Electronic Supplementary Material (ESI) for Materials Chemistry Frontiers.

Supporting information for

Amphiphilic-like carbon dots as antitumoral drug vehicles and phototherapeutical agents

Mariano Ortega-Muñoz[a], Paula Vargas-Navarro[a], Simona Plesselova[b], M. Dolores Giron-Gonzalez[b], Guillermo R. Iglesias[c], Rafael Salto-Gonzalez[b], Fernando Hernandez-Mateo[a], Angel V. Delgado[c], F. Javier Lapez-Jaramillo*[a] and Francisco Santoyo-Gonzalez*[a]

[a]Department of Organic Chemistry, Biotechnology Institute, School of Sciences, Campus Fuentenueva sn, University of Granada, 18071-Granada, Spain.
[b]Department of Biochemistry and Molecular Biology II, School of Pharmacy, Campus Cartuja 18071-Granada, University of Granada, Spain
[c]Department of Applied Physics, School of Sciences, Campus Fuentenueva sn, University of Granada, 18071-Granada, Spain

*Corresponding authors:
F. Javier Lopez-Jaramillo, fjljara@ugr.es
Francisco Santoyo-Gonzalez, fsantoyo@ugr.es

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1. Experimental procedures

Chemical and materials

Anhydrous citric acid (CA), anhydrous dimethyl sulfoxide (DMSO), hexan-1-amine, octadecan-1-amine, 1-octanol and dialysis membranes (14 000 Da cut-off) were obtained from Sigma-Aldrich (St Louis, MO, USA), 2-[2-[2-Chloro-3-[(1,3-dihydro-3,3-dimethyl-1-propyl-2H-indol-2-ylidene)ethylidene]-1-cyclohexen-1-yl]ethenyl]-3,3-dimethyl-1-propylindolium iodide (IR780) from Alfa Aesar (Kander, Germany) and 2-[2-[2-Chloro-3-[2-[1,3-dihydro-3,3-dimethyl-1-(4-sulfobutyl)-2H-indol-2-ylidene]-ethylidene]-1-cyclohexen-1-yl]-ethenyl]-3,3-dimethyl-1-(4-sulfobutyl)-3H-indolium hydroxide, inner salt, sodium salt (IR783) from Carbosynth (Berkshire, England, U.K.). Commercial reagents were used as received without further purification.

Cell cultures

Chinese hamster ovary (CHO-k1; ATCC nº CCL-61), mouse colon carcinoma (CT26.WT; ATCC nº CRL-2638), human skin malignant melanoma (G361; ATCC nº CRL-1424), human embryonic kidney (HEK-293; ATCC nº CRL-15739), human cervix adenocarcinoma (HeLa; ATCC nº CCL-2), human breast adenocarcinoma (MDA-MB-231; ATCC nº HTB-26), mouse neuroblast cells (Neuro-2a; ATCC nº CCL-131) and human breast adenocarcinoma (SK-BR-3; ATCC nº HTB-30) were provide by the Cell Culture Facility from Universidad de Granada. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FSB), 2 mM glutamine plus 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Cells were seeded in 48 well plates at a density of 1.5×10^4 cells per well and incubated at 37 °C for 24 h to reach a cell confluence of 80–90%.

Instrumentation

Fourier transform infrared (**FTIR**) spectra were collected using a PerkinElmer FT-IR Spectrum Two spectrometer. Electron microscopy analysis was carried out at Centro de Instrumentacion Cientifica, Universidad de Granada, using a high-resolution transmission electron microscope (HRTEM) (FEI TITAN G2) operated at 300 kV. X-ray photoelectron spectroscopy (XPS) analyses were carried out at Centro de Instrumentacion Cientifica, Universidad de Granada, with a Kratos Axis Ultra-DLD spectrometer using monochromatic Al Kα radiation. UV/Vis absorption spectra were collected using a Specord 200 Plus instrument (Analytik Jena). Photolumiscence spectra were conducted on F2000 fluorescence spectrophotometer (Hitachi).

Determination of the octanol-water partition coefficient (Kwo)

A volume of 1 mL of different solutions in water of the the salt form of LCDs at concentrations of 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3. 0.2 mg/mL was extracted with 1 mL of 1-octanol. In order to calculate Kow of the acidic form of LCDs they were obtained from the sodium salts by acidification with two drops of 5% HCl. After 1 h of shaking, phase separation was allowed to proceed for 5.5 h and the concentration of CDs in the aqueous phase was determined by interpolation of the fluorescence at 454 nm (Ex 326 nm) of the aqueous phase in the suitable calibration curve (concentration range from 1.2 to 0.05 mg/mL, $r^2 > 0.986$). The concentration in the organic phase was calculated as the difference between the concentration in the aqueous phase at the starting condition and after phase separation. Octanol-water partition coefficient was estimated as the common

logarithm of the slope of the plot of the concentration of CDs in the octanol phase against the corresponding equilibrium aqueous phase.

Formation of inclusion complexes with camptothecin (CPT)

Protocol A: a volume of 3 mL of a 0.5 mg/mL solution of CPT in Cl_3CH was combined with 3 mL of a 1mg/mL solution of the sodium salt of LCDs in water. The resulting two phases mixture was shaken at room temperature protected from light for 48 h. Afterwards phase separation was allowed to proceed and the remaining concentrations of CPT in the organic phase was estimated from interpolation of the fluorescence at 425 nm (Ex 365 nm) in a calibration curve.[1] The amount of CPT entrapped by the sodium salt of LCDs in the aqueous phase was estimated as the difference between the 0.5 mg/mL solution of CPT and the concentration of CPT in the organic phase. Results were further confirmed by determining the concentration of CPT in the organic phase resulting from the acidification of the aqueous phase with 5% HCl to yield the protonated form of LCDs (i.e. soluble in the organic phase) and extraction with Cl_3CH (3 mL).

Protocol B: a volume of 3 mL of a 0.5 mg/mL solution of CPT in Cl_3CH was combined with 3 mL of a 1 mg/mL solution of the sodium salt of LCDs in water. The mixture was rotavaporated to remove the organic solvent and force the entrapment of CPT. Then the aqueous phase was acidified with 5% HCl to yield the protonated form of LCDs and extracted with Cl_3CH (3 mL). The concentrations of CPT was estimated from interpolation of the fluorescence of the organic phase at 425 nm (Ex 365 nm) in a calibration curve.

Formation of the inclusion complex with IR780

Protocol A: a volume of 3 mL of a 0.5 mg/mL solution of IR780 in Cl_3CH was combined with 3 mL of **LCD-2** in Cl_3CH (img/mL) and shaken overnight at room temperature protected from light. After extraction with a saturated solution of HCO_3Na (3 x 6 mL) the aqueous phases were pooled and treated with 5% HCl to correct pH to 2 (to yield the protonanted from of LCDs) and then extracted with Cl_3CH (2 x 10 mL). The concentration of LCDs and NIR were estimated from the interpolation of the absorbance at both 250 nm and 326 nm for the LCD and 792 nm for IR780 in the suitable calibration curves in Cl_3CH.

Protocol B: a volume of 3 mL of a 0.5 mg/mL solution of IR780 in Cl_3CH was combined with 3 mL of LCD-2Na in H_2O and shaken overnight at room temperature protected from light. Then pH was corrected to 9 by addition of 10% Na_2CO_3 and the organic phase was discarded. The aqueous phase was acidified to pH 2 and extracted with Cl_3CH (2 x 10 mL). The concentration of LCDs and NIR were estimated as above.

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[1] Joykrishna Dey and Isiah M. Warner, “Spectroscopic and Photophysical Studies of the Anticancer Drug: Camptothecin,” *Journal of Luminescence* 71, no. 2 (March 1997): 105–14, doi:10.1016/S0022-2313(96)00125-1.

Cell viability using Flow cytometry[2]

G361 cells were incubated for 24 h with suitable amounts of either **LCD-2Na@CPT** or free CPT to yield final CPT concentrations up to 100 μg/mL. Then, cells were washed two times with phosphate saline buffer (PBS), trypsinized and pelleted. The pellet was washed two more times with PBS and re-suspended in PBS to yield 10^6 cells/mL. A volume of 200 μL of cells were incubated at room temperature with fluorescein diacetate (100 ng/mL, 20 μL) and propidium iodide (100 μg/mL, 20 μL) for 10 min and then analyzed by recording forward and orthogonal light scatter, red (>630 nm) and green (520 nm) fluorescence.
Propidium iodide is excluded by viable cells, but it binds to nucleic acids in dead or dying cells, fluorescing red. Fluorescein diacetate is uptaken by cells and retained by those with intact plasma membrane. It is not fluorescent and by action of the intracellular esterases it is converted to fluorescein Hence, viable cells fluoresce green.

Electrophoretic mobility and Z-potential determination

The different degree of hydrophobicity of the CDs could affect their electrical surface charge. In addition, one of the proposed applications is drug delivery, so the determination of the zeta potential of the carbon dots, and the effect of pH in the physiological range (pH 7-7.4) and close to cancer tissue values (pH 6.5) on this quantity, appeared also as an important characterization of the synthesized CDs. For that aim, we measured the electrophoretic mobility of the dots in PBS buffers at pH 6.5, 7.04, and 7.4. A Zetasizer Nano-ZS instrument (Malvern instruments, U.K.) was used, determining the mobilities of the three types of CDs in triplicate, with nine determinations in each sample. The zeta potential of the particles was calculated using the Henry formula[3].

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[2] K H Jones and J A Senft, “An Improved Method to Determine Cell Viability by Simultaneous Staining with Fluorescein Diacetate-Propidium Iodide.,” *Journal of Histochemistry & Cytochemistry* 33, no. 1 (January 1985): 77–79, doi:10.1177/33.1.2578146.

[3] H. Ohshima in *Electrical Phenomena at Interfaces: Fundamentals: Measurements, and Applications, H. Ohshima, K. Furusawa (Eds.), Marcel Dekker Inc. N. York, 2nd edition,1998 Chapter2, 19-56*

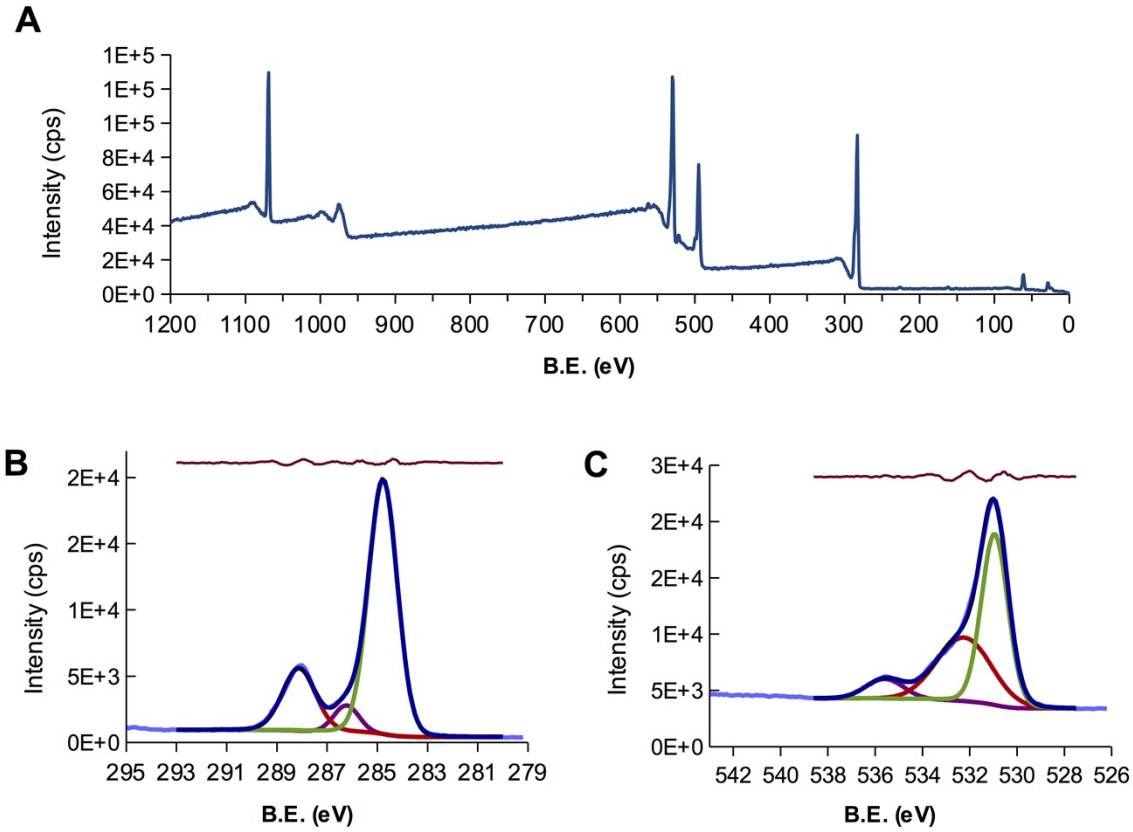

Fig. S1.- XPS survey spectrum (**A**) and high-resolution XPS spectra of C1s (**B**) and O1s (**C**) of the **CD-1Na** obtained by thermolysis of CA in DMSO after treatment with a solution of NaOH, dialysis and lyophilization. Experimental data are shown in light blue, envelope in black and residuals on top in brown

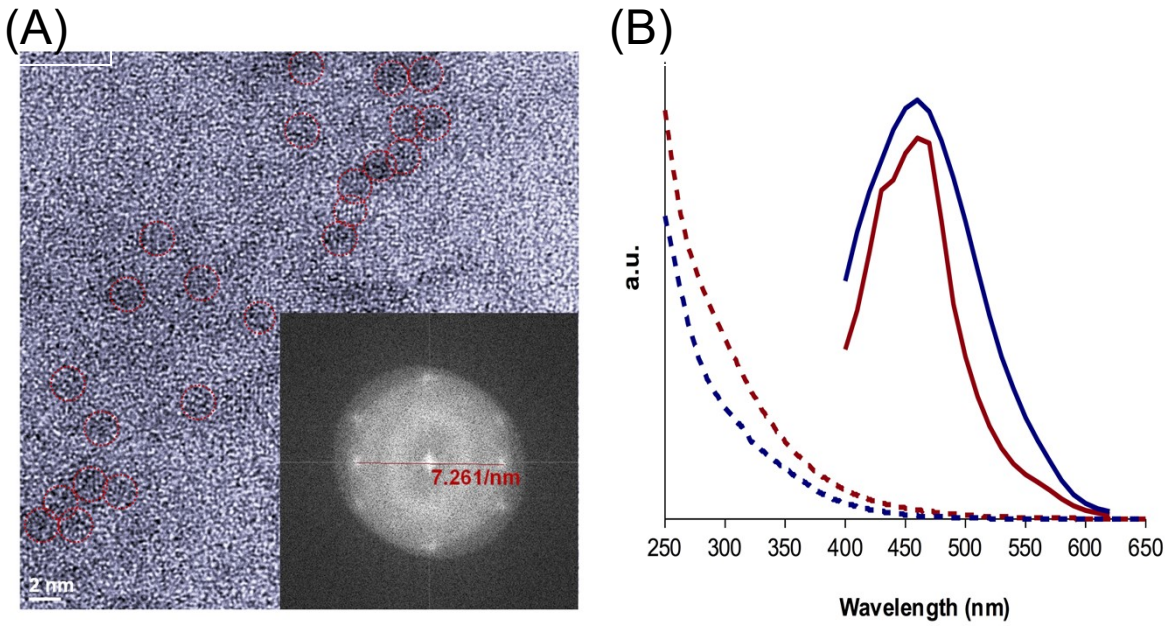

Fig. S2.- (**A**) HRTEM image of **CD-1Na** showing the estimation of the interplanar spacing. (**B**) Uv-Vis absorption (dash lines) and photoluminescence emission (excitation at 326 nm) spectra of **CD-1Na** (red) and **CDs** obtained by solvent free thermolysis of CA (blue).

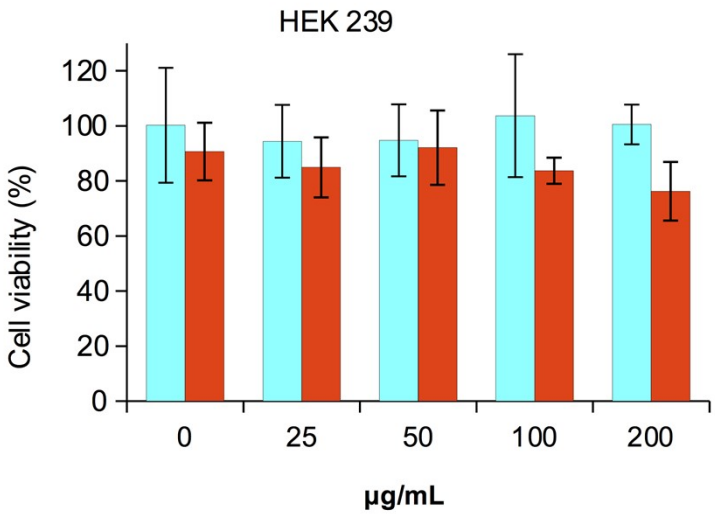

Fig. S3.- (A) Cytotoxicity assay on HEK 239 cells of **LCD-1Na** (cyan) and **LCD-2Na** (orange). Cell lines were incubated for 24 h and cytotoxicity was assayed by the MTT method. Results are means ± 1 standard deviation.

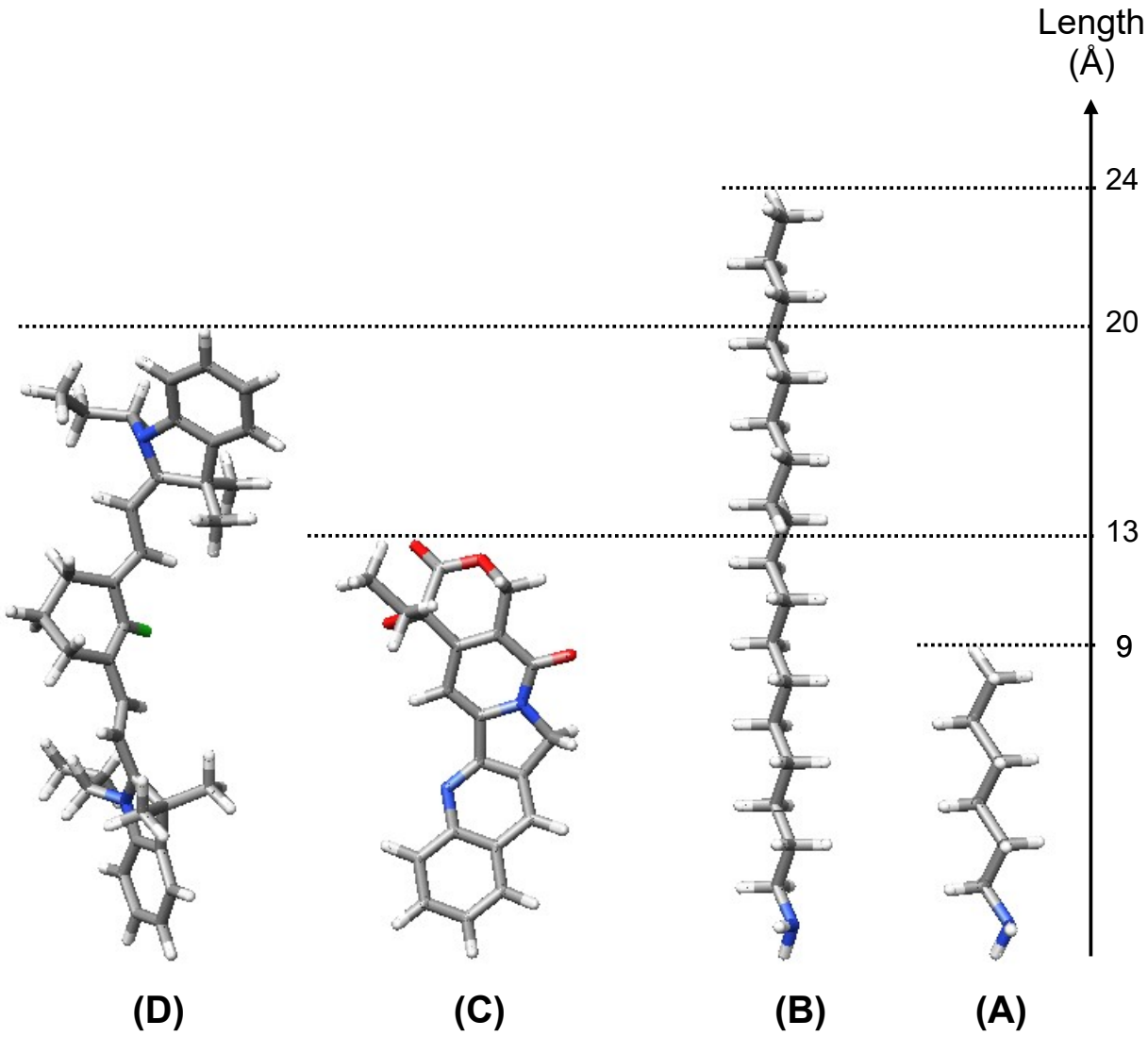

Fig. S4.- Length of the alkyl chain of **LCD-1** (A) and **LCD-2** (B), of CPT (C) and of IR-780 (D).

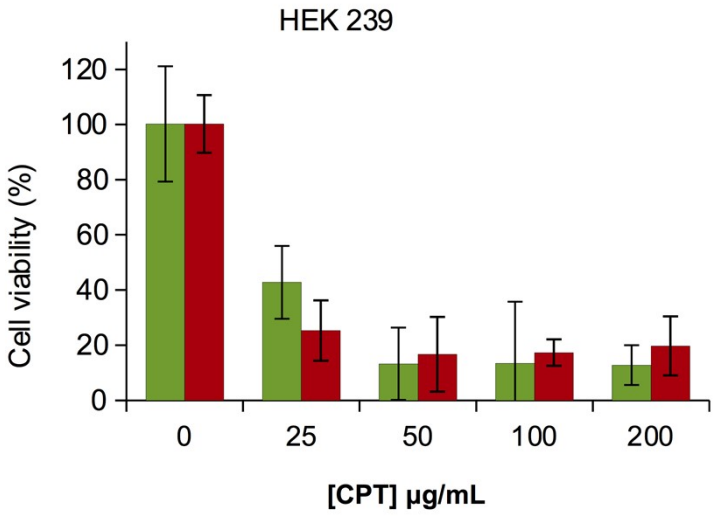

Fig. S5. Cytotoxicity of LCD-2Na@CPT (green) and free CPT (red) against the cell line HEK 239. For **LCD-2Na@CPT** values correspond to the load of CPT. Cell lines were incubated for 24 h with suitable amounts of either free CPT or **LCD-2Na@CPT** and cytotoxicity was assayed by the MTT method. Results are means ± 1 standard deviation. IC50 value was 78 µM for free CPT and 66 µM for **LCD-2Na@CPT**

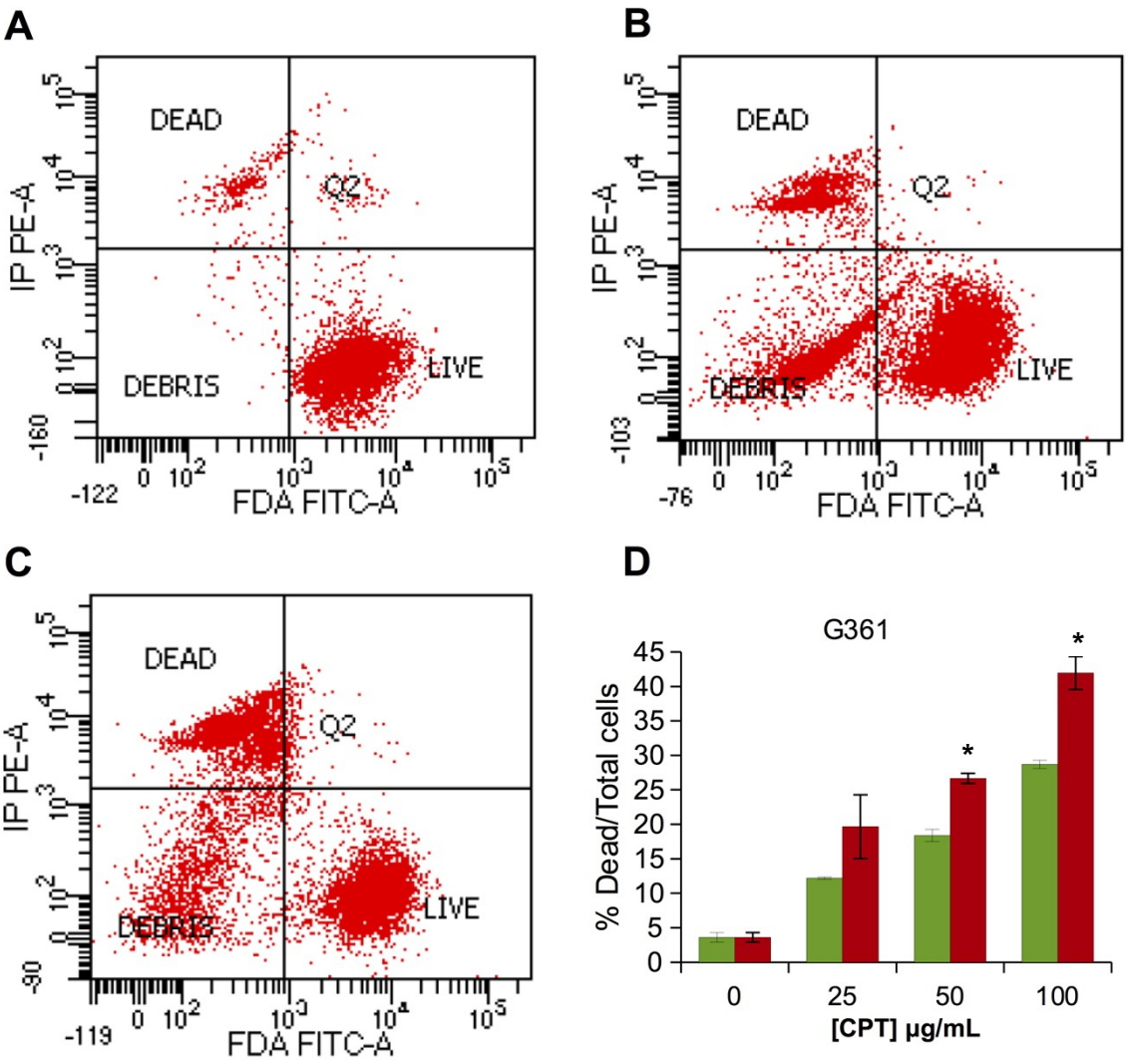

Fig. S6. Flow cytometry analysis of LCD-2Na@CPT and CPT against the cell line G361. (A-C) Flow cytometric differentiation of fluorescein diacetate and propidium iodide stained cells (example for a FACS sorted sample) for control cells (A) and cells incubated with 100µM of either **LCD-2Na@CPT** (B) or CPT (C). (D) Percentage of Dead/total cells after the incubation with free CPT (red) or **LCND-2Na@CPT** (green). * $p<0.05$ vs CPT incubated cells.

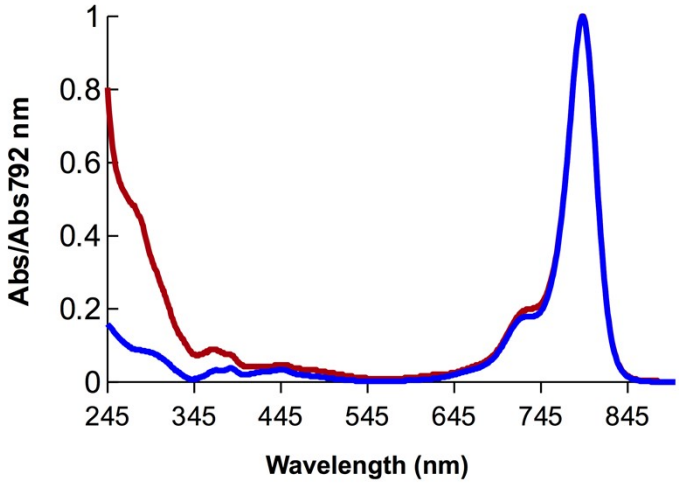

Fig. S7- UV-vis spectra of **LCD-2Na@IR780** normalized by the absorption at 792 nm (absorbance of IR780) from the complex obtained by two alternative procedures: i) combination of an aqueous phase containing **LCD-2Na** with an organic phase with IR780 (red) or ii) both **LCD-2** and IR780 in the organic phase (blue). The lower relative absorbance in the UV region (absorbance of LCDs) for the second approach (blue spectrum) indicates that the load of IR780 is higher.

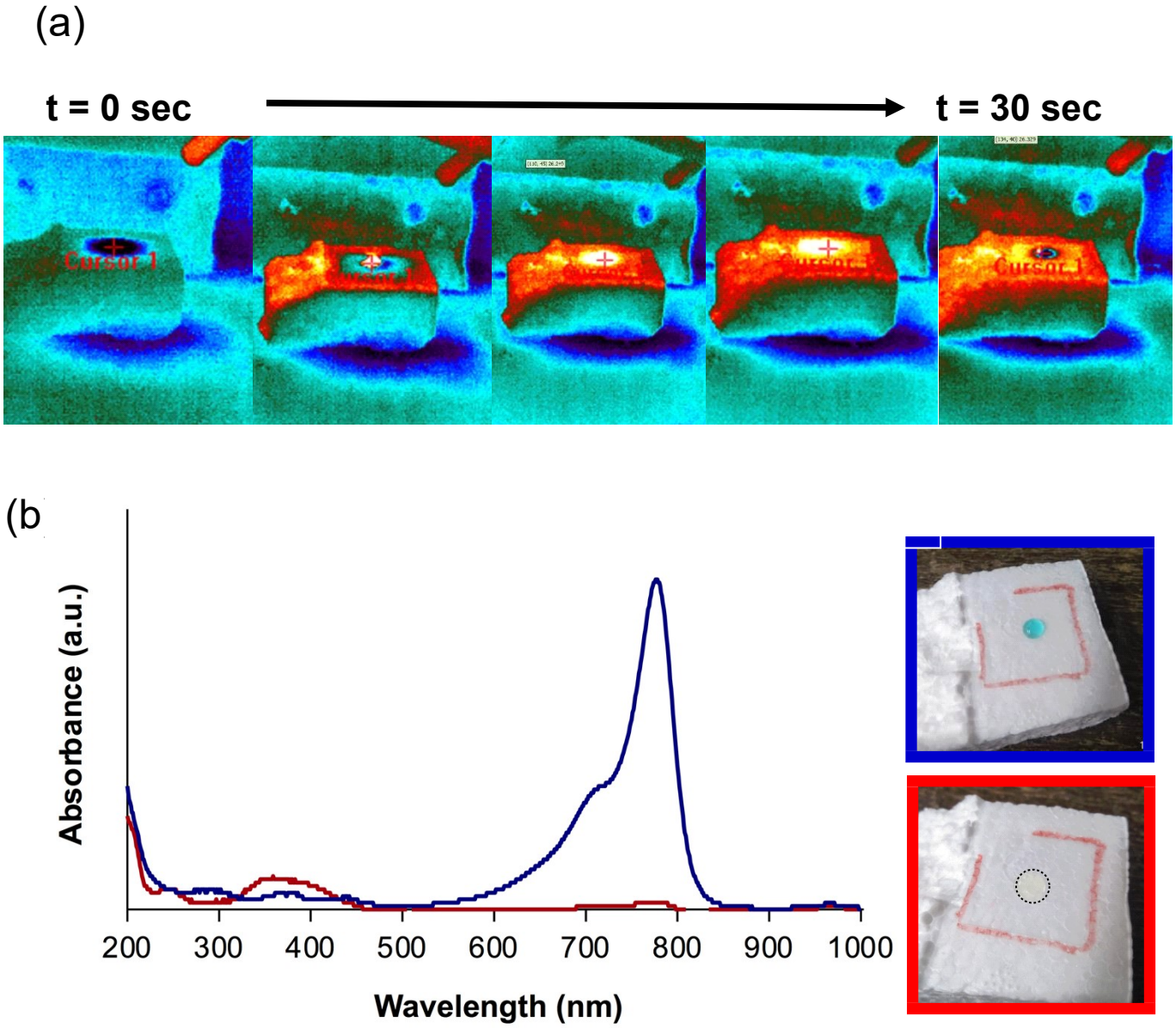

Fig. S8.- Irradiation of a 10 µL drop of a 13 nM solution of IR783 with with a 808 nm NIR laser at a power of 1.2 W/cm^2. (**A**) Thermal images of the drop along the first 30 second or irradiation. (**B**) UV-Vis spectra of the drop at the beginning (blue) and end (red) of the experiment. (**C**) Image of the drop at the beginning (blue frame) and end (red frame) of the experiment.

Table S1.- Quantification (%atomic concentration) by XPS of the most significant peaks detected in **CD-1Na,** the hydrophobic CDs **LCD-1**, **LCD-2** and their

hydrosoluble sodium salt **LCD-1Na** and **LCD-2Na**.

Sample	C1s	O1s	S2p	N1s	Na1s	O1s/C1 ratio	N1s/C1s ratio
CD-1Na	64.75	26.70	0.31	—	8.24	0.412	—
LCD-1	77.16	21.26	0.65	0.82	—	0.275	0.011
LCD-2	76.26	22.28	0.38	1.08	—	0.292	0.014
LCD-1Na	68.83	24.00	0.28	1.10	5.78	0.349	0.016
LCD-2Na	84.59	11.83	0.12	2.08	1.46	0.140	0.024

Table S2.- Fluorescent quantum yield (FLQY) of the hydrosoluble sodium salts of the CDots obtained in DMSO using as standard (QY=0.04) the sodium salt of AA-CD obtained from the free solvent pyrolysis of CA[4].

Sample	FLQY (%)
CD-1Na	4.1
LCD-1Na	4.6
LCD-2Na	3.1

[4] Mariano Ortega-Muñoz et al., “Acid Anhydride Coated Carbon Nanodots: Activated Platforms for Engineering Clicked (Bio)nanoconstructs,” *Nanoscale* 11, no. 16 (2019): 7850–56, doi:10.1039/C8NR09459D.

Table 3.- Electrophoretic mobility and Z-potential pf CD-1Na, LCD-1Na and LCD-2Na at three different pH values

Sample	pH	Mobility	Z-potential (eV)
CD-1Na	6.50	-0.75 ± 0.14	-14.0 ± 3
	7.04	-0.66 ± 0.09	-12.1 ± 1.6
	7.40	0.58 ± 0.06	-10.6 ± 1.1
LCD-1Na	6.50	-3.66 ± 0.20	-67 ± 4
	7.04	-3.30 ± 0.23	-60 ± 4
	7.40	-3.33 ± 0.12	-61.2 ± 2.2
LCD-2Na	6.50	-3.75 ± 0.24	-69 ± 4
	7.04	-3.72 ± 0.17	-68 ± 3
	7.40	-3.73 ± 0.17	-69 ± 3