Electronic Supplementary Material (ESI) for ChemComm.

SUPPLEMENTARY INFORMATION

Co-Delivery of Proteins and Small Molecule Drugs for Mitochondria-Targeted Combination Therapy

Si Si Liew,[a] Jia Zhou,[b] Lin Li [b]* and Shao Q. Yao [a]*

[a] Department of Chemistry, National University of Singapore
3 Science Drive 3, Singapore 117543 (Singapore)
E-mail: chmyaosq@nus.edu.sg

[b] Key Laboratory of Flexible Electronics (KLOFE) & Institute of Advanced Materials (IAM), Nanjing Tech University, Nanjing 211816 (P. R. China)
Email: iamlli@njtech.edu.cn

* To whom correspondence should be addressed
E-mail: chmyaosq@nus.edu.sg; iamlli@njtech.edu.cn

Table of Contents

1. General Information

2. Chemistry

2.1 Synthetic Scheme

2.2 Synthesis Procedures

2.3 Chemical Structures of Linkers Used

3. Protein Modifications

4. Nanogel Synthesis & Characterisation

5. Results & Discussion

6. ^{1}H and ^{13}C NMR

7. References

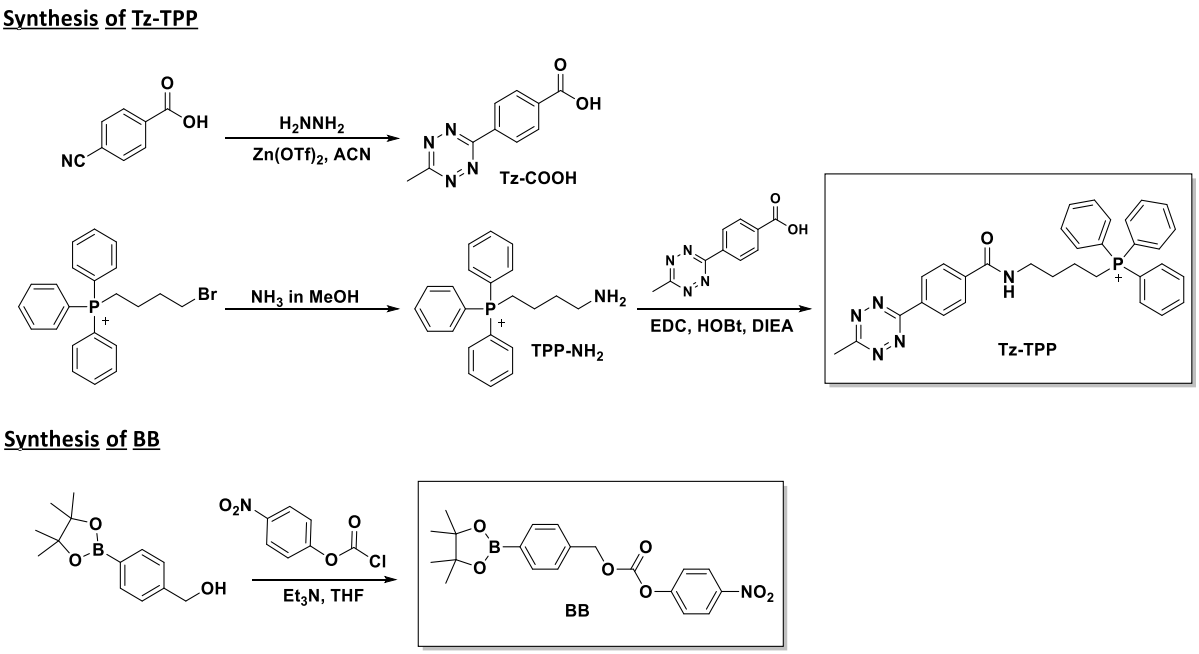
1. General Information

All commercial reagents were purchased from reputable vendors and used without further purification, unless indicated otherwise. All reactions were sealed with septa through which a nitrogen atmosphere was introduced unless otherwise stated. All non-aqueous reactions were carried out under a nitrogen/argon atmosphere in oven-dried glassware. Reaction progress was monitored by TLC on pre-coated silica plates (Merck 60 F254 nm, 250 μm thickness) and spots were visualized by UV light or appropriate staining ($KMnO_4$). Flash column chromatography was carried out using 200-300 mesh silica gel. All ^1H NMR and ^{13}C NMR spectra were carried out on a Bruker ACF-500 MHz NMR spectrometer. Chemical shifts were reported in parts per million (ppm) relative to residual solvent peaks. ^1H and ^{13}C chemical shifts (δ) were referenced to TMS or residual solvent peaks (Methanol-d_4 = 3.31 ppm) for ^1H NMR and (Methanol-d_4 = 49.10 ppm) for ^{13}C NMR. The following abbreviations were used for reporting ^1H NMR spectra: chemical shift (δ ppm), multiplicity (*s* = singlet, *d* = doublet, *t* = triplet, *q* = quartet, *dd* = doublet of doublets, *m* = multiplet), coupling constant (Hz), integration.

Cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen) and 1% (v/v) penicillin-streptomycin (Thermo). All cell lines were grown in a humidified incubator at 37 ºC with 5% CO_2 atmosphere. Protein concentration was determined by Nanodrop™ 2000c spectrophotometer (Thermo) and Pierce™ BCA Protein Assay Kit (Thermo). Zeba™ Spin Desalting Columns used for protein purification was purchased from Thermo Fischer. Illustra NAP™-5 Columns used for protein nanogel purification was purchased from GE Healthcare. Bovine Serum Albumin (BSA) was purchased from Bio Basic Inc. RNase A (Cat No: 101076) was purchased from MP Biomedicals, LLC. RNase A activity assay kit (RNaseAlert™ Substrate) was bought from Integrated DNA Technologies. NHS-PEG_4-TCO and NHS-PEG_4-DBCO were obtained from Click Chemistry Tools. Antibodies used for Western-blotting (WB) and Immunofluorescence assay (IF) were purchased from the following vendors: rabbit *anti*-TOMM20 (#ab186734, abcam), mouse *anti*-Actin (#ab8227, abcam), mouse *anti*-MTCO2 (#ab110258, abcam), HRP-conjugated goat *anti*-rabbit IgG (H+L) secondary antibody (#31460, Invitrogen), HRP-conjugated goat *anti*-mouse IgG (H+L) secondary antibody (#31430, Invitrogen), and FITC-conjugated goat *anti*-rabbit IgG (H+L) secondary antibody (#656111, Invitrogen). In-gel fluorescence scanning of SDS-PAGE gels was carried out with Typhoon 9410 fluorescence gel scanner (Amersham Biosciences). Confocal microscopy was performed on an Olympus FV3000 confocal microscope equipped with a 60× water-immersed objective. Transmission electron microscopy (TEM) images were collected on a JEOL 3011 transmission electron microscope with an accelerating voltage of 300 kV. Hydrodynamic size and zeta-potential were performed on a Litesizer™ 500 Particle Analyzer (Anton-Parr). Fluorescence spectra were measured on a BioTek Synergy 4 microplate reader.

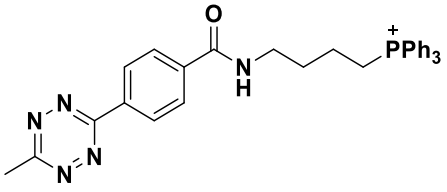
2. Chemical Synthesis

2.1 Synthetic Scheme



Scheme S1. *Synthesis of Tz-TPP* and *BB*.

2.2 Synthetic Procedures

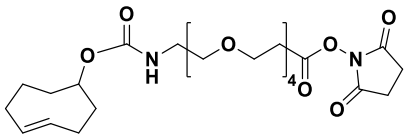


Tz-COOH[1] (50 mg, 0.24 mmol) was first dissolved in dry DMF. Next, DIEA (46 mg, 0.35 mmol) was added. After cooling in an ice bath, EDC.HCl (67 mg, 0.35 mmol) and HOBt (48 mg, 0.35 mmol) were then added into the solution, followed by TPP-NH_2[2] (104 mg, 0.25 mmol). The mixture was left to stir at room temperature overnight. The crude product was purified using column chromatography DCM:MeOH = 20:1, yielding Tz-TPP as a dark pink powder (30 mg, 20 %). ^{1}H NMR (500 MHz, CD_3OD) δ: 8.58 (d, J = 8.5 Hz, 2H), 7.97 (d, J = 8.5 Hz, 2H), 7.91-7.80 (m, 10H), 7.78-7.67 (m, 6H), 3.59-3.50 (m, 4H), 3.08 (s, 3H), 2.00-1.90 (m, 2H), 1.80 (dd, J = 15.7, 7.9 Hz, 2H). ^{13}C NMR (125 MHz, CD_3OD) δ: 167.72, 163.44, 134.99, 134.88, 133.43, 130.09, 127.47, 118.79, 118.11, 37.94, 29.75, 21.20, 20.79, 19.80, 19.30. ESI-HRMS: m/z $[M]^+$ calcd, 532.2261; found, 532.2267.

The synthesis of BB was adapted from previously published protocols.[3]

2.3 Chemical Structure of Linker Used in This Work

TCO-PEG$_4$-NHS:



Scheme S2. *Structure of linker used in this study.*

3. Protein Modifications

***Fluorescent Labeling of RNase A*:** 10 mg of RNase A was dissolved in 1 mL of $NaHCO_3$ solution (100 mM $NaHCO_3$, *p*H 9.0). 20 equiv. of freshly prepared FITC stock solution (10 mg/mL in DMSO) was added and the reaction was left to proceed at 4 ^oC overnight. Excess FITC was removed by Zeba™ Spin Desalting Columns (7K MWCO). The eluate was collected, and protein concentration determined using Pierce™ BCA Protein Assay Kit (Thermo). The FITC-labeled RNase A (RNase A^{FITC}) was stored in -20 ^oC prior to usage.

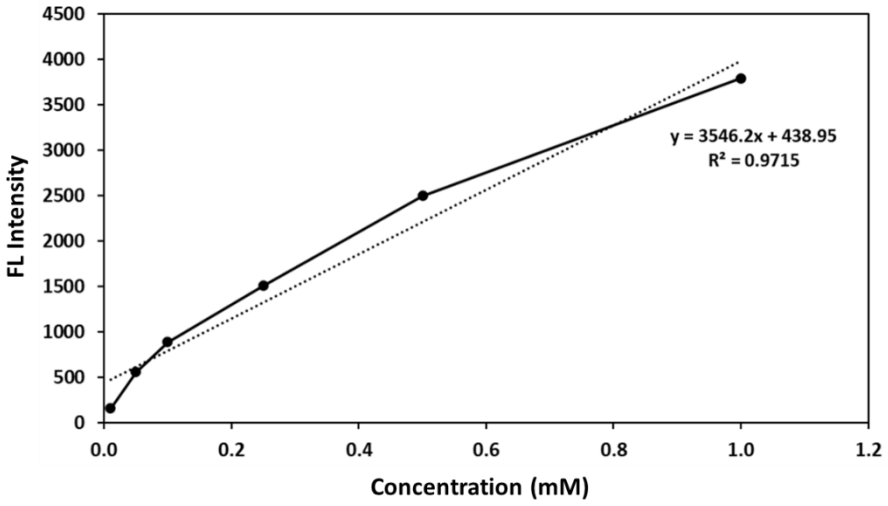
BB Labeling of RNase A[3]: RNase A or RNase A^{FITC} was dissolved in $NaHCO_3$ buffer solution (100 mM, *p*H = 8.5) at a protein concentration of 6 mg/mL. To the solution, 50 equiv. of BB (100 mM stock solution in DMSO) was added and reacted at room temperature for 16 h. The BB-labeled product (RNase A-BB or RNaseA-BB^{FITC}) was then purified by Zeba™ Spin Desalting Columns (7K MWCO). After purification, protein concentration was determined using Pierce™ BCA Protein Assay Kit (Thermo) before further use.

ARS Fluorescence Assay: To confirm successful BB modification on RNase A, 0.0025% w/v Alizarin Red S (ARS) solution was incubated with 1.0 mg/mL of RNase A-BB (final concentration) for 15 minutes. Other protein controls were carried out alongside, including native RNase A and RNase A-BB treated with 10 mM H_2O_2 at 37 ^oC for 3 h. ARS emission measurement was subsequently carried out on a BioTek Synergy 4 microplate reader (λ_{ex} = 490 nm, λ_{em} = 600 nm).

Determining ratio of BB to RNase A in RNase A-BB: The number of BB to RNase A ratio was determined via fluorescence "Turn-ON" using ARS dye. This method was previously reported for quantifying boronic acid functionalities.[4] A fluorescence "Turn-ON" calibration graph was first generated by adding different concentrations of phenyl boronic acid to 0.0025% *w/v* ARS solution.

Concentration of RNase A-BB used = 1 mg/mL ≈ 70 uM
Fluorescence intensity of 0.0025% ARS when treated with 1 mg/mL RNase A-BB = 2052
BB concentration in 1.0 mg/mL RNase A-BB can be determined by the equation,
$y = 3546.2\,x + 438.95$ obtained from the calibration graph (Graph S1).
$2052 = 3546.2\,x + 438.95$

x = 0.455 mM = 455 μM
Concentration of BB in 1 mg/mL of RNase A-BB = 455 μM
Hence, ratio of BB:RNase A = 455:70 ≈ **6.5**



Graph S1. *Calibration graph for BB concentration in response to phenyl boronic acid-triggered ARS dye fluorescence "Turn-ON".*

Note: This RNase A-BB labeling protocol was reproduced from a previously reported protocol, using the same reaction conditions.[3] In their study, the lysine labeling reactions provided RNase A–BB with an average of seven BB conjugations per protein molecule. Their data was calculated by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

4. Nanogel Synthesis and Characterisation

***General procedure for in situ polymerization and protein encapsulation*:** Monomers, *N*-(3-aminopropyl)methacrylamide hydrochloride (APM) (10% w/v in water) and acrylamide (AAM) (10% w/v in water), were first added to the protein (final concentration 1 mg/mL) in 50 mM phosphate buffer (*p*H = 7.0), followed by shaking at room temperature for 15 mins. Crosslinker *N*,*N*′-bis(acryloyl)cystamine (BAC) (10 % w/v in DMSO) was next added, followed by shaking for another 5 mins. *N*,*N*′-methylenebis(acrylamide) (Bis-AAM) was used as the GSH non-responsive crosslinker for negative control. Unless otherwise mentioned, monomer ratios were kept constant (APM/protein 5000:1, AAM/protein 2500:1 mol/mol; crosslinker/protein 1000:1 mol/mol). Ammonium persulfate (APS/protein 500:1 mol/mol) and tetramethylethylenediamine (TEMED/APS 2:1 w/w) were added to the protein/APM/AAM/crosslinker mixture. The reaction was tightly sealed and shaken at room temperature for 2 h. After which, the reaction was passed through a Illustra NAP^{TM}-5 column (GE Healthcare) to remove low molar mass reagents. The protein nanocapsules prepared with the above protocol were then subjected to SDS-PAGE gel analysis, DLS measurements, zeta potential measurements and TEM analysis. The protein nanocapsule concentration was determined using Pierce™ BCA Protein Assay Kit (Thermo). *Note: All protein nanocapsule concentrations were expressed using protein concentrations as reference. As such, 1 mg/mL of*

protein nanocapsule refers to BCA-determined 1 mg/mL concentration and not the actual weight of the protein nanocapsule.

***General procedure for TPP modification of protein nanocapsules*:** 0.5 mg/mL of protein nanocapsules was reacted with 20 equiv. of TCO-PEG_4-NHS (25 mM stock in DMSO) at rt for 16 h. The reaction was purified using Illustra NAP^{TM}-5 column (GE Healthcare) to remove excess small molecules. The eluate was then clicked with 20 equiv. of Tz-TPP (50 mM stock in DMSO) at room temperature for another 16 h. The reaction was subsequently purified using Illustra NAP^{TM}-5 column (GE Healthcare). The TPP-modified protein nanocapsule concentration was determined using Pierce™ BCA Protein Assay Kit (Thermo) before use. *Note: All protein nanocapsule concentrations were expressed using protein concentrations as reference. As such, 1 mg/mL of protein nanocapsule refers to BCA-determined 1 mg/mL concentration and not the actual weight of the protein nanocapsule.*

General procedure for in situ polymerization of dual-cargo protein nanocapsules: 20 equiv. of Dox.HCl (10 mM stock in water) were first added to RNase A-BB^{FITC} or RNase A-BB (final concentration 1.0 mg/mL) in 50 mM $NaHCO_3$ (*p*H = 8 to 9), followed by shaking at room temperature for 30 mins. Monomers, *N*-(3-aminopropyl)methacrylamide hydrochloride (APM) (10% w/v in water) and acrylamide (AAM) (10% w/v in water) and crosslinker *N*,*N*'-bis(acryloyl)cystamine (BAC) (10 % w/v in DMSO) were next added, followed by shaking for another 15 mins. *N*,*N*'-methylenebis(acrylamide) (Bis-AAM) was used as the GSH non-responsive crosslinker for negative control. Unless otherwise mentioned, monomer ratios were kept constant (APM/protein 4000:1, AAM/protein 2500:1 mol/mol; crosslinker/protein 1000:1 mol/mol). Ammonium persulfate (APS/protein 500:1 mol/mol) and tetramethylethylenediamine (TEMED/APS 2:1 w/w) were added to the protein/APM/AAM/BAC mixture. The reaction was tightly sealed and shaken at room temperature for 2 h. After which, the reaction was passed through a Illustra NAP^{TM}-5 column (GE Healthcare) to remove low molar mass reagents. The protein nanocapsules prepared with the above protocol were then subjected to DLS measurements, zeta potential measurements and TEM analysis. The protein nanocapsule concentration was determined using Pierce™ BCA Protein Assay Kit (Thermo) before use. *Note: All protein nanocapsule concentrations were expressed using protein concentrations as reference. As such, 1 mg/mL of protein nanocapsule refers to BCA-determined 1 mg/mL concentration and not the actual weight of the protein nanocapsule.*

Determining Dox to RNase A-BB ratio in dual-cargo protein nanocapsules: For 0.1 mg/mL $^{TPP}n_{ss}$(RNase A-BB^{FITC}/Dox) dual cargo nanocapsule, the fluorescence reading of Dox was 9960 (Fig. 3B).

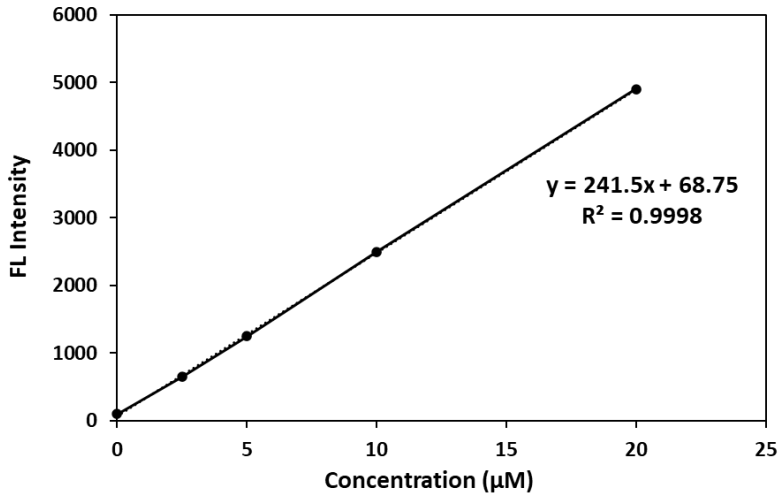
Dox concentration in 0.1 mg/mL $^{TPP}n_{ss}$(RNase A-BB^{FITC}/Dox) can be determined by the equation, $y = 241.5\,x + 68.75$ obtained from the calibration graph above.

$9960 = 241.5\,x + 68.75$

Dox concentration in 0.1 mg/mL $^{TPP}n_{ss}$(RNase A-BB^{FITC}/Dox) = 40.9 µM

RNase A-BB concentration in 0.1 mg/mL $^{TPP}n_{ss}$(RNase A-BB^{FITC}/Dox)

= 0.1 mg / 13.7 kDa = 7.3 µM
Ratio of Dox:RNase A-BB = 40.9 / 7.3 ≈ **5.60**



Graph S2. *Calibration graph for Doxorubicin.*

Determining cargo loading capacity in nanocapsule: Following $^{TPP}n_{ss}$(RNase A-BB/Dox) dual cargo nanogel synthesis and purification, the eluate obtained after purification was lyophilized to determine the overall nanocapsule weight. Prior to lyophilization, the concentration of protein (RNase A-BB) was determined using BCA Protein Assay Kit (Thermo) and converted into mass (mg). The concentration of Dox was determined *via* fluorescence measurement readings using Graph S2 and converted into mass (mg). The loading capacity of RNase A-BB and Dox was determined using %*wt/wt* of RNase A-BB or Dox in the nanocapsules after freeze drying.

	Mass (mg)	Loading Capacity	Input Amount (Final Concentration)	Loading Efficiency
Mass of dual cargo nanocapsule after lyophilization	5.01			
Mass of protein	0.60	12.0% *wt/wt*	1 mg/mL (1 mL)	60.0%
Mass of Dox	0.14	2.8% *wt/wt*	1.4 mM (1 mL)	16.9%

Table S1. *Loading capacity and loading efficiency of dual cargo nanocapsules.*

Determining number of TPP on dual-cargo protein nanocapsules: 0.5 mg/mL of protein nanocapsules was reacted with 20 equiv. of TCO-PEG$_4$-NHS (25 mM stock in DMSO) at rt for 16 h. The reaction was purified using Illustra NAP^{TM}-5 column (GE Healthcare) to remove excess small molecules. Instead of Tz-TPP, the eluate was clicked with 20 equiv. of Tz-TER (50 mM stock in DMSO) at room temperature for another 16 h. The reaction was subsequently purified using Illustra NAP^{TM}-5 column (GE Healthcare) and lyophilized.
The mass of RNase A is 13.7 kDa. Since the loading capacity of RNase A-BB in $^{TPP}n_{ss}$(RNase

A-BB/Dox) dual cargo nanocapsule was previously determined to be 12% *wt/wt*, we assume average mass of a n_{ss}(RNase A-BB/Dox) nanocapsule to be 114 kDa.

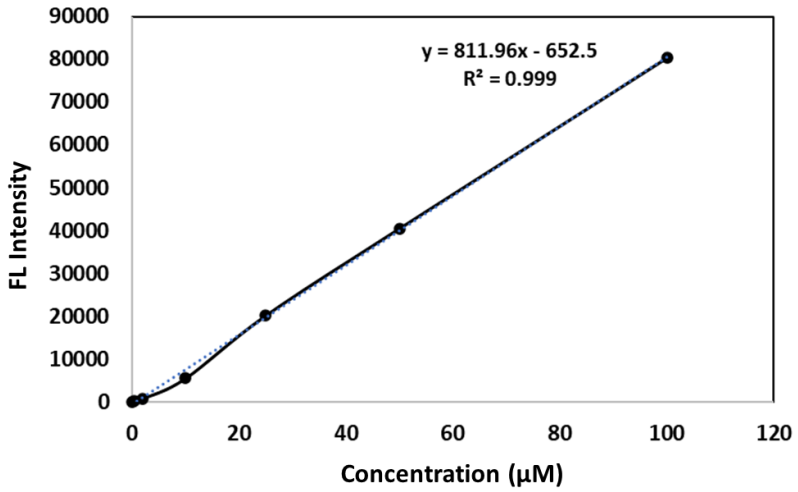
Number of mols of $^{TER}n_{ss}$(RNase A-BB/Dox) in 1 mg/mL solution (by weight of nanocapsule) = 1 mg/114 kDa = 8.77 µM.

Following 10x dilution, fluorescence intensity of 0.1 mg/mL $^{TER}n_{ss}$(RNase A-BB/Dox) is 45280. TER concentration can be determined from the calibration curve of Tz-TER (Graph S3) using the following equation obtained, y = 811.89x – 652.5.
45280 = 811.89x – 652.5, x = 56.5
Number of mols of Tz-TER in 1.0 mg/mL solution $^{TER}n_{ss}$(RNase A-BB/Dox)
= 10 x 56.5 µM = 565 µM

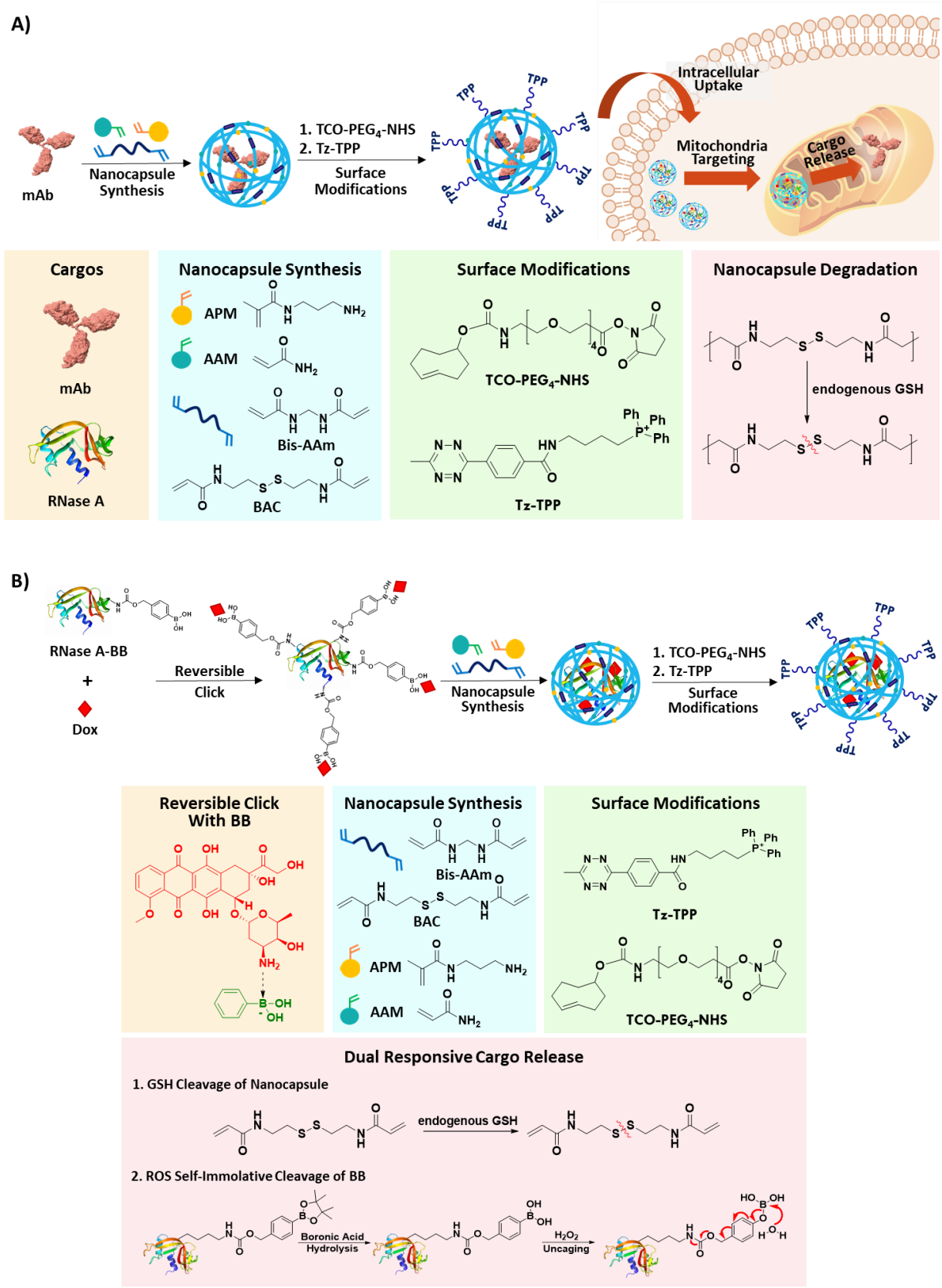
Average ratio of TER:$^{TER}n_{ss}$(RNase A-BB/Dox) = 565:8.77 = 64.4

Hence, we estimated that **64** Tz-TPP can similarly be clicked onto the surface of $^{TPP}n_{ss}$(RNase A-BB/Dox).

Note: The number of TPP modified on the surface of $^{TPP}n_{ss}$(RNase A-BB/Dox) can be tuned by varying the Tz-TPP ratio used.



Graph S3. *Calibration graph for Tz-TER.*



Scheme S3. *Schematic summarizing the preparation of A) TPP-modified protein nanocapsules and B) TPP-modified dual cargo protein nanocapsules.*

TEM measurement: TEM images were obtained on a JEOL 3011 transmission electron microscopy. 10 μL of 1 mg/mL protein nanocapsule was dropped on a copper grid. After 5 min, the solution was drawn off from the edge of the grid with filter paper. The grid was then stored for TEM observation. TEM images are acquired with an acceleration voltage of 300 kV and magnification of 10 000 to 30 000.

DLS measurement: DLS measurements were performed on a Litesizer^{TM} 500 Particle Analyzer (Anton-Parr). Native proteins and protein nanocapsules were prepared at 1 mg/mL in water and filtered through 0.22 mm membranes. Experiments were performed at 25 °C and 10 readouts were taken in three independent measurements for each sample.

Zeta potential measurement: Zeta-potential measurements were performed on a Litesizer^{TM} 500 Particle Analyzer (Anton-Parr). Native proteins and protein nanocapsules were prepared at 0.5 mg/mL in water and filtered through 0.22 mm membranes. Experiments were performed at 25 °C and readouts were taken in three independent measurements for each sample.

Fluorescence spectra measurement: Fluorescence measurements were performed on a BioTek Synergy 4 microplate reader. Proteins and protein nanocapsules were prepared at a concentration of 0.1-0.5 mg/mL in water. Experiments were performed at 25 °C and readouts were taken in three independent measurements for each sample (FITC: λ_{ex} = 490 nm, λ_{em} = 520 nm; Dox: λ_{ex} = 470 nm, λ_{em} = 595 nm).

In vitro release of* n_{ss}(RNase A^{FITC}) *under GSH stimulus: To 200 μL of n_{ss}(RNase A^{FITC}) solutions (0.5 mg/mL), GSH (final concentration 10 mM) was added. The mixture was incubated at 37 ºC. At different time points, a 20 μL aliquot was transferred to a microcentrifuge tube for storage at -80 ºC. After all the aliquots were collected, the degradation was visualized with SDS-PAGE gel electrophoresis/in-gel fluorescence scanning to determine the release profile. n(RNase A) sample was used as negative control. The same procedure was carried out for dual cargo n_{ss}(RNase A-BB^{FITC}/Dox). No DTT was added in the loading dye used for sample loading.

In vitro release of* $^{TPP}n_{ss}$*(anti-MTCO2) under GSH stimulus*:* To 100 μL of $^{TPP}n_{ss}$(*anti*-MTCO2) solution (0.1 mg/mL), GSH (final concentration 10 mM) was added. The mixture was incubated at 37 ºC for 36 h. The resulting samples were separated by 12% SDS-PAGE gel and visualised via Coomassie Brilliant Blue (CBB) staining as well as Western-blotting. No DTT was added in the loading dye used for sample loading. For WB, the samples were transferred to a PVDF membrane. The membrane was subsequently blocked by using 5% BSA (in 1x TBST buffer), followed by incubation with the appropriate HRP-conjugated secondary antibodies prior to image acquisition on Image Quant LAS 500. *anti*-MTCO2 antibody was loaded as control. Note: Upon successful release of antibody from the nanocapsule, positive signals should be observed on WB.

In vitro release of RNase A-BB^{FITC} from dual cargo nanocapsule under GSH stimulus[5]: 3 mL of 1 mg/mL $^{TPP}n_{ss}$(RNase A-BB^{FITC}/Dox) was added into the dialysis tubing (50 K MWCO) and

immersed in 20 mL of PBS containing GSH (10 mM) at 37 °C. At scheduled time intervals, the PBS was sampled. The fluorescent intensity of RNase A-BB^{FITC} was determined at the emission wavelength of 520 nm upon excitation at 490 nm.

In vitro release of Dox from dual cargo nanocapsule under GSH stimulus[5]: 3 mL of 1 mg/mL $^{TPP}n_{ss}$(RNase A-BB/Dox) was added into the dialysis tubing (10 K MWCO) and immersed in 20 mL of PBS containing GSH (10 mM) at 37 °C. At scheduled time intervals, the PBS was sampled. The fluorescent intensity of Dox was determined at the emission wavelength of 595 nm upon excitation at 495 nm.

***Live-cell imaging*:** For CLSM imaging of protein nanocapsule cellular uptake experiments, cells were seeded in Ibidi µ-Slide 8-well ibi-Treat imaging dish (Sciencewerke Pte. Ltd.) and grown overnight. Upon medium removal, cells were treated with ^{TPP}n(RNase A^{FITC}), $^{TPP}n_{ss}$(RNase A^{FITC}) or RNase A^{FITC} as the negative control (500 nM, 24 to 48 h). Following which, cells were washed with PBS buffer, followed by staining with MitoTracker^{TM} Deep Red (25 nM) or LysoTracker^{TM} Red DND-99 (100 nM) and Hoechst 33342 for 10 min before image acquisition on an Olympus FV3000 confocal microscope equipped with a 60× water immersed objective (Hoechst: λ_{ex} = 405 nm, λ_{em} = 415-475 nm; FITC: λ_{ex} = 488 nm, λ_{em} = 500-550 nm, LysoTracker^{TM} Red: λ_{ex} = 561 nm, λ_{em} = 580-650 nm, MitoTracker^{TM} Deep Red: λ_{ex} = 640 nm, λ_{em} = 655-755 nm, Dox: λ_{ex} = 470 nm, λ_{em} = 595 nm). The corresponding fluorescence intensity profiles were analyzed by Image *J* software. Note: The above protocol was similarly carried out for cellular uptake and subcellular localization studies of $^{TPP}n_{ss}$(*anti*-MTOC2) and $^{TPP}n_{ss}$(*RNase A-BB*^{FITC}*/Dox*).

Separation of mitochondrial fractions: Cells were seeded in 12-well plate (Greiner CELLSTAR®) and grown overnight. Upon medium removal, cells were treated with different protein nanocapsules (500 nM) for 48 h before washing with PBS buffer twice. After that, 200 µL of mitochondria isolation buffer (10 mM Tris-HCl, 0.3M sucrose, 0.5 mM EDTA, *p*H 7.4) with proteinase inhibitor was added to each well to lyse the cells. Mitochondria were released from cells by using 27G needle to facilitate cell breaking till 90% broken cells were observed under the microscope. To remove the unbroken cells, the resulting lysates were centrifuged (at 1000 g) at 4 ºC for 5 min before collection of supernatants to new centrifuge tubes. The supernatants were then centrifuged (at 8000 g) at 4 ºC for 20 min to obtain the new supernatant and pellets, which corresponded to the cytosolic and mitochondrial fractions, respectively. For the fluorescence measurement of mitochondrial and cytosolic fractions, 200 µL of PBS buffer was added to the pellet before the analysis. The supernatant was diluted to 200 µL using PBS buffer and run concurrently for comparison.

Western blotting analysis (WB). The obtained mitochondrial and cytosolic fractions were lysed in DTT-free loading buffer with proteinase inhibitor at 95 °C for 10 min. After that, the resulting samples were separated by 15% SDS-PAGE gel before being transferred to a PVDF membrane. The membrane was subsequently blocked by using 5% BSA (in TBST buffer), followed by incubation with the appropriate primary and secondary antibodies prior to image

acquisition on Image Quant LAS 500. *Anti*-TOMM20 (mitochondrial marker) and *anti*-actin (cytosolic maker) were applied to confirm the mitochondrial separation.

FACS experiments： HeLa cells were seeded in a 12-well plate (Greiner CELLSTAR®) and cultured overnight. Upon medium removal, the cells were treated with 500 nM of n(RNase A^{FITC}) in 0.5 mL DMEM at 37 ºC and 4 ºC. The cells were incubated for 24 h, and subsequently washed three times with PBS. The resulting cells were detached from the plate by treatment with 200 μL of 0.1% trypsin-EDTA at 37 °C for 2 min. The detached cells were collected by centrifugation. Upon further washing with cold PBS (500 μL) thrice, the cells were resuspended in PBS (1 mL). Cells were analyzed on a BD Accuri™ C6 cell analyzer (10,000 cells were counted for each event; in duplicates) using λ_{ex} = 488 nm, λ_{em} = 525 ± 40 nm (FL1) for FITC.

Intracellular ROS measurement: The intracellular ROS level of cancerous and non-cancerous cell lines were determined using 2',7'–dichlorofluorescin diacetate (DCFDA) staining, followed by flow cytometry analysis. Briefly, cells were seeded in a 12-well plate at a density of 150,000 cells per well one day before the experiment. Cells were washed once with PBS (500 μL), followed by 30 min. of incubation with 10 μM DCFDA in DMEM. The cells were harvested and washed with PBS before flow cytometry analysis. Cells were analyzed on a BD Accuri™ C6 cell analyzer (10,000 cells were counted for each event in duplicates) using λ_{ex} = 488 nm, λ_{em} = 525 ± 40 nm (FL1).

In vitro activity of RNase A: The enzymatic activity of n_{ss}(RNase A-BB/Dox) was studied *in vitro* first to evaluate whether RNase A function could be inhibited by both BB labeling and nanocapsule encapsulation, and subsequently restored by subsequent GSH and H_2O_2 release. Briefly, 0.5 μL of 0.26 μg/μL native RNase A, RNase A-BB, n_{ss}(RNase A-BB/Dox) and n(RNase A-BB/Dox) was treated with 10 mM GSH (24 h), followed by 10 mM H_2O_2 (12 h) at 37 ºC. The above mixture was diluted to 0.025 ng/mL and reacted with the RNaseAlert™ Substrate (IDT). Kinetic readings of the green-fluorescence substrate (λ_{ex} = 485 ± 20 nm, λ_{em} = 528 ± 20 nm) were recorded at 37 ºC by using a BioTek Synergy 4 microplate reader.

MTT Assay for combination therapy after intracellular delivery: To determine the viability of cells treated with $^{TPP}n_{ss}$(RNase A-BB/Dox), HeLa cells (10,000 cells per well) were incubated with each sample at the desired concentrations in a 96 well plate at 37 °C. Negative control experiments were done with ^{TPP}n(RNase A-BB/Dox), $^{TPP}n_{ss}$(RNase A-BB) and n_{ss}(RNase A-BB/Dox). 0 to 800 nM of n_{ss}(RNase A-BB/Dox) (final concentration) in 100 μL of DMEM was incubated with HeLa cells grown in a 96-well plate (~30% confluency) in triplicates. After 72 h incubation, the cells were washed with PBS thrice, replaced with fresh culture medium before viability measurement using the MTT colorimetric cell proliferation kit (Roche) following manufacturer's guidelines.

MTT Assay for cytotoxicity: To determine the toxicity of the nanocapsules, HeLa cells were treated with n(RNase A) or different ^{TPP}n(RNase A). 0 to 5 μM of ^{TPP}n(RNase A) (final

concentration) in 100 μL of DMEM was incubated with HeLa cells grown in a 96-well plate (~30% confluency) at 37 °C in triplicates. After 72 h incubation, the cells were washed with PBS thrice, replaced with fresh culture medium before viability measurement using the MTT colorimetric cell proliferation kit (Roche) following manufacturer's guidelines.

5. Results and Discussion

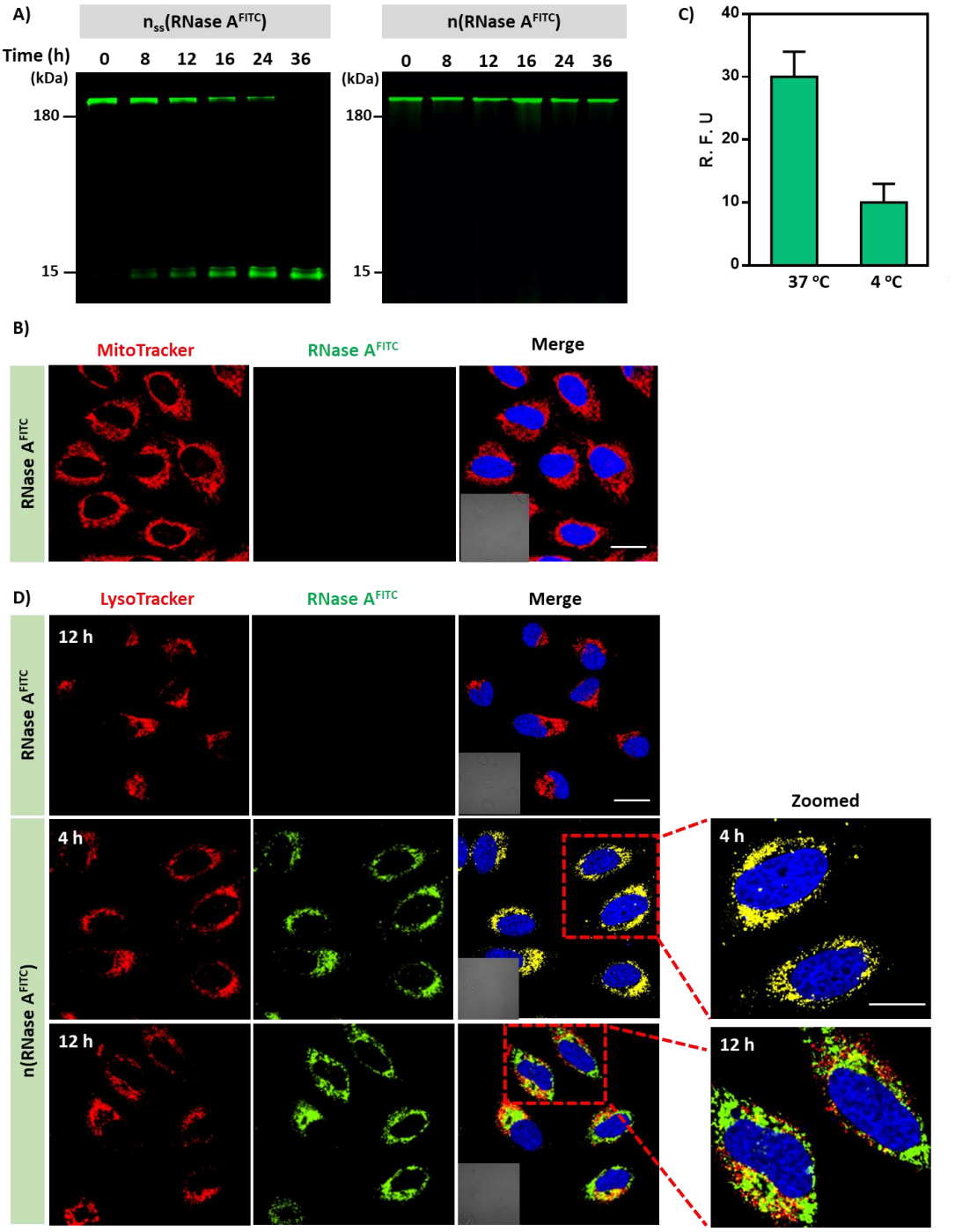


Fig. S1 A) *In vitro* release of RNase A^{FITC} from n$_{ss}$(RNase A^{FITC}) and n(RNase A^{FITC}) in the presence of GSH (10 mM, 37 °C) at different time points. B) CLSM images of live HeLa cells treated with RNase A^{FITC} (500 nM, 48 h). The cells were subsequently stained with MitoTracker Deep Red. (Blue: Hoechst; Green: FITC; Red: MitoTracker). Scale bar = 20 µm. C) FACS of HeLa cells treated with n(RNase A^{FITC}) (500 nM, 24 h) to determine the effect of temperature (37 °C and 4 °C) on endocytosis-dependent cellular uptake. D) CLSM images of live HeLa cells treated with n(RNase A^{FITC}) (500 nM, 4 h and 12 h). (Blue: Hoechst; Green: FITC; Red: LysoTracker). Scale bar = 20 µm.

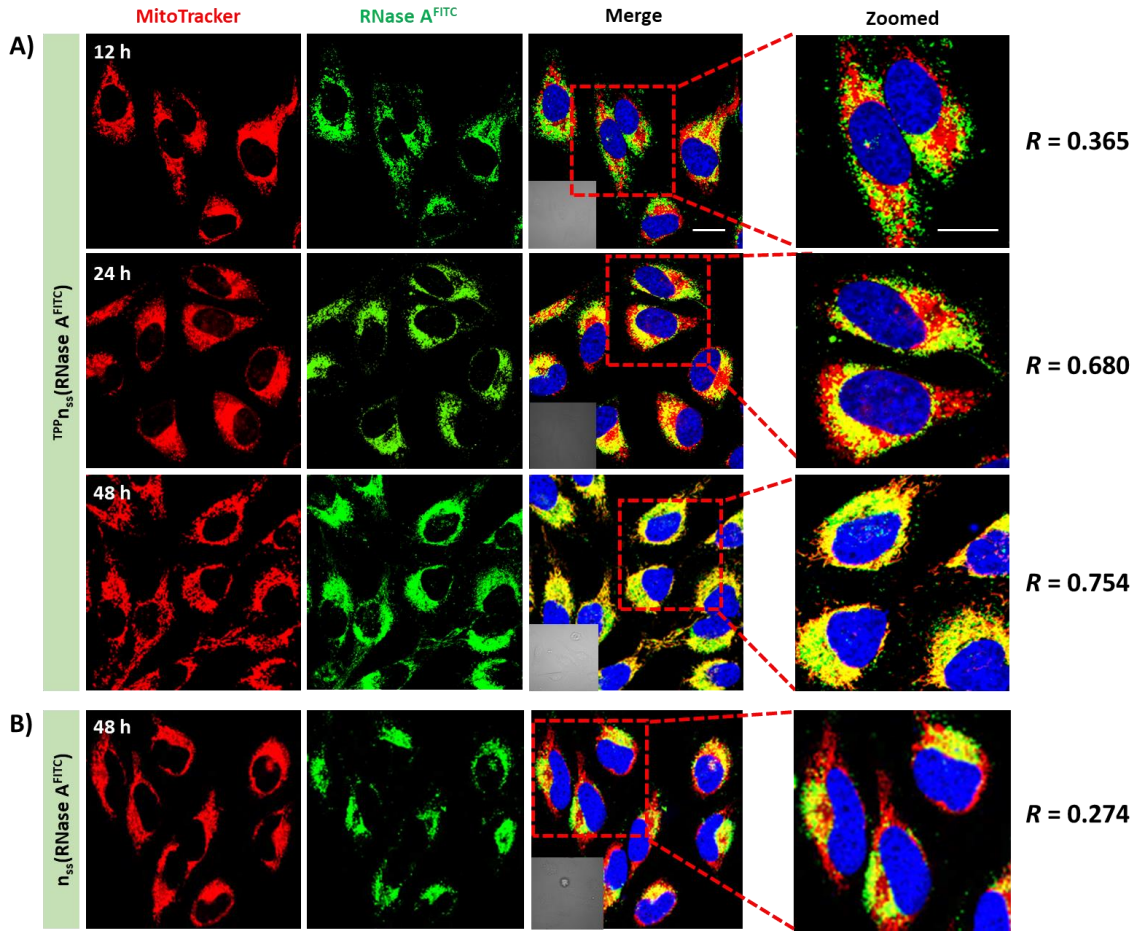


Fig. S2 A) CLSM images of live HeLa cells treated with $^{TPP}n_{ss}$(RNase A^{FITC}) and B) n_{ss}(RNase A^{FITC}) (500 nM, 12 to 48 h). The cells were subsequently stained with MitoTrackerDeep Red. (Blue: Hoechst; Green: FITC; Red: MitoTracker). Scale bar = 20 μm. The Global Pearson's *R* values for the merged images are indicated respectively. **Fig. S2A** (bottom row) and **Fig. S2B** were partially reproduced in **Fig. 2E**.

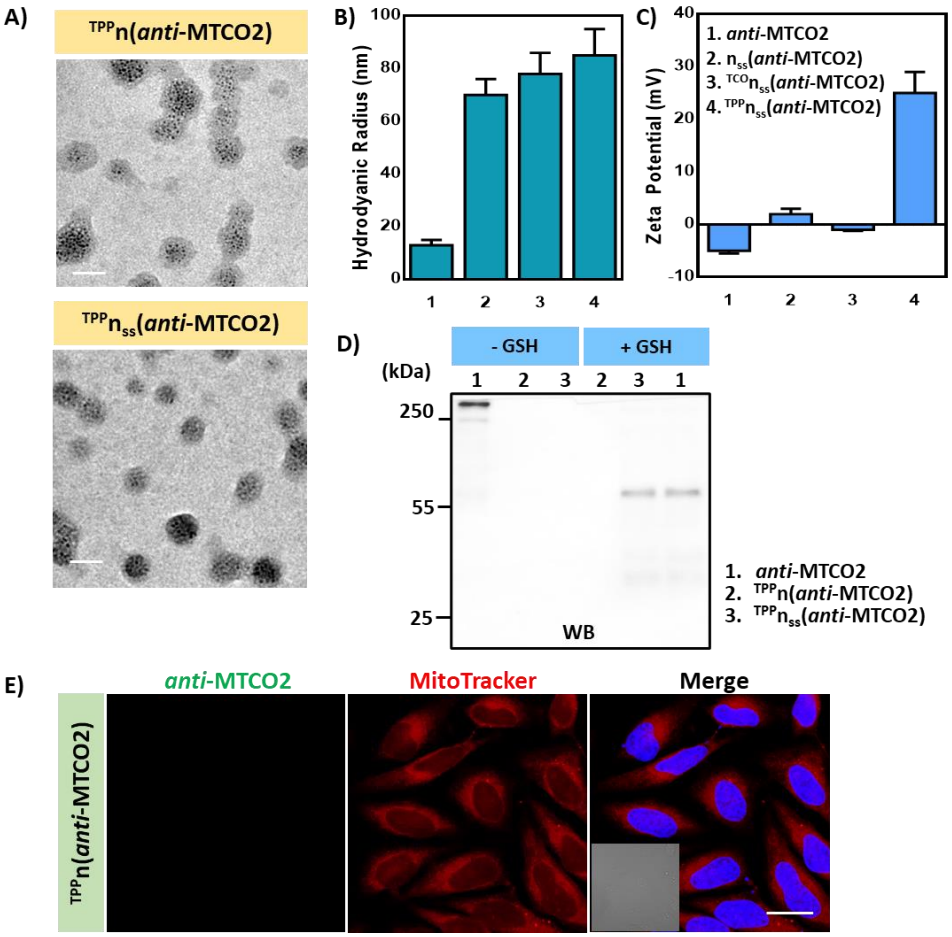


Fig. S3 A) TEM of ^{TPP}n(*anti*-MTCO2) and ^{TPP}n$_{ss}$(*anti*-MTCO2). Scale bar = 50 nm. B) Hydrodynamic radius and C) Zeta potential of 1. *anti*-MTCO2, 2. n$_{ss}$(*anti*-MTCO2), 3.^{TCO}n$_{ss}$(*anti*-MTCO2) and 4. ^{TPP}n$_{ss}$(*anti*-MTCO2). D) SDS-PAGE analysis followed by Western Blot of 1. *anti*-MTCO2 antibody, 2.^{TPP}n(*anti*-MTCO2), 3. ^{TPP}n$_{ss}$(*anti*-MTCO2) treated with/without 10 mM GSH (36 h, 37 °C). Samples were loaded onto SDS-PAGE gel using loading dye without DTT. Upon successful release of antibody from the nanocapsule, positive signals should be observed on WB. E) CLSM images of live HeLa cells treated with ^{TPP}n(*anti*-MTCO2) (500 nM, 48 h), prior to fixation and incubation with FITC-conjugated goat anti-mouse IgG (H+L) secondary antibody. Scale bar = 20 µm.

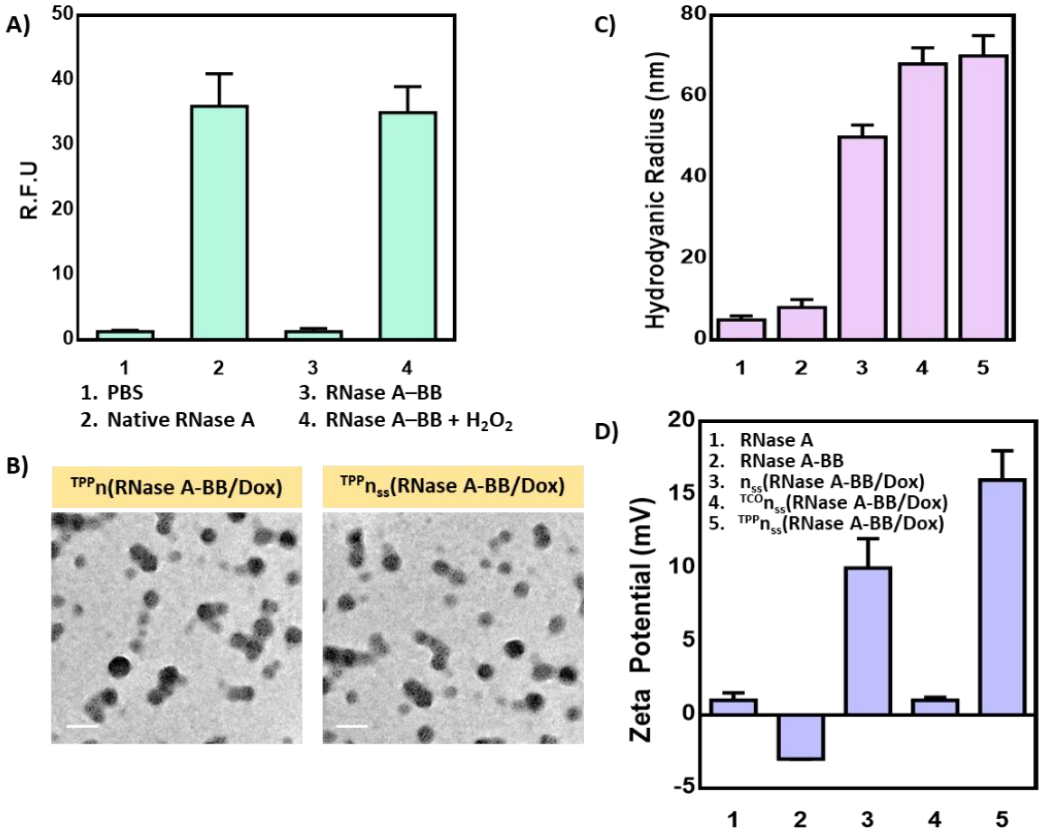


Fig. S4 A) Fluorescence of Alizarin Red S (ARS, 0.0025% w/v) in the presence of 1. PBS, 2. Native RNase A, 3. RNase A–BB and 4. RNase A–BB treated with H_2O_2 (10 mM, 37 °C, 3 h). B) TEM of ^{TPP}n(RNase A-BB/Dox) and $^{TPP}n_{ss}$(RNase A-BB/Dox). Scale bar = 100 nm. C) Hydrodynamic radius and D) Zeta potential of 1. RNase A, 2. RNase A-BB, 3. n_{ss}(RNase A-BB/Dox), 4.$^{TCO}n_{ss}$(RNase A-BB/Dox) and 5. $^{TPP}n_{ss}$(RNase A-BB/Dox).

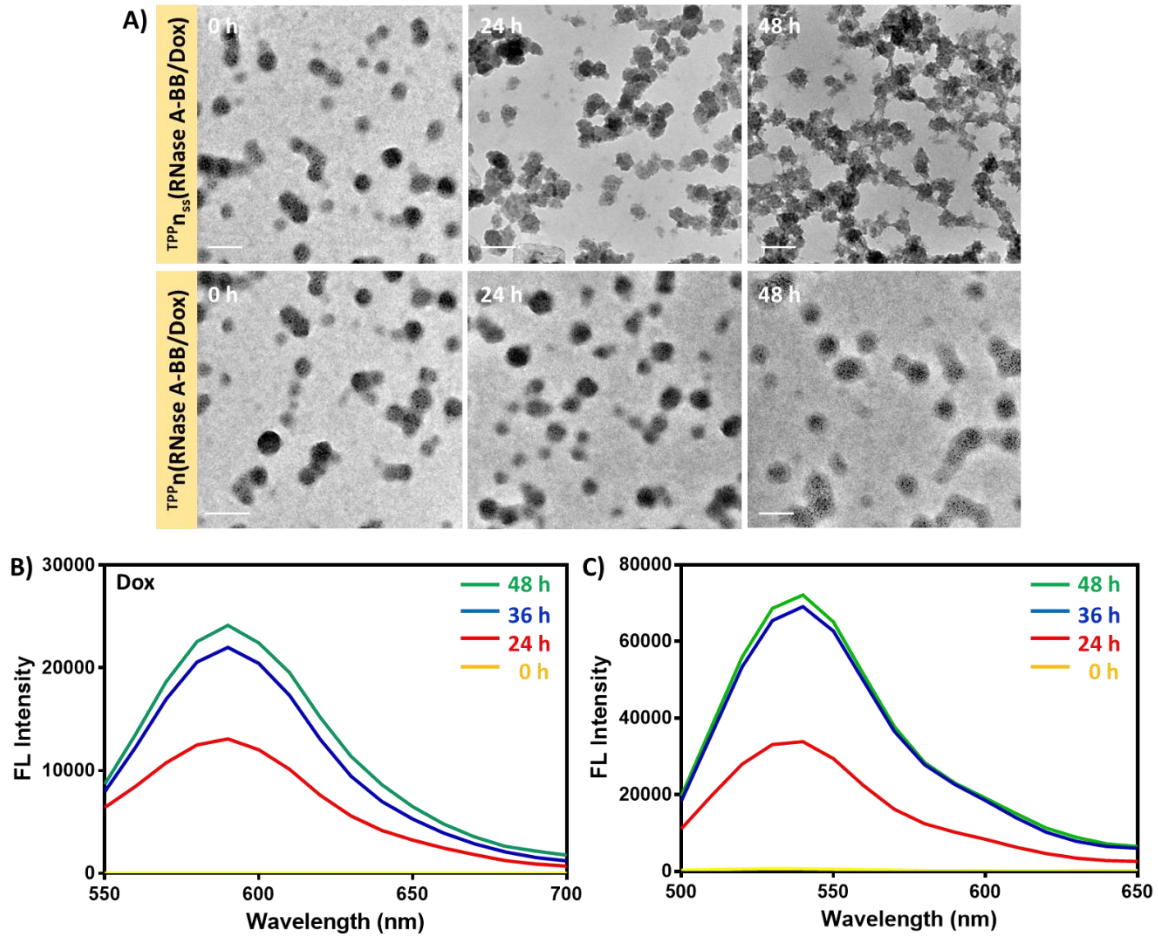


Fig. S5 TEM of $^{TPP}n_{ss}$(RNase A-BB/Dox) and ^{TPP}n(RNase A-BB/Dox) treated with GSH (10 mM, 37 °C, 0 to 48 h). Scale bar = 100 nm. B) Release profiles of Dox and C) RNase A^{FITC} from $^{TPP}n_{ss}$(RNase A-BB^{FITC}/Dox) treated with GSH (10 mM, 37 °C, 0 to 48 h). The release study was performed using a dialysis method.[5] The MWCO of the dialysis tubing were 50 K and 10 K for RNase A^{FITC} and Dox, respectively.

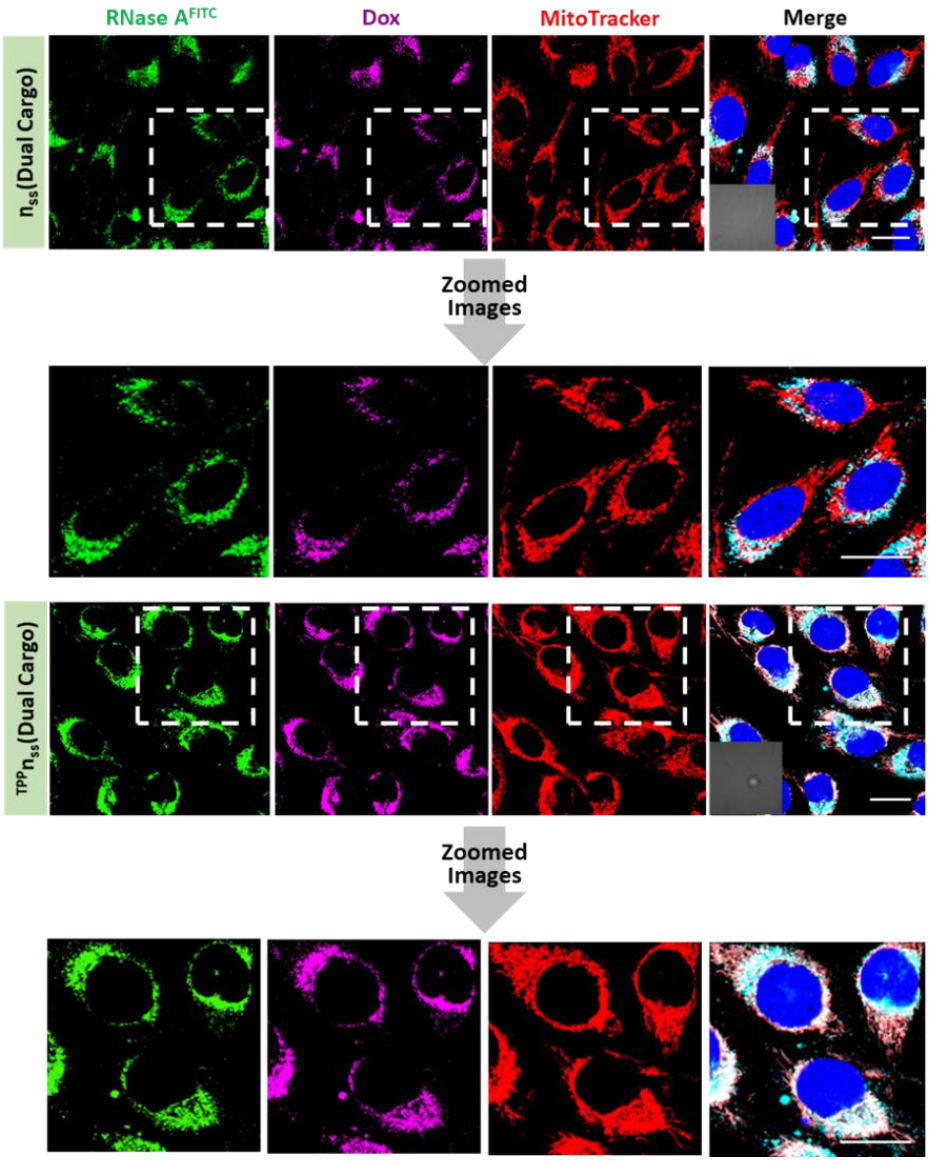


Fig. S6 CLSM of live HeLa cells treated with $^{TPP}n_{ss}$(RNase A-BB^{FITC}/Dox) and n_{ss}(RNase A-BB^{FITC}/Dox) (500 nM, 24h). (Blue: Hoechst; Green: FITC; Purple: Dox; Red: MitoTracker). Scale bar =20 μm. **Fig. S6** was partially reproduced in **Fig. 3C**.

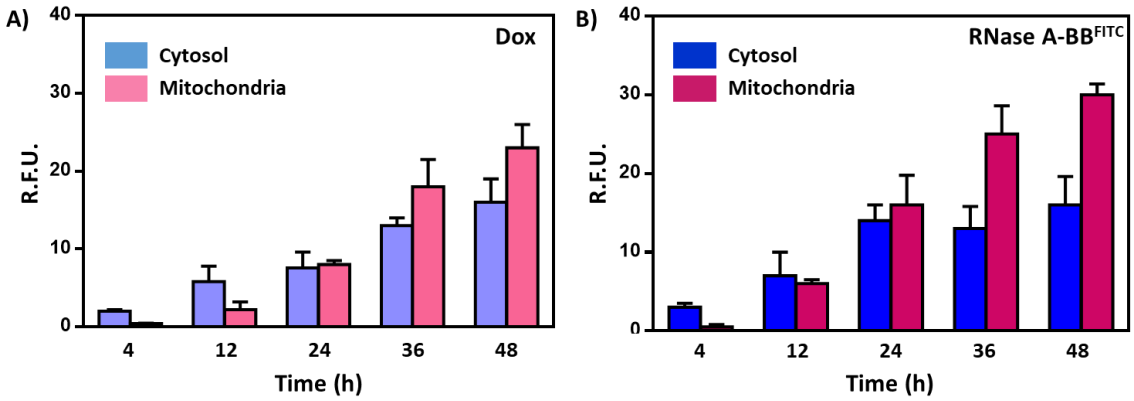


Fig. S7 A) Fluorescence measurement of Dox and B) RNase A-BB^{FITC} in cytosolic and mitochondrial fractions isolated from lysates of Hela cells treated with $^{TPP}n_{ss}$(RNase A-BB/Dox) (500 nM, 0 to 48 h).

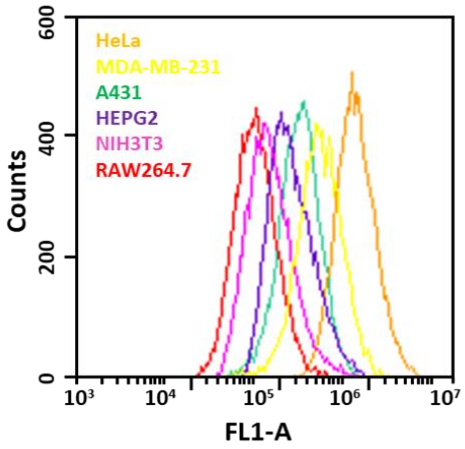


Fig. S8 FACS analysis of intracellular ROS levels in non-cancerous and cancer cells determined by using 10 µM DCFDA. The R.F.U of each sample was normalized against HeLa cells (set as 1).

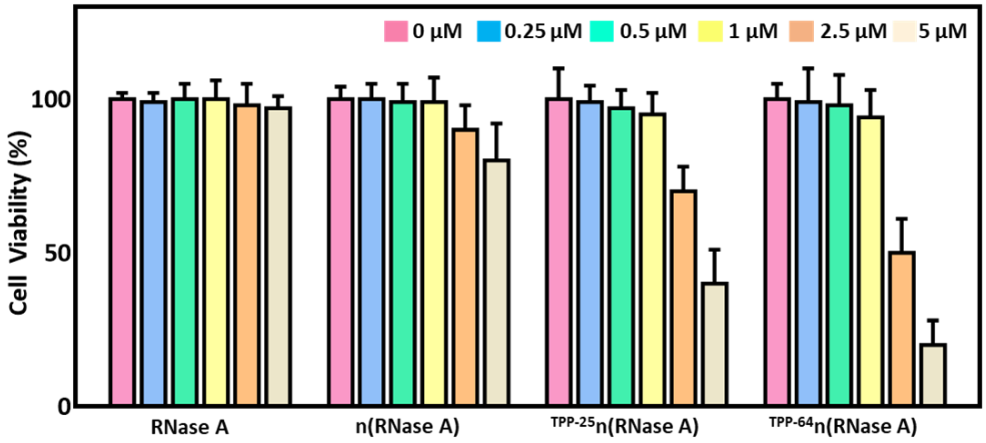
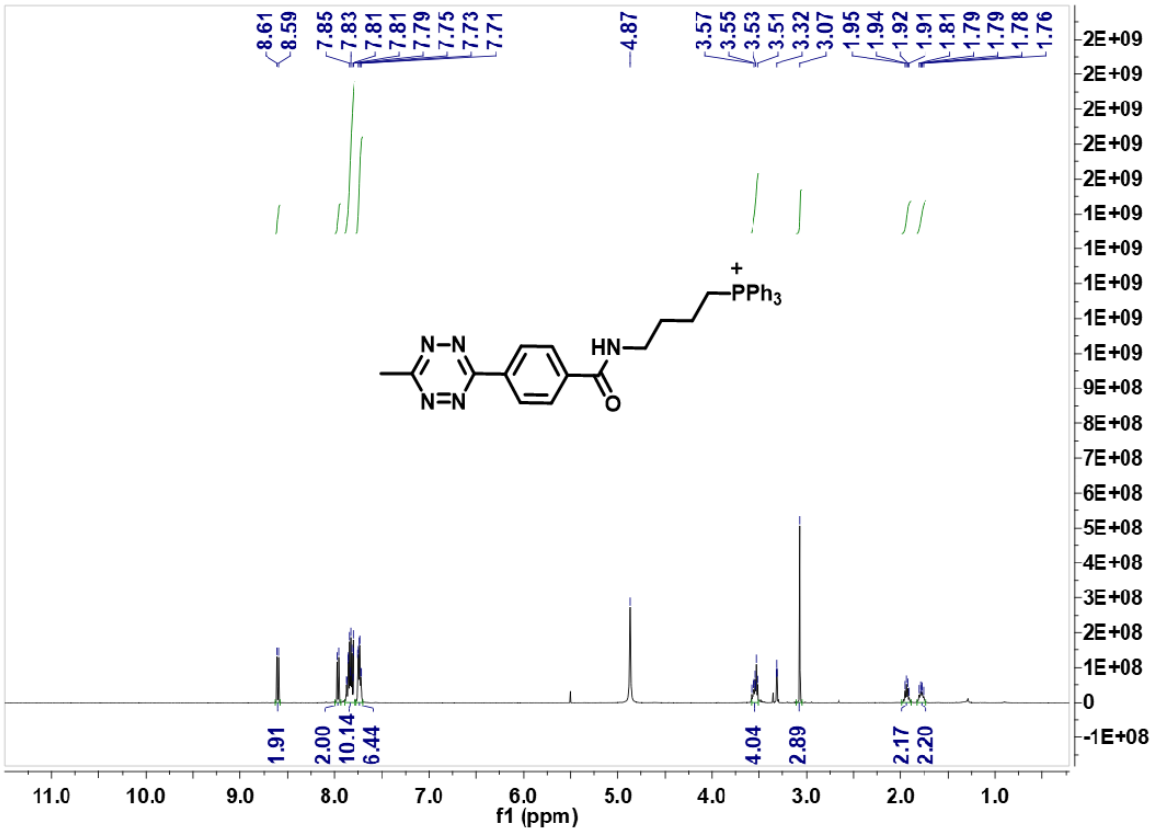
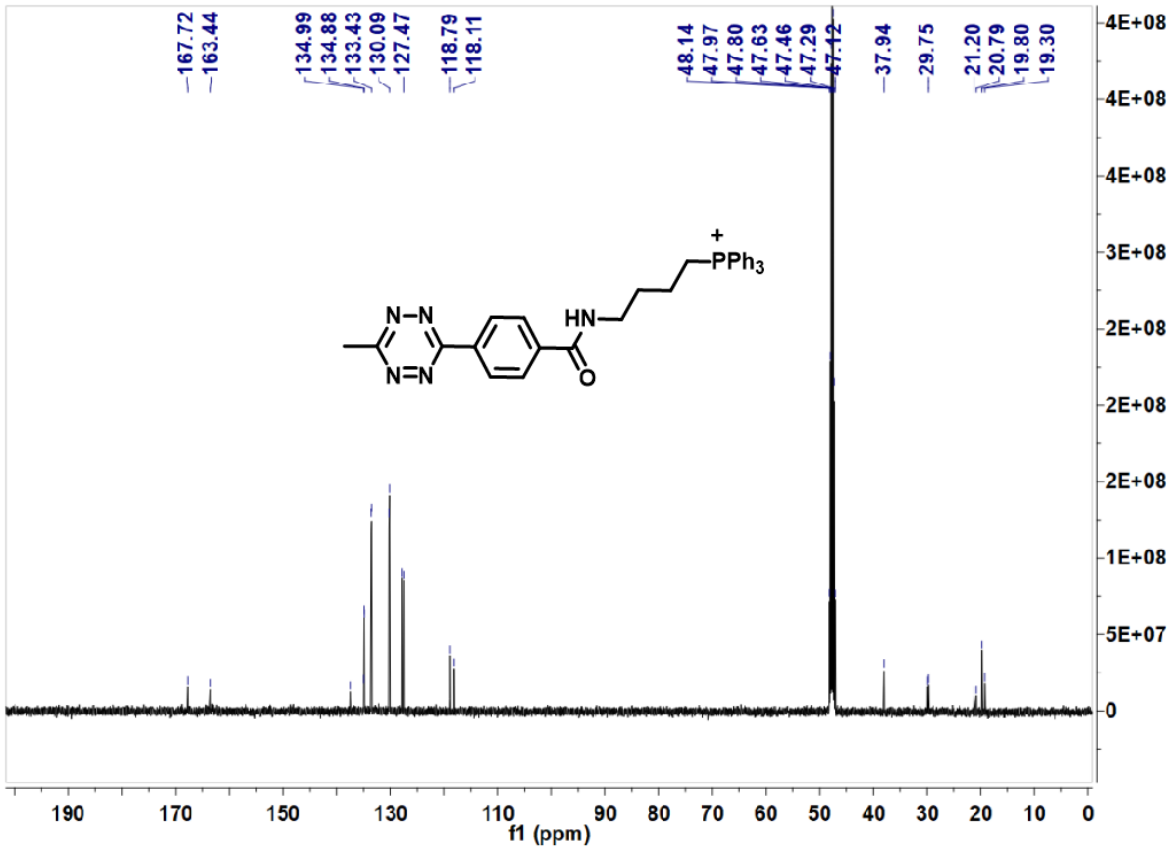


Fig. S9 Cytotoxicity of RNase A, n(RNase A) and TPP-modified n(RNase A) nanocapsules when incubated with HeLa cells (0 to 5 µM, 72 h). $^{TPP\text{-}25}$n(RNase A) and $^{TPP\text{-}64}$n(RNase A) refer to RNase A-loaded nanocapsules with 25 and 64 TPPs modified on them, respectively.

6. ^{1}H and ^{13}C NMR





7. References

1. B. Peng, A. G. Thorsell, T. Karlberg, H. Schuler and S. Q. Yao, *Angew. Chem. Int. Ed*. 2017, **56**, 248-253.
2. H. Yuan, H. Cho, H. H. Chen, M. Panagia, D. E. Sosnovik and L. Josephson, *Chem. Commun.* 2013, **49**, 10361-10363.
3. M. Wang, S. Sun, C. I. Neufeld, B. Perez-Ramirez and Q. Xu, *Angew. Chem. Int. Ed.* 2014, **53**, 13444-13448.
4. G. Springsteen and B. Wang, *Chem. Commun.* 2001, 1608-1609.
5. M. Liu, S. Shen, D. Wen, M. Li, T. Li, X. Chen, Z. Gu and R. Mo, *Nano Lett*. 2018, **18**, 2294-2303.