Supporting Information

Mitochondria Targeting Non-isocyanate-based Polyurethane Nanocapsules for Enzyme-Triggered Drug Release

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Cell studies for Nanocapsules

Cellular Uptake experiments of the nanocapsules (Single colour SIM/ Wide Field/ Deconvolved Microscopy):

Cells were seeded on Cover slips (22 mm X 22 mm, 170 ± 5 µm square Cover glasses) placed in six well plates in DMEM culture medium containing (10% FBS and 1% Penicillin Streptomycin) for 24 hours at 37 °C, 5% CO_2. After 24 hours when 70% Confluency was achieved the cells were washed with DMEM culture medium. Cells were then washed thrice with culture medium. After that cells were washed again with Phosphate Buffer Saline (2 times PBS). After carrying out the Live cell uptake of the nanocapsules for 4 hours, the cells were washed with DMEM media, then the cells were fixed with 4% PFA for 15 minutes and then washed thrice with PBS and two times and then the coverslips were mounted using Mounting medium (Vectashield h-1000).The Coverslips were then sealed using Nail varnish

and the sample were then imaged by SIM and Wide Field Fluorescence Microscopy (Deconvolved).

SIM Microscopy - Single colour, Colocalization and Dual colour experiments

Structured Illumination Microscopy (SIM) carried out using the Delta Vision OMX-SIM. Reconstruction of the acquired image was carried out using the inbuilt Soft Worx software. The Z stacks acquired during the Imaging are post-processed by using the Reconstruction option of Soft Worx. For single colour experiments, sample 1 nanocapsules were excited at 526 nm and the emission was collected at Alexa Flour 568 Channel (540 to 580 nm) of the Delta Vision OMX-SIM) and sample 2 nanocapsules were excited at 495 nm and the emission was collected at FITC Channel (500 to 550 nm). For both the nanocapsules the Structured Illumination (SI) experimental condition employed for running the SI experiment for Single Colour Experiments were mainly dependent on the Thickness of the Z stack (Sections 80 to 100), Section spacing (0.125 to 0.250), thickness of the sample (8 to 10).

Widefield Microscopy

Widefield Fluorescence Microscopy was carried out using OMX-SIM. The Widefield Microscopy technique involves improvement in resolution only after post processing the Z-stacks acquired. Processing of acquired data is done by using Fiji software. Deconvolution processing was carried out using the inbuilt Soft Worx software. The colocalization experiments were performed with Mito Tracker Green and Mito Tracker Deep Red. The Dual colour experiments were performed with Hoechst.

Concentration dependent Uptake Nancapsules in LN229 cells

Concentration dependent experiments were performed to determine the minimum dose of the drug required to be delivered to the biological system. Concentration dependent uptake experiments were carried out in order to determine the extent of uptake into LN229 cells (glioblastoma, brain cancer). The concentration uptake SIM and Widefield images indicated that both the nanocapsules localized mainly in the cytosol region in LN229 cells and the minimum concentration needed to work with for nanocapsules was around 10 μg.

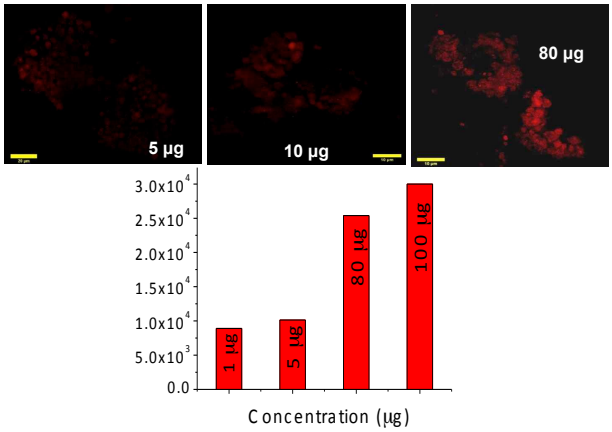

Figure S1. Loading dependent uptake of nanocapsule 1 in LN229 cells. Top: SIM images; Bottom: loading of nanocapsule 1 (µg) vs luminescent brightness (cd/m^2). Pseudocolor has been employed in these images. Scale bar 20 µm.

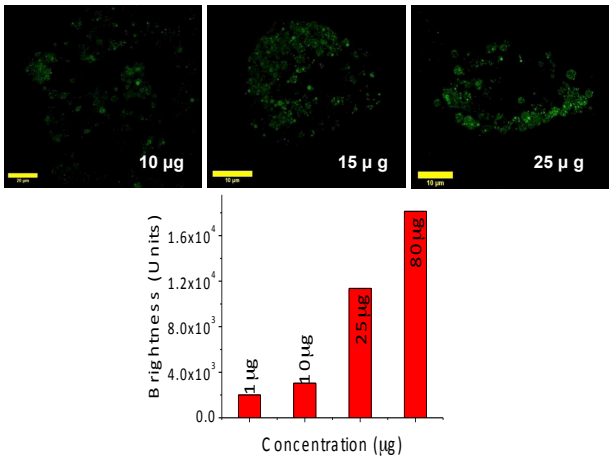

Figure S2. Loading dependent uptake of nanocapsule 2 in LN229 cells. Top: SIM images; Bottom: loading of nanocapsule 2 (µg) vs luminescent brightness (cd/m^2). Pseudocolor has been employed in these images. Scale bar 10 µm.

Colocalization of the nanocapsules (Mitochondria localization)

The Colocalization experiments were performed using commercial co-staining dyes. Due to the distinct spectral characteristics of the nanocapsules the colocalization were performed with a series of available Mito Trackers. For sample 1 nanocapsules, the colocalization experiment was carried out with Mito Tracker Deep Green. Dual colour or Multicolour imaging was carried out with Hoechst.

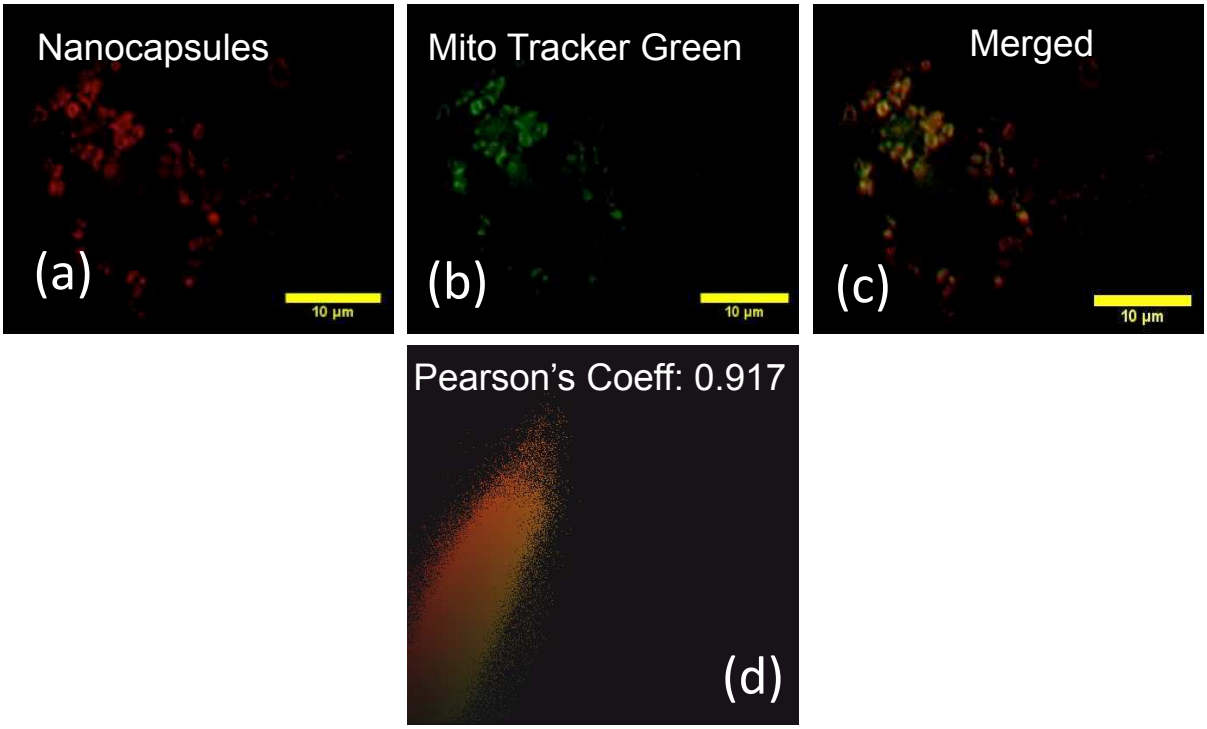

Figure S3. Colocalization experiments of intracellular localization of rhodamine 6G loaded nanocapsules using MitoTraker probes: Wide-field microscopy images of in cellular emission of sample 1 (panel a) with intensity along the traced line shown underneath. Emission from Mito Tracker Green (panel b). The overlap of the intensity is shown in panel c. panel c shows the overlap of the green and red fluorescence, indicating mitochondria localization of sample 2 nanocapsules. Panel d shows the Pearson co-efficient = 0.917. Scale bar 10 µm.

Dual colour imaging of nanocapsules (with Hoechst)

The Dual Colour experiments was carried out using Hoechst as the nuclear stain. LN229 cells were incubated with nanocapsules (15 µg) for 2 hours and then Hoechst (500 nM) was added and incubated further for another 30 minutes. The cells were washed regularly three times with DMEM culture media and PBS. The cellular uptake of both the probes were carried out in live cells and then the cells were fixed with 4% PFA and mounted and navigated initially for proper cell morphology by using Light Microscope and then imaged using SIM and WF micrscopyAs the nanocapsules does not exhibit nuclear localization, the samples could potentially be successfully used as a dual colour SIM probes in combination with Hoechst. The comparative Dual colour SIM and Dual colour Wide Field images are shown in Fig. S4.

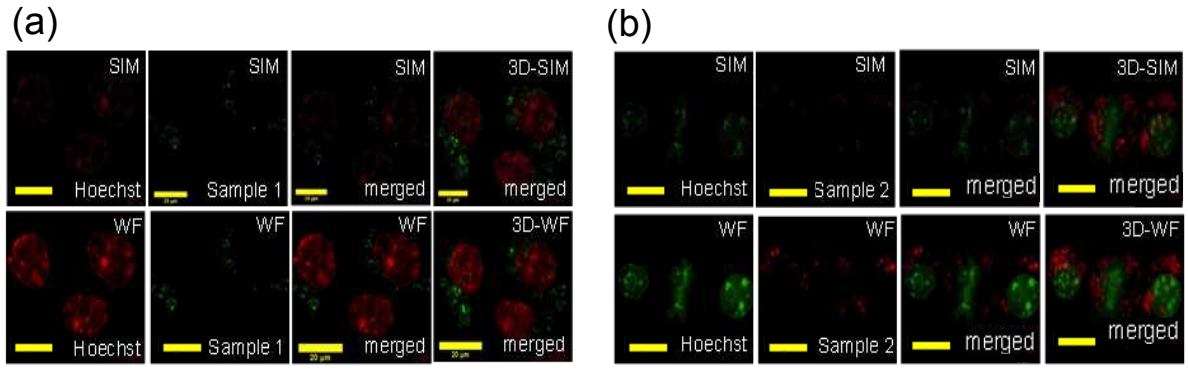

Figure S4. Dual colour SIM images and 3D SIM projections for the nanocapsule samples in combination with Hoechst: (Pseudo colour has been employed in all the images). (A) for sample 2 and (B) for sample 1. WF is wide field microscopy images. Scale bar 20 μm.

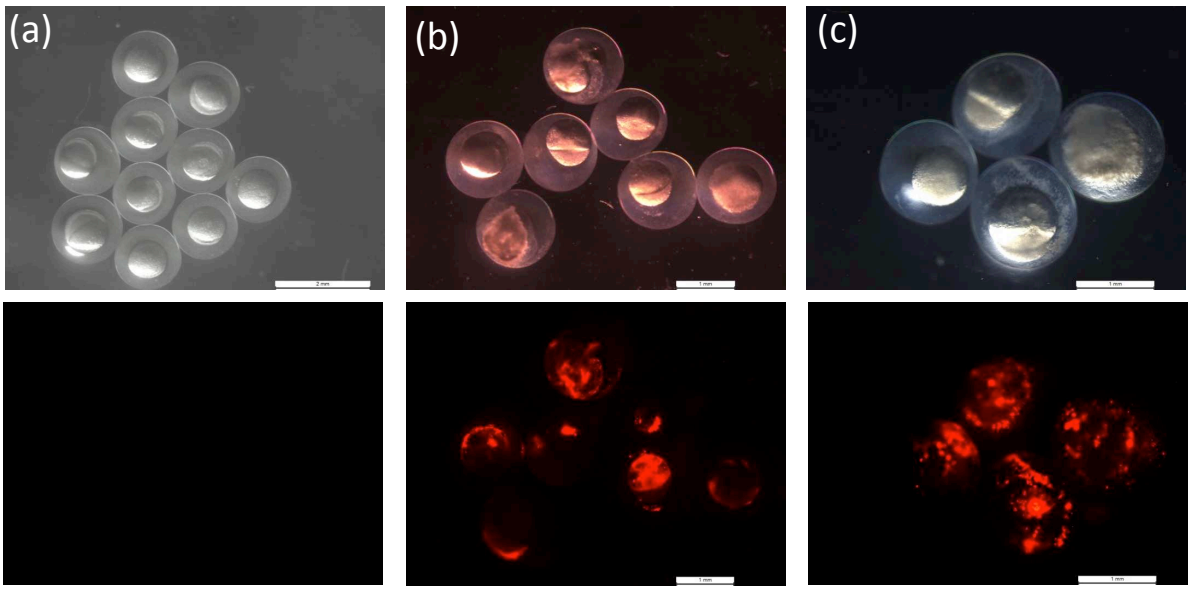

Figure S5. *In-vivo* release experiment in zebrafish model. Bright-field (upper) and fluorescence (lower) images of zebrafish embryo immediate after injection (a) control; (b) sample 2 and (c) sample 1. The injection was done at zero cell stage i.e, 0.5 hpf. In control experiment, the equivalent amount of empty (without any payload) nanocapsule was injected.

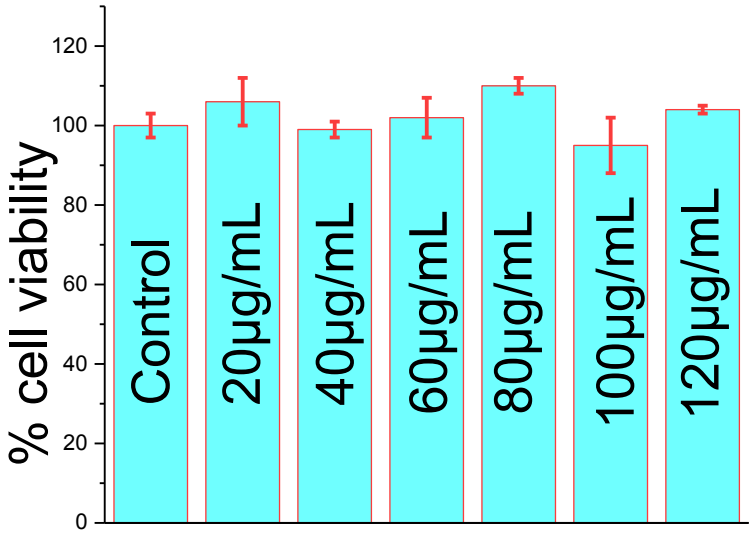

Figure S6. Cell viability of LN229 cells: Cells after 24 h exposure to a concentration range of sample 1, determined using the MTT assay. Data represent mean ± standard deviation of three replicates. The cell viability levels remained stable as compared to a control group, no decrease below 95 % was observed after exposure (24 h) to different concentrations of sample 1.

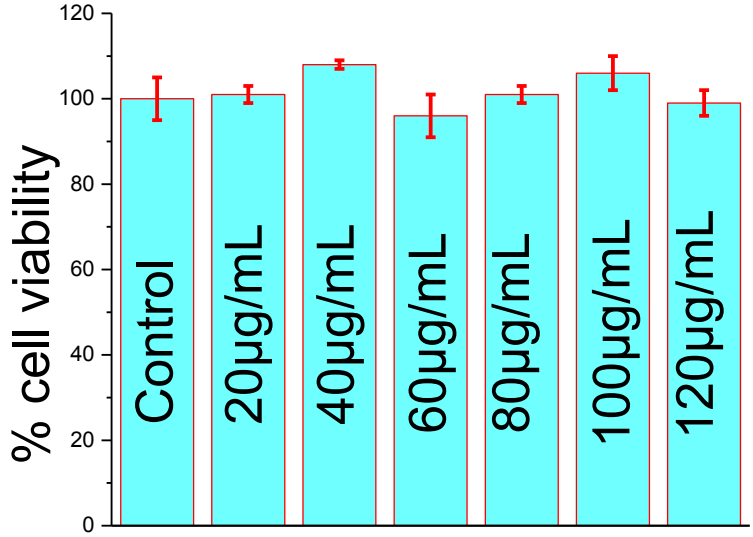

Figure S7. Cell viability of LN229 cells: Cells after 24 h exposure to a concentration range of sample 2, determined using the MTT assay. Data represent mean ± standard deviation of three replicates. The cell viability levels remained stable as compared to a control group, no decrease below 95 % was observed after exposure (24 h) to different concentrations of sample 2.

Calculation of shell thickness of the nanocapsules

HCImage image processing and image analysis software was used for quantitative analysis of the nanocapsules. Data were obtained by measuring over 250 nanocapsules (TEM images) and the average value was taken**.**

Solid state ^{13}C spectra of the sample1

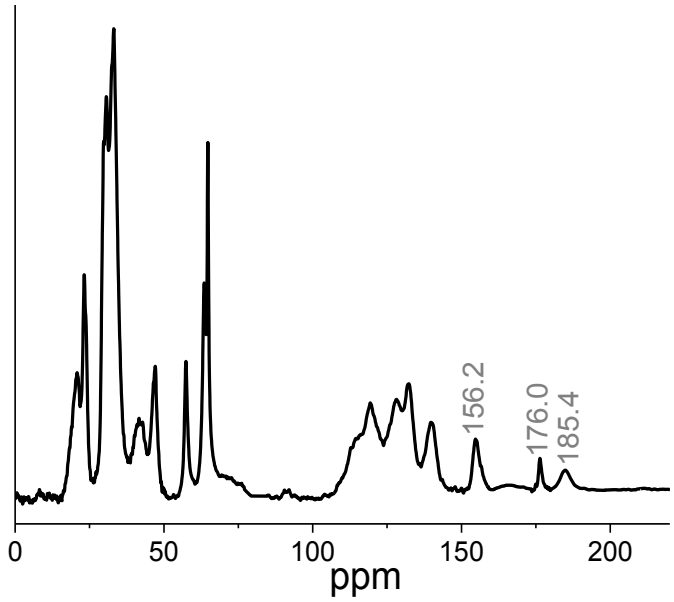

Figure S8. NMR (sample 1) showing the presence of and urethane linkages (the intense peak at 156.2 ppm corresponds to the carbonyl carbon of urethane linkages). The peak at 176.0 corresponds to the carbonyl carbon of the ester linkages and the peak at 185.4 corresponds to the carbonyl carbon of the amide linkages. The aromatic carbons of the triphenyl phosphonium ion are coming in the region of 108 to 142 ppm.

FT-IR spectra of the carbonate monomers

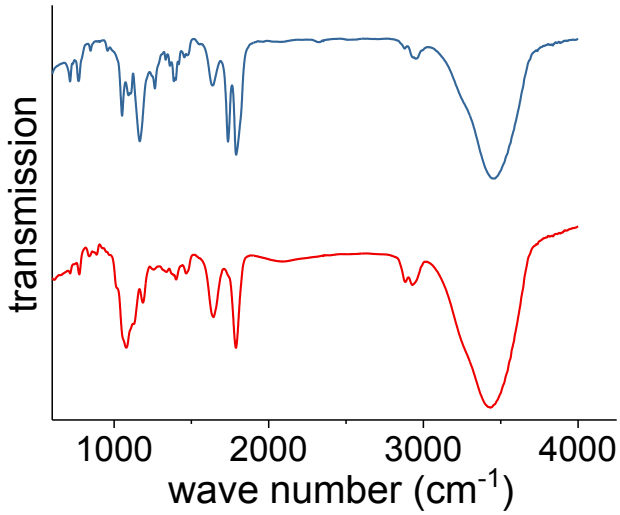

Figure S9. (a) Transmission FT-IR spectra of adipate bis carbonate (blue) and C10 diglycerol carbonate (red).

Evaluation of cell toxicity in LN229 cells

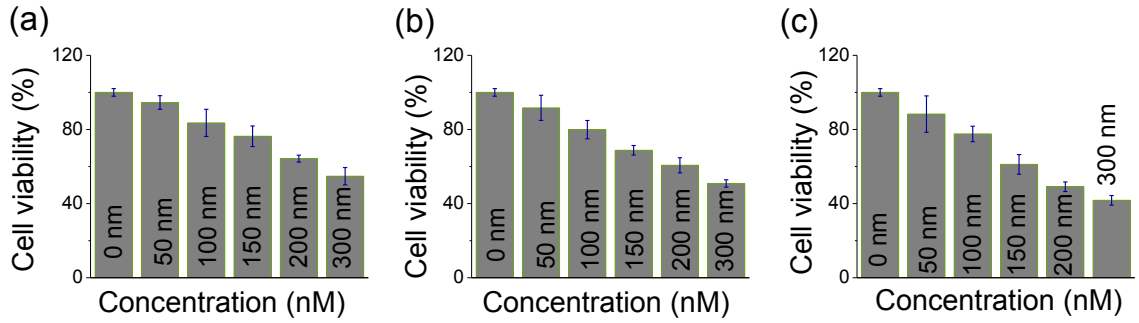

Figure S10. Evaluation of cell toxicity in LN229 cells after 24 h incubation: (a) nanocapsules with an esterified linker (sample 1, without post-grafting with triphenyl phosphonium ion); (b) dose-dependent decrease in cell viability with free doxorubicin; (c) dose-dependent decrease in cell viability with sample 1. These results indicate the enhanced cancer cell killing efficacy was achieved when using doxorubicin loaded and triphenyl phosphonium ion -functionalized NIPU nanocapsules (sample 1) for mitochondria-targeted delivery.