Electronic Supplementary Information

Nanocarriers for intracellular delivery of molecular payload triggered by visible light

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General

Amphiphilic β-cyclodextrin,[1] rhodamine B labelled amphiphilic β-cyclodextrin[2] and adamantane-terminated poly(acrylic acid)[3] were prepared according to the procedure reported earlier. 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 60 mM NaCl (Sigma Aldrich), milli-Q water (Pure lab UHQ water purification system) was used to prepared pH 7 buffer. DPBS (Dulbecco's phosphate buffered saline) was purchased from Sigma.

Preparation of CDV

2 mM 100 µL stock of amphiphilic β-cyclodextrin in chloroform was taken in a round bottom flask and evaporated under the stream of argon to form a thin layer on the walls of flask. 2 mL HEPES buffer (or water or DPBS) was added to obtain a dispersion of multilamellar vesicles (100 µM). The vesicles were then sonicated for 2 min and kept for stirring overnight. The solution was then repetitively filtered by a polycarbonate membrane of 100 nm pore size (AVESTIN) in a Liposofast manual extruder (AVESTIN) to obtain CDV. CDV were prepared in DPBS for cells experiments.

Preparation of PSV_{Tet}

PSV were prepared according to the procedure reported earlier.[4] After addition of EDC.HCl (9 mM) to PSV, 600 µM of 2,2'-(ethylenedioxy)bis(ethyleneamine) (TCI) was added for non-responsive PSV_{OetO}. Similarly, for responsive nano containers, after addition of EDC.HCl (9 mM) to PSV, 2-aminoethanethiol (600 µM) (TCI) was added to form PSV-SH and stirred overnight. 200 µM TCEP was added next day and the solution (PSV TCEP) was stirred for 4 h. 625 uM 3,6-Dichloro-1,2,4,5-tetrazine (BLD pharm) was then added to form PSV_{Tet}, the solution was then stirred for at least 2 h. The solution was then dialyzed in dialysis device (MWCO:3.5-5 kD; Float-A-Lyzer G2 Dialysis device) for 2 days with 3 solvent changes (every 8-10 h). To encapsulated molecular payload (FITC-phalloidin or pyranine) in the nanocontainers, molecular payloads were added during formations of CDV.

DLS and ζ-potential measurements

The measurements were performed on a Nano ZS Zetasizer (Malvern Instruments) at 25 °C. The samples were prepared in disposable 1 mL semi-micro PMMA cuvettes (BRAND) or in disposable DTS 1070 capillary cells (Malvern Instruments). Data analysis was performed with Zetasizer Software Version 7.12 (Malvern Instruments) and OriginPro 2023 10.0.0.154 (Academic).

Fluorescence measurement

Fluorescence measurements were carried out with a FP 6500 spectrofluorometer (JASCO) at 25 °C. Samples for spectroscopic measurements were prepared in quartz glass cuvettes (Hellma) and data analysis was done with Spectra Manager Version 2 (JASCO) and OriginPro 2023 10.0.0.154 (Academic).

FT-IR spectra

The samples in water were freshly prepared after dialysis (MWCO:3.5-5 kD; Float-A-Lyzer G2 Dialysis device). Sample were measured on Digilab FTS 3100 (Bio-RAD) and analysed with OriginPro 2023 10.0.0.154 (Academic).

Atomic force microscopy

The device that was used for acquiring images was Nanowizard3 (JPK Instruments AG, Berlin, Germany; software: JPK SPM desktop (version: 4.3.48)). The sample was taken and dried over cleaned quartz surface that were analysed using tapping mode. For analysis of images Gwyddion (version 2.55) was utilized.

Irradiation experiments

For photo-induced release experiments, a sample of the nanocontainers in buffer was irradiated from a distance of 5 cm at room temperature. In the case of cell experiments the HUVEC were irradiated from a distance of 5 cm at 37°C. The light source was a LED 515 nm (3W, 2.8 mW cm^{-2}) (3W515525m) purchased from Avonec GmbH.

Cell culture and transfection

HUVEC were acquired from PromoCell as cryoconserved pools (C-12203) and cultured on Corning CellBind dishes at 37°C and 5% CO_2 in 1:1 mixed medium comprising M199 medium (PAN-Biotech) supplemented with 10% FCS, 30 µg/ml gentamycin, 0.015 µg/ml amphotericin B, and ECGMII (PromoCell) supplemented with 30 µg/ml gentamycin, 0.015 µg/ml amphotericin B. Experiments were conducted with HUVEC passage 3-5. HUVEC were transfected with a plasmid coding for EGFP-Rab7 (Addgene #28047) using the Amaxa nucleofection system (Lonza) according to the manufacturer´s specifications. Per cuvette, cells from a confluent 20 cm^2 dish together with 1-7 µg plasmid DNA were resuspended in transfection buffer (4 mM KCl, 10 mM $MgCl_2$, 10 mM sodium succinate, 100 mM NaH_2PO_4, pH 7.4 adjusted with NaOH).

For live cell microscopy, transfected HUVEC were seeded on 8-chamber µ-slides (Ibidi, 80827) which were freshly coated with collagen from rat tail (Advanced Biomatrix, 5056) at a concentration of 50 µg/ml in 0.02 M acetic acid solution. Right before imaging HUVEC, the mixed medium was exchanged with Hank´s Balanced Salt Solution (Sigma, H6648), herein referred to as HBSS, supplemented with 20 mM HEPES pH 7.0-7.6 (Sigma, H0887), 1 mM $MgCl_2$ and 0.9 mM $CaCl_2$.

Cell uptake

The freshly dialyzed nanocontainers were incubated with HUVEC in the dark for 90 min at 37 °C and 5 % CO_2. After washing 4 times with serum free M199 medium cells were grown in mixed medium for 2 h and subsequently either analyzed via live cell microscopy or subjected to the release protocol.

Release and staining with Phalloidin-iFluor647

HUVEC were cultivated on collagen coated coverslips (12 mm diameter). Following the uptake protocol mixed medium was exchanged with HBSS, supplemented with 20 mM HEPES pH 7.0-7.6, 1 mM $MgCl_2$ and 0.9 mM $CaCl_2$ and the cells were irradiated with green light for 20 min at 37 °C. As control a nonirradiated sample was incubated in the dark. Subsequently medium was changed back to mixed medium and both samples were incubated at 37 °C and 5 % CO_2. After 3 h cells were fixed in 4 % PFA in PBS for 10 min at RT and permeabilized using 0.1 % Triton X-100 in PBS for 2 min. Unspecific binding was blocked by addition of 3 % BSA in PBS for at least 30 min, followed by incubation with 200 nM Phalloidin-iFluor647 (abcam ab176759) for 20 min at RT in 3 % BSA in PBS. DAPI staining was conducted

with a 0.1 µg/ml solution (Sigma, D9542) for 10 min at RT. After extensive washing, samples were mounted in mounting medium.

Microscopy and image analysis

Confocal microscopy was performed using an LSM 980 microscope (Carl Zeiss) equipped with a Plan-Apochromat 63x/1.40 Oil DIC M27 (Zeiss).

For uptake experiments multi-channel acquisition was achieved using 3 tracks for Hoechst, EGFP and Rhodamine B on a spectral GaAsP-PMT (Hoechst (excitation laser 353, detection wavelengths: 408-491), EGFP (excitation laser: 488, detection wavelengths: 499 – 534 nm), Rhodamine B (excitation laser: 543, detection wavelengths: 543 – 692)). Z-stacks (4 – 11 planes with 0.240 µm spacing) were acquired with 0.071 nm pixel size, 0.66 – 1.13 µs pixel integration time and 2x averaging at a bit depth of 8-bit.

Colocalization analysis of the obtained images was performed by using the Fij plugin JACoP.[5] The extent of cooccurrence of Rhodamine B with EGFP was quantified by calculating Manders coefficient 1 which determines the percentage of total signal from Rhodamine B overlapping with signal from EGFP. The extent of cooccurrence of EGFP with Rhodamine B was quantified by calculating Manders coefficient 2 which determines the percentage of total signal from EGFP overlapping with signal from Rhodamine B.[6, 7]

For release experiments multi-channel acquisition was achieved using 3 tracks for DAPI, FITC and AF647 on a spectral GaAsP-PMT (DAPI (excitation laser: 405, detection wavelengths: 412 – 490 nm), FITC (excitation laser: 488, detection wavelengths: 490 – 639), AF647 (excitation laser: 639, detection wavelengths: 648 – 692)). Z-stacks (5 planes with 0.190 µm spacing) were acquired with 0.071 µm pixel size, 0.25 µs pixel integration time and 2x averaging at a bit depth of 8-bit.

The obtained confocal images were analyzed using ImageJ. Actin was identified based on the iFluor647 signal of maximum intensity projections. Segmentation of actin structures was achieved by training a pixel classification model provided in Ilastik.[8] The images were analyzed using a custom macro in Fij.[9] Maximum intensity projections of the multicolor images were created. For actin segmentation the pixel classification model was applied through the ilastik plugin for Fiji. FITC signal was only measured within the segmented area. The respective mean grey value of all segmented pixel of each image was subsequently normalized to the segmented area in μm^2 of each image.

LDH cytotoxicity assay

HUVEC were seeded on two collagen coated 96-well plates (Corning Incorporated, REF 3595) and incubated at 37°C and 5% CO2 overnight. Thereafter, the medium was exchanged with M199 medium (PAN-Biotech) supplemented with 1% BSA, 30 µg/ml gentamycin, 0.015 µg/ml amphotericin B and the plates were irradiated with green light for 20 min at 37 °C. As control, nonirradiated plates was incubated in the dark. Subsequently both samples were incubated at 37 °C and 5 % CO_2 for 2 h before the lactate dehydrogenase (LDH) activity in the supernatant was measured according to the instructions of a cytotoxicity detection kit (Roche, Cat. No. 11644793001). The cytotoxicity assay was conducted three times.

Statistics

All statistics were performed using GraphPad PRISM (10.1.0). Asterisks mark statistically significant results: ****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$, ns $p>0.05$. Normal distribution was assessed by the Shapiro-Wilk or the D´Agostino & Pearson test. Non-parametric data were analyzed using a Mann-Whitney test.

Additional data

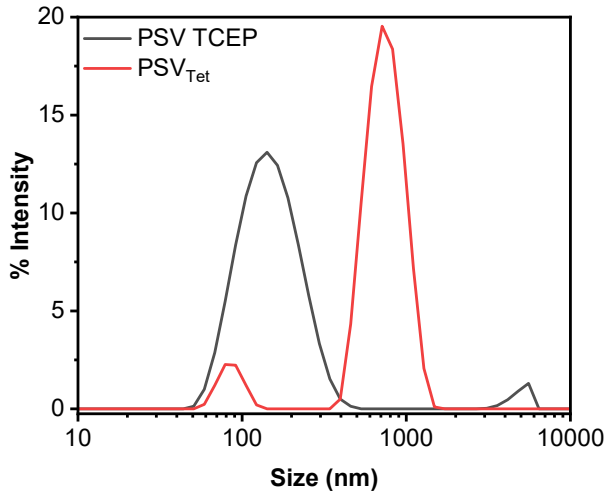

Figure S1. Variation of crosslinker concentration. DLS measurement of PSV_{Tet} and PSV TCEP with 200% (1200 µM) concentration of crosslinker with respect to the general procedure used (600 µM).

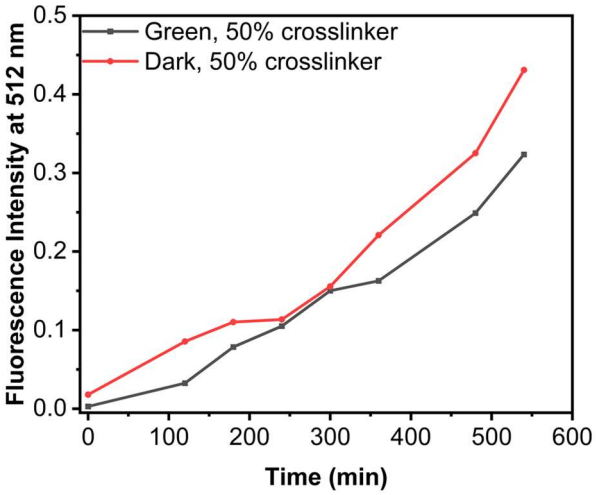

Figure S2. Variation of crosslinker concentration. Fluorescence spectroscopy measurement of release of pyranine of PSV_{Tet} made with 50% (300 µM) crosslinker concentration with one sample exposed to green light (515 nm) and the other sample kept in the dark for 540 min.

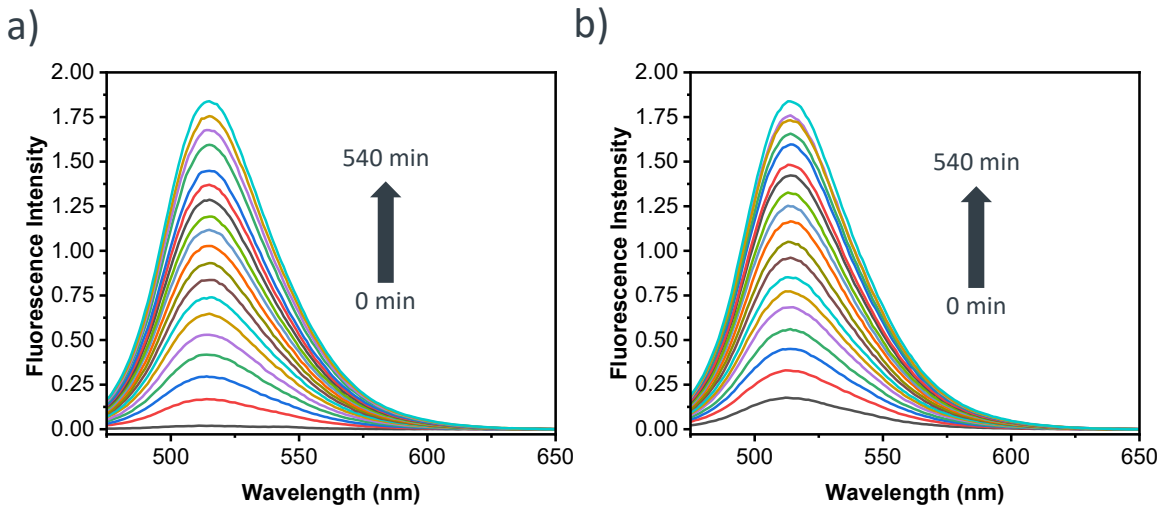

Figure S3. Phototriggered payload release. Fluorescence spectroscopy measurement of release of pyranine of PSV_{Tet} with 20 min (a) and 30 min (b) irradiation with green light (515 nm), respectively. The release was observed for the duration of 540 min.

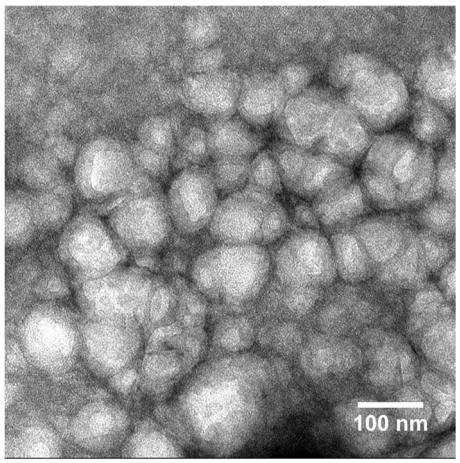

Figure S4. Microscopy of nanocontainers. TEM image of PSV_{Tet}. 5µL sample of PSV_{Tet} was kept on a TEM grid for 2 min and the excess was removed after 2 min. After drying the grid, the sample was analysed.

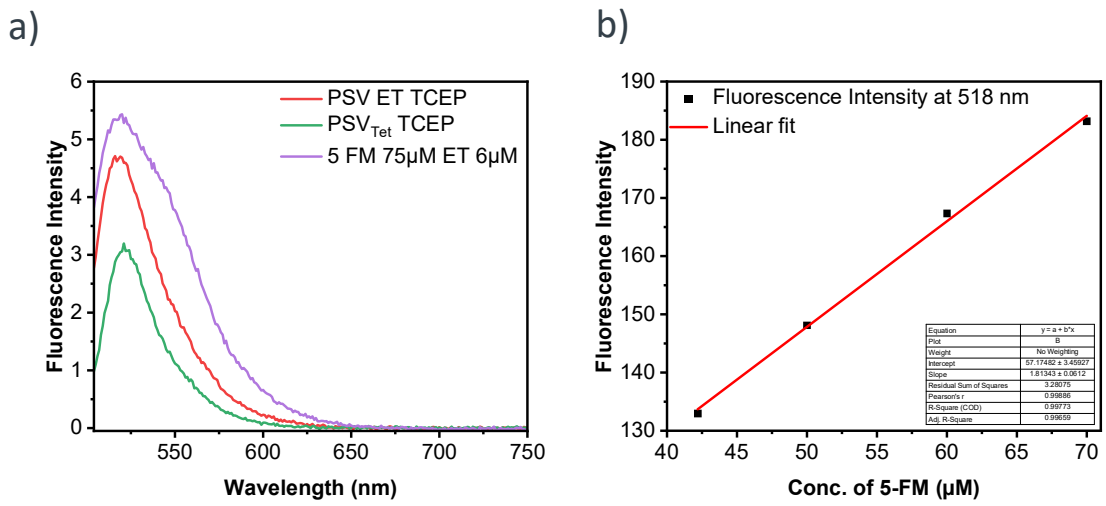

Figure S5. Determination of crosslinking efficiency. a) Fluorescence spectroscopy measurement of PdV ET TCEP, PSV$_{Tet}$ TCEP and 2-amino ethane-1-thiol with 75µM 5FM and b) Data and linear fit of fluorescence of different concentration of 5FM.

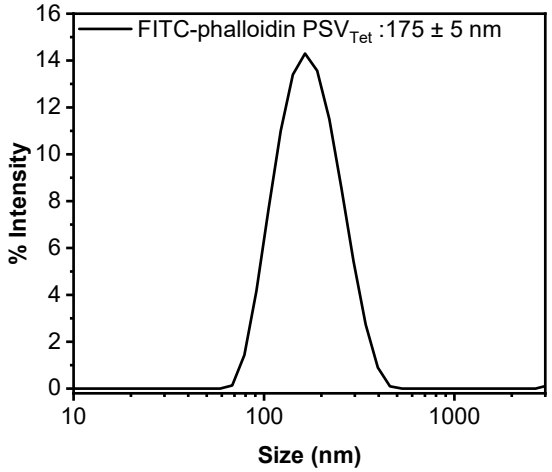

Figure S6. Size of loaded nanocontainers. DLS measurement of FITC-phalloidin containing PSV$_{Tet}$ before incubation into the cells. The data comprise of mean ± standard deviation for n = 3.

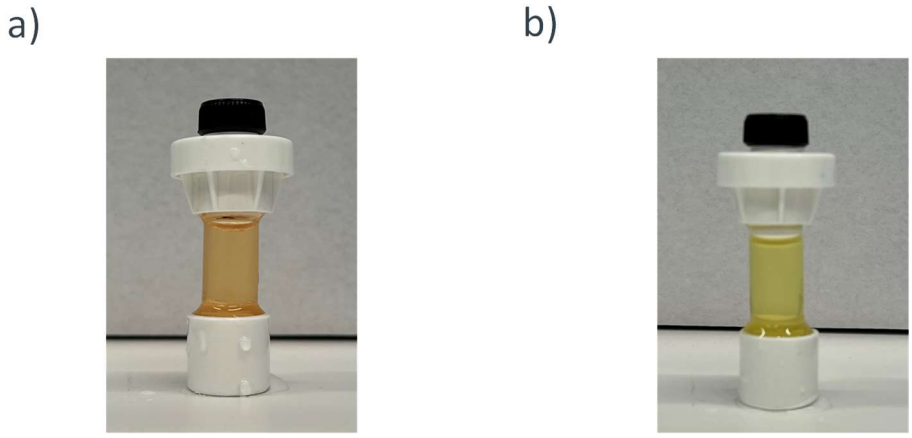

Figure S7. Optical images of photoinduced payload release. Images of PSV_{Tet} containing pyranine that were in dark (a) and 515 nm irradiated (b) conditions for 10 h respectively.

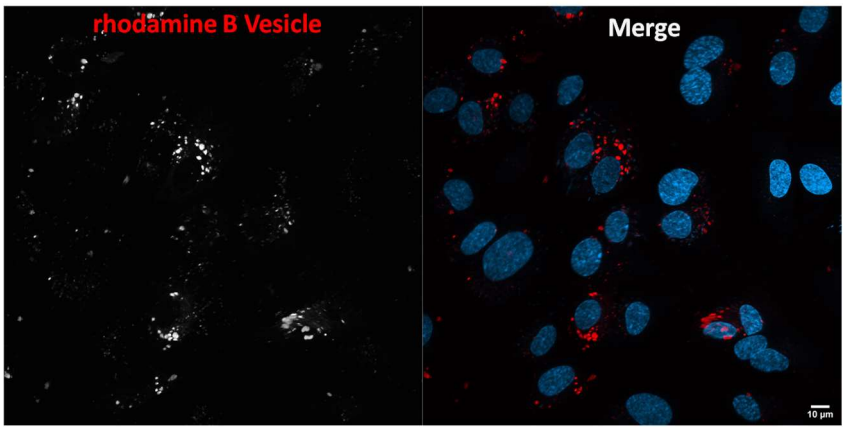

Figure S8. Cell uptake of nanocontainers. Rhodamine linked PSV_{Tet} in the HUVECs with labelled nuclei. First panel shows the fluorescence only from the rhodamine linked PSV_{Tet}, while the second one is merged with red fluorescence from rhodamine linked PSV_{Tet} and blue fluorescence from nuclei of the cells.

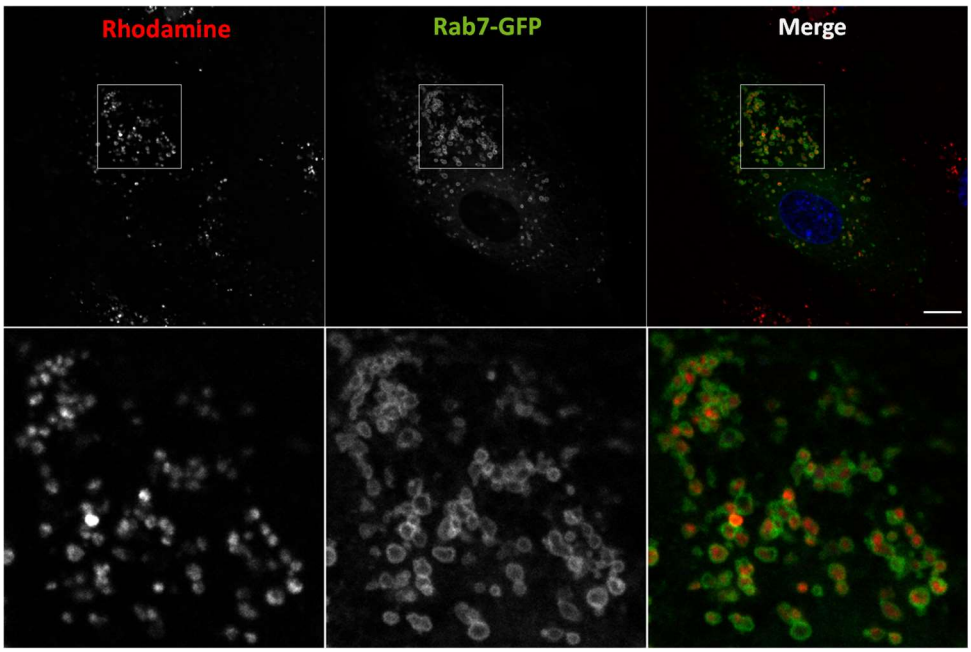

Figure S9. Cell uptake of nanocontainers. Rhodamine linked PSV_{Tet} colocalized with late endosomal marker Rab7-GFP in the HUVECs with labelled nuclei. In the first panel fluorescence from rhodamine linked PSV_{Tet} is shown, while in the second panel fluorescence from Rab7-GFP (late endosomal marker), and in the last panel the merge picture shows the red fluorescence from rhodamine linked PSV_{Tet}, and green fluorescence is from Rab7-GFP and blue fluorescence from labelled nuclei of the cell.

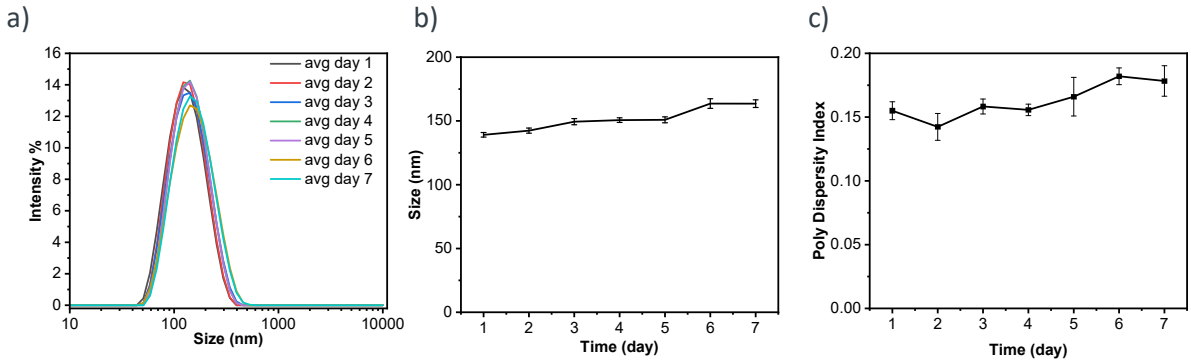

Figure S10. Colloidal stability of nanocontainers. a) % Intensity distribution of PSV_{Tet} on DLS b) Size (maximum) of PSV_{Tet} with mean ± standard deviation for n=3. c) PDI of PSV_{Tet} for the same data with mean ± standard deviation for n=3. All measurements were performed over the course of seven days starting from day after fresh preparation of PSV_{Tet}.

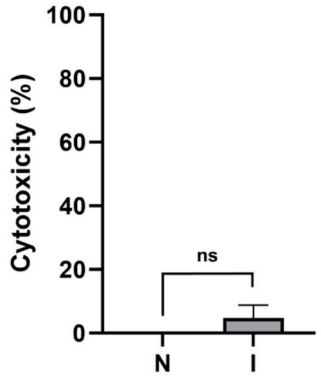

Figure S11. Irradiation with green light has no significant effect on viability of HUVEC. To assess phototoxicity, the LDH activity released from the cytosol into the supernatant was determined for HUVEC, either left untreated (N) or irradiated with green light for 20 min (I). The maximum amount of releasable LDH activity was assessed by lysis with 1 % Triton for 10 min and set to 100%. Bars indicate the mean. Error bars show the SEM. n=3. Significance was tested using a Mann-Whitney test. ****p≤ 0.0001, ***p≤ 0.001, **p≤0.01, *p≤0.05, ns p>0.05.

Notes and References

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