f-CNHs** at different concentrations.

[a]T_{onset}: temperature at which the thermal event starts; [b]$T_.$: temperature at which heat capacity (ΔC_p) at constant pressure. is maximum; [c]$\Delta T_{1/2}$: half width at half peak height of the transition; [d]ΔH: transition enthalpy normalized per mol of lipid bilayer system.

m: main transition; s: secondary transition

Sample	**Cf-CNH (mg/ml)**	**Tonset (°C)[a]**	**Tm (°C)[b]**	**ΔT1/2.m(°C)[c]**	**ΔHm (kJ/mol)[d]**	**Tonset.s(°C)**	**Ts (°C)**	**$\Delta T_{1/2}$ (°C)**	**ΔHs (kJ/mol)**
Heating									
DPPC:DPPG (9:1 molar ratio)	0	41.0	41.8	1.12	-349	34.1	35.8	1.98	-40
DPPC:DPPG (9:1 molar ratio)	0.1	41.1	42.3	1.42	-377	34.2	36.0	2.21	-41
DPPC:DPPG (9:1 molar ratio)	0.2	41.1	42.2	1.41	-337	34.2	36.0	2.70	-38
DPPC:DPPG (9:1 molar ratio)	0.5	41.2	42.3	1.59	-365	34.1	36.0	2.84	-36
DPPC:DPPG (9:1 molar ratio)	1	41.2	42.3	1.64	-389	34.1	36.0	3.19	-29
cooling									
DPPC:DPPG (9:1 molar ratio)	0	39.9	38.9	1.14	361	-	-	-	-
DPPC:DPPG (9:1 molar ratio)	0.1	40.0	38.8	1.39	396	-	-	-	-
DPPC:DPPG (9:1 molar ratio)	0.2	39.9	38.8	1.38	402	-	-	-	-
DPPC:DPPG (9:1 molar ratio)	0.5	40.1	38.8	1.58	395	-	-	-	-
DPPC:DPPG (9:1 molar ratio)	1	40.0	38.8	1.60	400	-	-	-	-

Table S2. The physicochemical characteristics of **f-CNHs** during stability studies.

T(days)	**Dh (nm)**	**SD**	**PDI**	**SD**	**I (KCps)**	**SD**	**ζ-potential (mV)**	**SD**
0	923.8	78.2	0.628	0.012	347.2	2.2	18.9	1.9
2	987.2	98.7	0.634	0.083	342.2	6.8	19.2	2.8
7	1256.3	109.2	1	0	289	8.2	17.6	1.8
21	2878.9	202.2	1	0	276.7	7.6	17.8	2.9
40	5184.3	347.8	1	0	234.9	6.8	13.8	4.5
after sonication								
20min	2293.7	11.1	0.709	0.087	282	0.9		
40min	1803.9	57	0.672	0.098	267.4	1.4	14.3	3.1

Table S3. The physicochemical characteristics and the %loading of complexes **f-CNHs**/insulin at different concentrations of the protein.

C ins (mg/mL)	**Dh (nm)**	**SD**	**PDI**	**SD**	**I (Kcps)**	**SD**	**ζ-potential (mV)**	**SD**	**%loading**	**SD**
0.001	2212.6	134.2	1	0	61.8	1.2	16.2	1.8	25.4	10.1
0.002	2216.7	128.7	1	0	71.2	1.8	15.2	1.2	27.2	12.8
0.005	1988.2	152.2	0.872	0.09	81.2	2.3	2.3	2.3	60.7	15.4
0.01	1782.2	167.2	0.802	0.029	125.2	1.9	-3.4	2.1	75.6	14.9
0.02	1702.2	187.2	0.735	0.035	191.2	3.4	-4.5	2.1	72.4	17.1
0.05	1687.2	156.2	0.702	0.02	221.2	1.8	-6.7	1.9	78.6	21.2

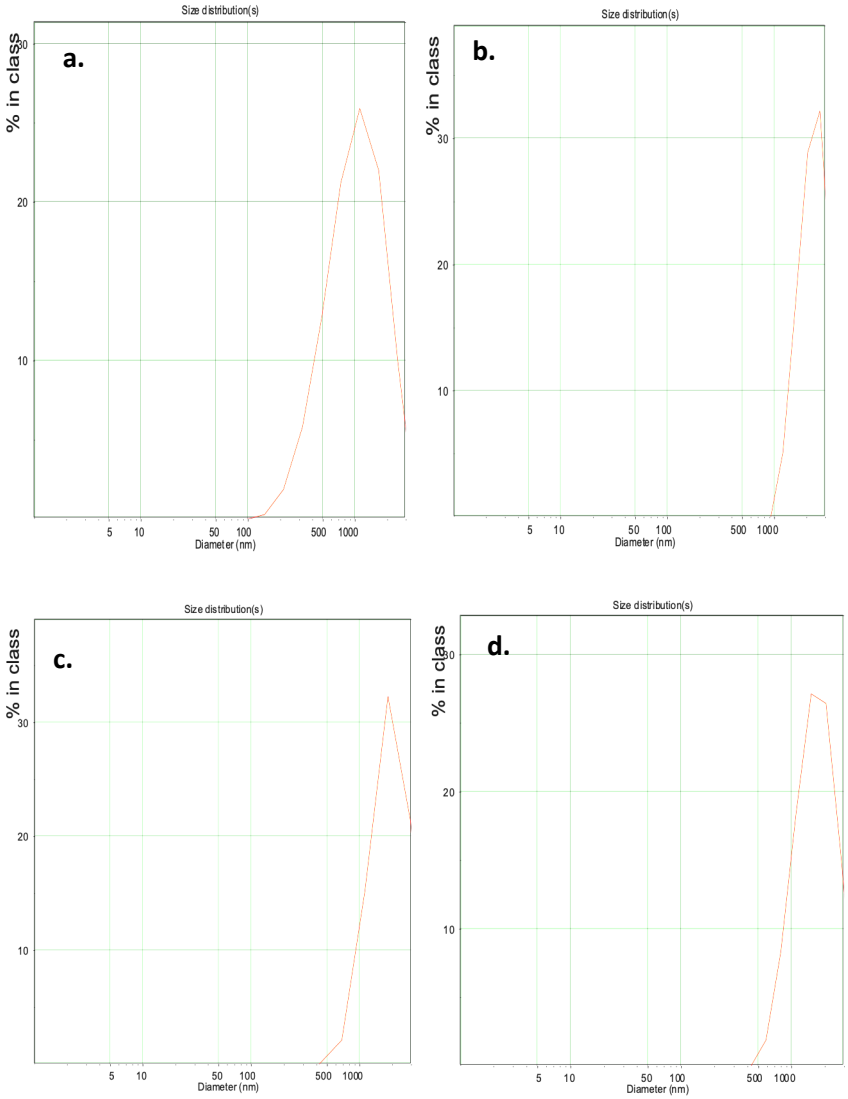

Figure S3. Size distribution of **f-CNHs** (1 mg/mL) a. the day of dispersion in HPLC grade water, b. after 40 days (at 4°C), c. after 20 min of bath sonication of aggregates and d. after 40 min of bath sonication of aggregates.

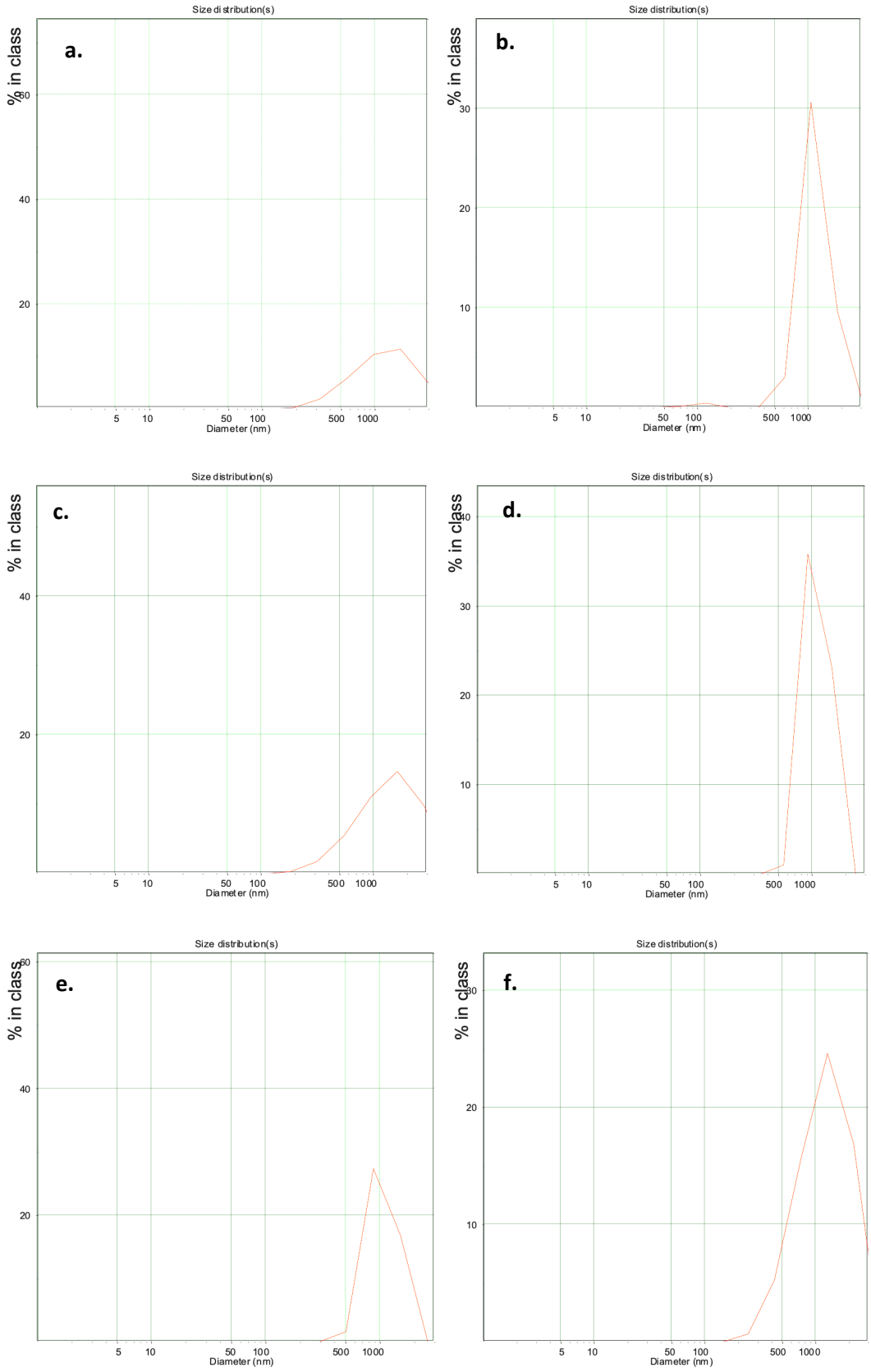

Figure S4. Size distribution of **f-CNHs** (1 mg/mL):insulin complexes at different concentrations of the protein a. 0.001 mg/mL, b.0.002 mg/mL, c.0.005 mg/mL, d. 0.01 mg/mL, e. 0.02 mg/mL and f. 0.05 mg/mL.

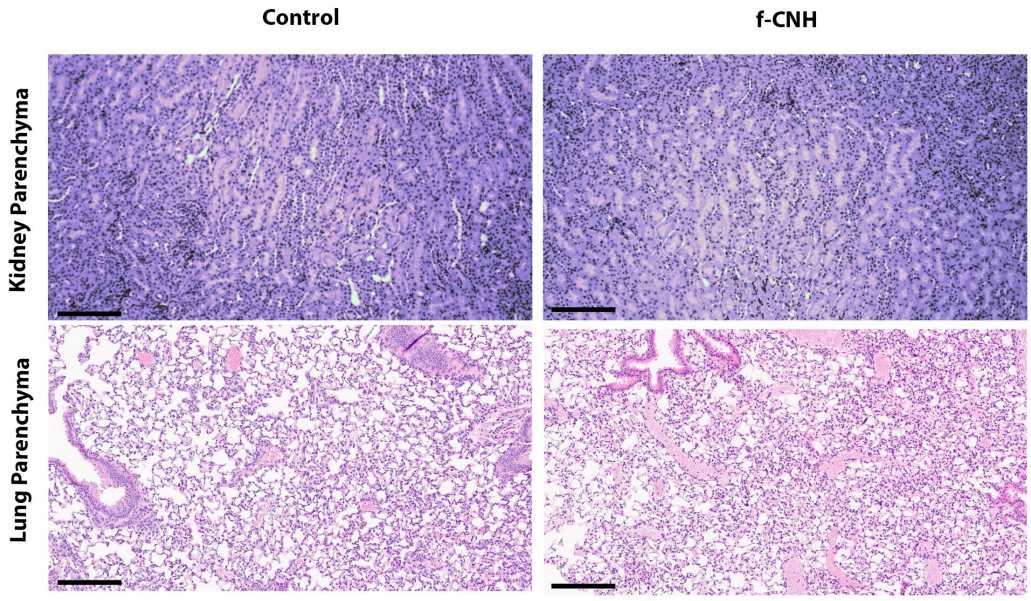

Figure S5: Hematoxylin and Eosin staining of the kidneys (upper panel) and lungs (lower panel) of mice treated with saline and f-CNHs respectively. Representative images of the two groups are presented. The bar indicates 2000 μm. The morphology of the lung and renal parenchymas at the cellular level did not reveal any tissue damage or necrosis. Moreover, we did not observe any infiltration of immune cells, indicative of possible inflammation.