Assembly, Postsynthetic Modification and Hepatocyte Targeting by Multiantennary, Galactosylated Soft Structures

Anisha Thomas,[a] Akansha Shukla,[b] Sri Sivakumar[b,c] and Sandeep Verma*[a,c]

[a]Department of Chemistry, Indian Institute of Technology Kanpur, Kanpur-208016 (UP), India, Fax: (+) 91-512-2597436, E-mail: sverma@iitk.ac.in

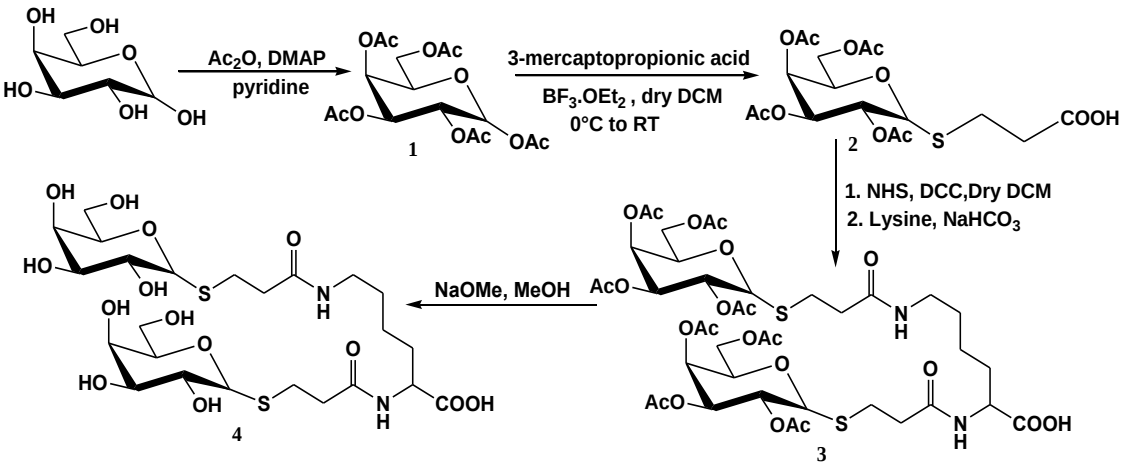
[b,c]Department of Chemical Engineering, Material Science Programme, Indian Institute of Technology Kanpur, Kanpur-208016 (UP), India,E-mail: srisiva@iitk.ac.in

[b,c]DST Thematic Unit of Excellence on Soft Nanofabrication, Indian Institute of Technology Kanpur, Kanpur-208016 (UP), India

Materials and Methods

Dichloromethane (DCM), ethyl acetate (EtOAc), methanol (MeOH) triethylamine (TEA), were distilled following standard procedures prior to use. N,N´-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), boron trifluoride etherate ($BF_3.EtO_2$), sodium methoxide (NaOMe), 3-mercaptopropionic acid (3-MPA) and L-Lysine.HCl, were purchased from Spectrochem Pvt. Ltd.; D-galactose was obtained from Sisco Research laboratories Pvt. Ltd.; Acetic anhydride (Ac_2O) and melamine were purchased from S.D. Fine-Chem Ltd., Mumbai and used as such without further purification. 1,4-diaminobutane (DAB) and tris(2-aminoethyl)amine (tren) were purchased from Lancaster. Galactose oxidase (GO) and peroxidase derived from horse radish (HRP) were purchased from Sigma Aldrich. ^1H and ^{13}C NMR spectra were recorded on JEOL-JNM LAMBDA 500 model operating at 500 and 100 MHz, respectively. Analysis of ^1H and ^{13}C NMR of final products were done by JEOL ECX-500 model operating at 500 MHz and 125MHz respectively. HRMS (ESI^+ and ESI^-) was recorded at IIT Kanpur, India, on Waters, Q-Tof Premier Micromass HAB 213 mass spectrometer using capillary voltage 2.6-3.2 kV.IR spectra were recorded as KBr pellets on a Perkin-Elmer Model 1320 spectrophotometer operating from 400 to 4000 cm^{-1}. For thin layer chromatography (TLC), Merck pre-coated TLC plates (MEARK 60 F254) were used, and compounds were visualized with a UV light at 254 nm. Chromatographic separations were performed on S. D. Fine-Chem 100-200 mesh silica gel.

Synthetic Scheme



Scheme S1: Synthesis of bis-galactose lysine

Synthesis of 1,2,3,4,6-penta acetyl-β-D-galactopyranoside (1)

D-galactose (5.0 g, 28 mmol, 1 eq) was suspended in dry pyridine (100 ml) and cooled to 0°C in an ice bath.[1] Acetic anhydride (17.5 g, 170 mmol, 6.1 eq) was added dropwise to the suspension while maintaining the temperature at 0°C. 4-Diaminomethylpyridine (DMAP) (0.17 g, 1 mmol, 0.05 eq) was added to the mixture as catalyst. The reaction was maintained at 0°C for ~1 h, after which it was stirred for 12 h at room temperature. Excess solvent was evaporated and the residue was dissolved in EtOAc (15 ml) and washed with water (3x30 ml) and saturated aqueous potassium hydrogen sulfate (3x30 ml) to remove excess pyridine. Combined organic layer was dried over anhydrous sodium sulfate and the gummy mass was used in the next step without further purification. R_f: 0.5 (50% EtOAc/ hexane). Yield: ~99%. ^{1}H NMR (500 MHz, $CDCl_3$, 25°C, TMS) δ (ppm): 6.36 (d, 1H), 5.48 (d, 1H), 5.32-5.29 (m, 2H), 4.33 (t, 1H), 4.08 (m, 2H), 2.14, 2.03, 2.00, 1.99 (m, 15H).

Synthesis of 3-(2,3,4,6-tetra acetyl-α-D-galactopyranosyl)thiopropionic acid (2)

2 was synthesized by a previously reported procedure.[2] **1** (5g, 13 mmol, 1 eq) was dissolved in dry DCM and cooled to 0°C in an ice bath. 3-MPA (1.63g, 15 mmol, 1.2 eq) was added dropwise to the solution followed by $BF_3.EtO_2$ (3.64g, 25mmol, 2 eq). The temperature of the reaction was

maintained at 0°C for ~1 h followed by stirring under inert nitrogen atmosphere at room temperature for 12 h. After completion of the reaction, the DCM layer was diluted with more DCM (15ml), and washed with water (3x30 ml) and brine (3x30 ml). The organic layer was then dried over anhydrous sodium sulfate, filtered and concentrated to dryness over reduced pressure. The resulting gummy substance was purified using column chromatography on silica gel (MeOH/DCM 1.5% v/v; R_f: 0.5 (4% MeOH/DCM) to yield a yellowish gummy liquid. ^{1}H NMR (500 MHz, $CDCl_3$, 25°C, TMS) δ (ppm): 6.36 (d, 1H), 5.48 (d, 1H), 5.32-5.29 (m, 2H), 4.33 (t, 1H), 4.08 (m, 2H), 2.14, 2.03, 2.00, 1.99 (m, 15H) 5.43 (m, 1H), 5.26-5.15 (m, 2H), 4.54 (m, 1H), 4.19-4.03 (m, 2H), 3.01-2.84 (m, 2H), 2.81-2.65 (m, 2H), 3.14 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, 25°C, TMS) δ (ppm): 176.67, 170.2, 84.70, 83.04, 74.52, 71.89, 68.01, 67.11, 62.03, 35.36, 34. 57, 25.46, 20.7, 19.9; m/z (HRMS) Calculated: $[M+NH_4]^+$ 454.1383, Found: 454.1385.

Synthesis of N^2,N^6-Bis[(2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl)thio]propionyl lysine (3)

NHS (1g, 5 mmol, 1.1 eq) and **2** (2g, 4.5 mmol, 1 eq) were dissolved in dry DCM under N_2 atmosphere and cooled to 0°C using an ice bath. DCC (0.63g, 5mmol, 1.1 eq) was dissolved in dry dichloromethane under N_2 atmosphere and added to the reaction mixture slowly at 0°C. The reaction was stirred at 0°C for ~1 h and at room temperature for 12 h. On completion, the white precipitate of N, N´-dicyclohexylurea (DCU) was filtered off and the filtrate was washed with water (2x15 ml) and brine (2x15 ml) and dried over anhydrous sodium sulfate. The combined organic layer was then evaporated and concentrated under reduced pressure. The sticky solid was used without further purification for conjugation with L-lysine. R_f: 0.6 (5% MeOH/DCM).

The active ester (1.0g, 2 mmol, 1 eq) was dissolved in 1,4-dioxane (10 ml) and stirred in a round bottom flask. L-Lysine (0.17g, 0.9 mmol, 0.5 eq) and $NaHCO_3$ (0.4g, 5 mmol) were dissolved in equal volume of distilled water (10 ml), and added to the round bottom flask containing the active ester. The reaction mixture was stirred for 6 h at room temperature. The dioxane-water layer was evaporated under vacuum. The residue was dissolved in EtOAc (10 ml), acidified using 1N HCl (2x10 ml) and washed with water (2x10 ml) and brine (2x10 ml). The organic layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The resulting sticky compound was purified using column chromatography on silica gel

(MeOH/DCM, 5% v/v); R_f: 0.5 (10% MeOH/DCM). ^{1}H NMR (500 MHz, $CDCl_3$, 25°C, TMS) δ (ppm): 5.73 (m, 1H), 5.4-5.39 (m, 2H), 5.27-5.16 (m, 3H), 5.02-4.99 (m, 1H), 4.56-4.48 (m, 2H), 4.14-4.07 (m, 2H), 3.92 (m, 1H), 3.51-3.47 (m, 3H), 3.00-2.67 (m, 6H), 2.53-2.46 (m, 4H), 2.13, 2.02, 1.94 (s, 24H), 1.38-1.15 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$, 25°C, TMS) δ (ppm): 170.75, 84.5, 74.69, 71.8, 68.19, 67.83, 67.47, 67.15, 61.82, 52.4, 39.17, 36.9, 31.69, 29.76, 29.01, 26.15, 20.91, 20.85, 20.77, 20.68. m/z (HRMS) Calculated $[M+H]^+$: 983.3001, Found 983.3006.

Synthesis of N^2,N^6-Bis[(2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl)thiopropionyl]-L-lysine (4)

3 (0.23g, 0.24 mmol, 1 eq) and NaOMe (0.13g, 2.4 mol, 10 eq) were dissolved in MeOH (10 ml) and stirred at room temperature for ~6 h The reaction mixture was neutralized by Amberlite resin activated using 1N HCl. The resulting organic layer was then dried over anhydrous sodium sulfate and concentrated under reduced pressure to obtain the compound in nearly quantitative yield. ^{1}H NMR (500 MHz, $CDCl_3$, 25°C, TMS) δ (ppm) 5.73 (m, 1H), 5.4-5.39 (m, 2H), 5.27-5.16 (m, 3H), 5.02-4.99 (m, 1H), 4.56-4.48 (m, 2H), 4.14-4.07 (m, 2H), 3.92 (m, 1H), 3.51-3.47 (m, 3H), 3.00-2.67 (m, 6H), 2.53-2.46 (m, 4H), 2.13, 2.02, 1.94 (s, 24H), 1.38-1.15 (m, 6H) ^{13}C (100 MHz, $CDCl_3$, 25°C, TMS) δ (ppm) 170.75, 84.5, 74.69, 71.8, 68.19, 67.83, 67.47, 67.15, 61.82, 52.4, 39.17, 36.9, 31.69, 29.76, 29.01, 26.15, 20.91, 20.85, 20.77, 20.68; m/z (HRMS) Calculated $[M+H]^+$: 983.3001, Found 983.3006.

Scanning Electron Microscopy (SEM)

10 µl aliquots of samples (0.5 mM in water and incubated at room temperature for 24 h) were deposited onto a copper stub and air dried. These were dried *in-vacuo* for 30 min prior to imaging. This was then gold coated and scanned using an FEI QUANTA 200 microscope equipped with a tungsten filament gun, operating at WD 10.6 mm and 20 kV.

Fluorescence Microscopy

3 (0.65 mg) was dissolved in 1 ml sterile water to obtain a 1 mM solution. To this, 10 µl of rhodamine B dye solution (1 mM in water) was added. It was incubated at 37 °C and 10 µl of the solution was loaded onto a glass slide at specific time intervals and dried under a tungsten lamp.

Dye stained structures were examined under a fluorescent microscope (Leica DM2500M), provisioned with a Rhodamine filter (absorption 540 nm/emission 625 nm).

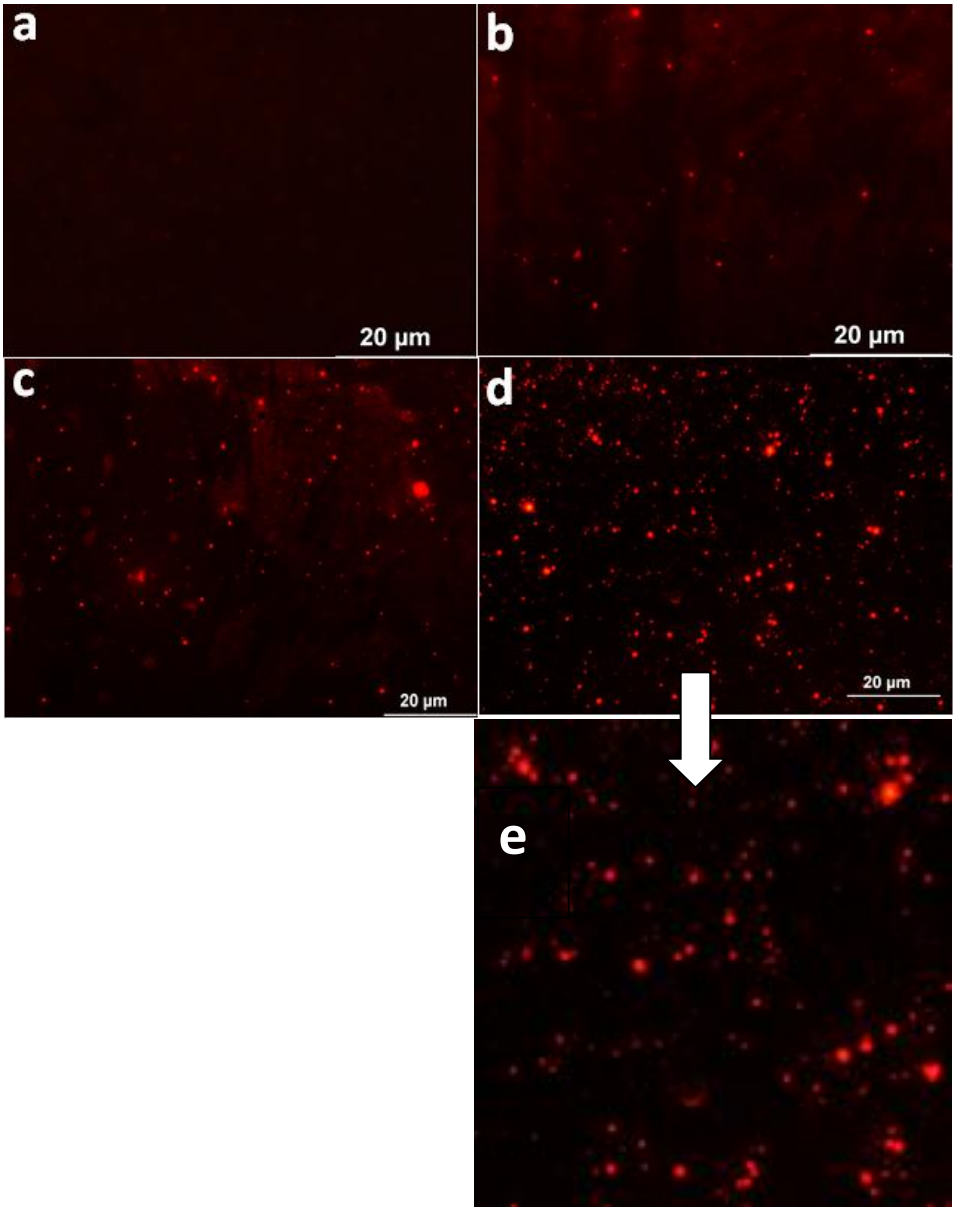


Figure S1: Rhodamine B stained vesicles of **3** (1 mM solution in water) as shown by the bright red dots after **a**) 0 h; **b**) 4 h; **c**) 8 h and **d**) 24 h of incubation at 37 °C; e) zoomed in section of (d).

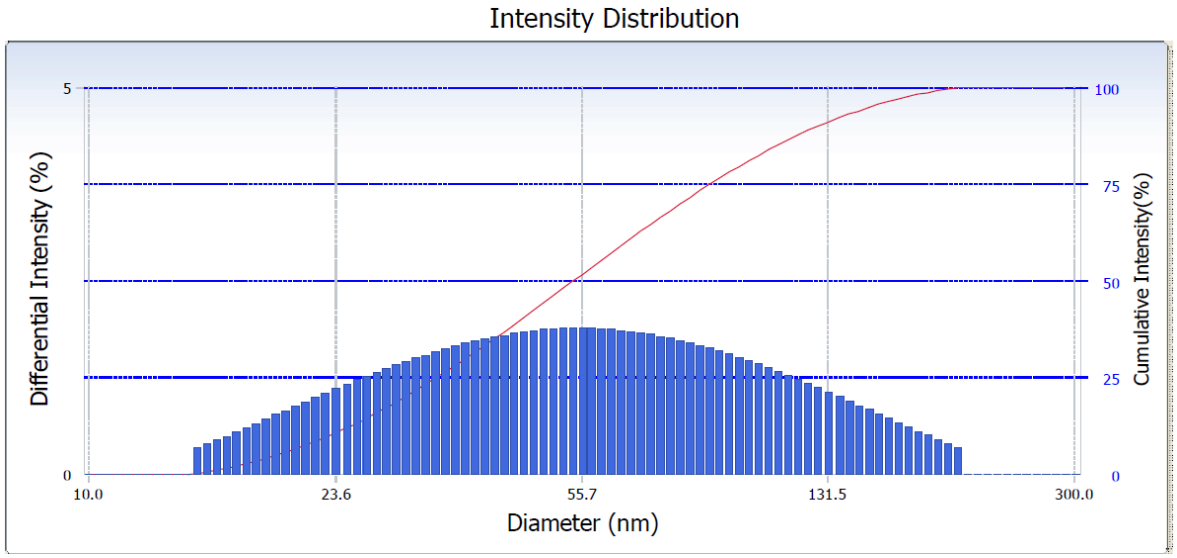


Figure S2: DLS data of vesicles of **3** (1 mM solution in water) after 24 h incubation at 37 °C.

Atomic force Microscopy (AFM)

10 µl aliquots of samples (0.5 mM in water and incubated at room temperature for 24 h) were deposited onto a glass surface and air dried. Scanning was carried out at room temperature using an Agilent Technologies AFM (5500 AFM/SPM) operating under the acoustic AC mode (AAC) force constant was 0.6 N/m. The sample was mounted on the XY stage of the AFM and the integral video camera (NAVITAR, Model N9451A-USO6310233 with the Fiber-light source, MI-150 high intensity illuminator from Dolan-Jenner Industries) was used to locate the regions of interest. Silicon nitride cantilevers with a resonant frequency of 150 kHz was used. The average dimensions: thickness, width and length of the cantilever were approximately 2.0, 51 and 446 µm respectively. The scanner model N9524AUSO7480132.xml/N9520A-USO7480152.xml was calibrated and used for imaging. The images were taken in air at room temperature, with a scan speed of 1.5-2.2 lines/s. Data acquisition and analysis was carried using Pico View® 1.8 and Pico Image® Basic software respectively.

Enzymatic studies

Experimental Procedures

All solutions for the enzymatic study were prepared using HPLC grade water. 1mg/ml of GO was prepared in acetate buffer (pH 5.2, 10 mM) 0.5 mM solution of 3 in acetate buffer was prepared. 50 µl of GO solution was added to 100 µl of 3 and incubated for 15 min (I). 1 mg of HRP in 10 ml of acetate buffer (pH 5.2, 10 mM) was prepared. 1 mg of NBD in 10 ml of HPLC grade acetonitrile was prepared. 20 µl of POD solution was added to 20 µl of dye solution.[2] (II)

Fluorescence spectroscopy

60 µl of II was added to 300 ml of I (after I being incubated for 15 min) and incubated for 10 min at room temperature. This solution was excited at 470 nm and its emission was measured at 560 nm. All the experiments were performed with this basic volume and the slit width for both excitation and emission was set at 10.

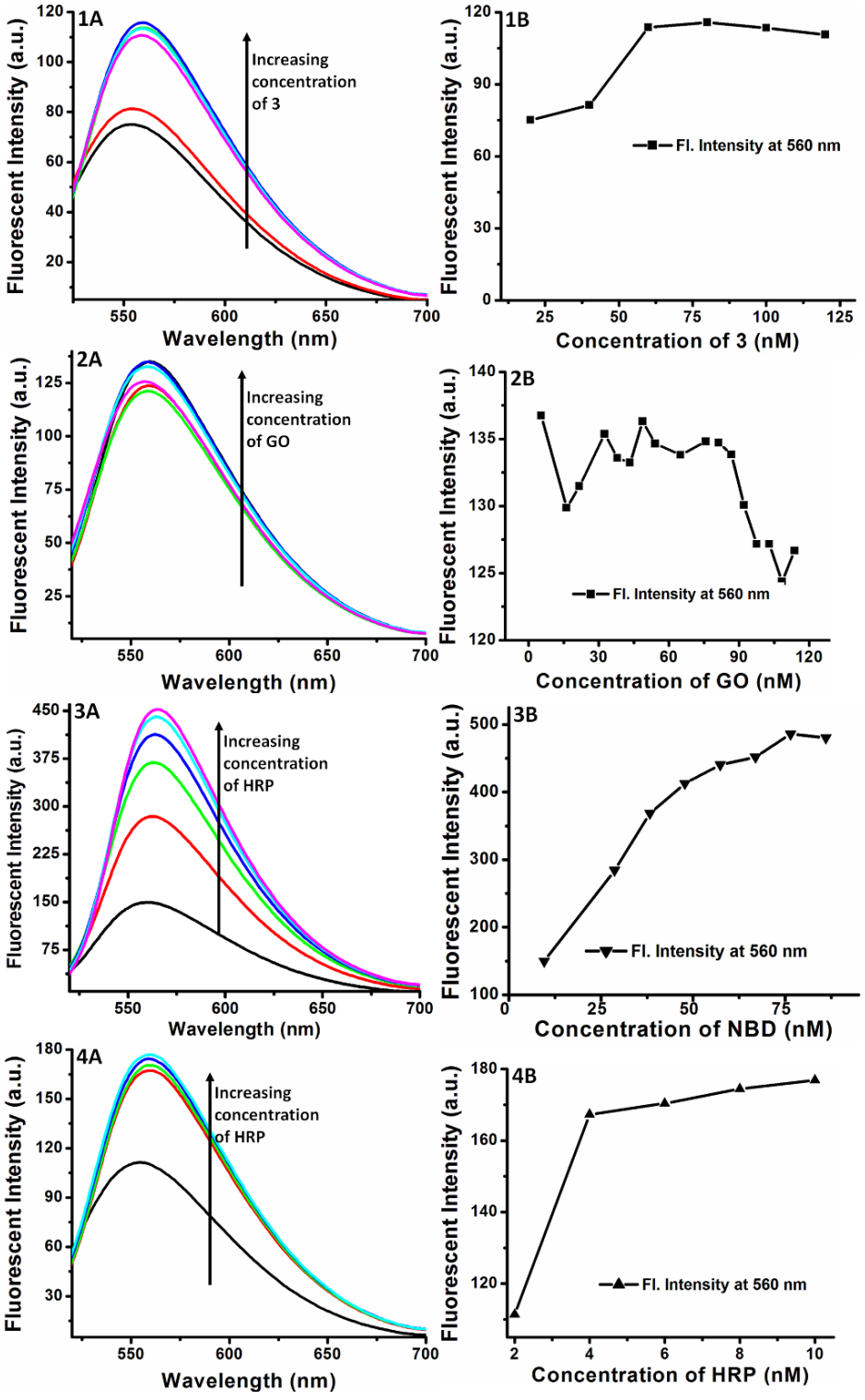


Figure S3: 1A) Fluorescent intensity (intial conc. of **3**: 20 nM) after subsequent 20 nM additions of **3** (keeping concentrations of GO, NBD and HRP constant); **1B)** Maximum fluorescent intensity at 560 nm on subsequent 20 nM additions of **3**; **2A)** Fluorescent intensity (intial conc. of GO: 5.4 nM) after subsequent 5.4 nM additions of GO (keeping concentrations of **3**, NBD and HRP constant); **2B)** Maximum fluorescent intensity at 560 nm after subsequent 5.4 nM additions of GO; **3A)** Fluorescent intensity (initial conc. of NBD: 10 nM) after subsequent 10 nM additions of NBD ((keeping concentrations of **3**, GO and HRP constant); **3B)** Maximum fluorescent intensity at

560 nm with subsequent 10 nM additions of NBD; **4A)** Fluorescent intensity (initial conc. of HRP: 2 nM) after subsequent 2 nM additions of HRP ((keeping concentrations of **3**, GO and NBD constant); **4B)** Maximum fluorescent intensity at 560 nm with subsequent 2 nM additions of HRP.

TEM of 3-AuNC complex:

A 10 μL aliquot of **3-AuNC** (1 mM of **3**; 1:10 w/w) after 16 h of incubation in water was placed on a 400 mesh copper grid. It was dried under a tungsten filament lamp for 30 min. The samples were viewed, without staining, using FEI Technai 20 U Twin transmission electron microscope operating at 120 keV.

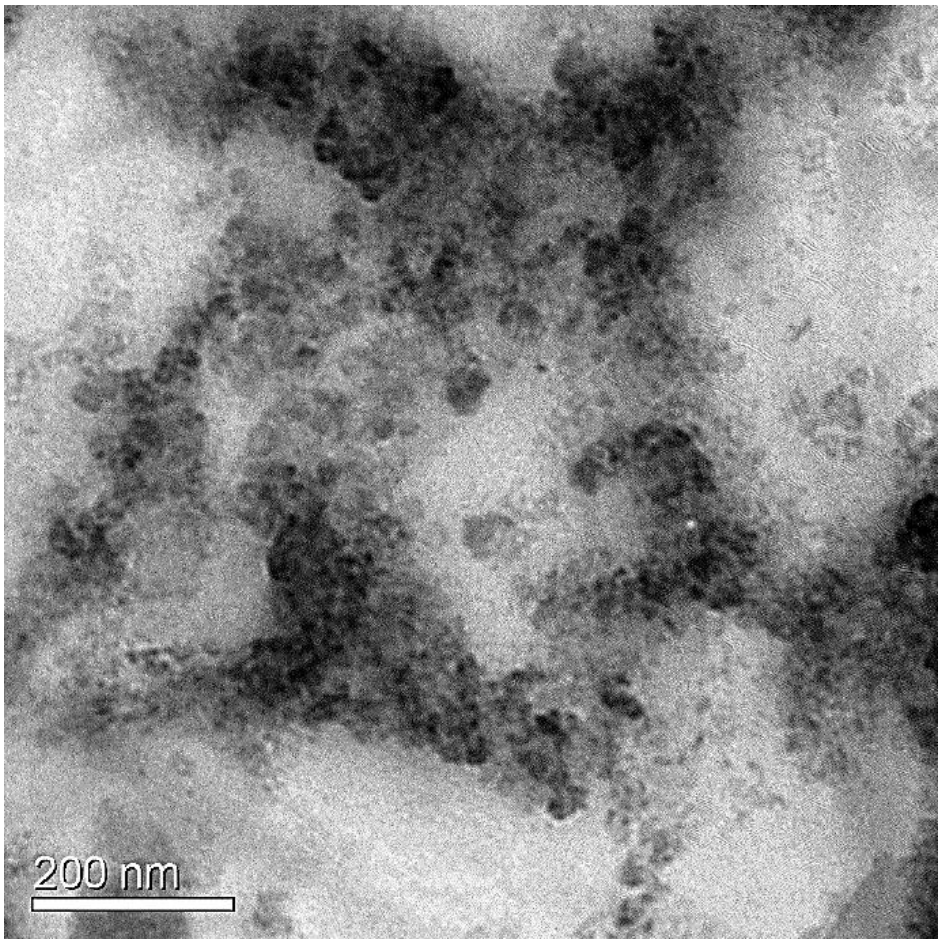


Figure S4: TEM micrograph of **3**-AuNC complex.

Cell culture Studies

Materials

FITC-phalloidin (Fluorescein isothiocyanatephalloidin), trypsin-EDTA, Dulbecco's modified eagle's medium (DMEM), penicillin-streptomycin antibiotic, (3-(4,5-Dimethylthiazol-2-yl)-2,5-iphenyltetrazolium bromide (MTT), and gelatin (from cold water fish skin) were purchased from Sigma Aldrich and used without further purification. Deep red plasma membrane stain (Catalog no. C10046) was purchased from Molecular Probes from Life Technologies. (Dimethyl sulfoxide (DMSO) was obtained from Merck's chemicals, India. Fetal bovine serum brought from Gibco® Life technologies, India. Hep G2, a perpetual cell line derived from the liver tissue, hepatocellular carcinoma, was purchased from the National Center for Cell Sciences Pune, India.

In Vitro Cell Viability/Cytotoxicity Studies

Cytotoxicity studies were carried out using filter-sterilized **3**-AuNC complex to determine if the complex can be used for biological applications. Hep G2 cells were used as the model cells and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to determine the cell viability.

MTT assay

To verify the biocompatibility of **3**-AuNC complex, *in-vitro* biocompatibility studies of **3**-AuNC was carried out with Hep-G2 cells by the MTT assay[3] which is a quantitative colorimetric assay which gives the extent of cell proliferation. Cells were maintained with Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum in a humid incubator (37°C and 5% CO_2). Cells (10^4 cells/well) were plated onto 96 well glass-bottom tissue culture plates at an initial confluence of 70%. After 14 h, **3**-AuNC complex was added to all the wells, including the control wells (i.e. only cells), incubated for 16 h at 37°C in a 5% CO_2 humidified incubator followed by removal of media. 0.5 mg/ml of MTT in DMEM basal was prepared in a dark environment. After discarding the old media, 200 µL of the freshly prepared MTT solution was added to each of the cell containing wells followed by incubation for 4 to 5 h. After incubation, the basal DMEM (having MTT) was removed and 200 µL of DMSO (dimethyl sulfoxide) was added. The cell viability was determined by measuring their absorbance at 570 nm which is

directly proportional to the number of live cells. All the in-vitro cytotoxicity experiments were performed in quintuplicates and the best three wereselected to plot the MTT assay.

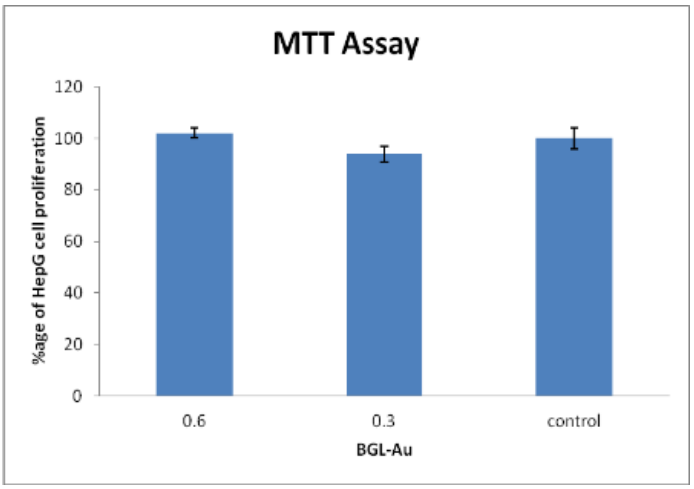


Figure S5: Graph showing the MTT assay for **3**-AuNC complex in Hep G2 cell line at 0.3 and 0.6 mg/ml.

Cellular Uptake Studies

To study the cellular uptake of **3**-AuNC complex using confocal laser scanning microscopy, they were added to the cell culture media at a concentration of 0.6 mg/ml. The cells (10^4 cells/well) were seeded on a sterilized glass cover slip (13 mm, 0.2% gelatin coated) for 15 h. **3**-AuNC complex was added for an incubation time of ~16 h at 37°C with 5% CO_2 humidified incubator after which the cells were washed thrice with PBS buffer and fixed with 4% formaldehyde solution for 20 min. After washing, the cells were stained with deep red plasma membrane dye and again washed with PBS buffer. The coverslips were then mounted onto slides coated with buffered mounting medium to prevent fading and drying. It was then observed under confocal laser scanning microscopy (CLSM).

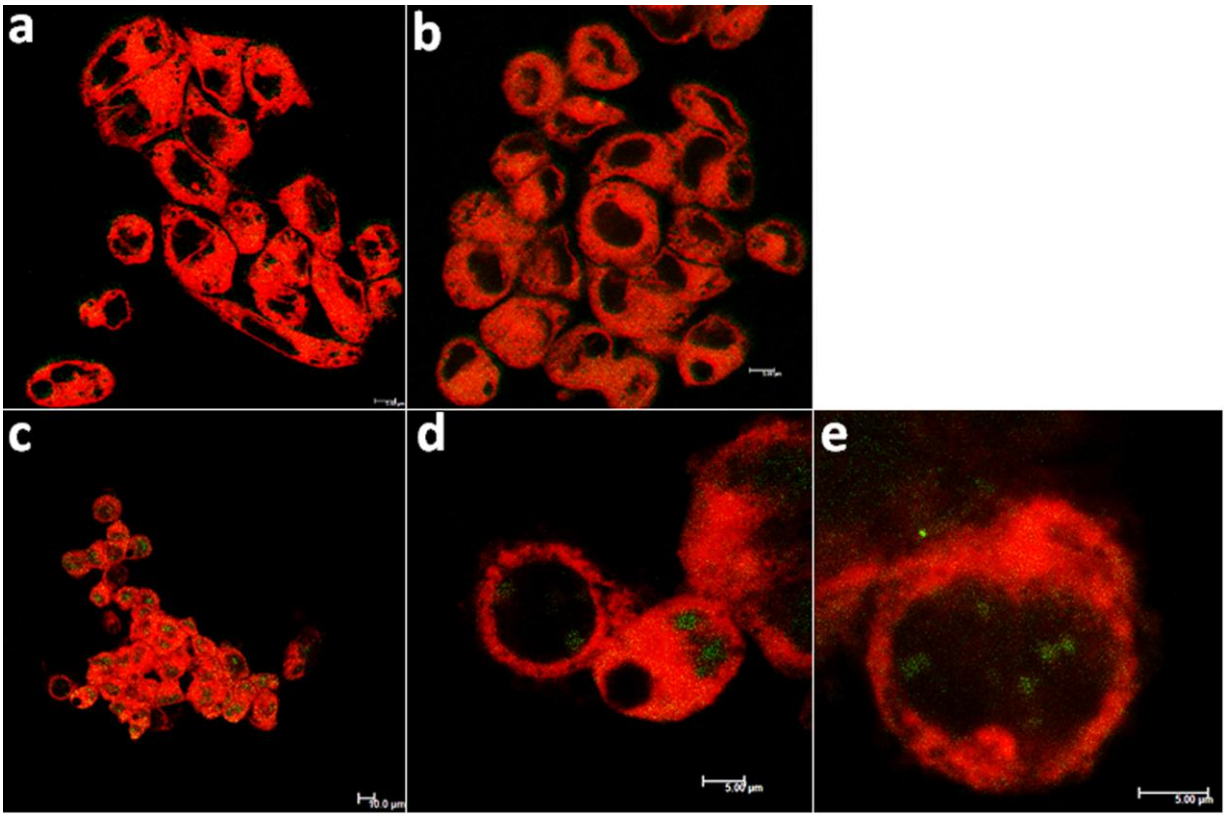


Fig. S6 Confocal laser scanning microscope images of Hep G2 cell lines (λ_{em} = 510 nm); cell membrane stain deep red plasma membrane dye was used as a reference and it exhibits red emission (λ_{em} = 633 nm); **a, b)** Hep G2 cells (control) showing green autofluorescence **c, d, e)** Images of Hep G2 cells incubated with **3**-AuNC for 16 h.

References

1. A. L. Martin, B. Li and E. R. Gillies, *J. Am. Chem. Soc*., 2008, **131**, 734.
2. S. Espuelas, P. Haller, F. Schuber and B. Frisch, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 2557.
3. J. Meyer, A. Büldt, M. Vogel and U. Karst, *Angew. Chem., Int. Ed*., 2000, **39**, 1453.
4. T. Mosmann, *J. Immunol. Methods*, 1983, **65**, 55.