C_{60}-β-cyclodextrin conjugates for enhanced nucleus delivery of doxorubicin

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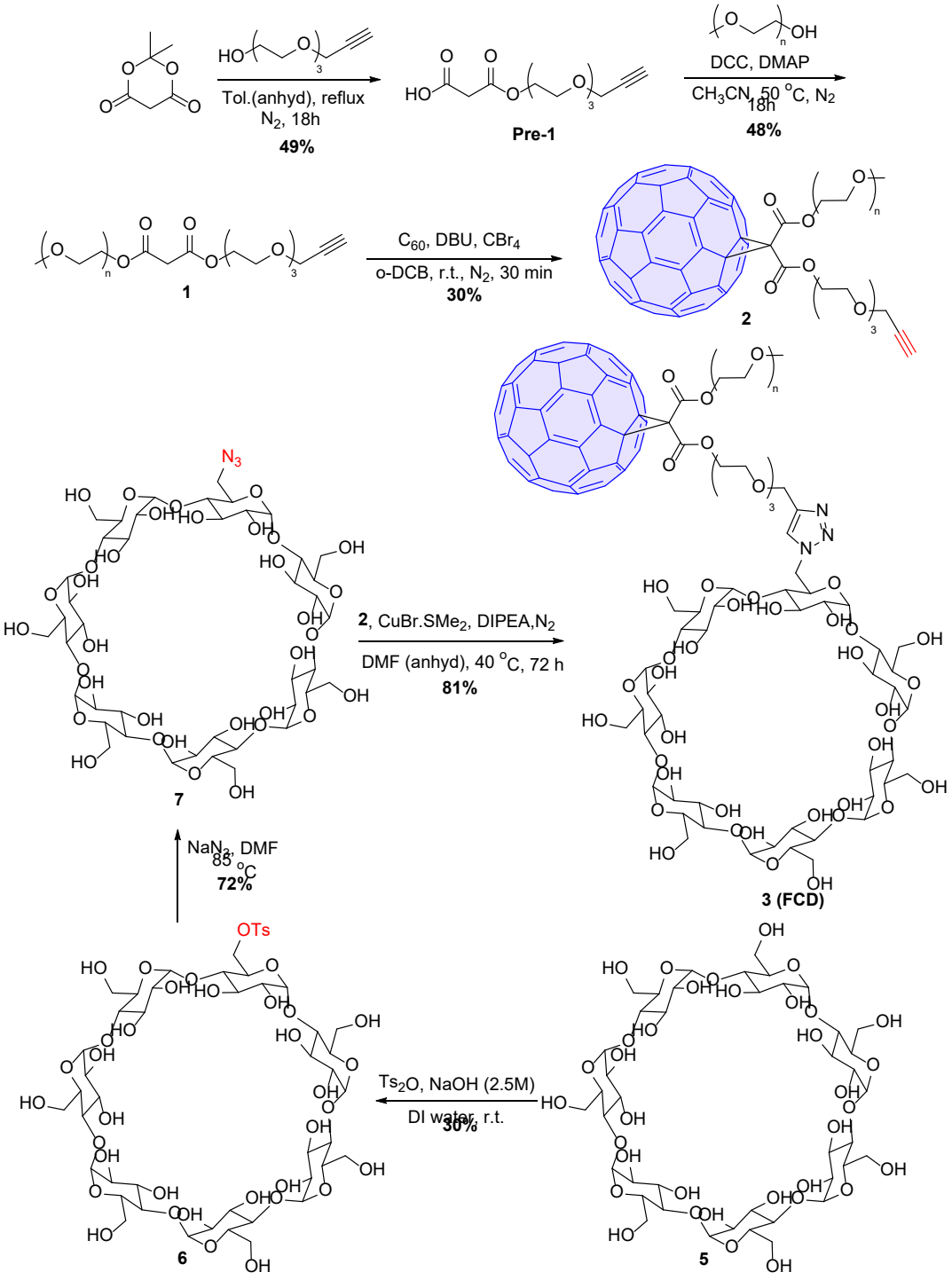
Materials & Instruments

Triethylene glycol mono (2-propynyl) ether (96%) was purchased from TCI America. Isopropylidene malonate (Meldrum's acid, 98%), polyethylene glycol monomethyl ether MW~750, N,N'-dicyclohexylcarbodiimide (DCC, 99%), 4-dimethylaminopyridine, 4-toluenesulfonylchloride, 18-crown-6 (99%) were purchased from Alfa Aesar. Glutaraldehyde and paraformaldehyde were purchased from Electron Microscopy Sciences. C_{60} was purchased from Henan Fullerene Co. Ltd. Carbon tetrabromide and β-cyclodextrin was purchased from TCI Chemicals. 1,8-diazabicyclo[5.4.0]undec-7-ene was purchased from Oakwood Chemical and was distilled before use. Sodium hydroxide was purchased from Fischer Scientific. Sodium azide, copper (I) bromide dimethyl sulfide complex ($CuBr.SMe_2$, 99%), N,N-diisopropylethylamine (DIPEA, 99.5%) were purchased from Acros Organics. Hydrogen peroxide and triethylamine were purchased from Fischer Chemical. Doxorubicin hydrochloride was purchased from Tocris Bioscience. DAPI was purchased from Sigma Aldrich. LysoTracker Deep Red and Thapsigargin were purchased from Invitrogen. Dialysis membranes were purchased from Spectra/Por. HeLa cells were purchased from commercial cell line (ATCC) and cultured in EMEM medium.

Chromatographic purifications were carried out with standard 230-400 mesh silica gel. NMR spectra were obtained at 25 °C with 400, 500 or 600 MHz spectrometers. Dynamic light scattering experiments were performed using a Malvern Zetasizer Nano ZS90 instrument with a standard laser source (4mW, 633nm). Dialysis membrane: Spectra/Por standard grade regenerated cellulose dialysis membranes (MWCO: 1 kD and 2 kD) were stored in 0.5% sodium azide solution. Prior to use, dialysis bags of desired sizes were soaked in DI water for at-least 30 minutes before loading the sample. UV-vis measurements were made using an Agilent Cary 60 instrument. Fluorescence microscope images were taken using a Leica DMi8 fluorescent microscope equipped with an 100x oil objective (HC PL APO, n.A. = 1.47). Absorbances from

a 96-well plate were measured using a Spectramax iD3 multi-mode microplate reader. TEM measurements were performed on a Nion UltraSTEM 100 with an aberration corrector at an operating voltage of 60 kV. TEM images with HeLa cells were captured using a Philips CM12 TEM with an AMT (Advanced Imaging Technique) XR111 digital camera at 80 kV.

Synthetic route to FCD:



Scheme 1. Synthesis of C_{60}-β-cyclodextrin conjugate (FCD).

Synthesis of compound Pre-1:

In a 50 mL Schlenk flask, Meldrum's acid (0.620 g , 4.30 mmol, 1.00 eq) was taken in 12 mL anhydrous toluene and flushed with nitrogen six times. Then, triethylene glycol mono(2-propynyl)ether (0.800 g, 4.25 mmol, 0.990 eq) dissolved in 4 mL anhydrous toluene and bubbled with nitrogen was added to the Schlenk flask and the reaction was refluxed for 18 hours. After 18 hours, the reaction mixture was extracted three times with $NaHCO_3$ solution and the aqueous layer was collected. The aqueous layer was acidified with HCl. Then, it was extracted three times with Et_2O and the combined organic layer was dried using Na_2SO_4 and concentrated under vacuum to give compound **Pre-1** as an yellow oil (0.560 g, 2.09 mmol, 49%).

Characterization data for compound Pre-1:

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.34-4.32 (m, J = 5 Hz, 2H), 4.23 (d, 2H), 3.73-3.65 (m, 10H), 3.45 (s, 3H), 2.45-2.44 (t, J = 5 Hz, 1H).
^{13}C NMR (126 MHz, $CDCl_3$) δ 169.30, 167.05, 79.27, 74.82, 70.55, 70.45, 70.21, 69.03, 68.63, 64.71, 58.29, 40.85.

Synthesis of compound 1:

In a 100 mL Schlenk flask, PEG 750 monomethyl ether (1.46 g , 1.95 mmol, 0.950 eq) was taken in 16 mL acetonitrile. To this solution, compound **Pre-1** (0.550 g, 2.05 mmol, 1.00 eq) dissolved in 2 mL acetonitrile was added, followed by DMAP (0.0120 g, 0.100 mmol, 0.0500 eq). Then, this mixture was flushed with nitrogen six times. After this, the Schlenk flask was placed in an oil bath at 50 ^{o}C and stirred. To this stirring mixture, DCC (0.444 g, 2.15 mmol, 1.05 eq) in 2 mL acetonitrile was added dropwise under nitrogen and stirring was continued. After 18 hours, the reaction mixture was concentrated under vacuum and directly loaded into a silica column which was packed in DCM. The desired product eluted in 3% MeOH/DCM as eluant. The fractions were combined and concentrated to give compound **1** as a yellow oil (0.940 g, 0.940 mmol, **48%**).

Characterization data for compound 1:

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.30-4.28 (m, 4H), 4.20-4.19 (d, J = 5 Hz, 2H), 3.71-3.62 (m, 70H), 3.55-3.53 (m, 2H), 3.44 (s, 2H), 3.37 (s, 3H), 2.44 (t, J = 5 Hz, 1H).
^{13}C NMR (126 MHz, $CDCl_3$) δ 166.41, 79.58, 74.54, 71.87, 71.87, 70.54, 70.51, 70.45, 70.40, 69.04, 68.79, 64.51, 58.97, 58.34, 41.21.

Synthesis of compound 2:

In a 300 mL round-bottomed flask, C_{60} (0.262 g, 0.364 mmol, 1.00 eq) was taken. Then, compound **1** (0.400 g, 0.400 mmol, 1.10 eq) and 100 mL of o-DCB was added to it, followed by the addition of CBr_4 (0.120 g, 0.364 mmol, 1.00 eq). Finally, DBU (0.0830 g, 0.545 mmol, 1.50 eq) was added. The entire solution was wrapped in an aluminium foil and bubbled with nitrogen for 15 minutes. Then, the solution was stirred at r.t. and the reaction was monitored using TLC. After 30 mins, appearance of bis-adduct was observed and the reaction mixture was directly loaded in a silica column packed in toluene. Unreacted C_{60} eluted as a purple band. After all the unreacted C_{60} eluted, DCM was used to elute the column, followed by a gradient elution using MeOH/DCM. The desired monoadduct eluted using 3% MeOH/DCM as a brown band. The fractions were collected and concentrated under vacuum to give compound **2** as a brown solid (0.520 g, 0.181 mmol, **50%**).

Characterization data for compound 2:

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.65-4.63 (m, 4H), 4.20-4.19 (d, J = 5 Hz, 2H), 3.88-3.62 (m, 72H), 3.37 (s, 3H), 2.44 (s, J = 5 Hz, 1H).
^{13}C NMR (126 MHz, $CDCl_3$) δ 163.46, 146.13, 145.23, 145.14, 144.86, 144.73, 144.58, 143.85, 143.05, 142.98, 142.94, 142.16, 141.82, 140.89, 139.04, 79.61, 74.63, 71.89, 71.40, 70.67, 70.64, 70.60, 70.56, 70.52, 70.47, 70.45, 69.07, 68.74, 68.73, 68.65, 66.20, 66.14, 59.00, 58.38.

Synthesis of compound 3:

Compound **2** (0.0295 g, 0.0172 mmol, 1.00 eq.) was dissolved in 2 mL dry DMF in a 10 mL Schlenk flask. β-CD-6-azide (0.0400 g, 0.0344 mmol, 2.00 eq.), $CuBr.SMe_2$ (0.00530 g, 0.0258 mmol, 1.5 eq.) and DIPEA (0.0334 g, 0.258 mmol, 15.0 eq.) were added sequentially. The mixture was lightly sonicated and subjected to three cycles of freeze-pump-thaw, and then the flask was refilled with nitrogen. The solution was stirred at 40 °C for 72 hours. The reaction mixture was then poured in cold diethyl ether and the brown precipitate was filtered and extracted three times with $CHCl_3$/water mixture. The brown aqueous layer was collected and dialyzed using a membrane with MWCO= 2 kD against DI water for 72 hours, with water changed three times a day. The final solution was lyophilized to give the compound **3** (FCD) as a brown solid (0.0400 g, 0.0139 mmol, **81%**).

Characterization data for compound 3:

^{1}H NMR (500 MHz, DMSO) δ 8.00 (s, 1H), 5.88-5.64 (m, 14H), 5.04-4.76 (m, 7H), 4.62-4.46 (m, 10 H), 4.23 (s, 2H), 3.80-3.29 (m, mixed with water peak), 3.24 (s, 3H).

Synthesis of compound 4:

Compound **3** was (0.027 g, 0.0094 mmol) was dissolved in 20 mL toluene. To this solution, 18-crown-6 was added, followed by 2 mL 50% NaOH solution. This mixture was stirred for 4 hours until the solution became colorless. 1.5 mL 30 wt% H_2O_2 was added followed by addition of 20 mL water. The mixture was further stirred at room temperature overnight. It was then extracted and the brown aqueous layer was subjected to dialysis using a membrane (MWCO= 2kD) for 72 hours, with water changed three times a day. Then, the dialyzed solution was freeze-dried to obtain compound **4** (hFCD) as a light brown fluffy solid in quantitative yield.

Characterization data for compound 4:

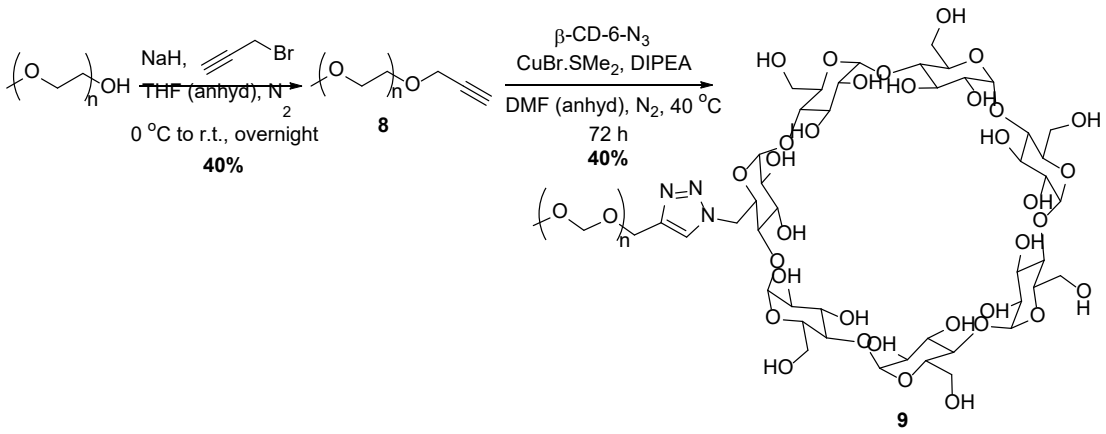
This spectra was taken in a mixture of 2:1 D_2O:DMSO-d6. ^{1}H NMR (500 MHz, D_2O) δ 7.92 (s, 1H), 5.00-4.81 (m, 7H), 3.20 (s, 3H).

Synthesis of compound 6:

This is a known compound and the synthesis followed the reported procedure with minor revision.[1,2] In a 300 mL round-bottomed flask with 95 mL deionized water, β-cyclodextrin (10.5 g, 9.30 mmol, 1.00 eq) and Ts_2O (4.46 g, 13.7 mmol, 1.47 eq) were added and stirred for 2 hours at room temperature. Then, NaOH (4.5 g in 45 mL water) was added to the flask, stirred for 10 mins and filtered. The filtrate was collected and neutralized using NH_4Cl and kept at 4 °C. The crude product precipitated out and was collected by vacuum filtration. The crude solid was re-crystallized in 1:1 MeOH/water mixture to obtain compound **6** as a white solid (3.59 g, 2.79 mmol, **30%**).

Synthesis of compound 7:

In a round-bottomed flask containing compound **6** (1.31 g, 1.02 mmol, 1.00 eq), NaN_3 (0.990 g, 15.2 mmol, 15.0 eq) was added followed by 130 mL of DMF. The mixture was heated at 80 °C and stirred overnight. Then, the DMF was concentrated under vacuum. The crude white solid was dissolved in a little volume of water and precipitated into 70% acetone water mixture and kept at 4 °C. Compound **7** precipitated out as a white solid which was collected by vacuum filtration to yield the pure product (0.850 g, 0.733 mmol, 72%). This is a reported compound.[3,4]



Scheme 2. Synthesis of methoxy PEG 750-β-cyclodextrin conjugate (CD).

Synthesis of compound 8:

In a round-bottomed flask, PEG 750 (5.0 g, 6.7 mmol, 2.2 eq.) was taken to which 20 mL of dry THF was added and flushed with nitrogen thrice. The flask was then kept in an ice-bath for 15 minutes and allowed to cool to 0 °C. Then, under nitrogen flow, sodium hydride (0.12 g, 3.0 mmol, 1.0 eq.) was added to the flask, the ice-bath was removed and the mixture was allowed to come to room temperature. Following this, propargyl bromide (0.45 g, 3.0 mmol, 1.0 eq.) was added to the flask and the mixture was stirred at room temperature overnight. Then, the stirring was stopped, the mixture was filtered, reaction mixture was concentrated under vacuum and loaded in a silica gel column which was eluted using a DCM/MeOH mixture. The desired product eluted in 3% MeOH/DCM which was concentrated to give a light yellow oil as the product (1.1 g, 1.5 mmol, **40%**).

Characterization data for 8:

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.17 (d, 2H), 3.68-3.58 (m, 62H), 3.52-3.51 (m, 2H), 3.35 (s, 3H), 2.42 (t, J = 5 Hz, 1H).

Synthesis of compound 9:

Compound **9** (CD) was synthesized from the Click partners, compound **8** and β-CD-6-azide. Compound **8** (0.116 g, 0.147 mmol, 2.00 eq.) was dissolved in 2 mL. dry DMF in a 10 mL. Schlenk flask. Then, β-CD-6-azide (0.0850 g, 0.0740 mmol, 1.00 eq.) was added, followed by $CuBr.SMe_2$ (0.0460 g, 0.222 mmol, 3.00 eq.) and DIPEA (0.0286 g, 2.22 mmol, 30.0 eq.). The mixture was sonicated briefly and subjected to three cycles of freeze-pump-thaw. Then, the reaction mixture was stirred at 40 °C for 72 hours. The reaction

mixture was then precipitated in cold Et_2O, filtered and dissolved in water. The solution was dialyzed using a membrane with MWCO= 1kD against DI water for 72 hours, water was changed three times a day, and then freeze-dried to give the product as a white fluffy solid (0.0430 g, 0.0222 mmol, **30%**).

Characterization data for compound 9:

^{1}H NMR (500 MHz, DMSO) δ 8.02 (s, 1H), 5.79-5.64 (m, 14H), 5.04-4.76 (m, 7H), 4.49-4.46 (m, 6H), 3.24 (s, 3H).

General procedure of Doxorubicin loading in the DDS

The DDSs and Doxorubicin hydrochloride (Dox.HCl) were added in water:DMSO (9:1) mixture and stirred at 40 ºC for 15 minutes. Then, Et_3N (3 eq. w.r.t. Dox.HCl) was added and the mixture was stirred for 24 hours. After 24 hours, stirring was stopped and then the mixture was dialyzed overnight against water:DMSO (9:1) and then against pure DI water for another 48 hours with changing water three times a day. For Dox@CD, the solutions were filtered as well. Then, the solutions were lyophilized to get the product as a reddish powder.

The loading of doxorubicin in the carriers was quantified using UV-visible absorbance at 495 nm and calculating the concentration of doxorubicin using a standard plot that was prepared using a series of known concentrations of the drug. Further from the absorbance, encapsulation efficiency (EE) and loading efficiency (LE) data were also calculated, as shown in Fig. 3c. EE and LE have been calculated as defined below.

$$\text{EE} = \frac{Weight\ of\ drug\ taken\ up\ by\ the\ carrier}{Weight\ of\ drug\ initially\ fed} * 100\ \%$$

$$\text{LE} = \frac{Weight\ of\ drug\ taken\ up\ by\ the\ carrier}{Weight\ of\ drug\ taken\ up\ by\ the\ carrier + Weight\ of\ carrier} * 100\ \%$$

General procedure of cellular uptake experiments

HeLa cells were cultured in EMEM supplemented with 10% FBS and penicillin/streptomycin in a 37°C incubator under 5% CO_2. On day 1, 5 x 10^4 cells were plated on 35 mm glass-bottom dishes in 1.5 mL EMEM culture medium. 24 hours later, the old medium was discarded and the cells were then treated with 10 µM of the drug loaded DDS (Dox@CD, Dox@FCD or Dox@hFCD dissolved in EMEM) for 4 hours. For the thapsigargin experiment, the cells were incubated for 45 minutes with 0.5 µM thapsigargin for about an hour and then incubated with the drug loaded DDSs for 4 hours. Then, the medium containing the respective molecules was discarded and the cells were incubated in fresh medium containing DAPI (10 µg/mL) at 37 ºC for 30 minutes. After that, the medium containing DAPI was removed, and cells were washed with extracellular (XC) imaging buffer (125 mM NaCl, 2.5 mM KCl, 15 mM HEPES, 30 mM glucose, 1 mM $MgCl_2$, 3 mM $CaCl_2$, and pH 7.3) three times. Finally, fresh XC buffer was added and the dishes were immediately imaged using a Leica DMi8 fluorescent microscope equipped with an 100x oil objective (HC PL APO, n.A. = 1.47).

For lysosomal uptake experiment, the cells were seeded and allowed to grow for 24 hours as described above, following which they were incubated with Dox@CD, Dox@FCD and Dox@hFCD for different time points (1 h, 2.5 h and 3.5 h). Then, the medium containing the drug loaded DDSs were discarded and the cells were further incubated for another 30 minutes with LysoTracker Deep Red (0.1 µM) and DAPI (10 µg/mL). Then, the washing steps, as described above, were followed prior to imaging the dishes using the same instrument as mentioned above.

Fluorescence signals that were present from cross-talk between DAPI and doxorubicin due to the broad emission of doxorubicin were corrected using doxorubicin fluorescence and applied to the DAPI channel. Box plots were constructed to quantify the nucleus enrichment of doxorubicin. Enrichment was defined as

$$\frac{mean\ signal\ intensity\ in\ nucleus}{mean\ signal\ intensity\ in\ cytosol} - 1$$

For the group without TG treatment, mean and standard deviations were 0.26 ± 0.26 for Dox@CD, 0.85 ± 0.24 for Dox@FCD and 3.13 ± 0.92 for Dox@hFCD. With TG, the values were 0.12 ± 0.26 for Dox@CD, 0.47 ± 0.26 for Dox@FCD and 1.11 ± 0.53 for Dox@hFCD. Statistical analysis was done using the Paired Comparison Plot app in Origin. One-way ANOVA was performed and the Tukey mean comparison test was applied.

General procedure of cell viability study

Cell viability assay was performed using HeLa cells with Prestoblue dye. HeLa cells were seeded onto 96 well plates in 100 µL EMEM culture medium (each well containing 2.5 x 10^3 cells), and incubated at 37 ºC incubator under 5% CO_2 for 24 hours. Then, the cells were treated with the DDS or doxorubicin-loaded DDS at different concentrations (1, 4, 8, 16, 32 µM for each) for 48 hours and 72 hours. After incubating at 37 ºC for the respective duration, the medium was discarded and XC imaging buffer (125 mM NaCl, 2.5 mM KCl, 15 mM HEPES, 30 mM glucose, 1 mM $MgCl_2$, 3 mM $CaCl_2$, and pH 7.3) containing Prestoblue (10% v/v) was added to the wells and incubated for 30 minutes. Then, the absorbance from the wells were read using a SpectraMax iD3 plate reader at 570 nm, and a reference wavelength at 600 nm. The relative cell viability was calculated by blank corrected absorbances using the formula. Cell viability (%) = ($OD_{570-600}$ treated wells/$OD_{570-600}$ control) * 100%.

TEM sample preparation

Samples were prepared by roughly following the protocol outlined here.[5] HeLa cells (1 x 10^6) were treated with FCD and hFCD at a concentration of 16 µM for 24 hours. The medium was then discarded and cells were detached from the dishes and transferred to vials and fixed using 2.5% glutaraldehyde/4% paraformaldehyde in 0.1M cacodylate buffer. Samples were subsequently dehydrated in a graded series of acetone and embedded in Embed812 resin. 90 nm thin sections were cut on a Leica UC6ultramicrotome and stained with saturated solution of uranyl acetate and lead citrate. Images were captured with an AMT (Advanced Microscopy Techniques) XR111 digital camera at 80 kV on a Philips CM12 TEM.

Artificial Lysosomal Fluid (ALF)

ALF was synthesized following the protocol outlined here.[6] The DDSs were soaked in ALF for 24 hours and then their absorbances and aggregate sizes were recorded using UV-vis and DLS respectively.

System	Dox:DDS equivalents	EE (%)	LE (%)	Dox:DDS (after encapsulation)
Dox@CD	10:1	8	20	0.9
Dox@FCD	1.1:1	90	18	1.2
Dox@hFCD	1.25:1	85	17	1.0

Table S1. Optimized ratio for doxorubicin loading in the DDSs, with the corresponding EE, LE, and doxorubicin/DDS molar ratio.

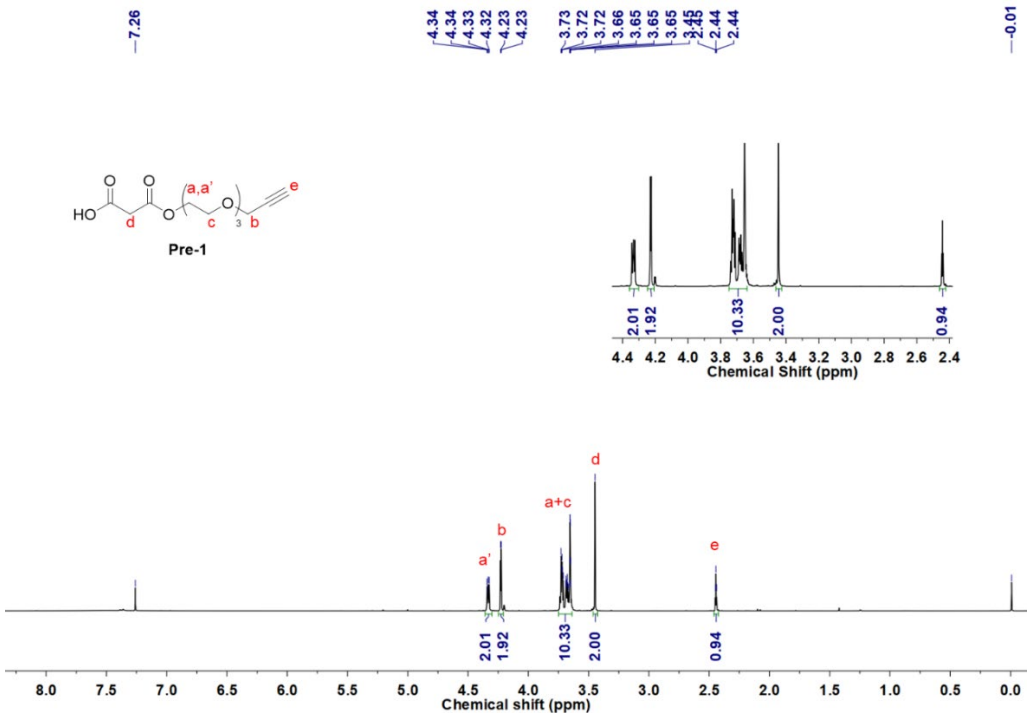


Figure S1. ^{1}H NMR spectrum of **Pre-1.**

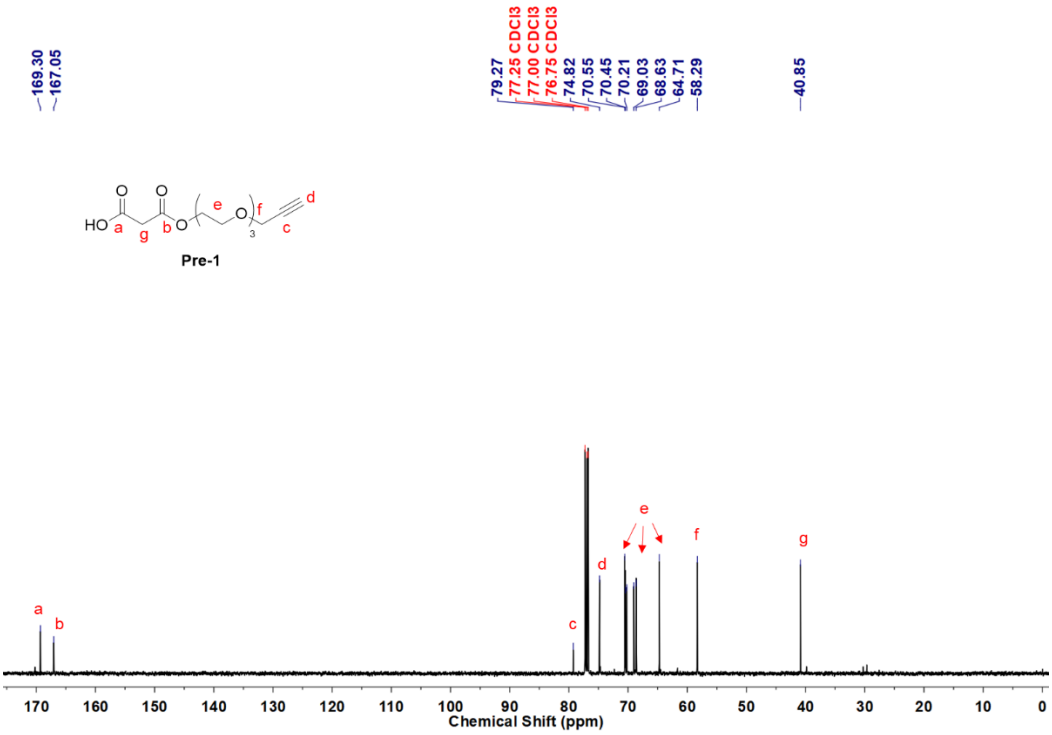


Figure S2. ^{13}C NMR spectrum of **Pre-1.**

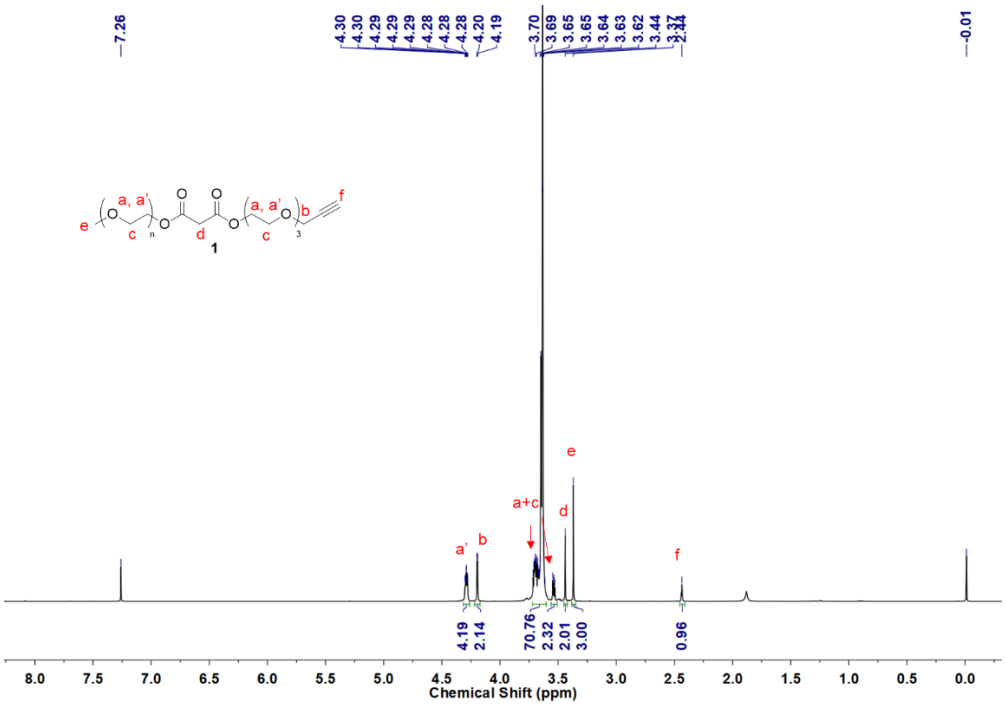


Figure S3. ^{1}H NMR spectrum of **1.**

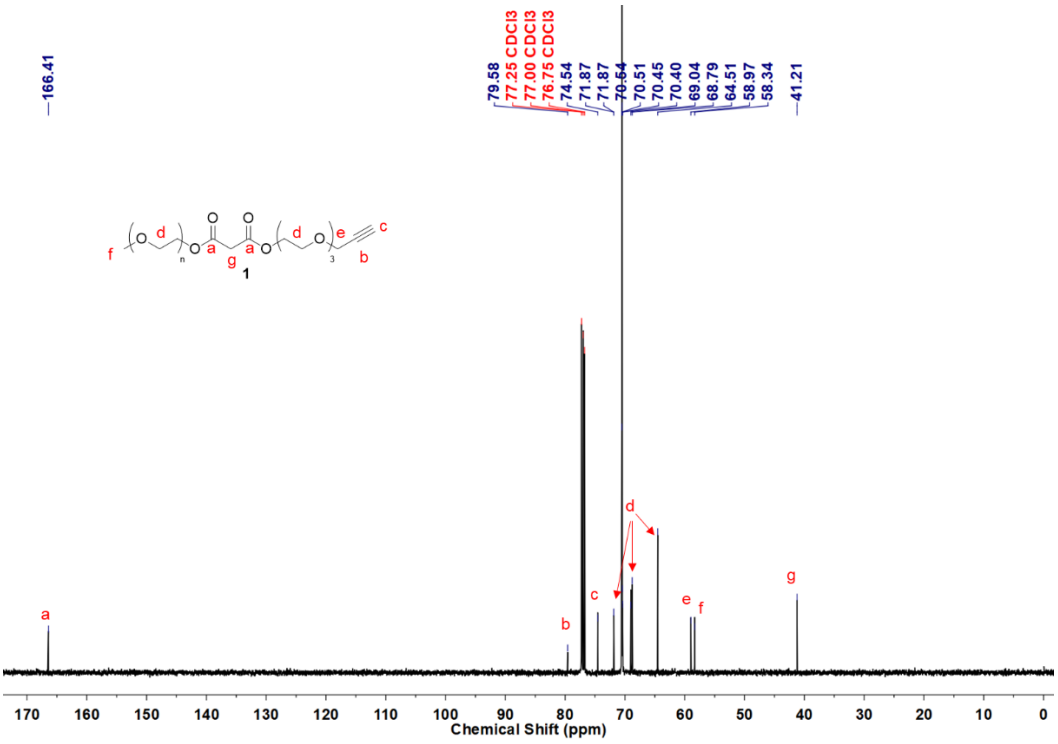


Figure S4. ^{13}C NMR spectrum of **1.**

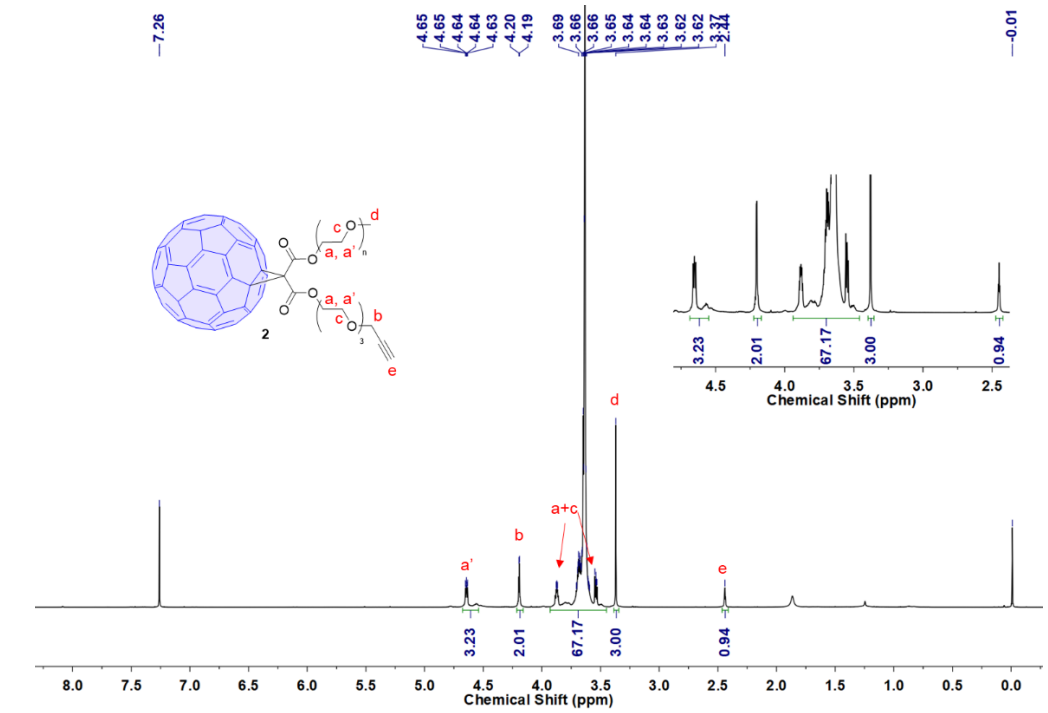


Figure S5. [1]H NMR spectrum of **2.**

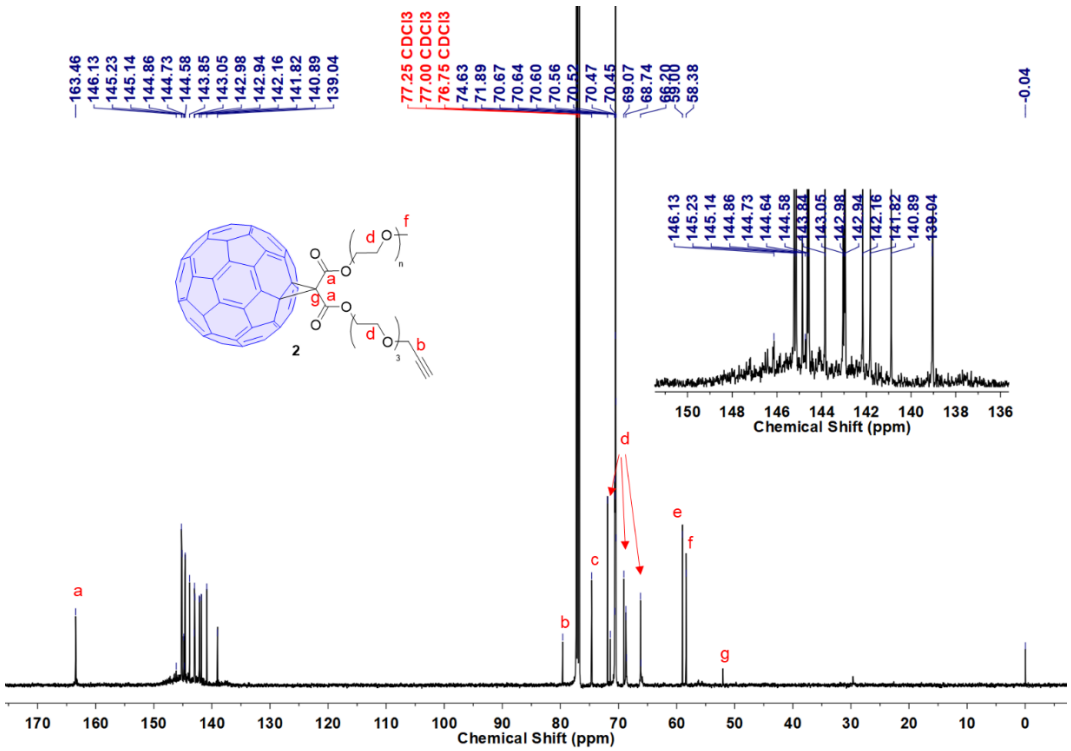


Figure S6. [13]C NMR spectrum of **2.**

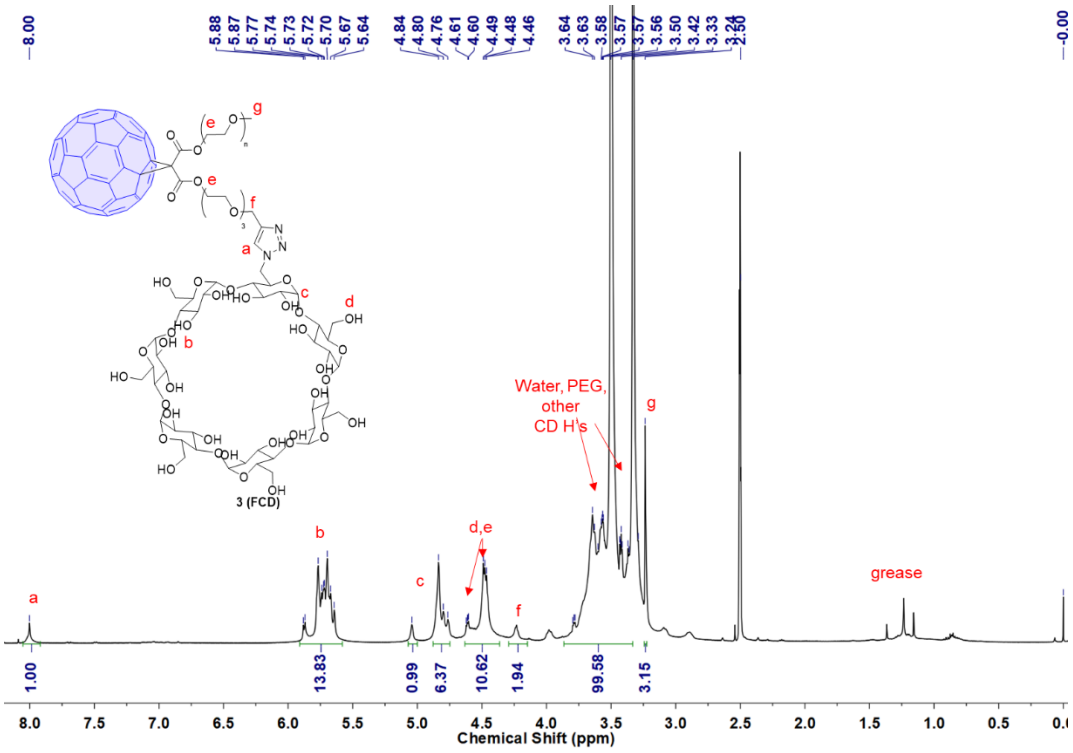


Figure S7. ^{1}H NMR spectrum of **3** (FCD).

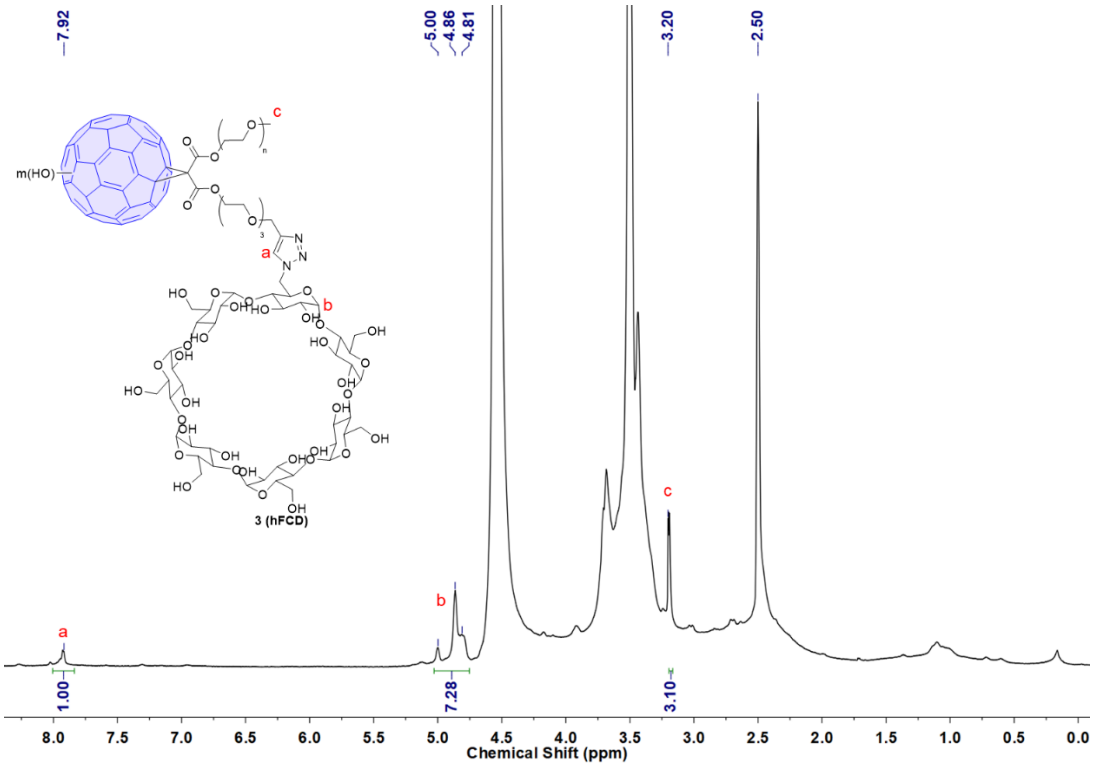


Figure S8. ^{1}H NMR spectrum of **4** (hFCD).

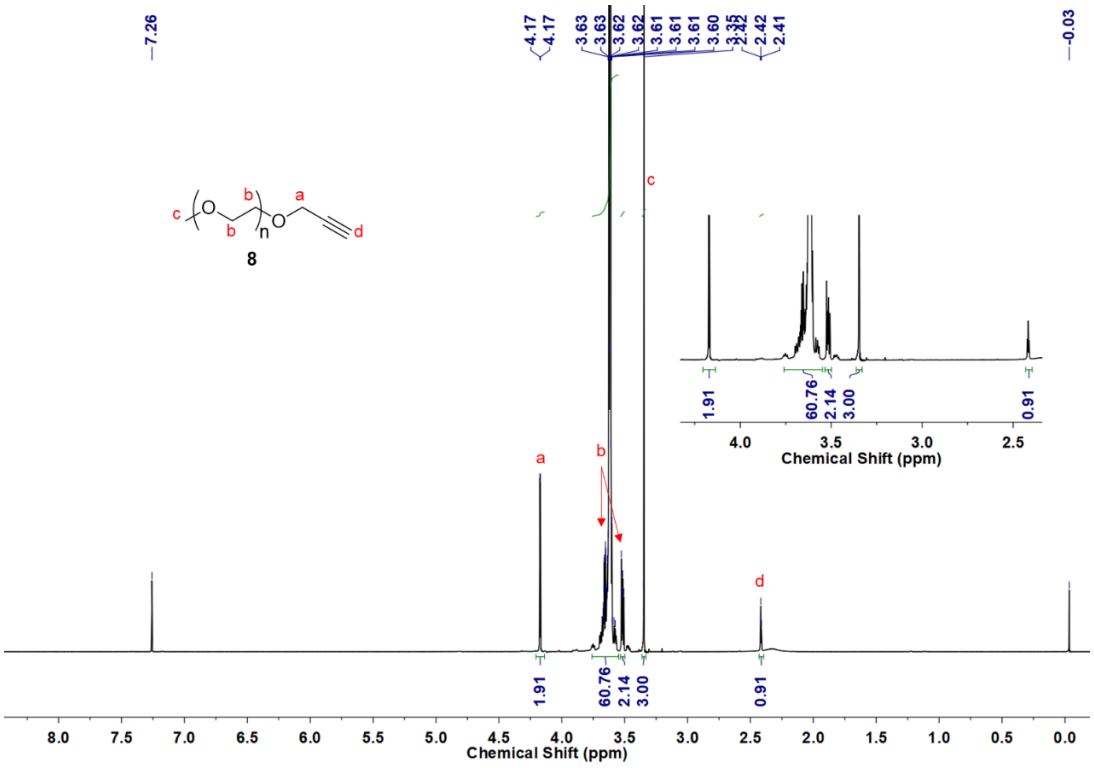


Figure S9. ^{1}H NMR spectrum of **8**.

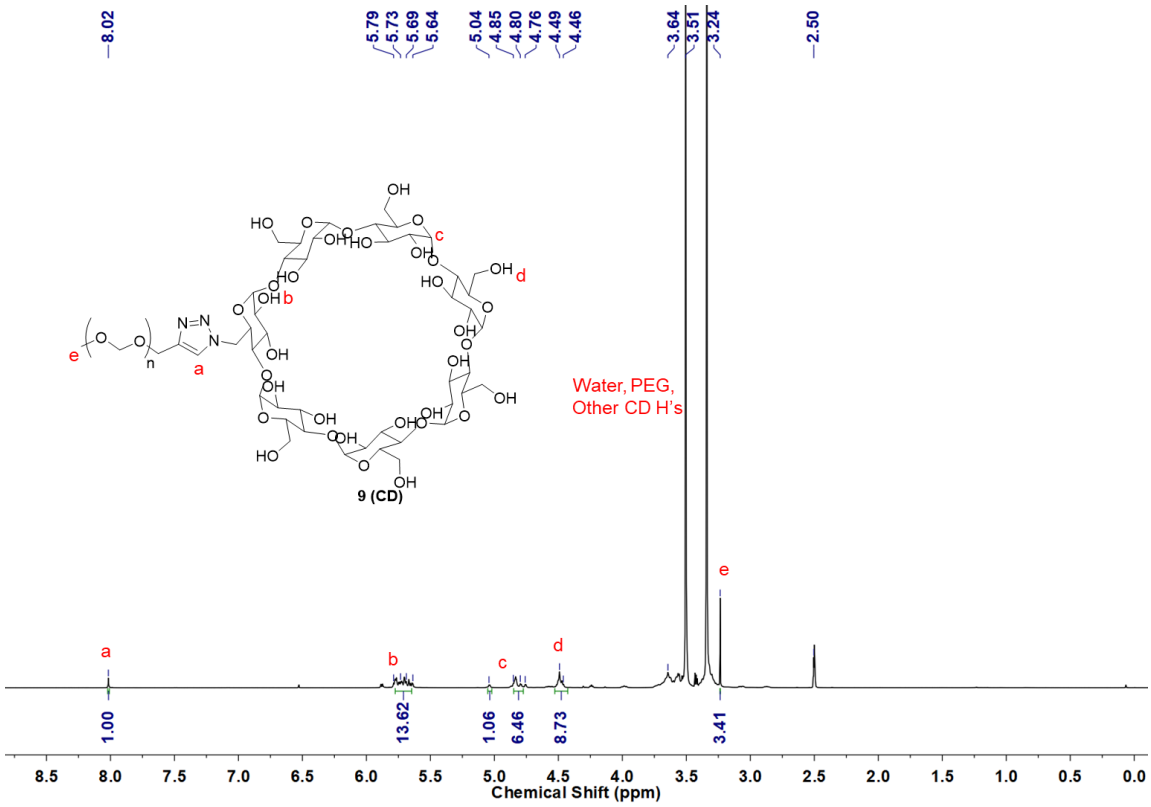


Figure S10. ^{1}H NMR spectrum of **9** (CD).

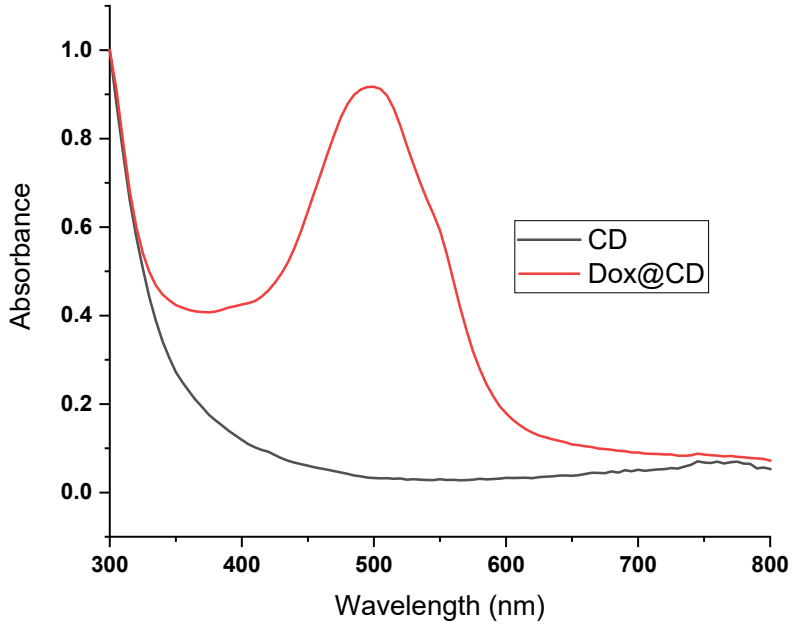


Figure S11. UV-vis spectra of CD and Dox@CD.

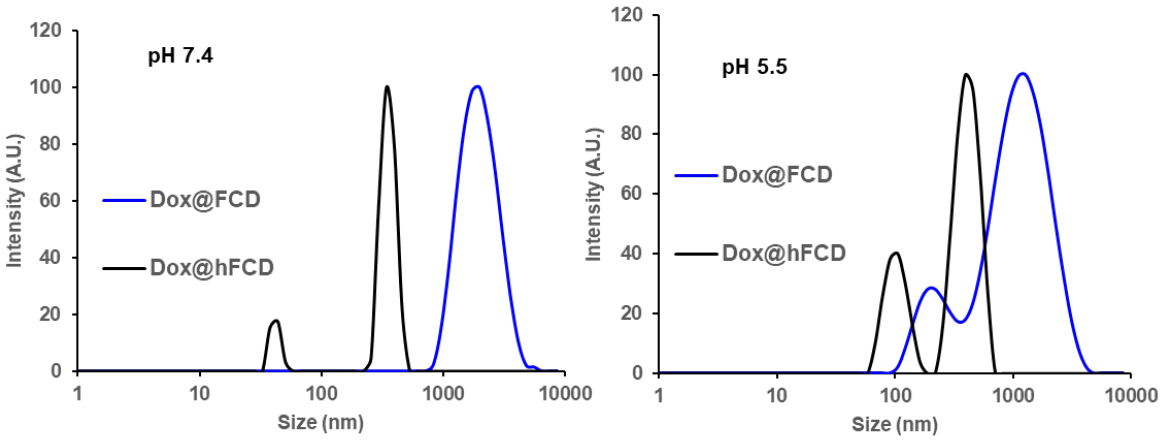


Figure S12. DLS data of DDSs and doxorubicin loaded DDSs at two different pH conditions.

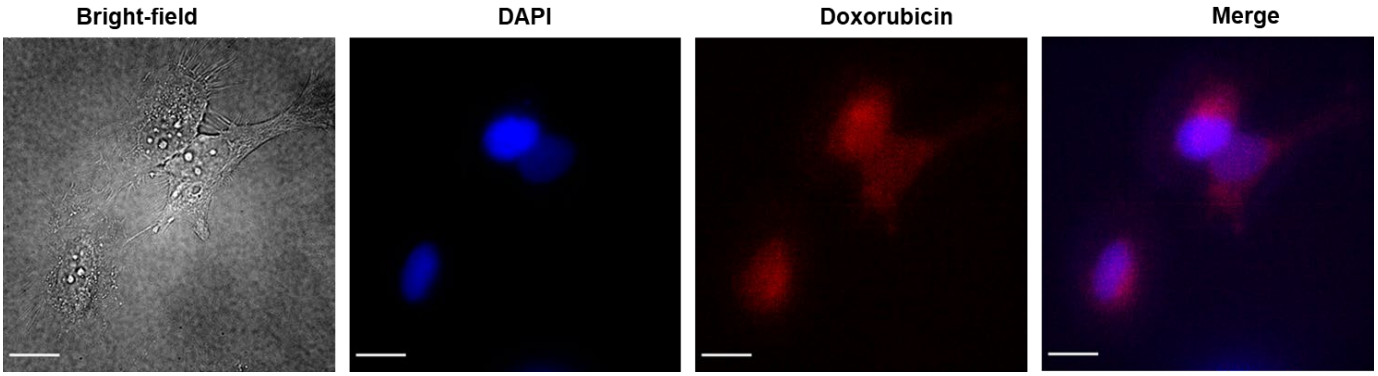
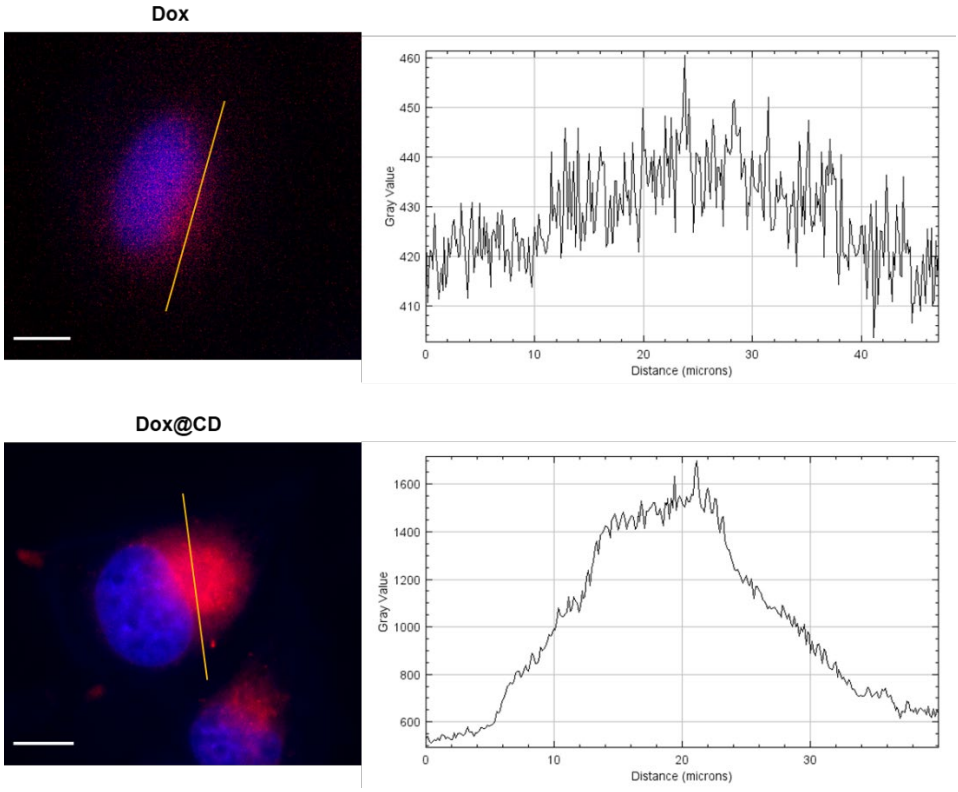


Figure S13. Cell uptake data of native doxorubicin in HeLa cells. Scale bar is 20 microns.



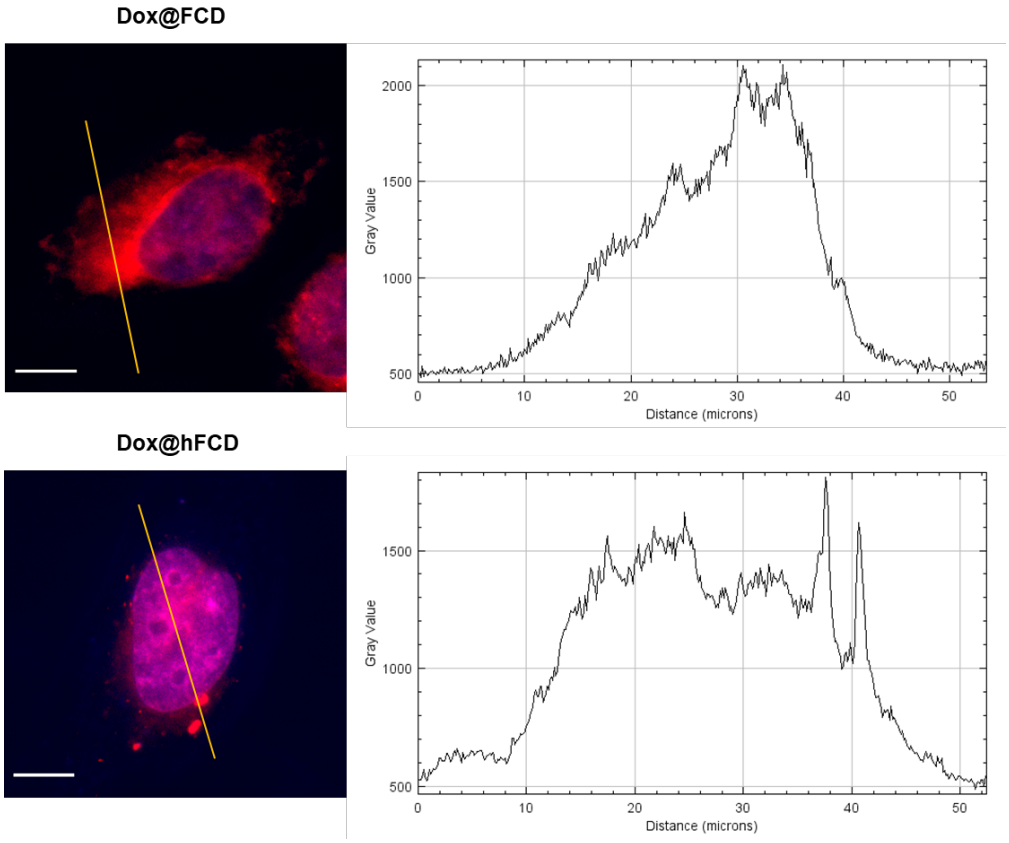


Figure S14. Dox@DDSs (zoom-in) in cells and their corresponding signal intensities. Scale bar: 10 μm.

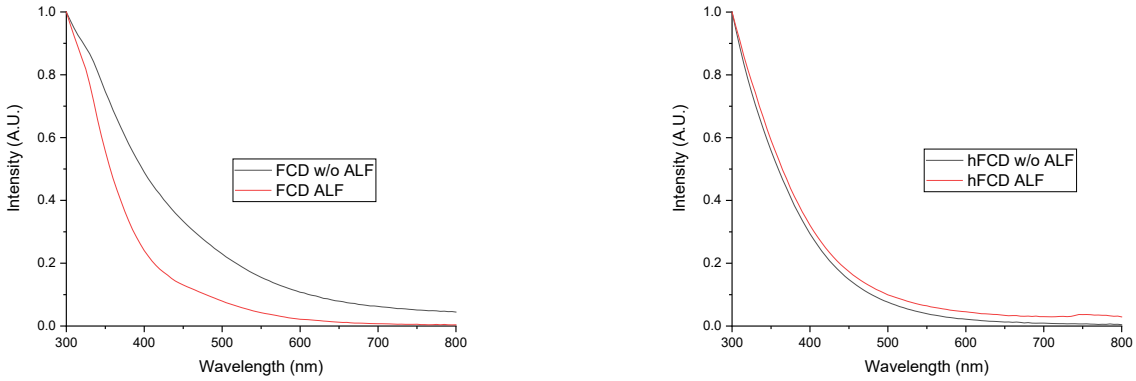


Figure S15. UV-vis spectra of FCD (left) and hFCD (right) solutions in ALF taken after 24 hours.

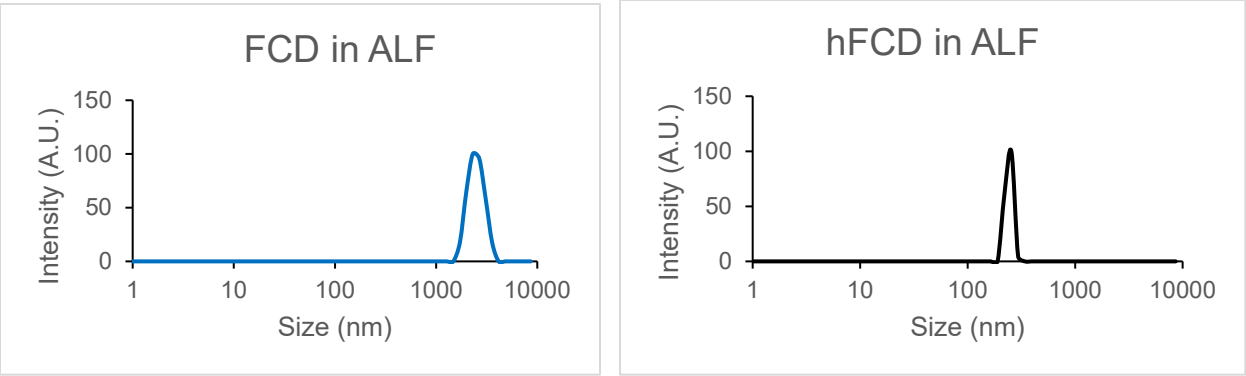


Figure S16. DLS aggregate sizes of FCD (left) and hFCD (right) solutions in ALF taken after 24 hours.

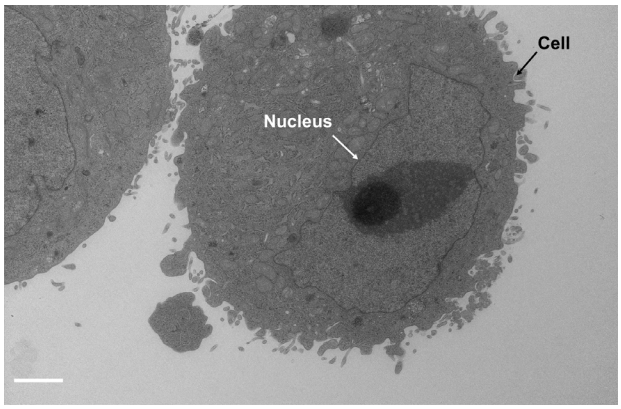


Figure S17. TEM image of HeLa cells showing the cell and the nucleus.

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