Electronic Supplementary information

Synthesis, Self-Assembly, and Photocrosslinking of Fullerene-Polyglycerol Amphiphiles as Nanocarriers with Controlled Transport Property

Ievgen Donskyi, Katharina Achazi, Virginia Wycisk, Christoph Böttcher, and Mohsen Adeli

Contents

1. Experimental section

1.1. Materials

Fullerene (SigmaAldrich, 98%) and potassium tert-butoxide in THF (1M) (SigmaAldrich) were used without further purification. Cy5-COOH was purchased from IC Discovery company. Glycidol (SigmaAldrich) was dried with CaH_2, distilled before use, and stored at 4 ^{0}C. All other reagents and solvents were purchased from different commercial suppliers and used as received, unless otherwise stated. Anhydrous solvents were either obtained from MBraun MB SPS-800 solvent purification system or purchased as ultra dry solvents from Acros Organics company. Water was used from Milli-Q® Advantage A10 Water Purification System in all experiments. Phosphate-buffered saline (PBS) (10X) pH 7.4 (ThermoFisher Scientific) was diluted 10 times with Millipore quality water. Sephadex G-25 Superfine (GE Healthcare Life Sciences) was mixed with PBS solution for 3 hours before preparation of the SEC columns. Benzoylated cellulose dialysis tubes (width: 32 mm, MWCO > 1000-2000 g/mol) from Sigma-Aldrich were used for purifiction of the synthesized compounds.

1.2. Methods

NMR. NMR spectra were performed on a Brucker AMX 500, Brucker Avance 400 spectrometer or Jeol ECP 500. Inverse-gated ^{13}C NMR was performed on Brucker Avance 400 or Brucker Avance 500 spectrometers. For internal calibration tetramethylsilane was used at 12 MHz with complete proton decoupling. The degree of branching was calculated according to the inverse-gated ^{13}C NMR data using an equation from Frey et al.[1]

GPC. GPC measurements were performed using an Agilent 1100 solvent delivery system with a manual injector, isopump, and Agilent 1100 differential refractometer. The Brookhaven BI-MwA7-angle light scattering detector was coupled to a size exclusion chromotography (SEC) to measure the molecular weight for each fraction of the polymer that was eluted from the SEC columns. For separation of the polymer samples, three 30 cm columns were used (10μm PSS Suprema columns with pore sizes of 100 Å, 1000 Å, 3000 Å). Water was used as mobile phase;

the flow rate was set at 1.0mL/min. All columns were held at room temperature. For each measurement, 100 μL of samples with concentration of 5 mg/mL solution was injected. For acquisition of the data from seven scattering angles (detectors) and differential refractometer WinGPC Unity from PSS was used. Molecular-weight distributions and molecular weights were determined by comparison with Pullulan standards (10 different sizes from 342 to 710,000 g/mol). Water was used as a solvent with 0.1 M $NaNO_3$.

DLS experiments. DLS experiments were performed on Malvern Zetasizer Nano machine (Brookhaven Instruments Corp.) at 25 ^{0}C. Millipore quality water was used in all the experiments. General purpose method (NNLS) was used for correlation function to get the distribution of the solute's diffusion coefficients (D). The value of hydrodynamic diameter was determined using Stokes-Einstein equation. For performing different runs, mean diameter values were measured. CAC was measured using data of Baret et al.2 Measurements were performed with UV Micro Disposable Cuvettes (Brand ©).

TGA. TGA experiments were performed on a LINSEIS STA PT1600 (TG – DTA/DSC) machine in air atmosphere. The heating rate was set to 10 degrees per minute. Calibration curves were measured for each sample. Measurements were performed in Al_2O_3 crucibles. Mass of samples varied from 7 to 15 mg.

UV-Vis. UV/Vis-measurements were recorded on a Perkin Elmer Lamda 950 UV/Vis/NIR spectrophotometer in the range of 750-280 nm. Samples were measured in UV Micro Disposable Cuvettes (Brand ©). Millipore quality water was used in all the measurements.

Fluorescence measurements were performed on the Jasco FP-6500 fluorometer in the range of 500-750 nm. MilliQ quality water was used for all the experiments. Measurements were performed in quartz cuvettes with square apertures. The width of the excitation and emission bands varied between 3 and 5 nm and the excitation wavelength was set to 620 nm.

Dye purification. Purification of Cy5-CH_3 was done by an automated flash chromatography on a Combi Flash R_f (Teledyne ISCO) on normal phase material (silica gel, 30 μm).

UV irradiation. [2+2] photo crosslinking reaction was performed by UV irradiation with the help of an USHIO super high mercury lamp (USH 102d, 100 W).

Encapsulation stability experiments. After the encapsulation of the dye, the samples were stirred at 37 °C for 40 hours at c=10μM. The stability of the encapsulation was performed by measuring the UV spectra of encapsulated dye after additional purification with a Sephadex column to separate the released and encapsulated parts in the media dye.

Cellular uptake studies. Cellular uptake of Cy5-loaded carriers in A549 lung carcinoma cells (DSMZ no.: ACC 107) was monitored by confocal laser scan microscopy (cLSM). A549 cells were maintained in DMEM, low glucose, GlutaMAX™ supplement, pyruvate (Gibco, Life Technologies GmbH, Darmstadt, Germany) supplemented with 10% fetal bovine sera (Biochrom GmbH, Berlin, Germany), 100 units/ml Penicillin and 100 µg/ml Streptomycin (Gibco, Life Technologies GmbH, Darmstadt, Germany) at 37 °C, 5% CO_2, and high humidity. Serial dilutions of the carriers were prepared in Milli-Q water, which obtained the following stock concentrations: 1.200 µM and 100 µM. Dilution of Cy5-CH_3 dye was performed till the dye concentration was same as in NCAs. For the uptake studies, 27,000 A549 in 270 µl supplemented DMEM low glucose without phenol red were seeded in each well of an 8-well ibiTreat µ-Slide (ibidi GmbH, Martinsried, Germany). After 24 h, 30 µl of carrier dilution or dye were added per well. The carrier containing media was replaced after approximately 4 h by fresh medium before taking images. Cell nuclei were stained with 1 µg/ml Hoechst 33342 (Life Technologies GmbH, Darmstadt, Germany). For labeling the specific structures (Lysosomes, Endoplasmic reticulum, Golgi and Mitochondria) CellLight® GFP reagents (Life Technologies GmbH, Darmstadt, Germany) were used according to the manufactures protocol. Images were taken by a Leica (DMI6000CSB stand) confocal laser scan microscope (Hoechst 33342: excitation 405 nm, emission: 416 nm – 487 nm; GFP: excitation 488 nm, emission 495 nm – 587 nm; Cy5: excitation 633 nm, emission 640 nm – 732 nm) and processed by the Leica LAS X software.

Flow cytometry. For flow cytometry, 150,000 A549 cells were seeded in each well of a 24-well plate and cultured for 24 h before adding the Cy5-loaded carriers for 10 min, 2 h, 4 h, 6 h and 24

h, respectively. Cy5-dye without carrier as well as non-treated cells severed as controls. For the analysis, cells were washed 3 times with PBS and detached by trypsin. The detached cells were transferred to a reaction tube tube and centrifuged at 200 xg for 5 min. Supernatants were discarded and cells were resuspended in PBS. 10,000 cells were analyzed per sample by a BD ACCURI C6 flow cytometer (Becton Dickinson, Heidelberg, Germany) and the mean intensity of the cells in each sample was determined by the BD Accuri C6 software.

Cryogenic transmission electron microscopy (cryo-TEM). For the cryo-TEM experiments FPAs (40 μM) were dissolved in PBS. Perforated (1 μm hole diameter) carbon film-covered microscopical 200 mesh grids (R1/4 batch of Quantifoil, MicroTools GmbH, Jena, Germany) were hydrophilized by 60 s glow discharging at 8 W in a BALTEC MED 020 device. Then 5 μl of the corresponding sample were pipetted to the hydrophilized grid and the supernatant fluid was immediately removed with a piece of filter paper until an ultrathin layer of the sample solution was obtained that spanned the holes of the carbon film. The sample was instantly vitrified by plunging the grids into liquid ethane using a guillotine-like apparatus. The vitrified sample was subsequently transferred under liquid nitrogen into a Tecnai F20 TEM (FEI Company, Oregon) equipped with field emission gun and operating at 160 kV by the use of a Gatan tomography cryo-holder (Model 914). Microscopy was carried out at a 94 K sample temperature using the low-dose protocol of the microscope. Micrographs were taken with an FEI Eagle 4k × 4k CCD camera using the twofold binning mode. To generate stereo images the same image section was recorded at two different tilt angles (4° and -4°) using the compustage of the microscope. The resulting micrographs were aligned using the software StereoPhoto Maker (Masuji Suto, Japan).

1.3. Synthesis

1.3.1. Synthesis of Fullerene-Polyglycerol Amphiphiles (FPAs)

Fullerene (1 eq.) was added to a round-bottom flask connected to argon and a vacuum inlet. Potassium tert-butoxide in THF (1 M) (n eq.) was added to the fullerene and stirred under argon atmosphere for 30 minutes at room temperature. Then mixture was heated up to 80 °C and

glycidol (200 eq.) was added dropwise. After adding part of the glycidol the temperature was increased to 100 °C. Then the rest of the glycidol was added and the mixture was stirred for over 6 hours. After that the mixture was cooled down to room temperature and the product was dissolved in methanol and precipitated in acetone. The precipitate was re-dissolved in water and dialyzed against water using dialysis bag with MWCO > 2000 for 3 days. Then the water was evaporated and the product was lyophilized.

Table S1. Initial ratios for the synthesis of FPAs.

Compound	Fullerene	KTBO	Glycidol
FPAs	0.3 g, 0.416 mmol, 1 eq.	0.8 mmol, 2 eq.	5 ml, 0.083 mol, 200 eq.
FPAs-**2**	0.3 g, 0.416 mmol, 1 eq.	1.2 mmol, 3 eq.	5 ml, 0.083 mol, 200 eq.
FPAs-**3**	0.3 g, 0.416 mmol, 1 eq.	2 mmol, 5 eq.	5 ml, 0.083 mol, 200 eq.

Table S2. Calculation of the number of branches of PG attached to the fullerene core.

Compound	(A) Glycidol/KTBO $_{\text{theoretical}}$	(B) PG/KTBO $_{\text{from NMR}}$	(A)/(B)	Average amount of branches
FPAs	100	273	0.36	2
FPAs-**2**	66	208	0.32	3
FPAs-**3**	40	133	0.30	5

The close values of the (A)/(B) ratio showed a direct dependence of the amount of PG groups attached to the fullerene core on the initial amount of the potassium tert-butoxide (2, 3, or 5 eq.) that was added to the fullerene during the reaction.

FPAs: ^{1}H-NMR (700 MHz, ($DMSO_{d6}$): δ = 4.4 – 4.8 (secondary PG-groups), 3.1 – 4.0 (primary PG-groups), 2.5 (methyl groups of DMSO), 1.2 (tert-butoxide).
Inverse-gated ^{13}C-NMR (700 MHz, ($DMSO_{d6}$): δ = 83.0 – 61.0 (corresponding to different structural units of PG) 40.5 – 39.5 (methyl groups of DMSO).

DB=0.54.

SEC: M_n=8400 Da, M_w=11700 Da, M_w/M_n= 1.4.

FPAs-**2**: ^{1}H-NMR (700 MHz, ($DMSO_{d6}$): δ = 4.4 – 4.8 (secondary PG-groups), 3.1 – 4.0 (primary PG-groups), 2.5 (methyl groups of DMSO), 1.2 (tert-butoxide).

SEC: M_n=4900 Da, M_w=6800 Da, M_w/M_n =1.4.

FPAs-**3**: ^{1}H-NMR (700 MHz, ($DMSO_{d6}$): δ = 4.4 – 4.8 (secondary PG-groups), 3.1 – 4.0 (primary PG-groups), 2.5 (methyl groups of DMSO), 1.2 (tert-butoxide).

SEC: M_n=3000 Da, M_w=3900 Da, M_w/M_n =1.3.

1.3.2. Synthesis of Cy5-CH_3

A solution of 1,3,3-trimethyl-2-methyleneindolenine (90 mg, 0.518 mmol), 3-anilino acroleine anil (50 mg, 0.225 mmol) and sodium acetate (80 mg, 0.972 mmol) in acetic anhydride (6 mL) and acetic acid (0.182 mL) was heated and stirred at 100 °C for 20 min. Toluene was added and the mixture was concentrated in vaccuum. The residue was purified by automated flash chromatography using CH_2Cl_2/methanol (0-20 % methanol) and afforded 82 mg (83 %) of a blue solid.

^{1}H NMR (400 MHz, $MeOD_{d4}$): δ = 8.24 (t, J = 12 Hz, 2H, CH), 7.48 (d, J = 8 Hz, 2H, CH_{ar}), 7.41 (t, J = 8 Hz, 2H, CH_{ar}), 7.28 (t, J = 8 Hz, 4H, CH_{ar}), 6.61 (t, J =12 Hz, 1H, CH), 6.26 (d, J = 16 Hz, 2H, CH), 3.61 (s, 6H, CH_3), 1.72 (s, 12H, CH_3).

MS m/z 383.2482 ($C_{27}H_{31}N_2^+$ calculated 383.2482).

1.3.3. Photo crosslinking of FPAs

The [2+2] photo crosslinking reaction was performed using different times of exposure of the samples to the UV light. The times of irradiation for partial and full crosslinking were 3 and 7 hours, respectively. The stirring speed of the system was set to 150 rpm.

1.3.4. Transport of dyes

Hydrophobic and hydrophilic Cy5 dyes were dissolved in methanol, 0.5 mg of each dye were transferred to the vials, and the solvent was evaporated. Thus the thin layer of the dyes was evenly distributed on the surface of vials. FPAs with 2 mg/ml concentration in PBS were added to the vials and stirred for 72 hours at 1200 rpm. Then SEC columns were prepared and used to separate FPAs with encapsulated dyes from non encapsulated dyes. The amount of encapsulated dyes was studied by UV/Vis spectrophotometer in the range between 600 nm and 750 nm (absorption maximum of the dyes was 650 nm). Extinction coefficients of the hydrophobic and hydrophilic dyes were respectively 202,000 (methanol) and 242,000 (PBS). The transport capacity and transport efficacy were measured according to the mol ratio of the encapsulated dye per mol of carrier and to the mol ratio of the encapsulated dye per mol of dye, correspondingly.

2. Characterization of FPAs

2.1. Characterization by NMR

A small signal for tert-butoxy at 1.2 ppm confirmed the nucleophilic addition of potassium tert-butoxide (KTBO) to fullerene and production of the anionic centers onto its surface to initiate the ring-opening polymerization of glycidol. Signals at 3.1-3.9 and 4.4-4.8 ppm corresponded to polyglycerol primary and secondary PG-groups, respectively (Figure S1).

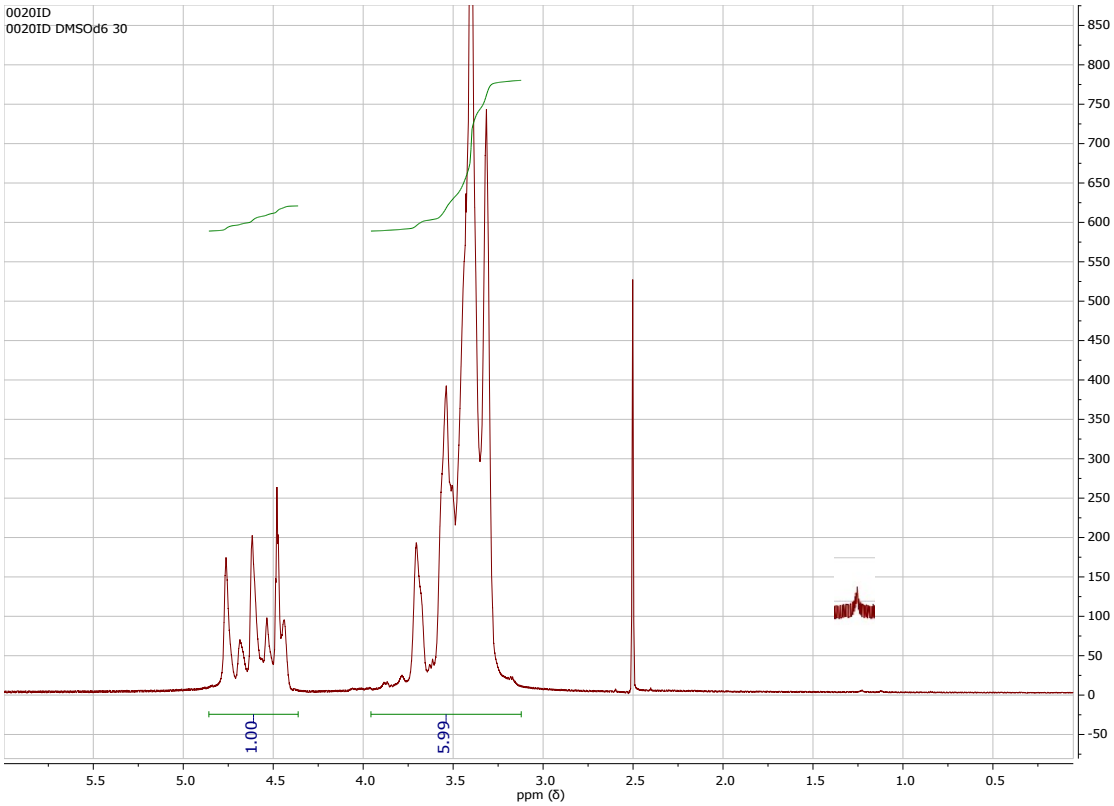


Figure S1. ^{1}H NMR spectrum of the synthesized FPAs in $DMSO_{d6}$.

Inverse-gated ^{13}C NMR spectrum in DMSO was performed to calculate the degree of branching (DB) of the polymer. According to Frey et al,[1] DB can be calculated with Equation (1):

$$DB = \frac{2D}{2D + L13 + L14} \quad (1)$$

where D, L_{13}, and L_{14} represent the parts corresponding to dendritic, linear 1,3- and 1,4-units, respectively (Figure S2). The value of DB was 54%.

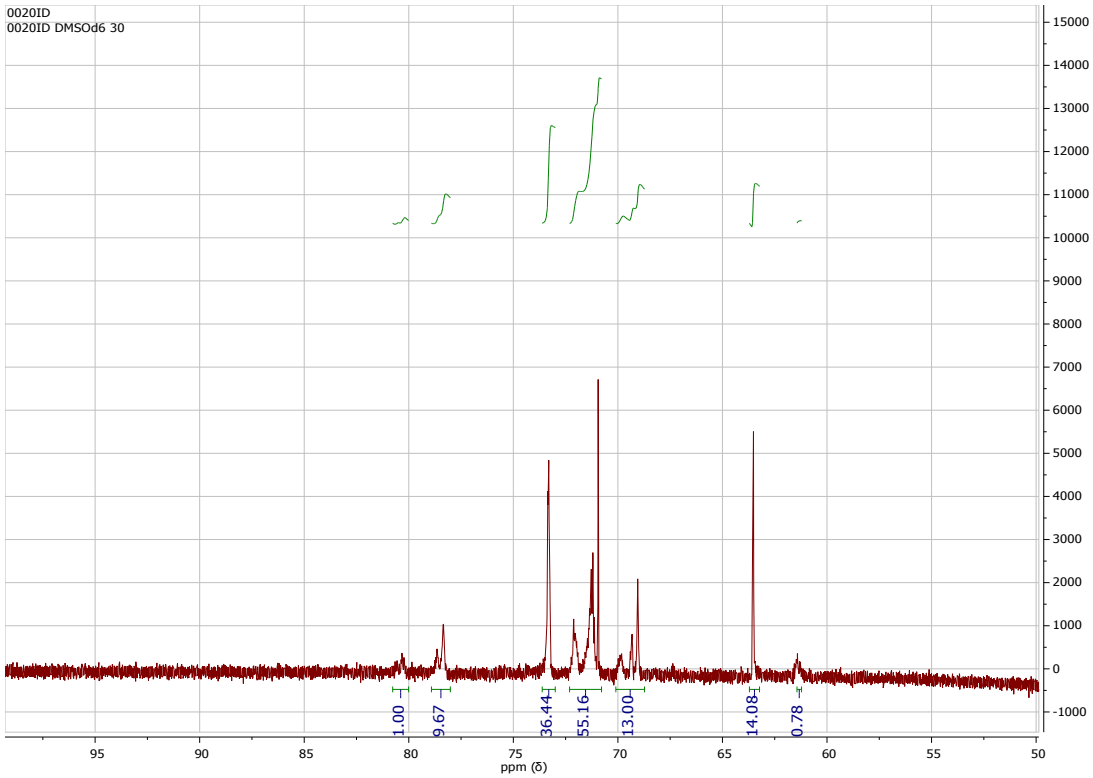


Figure S2. ^{13}C NMR spectrum of synthesized FPAs in $DMSO_{d6}$.

2.2. Characterization by GPC

According to the GPC diagram (Fig. S3), monomodal distribution of the molecular weight proved that products (FPAs, FPAs-**2**, FPAs-**3**) were free from impurities or sideproducts. The difference in the resulting molecular weight of the synthesized FPAs can be explained by the difference in the hydrodynamic radius of FPAs. It was found that FPAs with lower ratios of added KTBO (**1**) and therefore lower number of branches on the surface of the fullerene showed a higher hydrodynamic radius due to the bigger PG chains. However, the synthesized compounds with higher ratios of added KTBO (**2** and **3**) and, thus, a higher number of PG branches showed lower hydrodynamic radii.

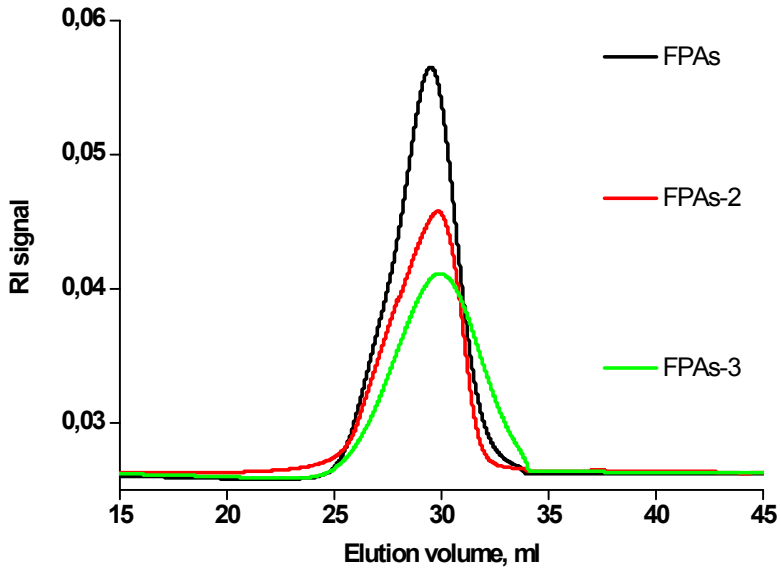


Figure S3. GPC diagram of the FPAs using Agilent 1100 solvent delivery system.

2.3. Characterization by TGA

Since FPAs show different thermal decompositions, their fullerene content was measured by TGA. According to this analysis, the amount of fullerene in the synthesized FPAs was 15 wt% (Figure S4).

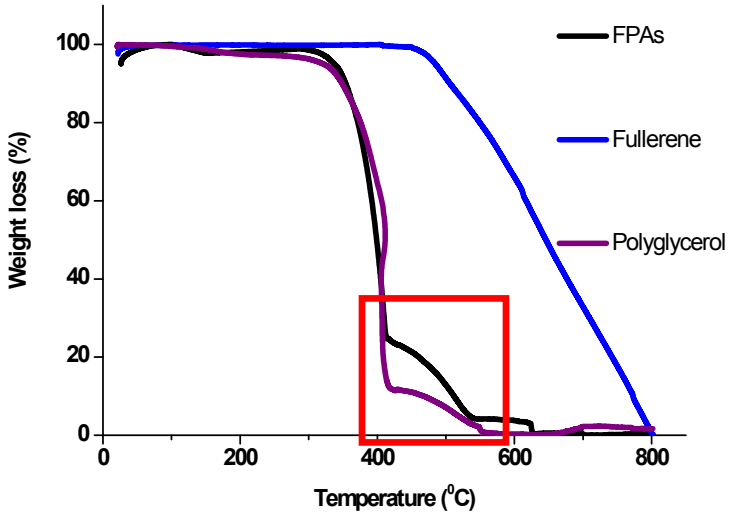


Figure S4. TGA diagrams of pure fullerene, PG and FPAs.

2.4. Characterization by UV/Vis

In the UV spectra of FPAs, the maximum absorption centered at 280 nm, which is a well-known characteristic for fullerene[3] and proves conjugation of the fullerene to polyglycerol. Obviously, there was no significant absorption for polyglycerol in the UV/Vis region (Figure S5).

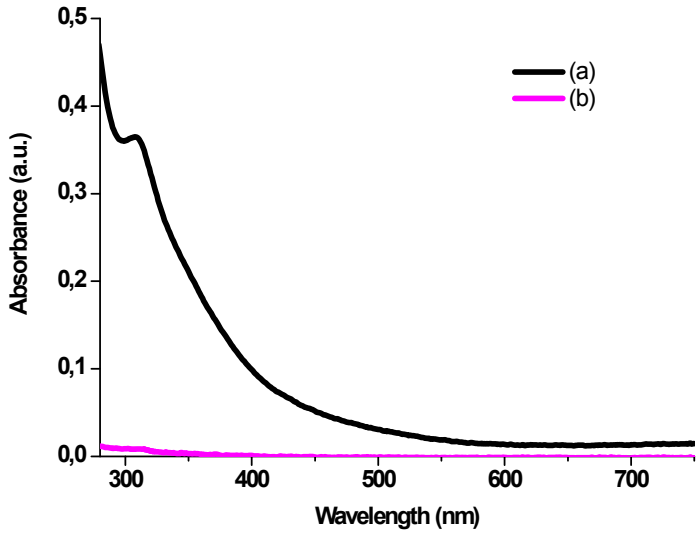


Figure S5. UV spectra of (a) FPAs without UV irradiation and (b) hyperbranched PG.

3. Photocrosslinking FPAs

The irradiation of the PBS solution of the FPAs (2 mg/ml) by a UV lamp with 100 W resulted in a decreased λ_{max} of fullerene segment. This indicates that the assembled fullerene moieties were photo crosslinked by [2+2] cycloaddition reaction.[4] Since the polyglycerol branches were big enough to hinder direct contact between fullerene segments, longer times than those reported in the literature for fullerene derivatives with small molecules on their surfaces are needed.[4] There is a direct correlation between the irradiation time and intensity of the λ_{max} of fullerene. Minimum absorption for fullerene segments was observed after 7 h of irradiation. Therefore, the extent of the photo crosslinking directly depends on the irradiation time and can be tuned by manipulating this factor (Figure S6).

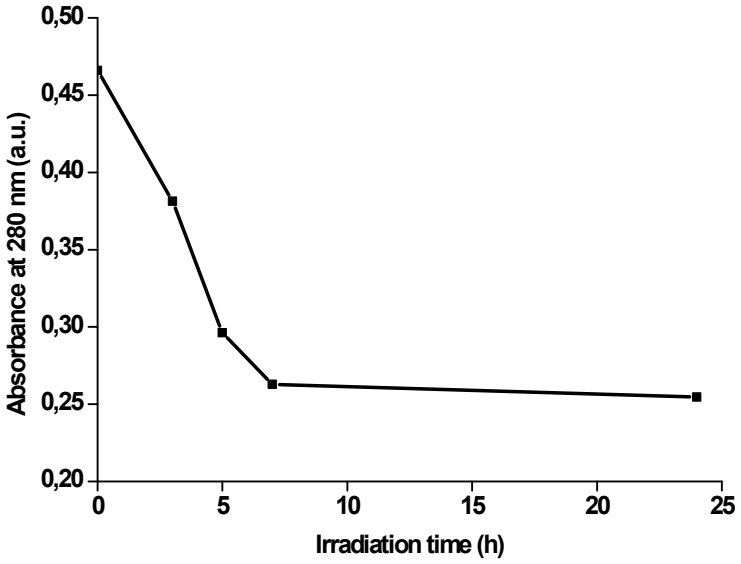


Figure S6. Intensity of the λ_{max} of FPAs in PBS after different irradiation time.

4. Study of solution behavior

4.1. DLS measurements

To study the behavior of crosslinked and non-crosslinked samples in solutions, DLS diagrams of FPAs in PBS and DMF were measured (Figure S7). Non-crosslinked fullerene-polyglycerol assemblies (NCAs) dissolved in PBS showed that the size of particles was around 20 nm, which corresponded to the aggregation of several FPAs forming oligomeric aggregates. With irradiation and, hence, formation of partially crosslinked fullerene-polyglycerol assemblies (PCAs) and fully crosslinked fullerene-polyglycerol assemblies (FCAs), the size of the particles increased. The size of the PCAs was slightly bigger than the size of NCAs (Figure S7a). In the case of FPAs dissolved in DMF (Figure S7b), NCAs dissociated to the individual particles with several nanometer sizes, which confirmed there were noncovalent interactions between the amphiphiles in aqueous solutions. However, this was not the case for the FCAs. Larger sizes of FCAs occurred in both DMF and PBS due to their higher stability, which was induced by the [2+2] cycloaddition reaction that had formed covalent bonds between the fullerene moieties.

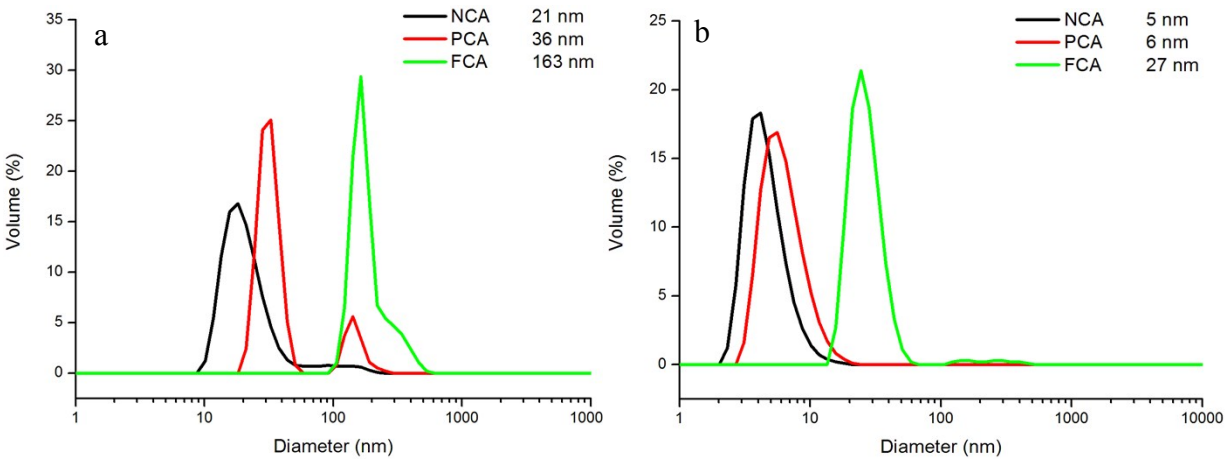


Figure S7. DLS diagrams of NCAs, PCAs, and FCAs in (a) PBS and (b) DMF.

4.2. Cryo-TEM images

The DLS data was proven by Cryo-TEM measurements of FPAs dissolved in PBS (Fig. S8). Cryo-TEM images of FCA (40 μM) showed particles with diameters in the range of 100 to 500 nm. It was also found that inside the aggregates of FCAs there were areas where fullerenes covalently bounded to each other, which was clearly visible. These observations proved the overall feasibility of [2+2] photo crosslinking the fullerenes with each other.

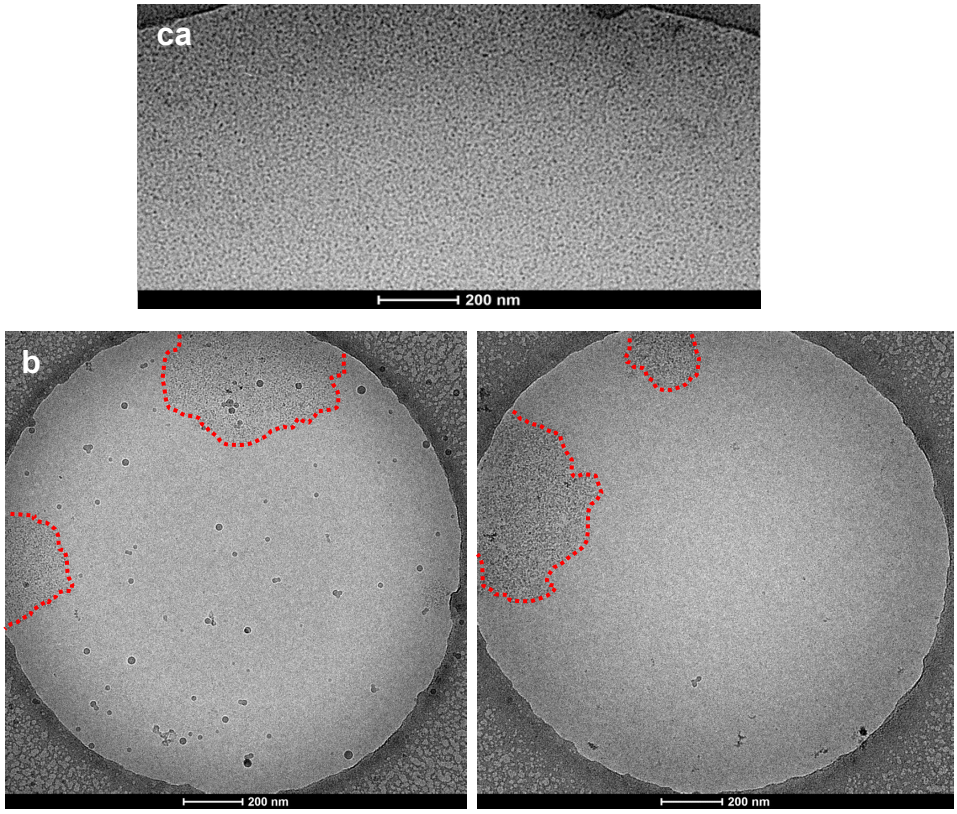


Figure S8. (a) Cryo-TEM image of FPAs (120 μM) in PBS. Individual dark spherical spots with an approximate diameter of 6 nm correspond to the oligomeric FPAs assemblies, (b) and (c) Cryo-TEM images of FPAs in PBS after UV irradiation. Crosslinked assemblies ranging from 100-500 nm composed of FPAs particles of several nanometers can be observed in these images.

5. Encapsulation studies

5.1. Transport capacity and efficiency of FPAs

Encapsulation of Cy5-COOH and Cy5-CH_3 afforded different transport efficacies for the FPAs. For hydrophilic dye with increased UV irradiation, the amount of encapsulated dye increased, but the inverse behavior was observed for hydrophobic dye (Figure S9, Table S2).

Table S3. Transport capacity and efficiency (in mmol/mol) for different irradiated and non-irradiated assemblies.

Sample	TC $_{Cy5\text{-}COOH}$	TC $_{Cy5\text{-}CH3}$	TE $_{Cy5\text{-}COOH}$	TE $_{Cy5\text{-}CH3}$
NCAs	2.3	23.1	2.3	14.8
PCAs	5.5	21.5	5.4	13.6
FCAs	28.9	14.5	28.6	9.2

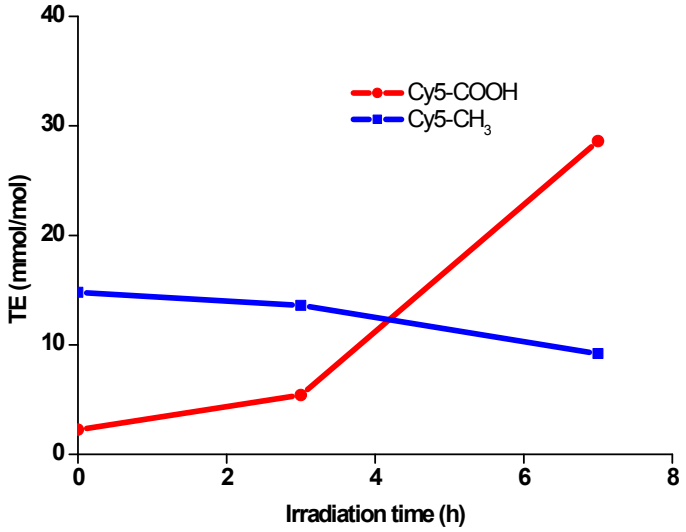


Figure S9. Transport efficiency (TE) of FPAs for Cy5-COOH and Cy5-CH_3 versus irradiation time.

Encapsulation values of the hydrophilic dye showed a direct dependence upon the photo crosslinking of the FPAs. With increased irradiation times, the amount of encapsulated dye also increased (Figure S10).

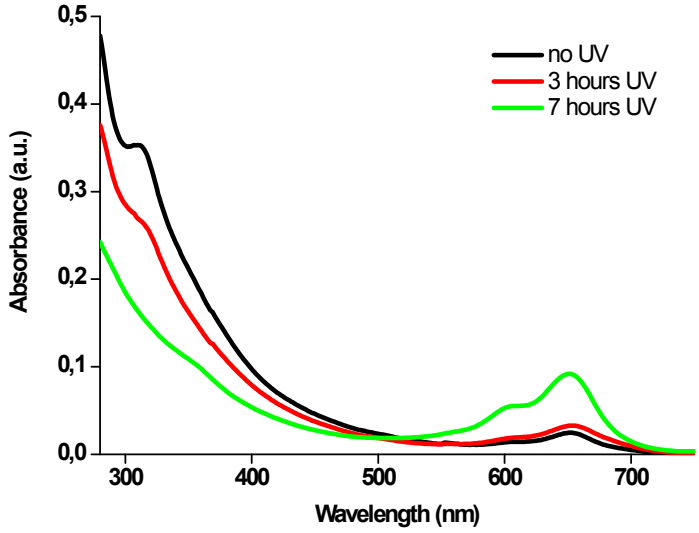


Figure S10. UV spectra of FPAs encapsulated with Cy5-COOH.

5.2. *Release of encapsulated dye*

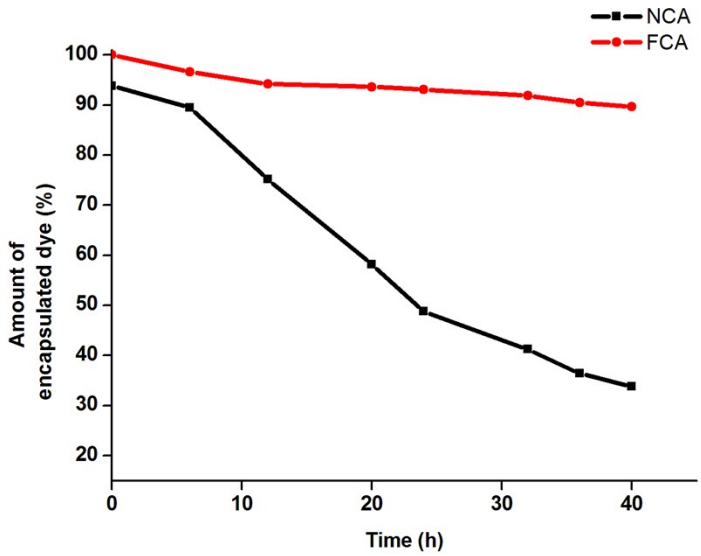


Figure S11. Release of the encapsulated Cy5-CH_3 by FCAs versus NCAs.

5.3. Confocal microscopy studies

Fluorescence spectra of encapsulated hydrophilic dye showed intense emission signals with a maximum at 664 nm. The fluorescence spectrum of hydrophobic Cy5 dye showed a maximum

emission signal at 644 nm (Fig. S11). The variation in the emission maxima was explained by the difference in the structures of Cy5-CH_3 and Cy5-COOH.

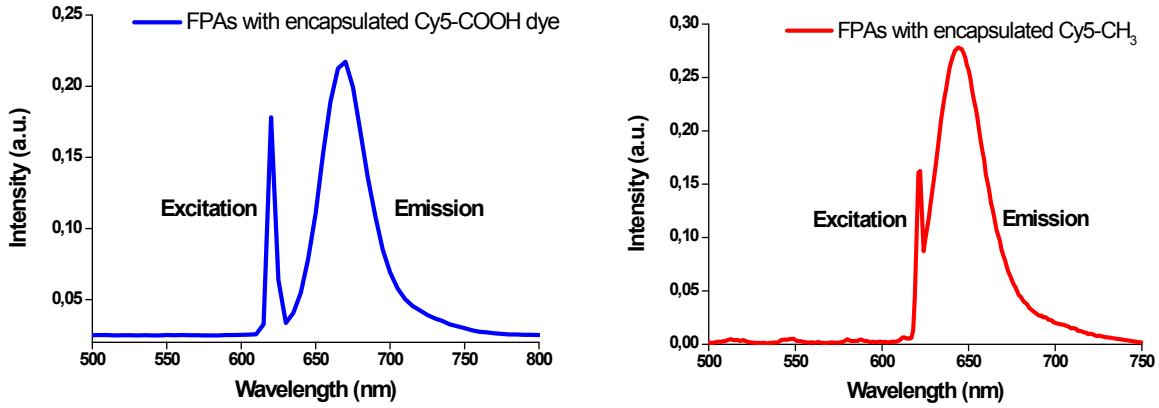
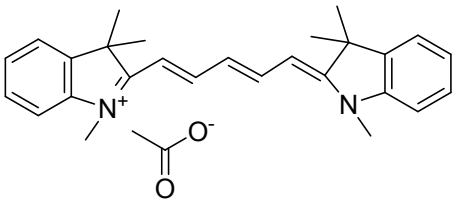


Figure S12. Fluorescense spectra of FPAs encapsulated with Cy5-COOH and Cy5-CH_3.



Chemical Formula: $C_{29}H_{34}N_2O_2$
Exact Mass: 442,2620
Molecular Weight: 442,6030

Figure S13. Chemical structure of synthesized Cy5-CH_3.

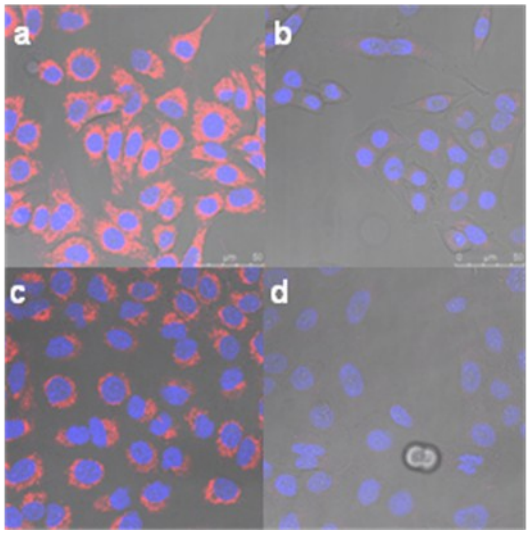


Figure S14. Confocal microscopy images of the A549 cells incubated with 10 μM NCAs and FCAs loaded with Cy5-CH_3 after (a) and (b) 4 h and (c) and (d) 24 h, respectively.

5.4. Flow cytometry

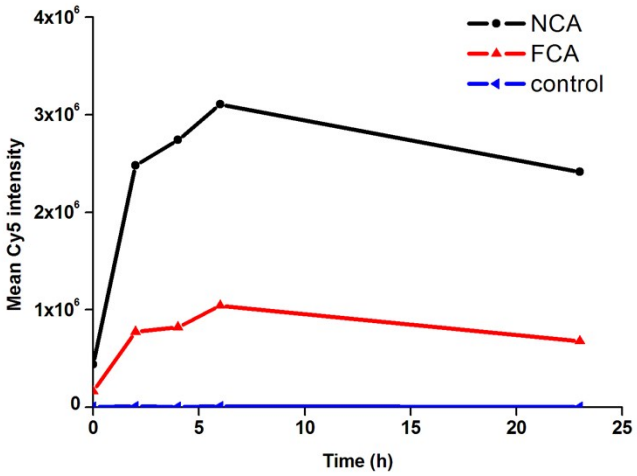


Figure S15. Flow cytometry of encapsulated Cy5-CH_3 in NCA and FCA.

6. References

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