Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

In situ activation of a doxorubicin prodrug using imaging-capable nanoparticles

Irfan Khan[a], Paul F. Agris[a], Mehmet V. Yigit[a] and Maksim Royzen[a]**

University at Albany, Department of Chemistry and The RNA Institute,

1400 Washington Ave., Albany, NY 12222, USA.

Email: mroyzen@albany.edu or myigit@albany.edu

Table of contents: **Page:**

Materials and Methods:

Materials.

Doxorubicin was purchased from LC Laboratories (Woburn, MA), cat.# D-4000. Bis-PEG3-NHS ester was purchased from Broadpharm, cat.# BP-20611. Cy5.5-mono NHS ester was purchased from GE Healthcare, cat.# PA15601. All other chemicals were purchased from Krackeler Scientific and used without further purification. Chromatographic purifications were conducted using SiliaSphere™ spherical silica gel 5μm, 60 Å silica gel (Silicycle). Thin layer chromatography (TLC) was performed on SiliaPlate™ silica gel TLC plates (250 μm thickness) purchased from Silicycle. Preparative TLC was performed on SiliaPlate™ silica gel TLC plates (1000 μm thickness). HPLC purification was performed using Phenomenex Luna 5u C18(2) semi-preparative column (250 x 10 mm). ^{1}H and ^{13}C NMR spectroscopy was performed on a Bruker NMR at 400 (^{1}H), 100 (^{13}C) MHz and 162 (^{31}P) MHz. All ^{13}C NMR spectra were proton decoupled. Fluorescence microscopy experiments were carried out using Zeiss LSM 710 Pascal laser confocal microscope (Carl Zeiss Microscopy, Thornwood, NY, USA). Image acquisition and analyses were performed using Zeiss ZEN 2012 Confocal Microscopy Software (Release 2.02). Caspase-3 assay kit that was purchased from ENZO Life Sciences, cat.# BML-AK703. The MTT reagent was purchased from Sigma-Aldrich, cat.# M5655. Nanoparticle purifications were done using Sephadex PD-10 columns, purchased from GE Healthcare, cat.# 17-0851-01.

Methods.

Cell culture. MDA-MB 231 cells were purchased from ATCC (cat.# HTB-26), and propagated in Dulbecco's modified Eagle's medium (DMEM; Meditech, Inc. Corning, Manassas, VA) containing 5% fetal bovine serum (FBS; HyClone, Logan Utah), supplemented with 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Tech Corp., Grand Island, NY) at 37°C in a 5% CO_2 incubator.

Synthesis of dextran-coated magnetic nanoparticles (MNPs). The magnetic nanoparticles were synthesized using our previously published protocol.[1-3] Briefly, (18 g) of Dextan-T10 (Pharmacosmos, Holbaek, Denmark) was mixed in 60 mL of double-distilled water and stirred in

a round bottom flask in an ice bath. $FeCl_3.6H_2O$ (1.3 g) was added while flushing nitrogen gas into the reaction mixture. $FeCl_2.4H_2O$ (0.8 g) was added into the reaction mixture and then 30 mL of concentrated cold NH_4OH (~28%) was added to the stirring mixture. The temperature was increased to 75–85 ºC for an hour. The mixture was cooled to room temperature afterwards and concentrated to 40 mL using Amicon Ultra centrifugal units (MWCO 100 kDa; Millipore, Billerica, MA, USA). The dextran coating on the nanoparticles was cross-linked with the addition of 70 mL of 5 M NaOH and 28 mL of concentrated epichlorohydrin (cat.# 45330-1L-F, Sigma Aldrich) and the mixture was stirred for 8 hours. Concentrated NH_4OH (120 mL) was added to the stirring mixture. The nanoparticle solution was purified using a dialysis bag (MWCO 14 kDa) against water and suspended in 20 mM citrate buffer (pH 8.0). The nanoparticle concentration was determined based on iron concentration, measured by UV-vis spectroscopy and determined to be ~10.0 mg/ml^{-1} Fe.

Calculation of the number of amine groups per nanoparticle. N-Succinimidyl-3-(2-pyridyldithio)propionate (SPDP) is a bifunctional linker which can be used to estimate the number of amine per nanoparticle by coupling to the nanoparticles.[1-6] Stock solution of SPDP (150 mM) was prepared by dissolving SPDP in 1 ml of DMSO. 0.5 ml of nanoparticle (3.7 mg/ml) in PBS (pH 7.4) is mixed with excess SPDP (10 µl of 150 mM). The mixture was left at room temperature for 3 hours. SPDP binds to amine termini of nanoparticles through reactive N-hydroxysuccinimide (NHS) ester moities. Nanoparticles were purified using disposable desalting PD-10 column using PBS (7.4) buffer. The number of SPDP was determined by UV-Vis spectroscopy by monitoring cleaved byproduct, pyridine-2-thione (P2T), of SPDP using a disulfide reducing agent. Briefly, 400 µl of purified SPDP functionalized nanoparticles was mixed with 25 µl of 60 mM tris(2-carboxyethyl) phosphine hydrochloride (TCEP) which cleaves the disulfide bond of the SPDP. The nanoparticles solution was centrifuged using 1 ml 10K cut off amicon tubes. The supernatant contain P2T was collected and UV-Vis spectrum of the supernatant was determined. The product contains pyridine-2-thione (P2T) which has a distinct 343 nm absorbance peak with an extinction coefficient of 8100 M^{-1} cm^{-1}. Number of SPDP per nanoparticle is calculated according to the amount of P2T in the supernatant. The average number of SPDP per nanoparticle is determined to be around 180. In order to check if the 343 nm peak is result of SPDP on the surface, a fraction of SPDP functionalized nanoparticles was

centrifuged in small 1 ml 10K cut off amicon tubes without TCEP treatment. The supernatant was collected and UV-Vis spectrum of the supernatant was determined. The product did not exhibit any 343 nm absorbance peak due to absence of pyridine-2-thione (P2T) in the solution. Two results taken together suggest that the number of P2T is equivalent to amount of SPDP coupled to nanoparticles and used to estimate the amine content of the nanoparticles.

Estimation of the number of cy5.5 and tetrazine groups per nanoparticle. Cy5.5-NHS and Tet-NHS have similar reactivity against to the nanoparticle's amine termini. The cy5.5 content was calculated spectrophotometrically. The number of cy5.5 was calculated by monitoring the 675 nm absorbance peak (ε_{675}=250,000 $M^{-1}cm^{-1}$) of the MNP-cy5.5-tet. The nanoparticle concentration were determined according to the previously published report.[4] In this study, the MNPs treated with cy5.5-NHS only incorporated 56 units of the dye per nanoparticle. The number of conjugated tetrazine groups is estimated indirectly, since tetrazine has a weak absorbance at 500 nm. When MNPs were treated with a 1:1 mixture cy5.5-NHS:tetrazine-NHS, the number of conjugated cy5.5 per nanoparticle was calculated to be 24 (Figure 3d) therefore the number of tetrazine was estimated as 32 (56-24=32). When MNPs were treated with a 1:9 mixture cy5.5-NHS:tetrazine-NHS, the number of cy5.5 per nanoparticle was calculated to be 8 (Figure 3d) therefore the number of tetrazine was estimated as 48 (56-8=48).

Conjugation of tetrazine and cy5.5 groups to iron oxide nanoparticles. Aliquots of 10 mM stock solutions of tetrazine-NHS ester and cy5.5 mono-NHS ester in DMSO were added to the 0.5 mL PBS solutions of the nanoparticles (~10.0 mg ml^{-1} Fe). Tetrazine to cy5.5 ratios of 9:1, 7:3, 1:1 3:7, 1:9 were used, with a combined volume of 100 μL. After 24 h incubation the conjugated nanoparticles were purified using Sephadex PD-10 column against H_2O.

Treatment of MDA-MB-231 breast cancer cells with MNP-cy5.5-tet and DOX-TCO. Plated ~1000 MDA-MB-231 cells on MatTek glass bottom plates for 24 h. The cells were treated with 2 μM **MNP-cy5.5-tet** for 24 h. Cellular nanoparticle internalization was confirmed by fluorescence microscopy in cy5 channel. The cells were subsequently treated with 0.2 μM DOX-TCO for 48 h. Cellular uptake of DOX-TCO was confirmed by fluorescence microscopy in red

channel. The medium was replaced with fresh DMEM and the cells were grown for another 48 h. Caspase-3 activity assay was performed to measure apoptosis.

Cytotoxicity Assay. The colorimetric MTT assay, adopted from previously published work, was used to evaluate the cytotoxicities of DOX or DOX-TCO.[7] Day one: using 96-well format, plated ~100 cells/well in 100 μL DMEM and incubated for 24 h. Day two: DMEM was removed and the cells were treated with variable concentrations of DOX and DOX-TCO in 100 μL DMEM for 48 h. Day four: the medium was replaced with 100 μL of fresh DMEM and the cells were incubated for 48h. Day six: DMEM was removed and the cells were incubated with 100 μL of MTT solution (0.6 mg/mL in DMEM) per well for 4 h at 37 °C. The MTT solution was then replaced with 100 μL of DMSO containing 4% aqueous ammonia per well to dissolve the purple formazan crystals. After 30 min, the absorbance of each well at 550 nm was recorded using BioTek Synergy HT multi detection microplate reader.

Caspase3 Activity Assays. Ability of the nanoparticles to activated **MNP-cy5.5-tet** and trigger apoptosis was assayed using Caspase-3 assay kit that was purchased from ENZO Life Sciences. MDA-MB-231 cells treated with **MNP-cy5.5-tet** and subsequently DOX-TCO were lyzed using the lysis buffer provided in the kit and processed following the kit's protocol. The kit also included positive and negative controls. Fluorimetric measurements (ex. 360 nm, em. 460 nm) were done to determine aspase-3 activity. All Caspase assays were done in triplicate.

Statistical Analysis. Data were expressed as mean ± SD. Statistical differences were analyzed by the Student's t-test (http://graphpad.com). A value of $P < 0.05$ was taken as statistically significant. MTT and caspase-3 experiments were performed in triplicate.

Stability of DOX-TCO in the lysate of MDA-MB-231 cells. 300 μL of lysate of MDA-MB-231 cells was obtained upon treating ~3 million cells with the MicroRotofor™ Cell Lysis Kit, purchased from Bio-Rad. 6 μL of 10 mM DOX-TCO was added to the lysate to achieve 0.2 μM solution of the pro-drug. The cell lysate solution was stored at 37 °C. The pro-drug's stability was analyzed by RP-HPLC on the daily basis for four days and compared to the spectra of the DOX and DOX-TCO standards, as shown in Figure S3. The HPLC spectra contain multiple

peaks that arise from the endogenous cellular content. There is a shoulder next to the DOX-TCO peak that over time separates into a separate peak. The spectra clearly indicate that TCO does not spontaneously hydrolyze from the prodrug converting it into DOX.

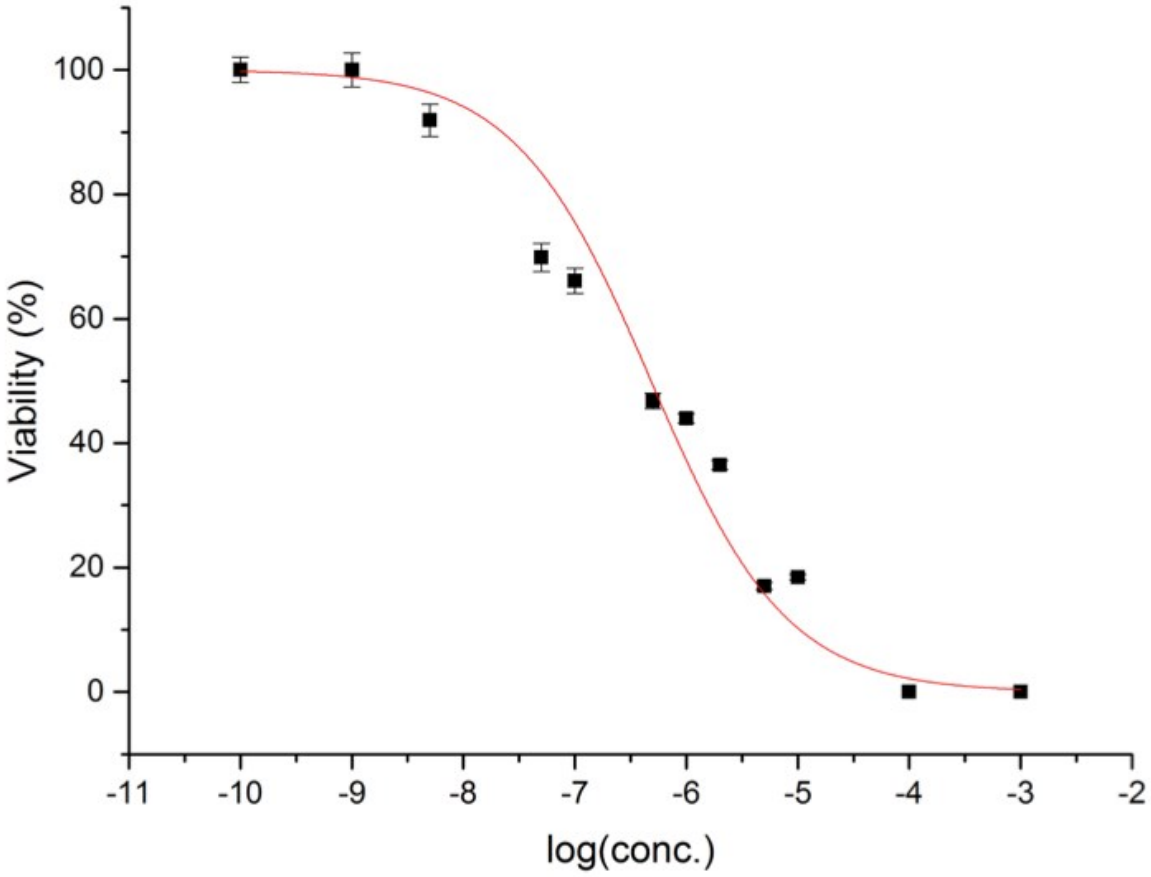


Fig S1. MTT Assay results for MDA-MB-231 cells treated with variable concentrations of DOX (IC_{50} = 97 nM).

Fig S2. MTT Assay results for MDA-MB-231 cells treated with variable concentrations of DOX-TCO (IC_{50} = 480 nM).

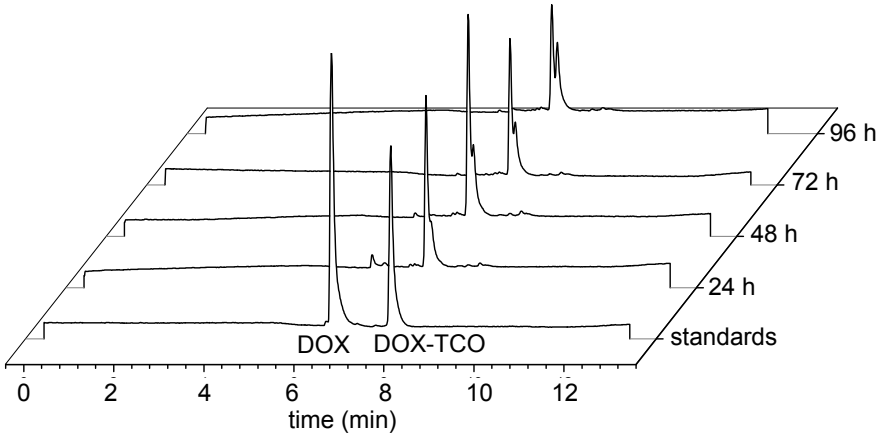
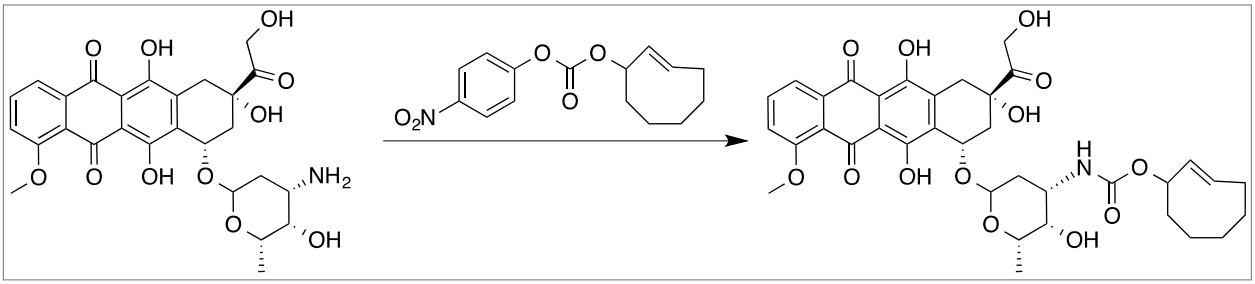


Fig S3. Stability DOX-TCO in the lysate of MDA-MB-231 cells.

Synthesis of DOX-TCO.

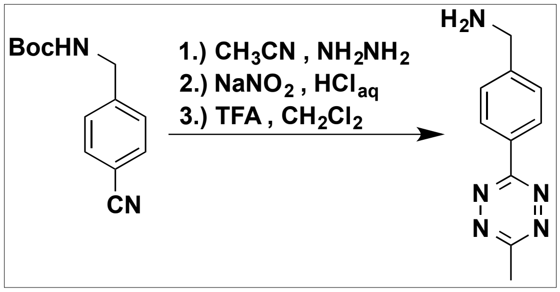


Doxorubicin **DOX-TCO**

Doxorubicin hydrochloride (45 mg, 0.078 mmol), *p*-nitrophenyl carbonate of TCO (20 mg, 0.069 mmol), and triethylamine (105 μL) were dissolved in DMF (3 mL). The reaction mixture was stirred at RT under nitrogen atmosphere for 18 h. The title product was purified as a pink powder by preparative TLC using a 1:9 mixture of MeOH and CH_2Cl_2 as mobile phase. Yield = 31 mg (56%).

^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.04 (d, *J* = 6.9 Hz, 1H), 7.79 (7, *J* = 8.2 Hz, 1H), 7.40 (d, *J* = 8.2 Hz, 1H), 5.77 (q, *J* = 13.6 Hz, 1H), 5.52-5.45 (m, 2H), 5.30-5.25 (m, 2H), 5.11-5.09 (m, 1H), 4.76 (s, 2H), 4.53 (s, 1H), 4.16 (q, *J* = 5.4 Hz, 1H), 4.09 (s, 3H), 3.88 (bs, 1H), 3.28 (d, *J* = 19.1 Hz, 1H), 3.04-2.99 (m, 2H), 2.50-2.40 (m, 1H), 2.34 (d, *J* = 15.0 Hz, 1H), 2.18 (d, *J* = 15.0 Hz, 1H), 2.10-1.76 (m, 8H), 1.70-1.55 (m, 3H), 1.55-1.40 (m, 1H), 1.31 (d, *J* = 6.8 Hz, 4H), 1.1-1.0 (m, 1H), 0.80-0.70 (m, 1H).
^{13}C NMR ($CDCl_3$, 100 MHz) δ 213.85, 186.68, 186.33, 174.25, 172.02, 169.49, 160.84, 156.10, 155.41, 154.85, 135.64, 135.18, 133.51, 131.72, 131.01, 120.56, 119.64, 118.36, 111.32, 111.13, 100.66, 71.98, 69.42, 65.44, 56.55, 46.87, 44.30, 36.62, 35.66, 33.74, 30.30, 29.59, 25.55, 25.32, 17.85, 16.75.

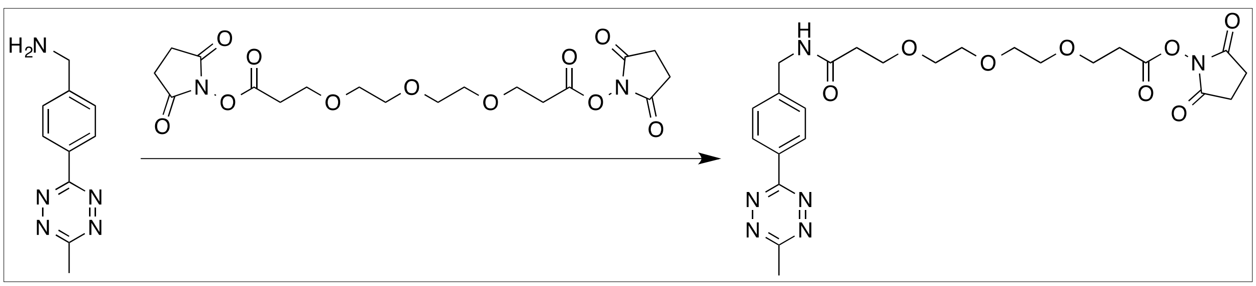
HRMS (ESI) *m/z*: calcd. for $C_{36}H_{42}NO_{13}$ $[M+1]^+$ 696.2656; found 696.2664



The tetrazine was synthesized by the procedure described in the literature.[8]

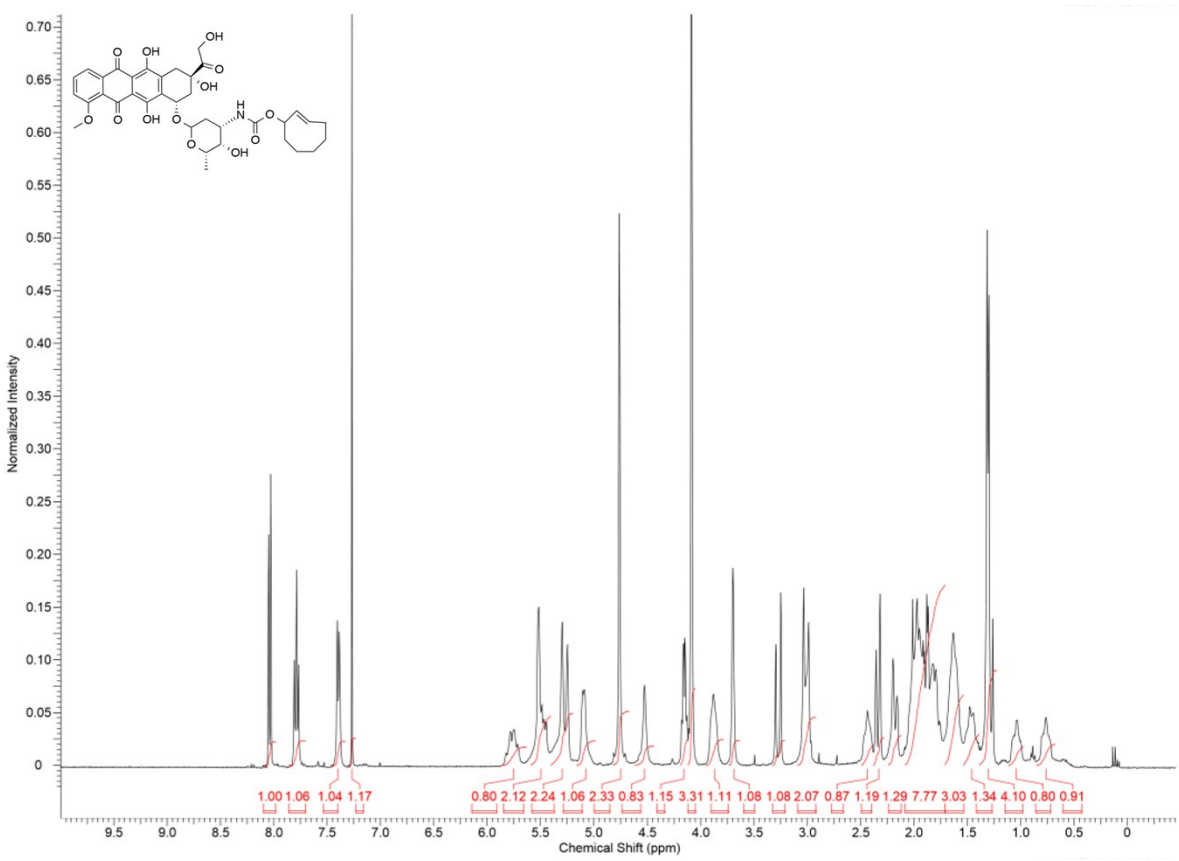
^{1}H NMR (CD_3OD, 400 MHz) δ 8.62 (d, *J* = 9.6 Hz, 2H), 7.72 (d, *J* = 8.2 Hz, 2H), 4.27 (s, 2H), 3.05 (s, 3H).
^{13}C NMR (CD_3OD, 100 MHz) δ 169.20, 165.06, 138.93, 134.47, 130.96, 129.58, 44.07, 21.25.
HRMS (ESI) *m/z*: calcd. for $C_{10}H_{12}N_5$ $[M+1]^+$ 202.1093; found 202.1112.

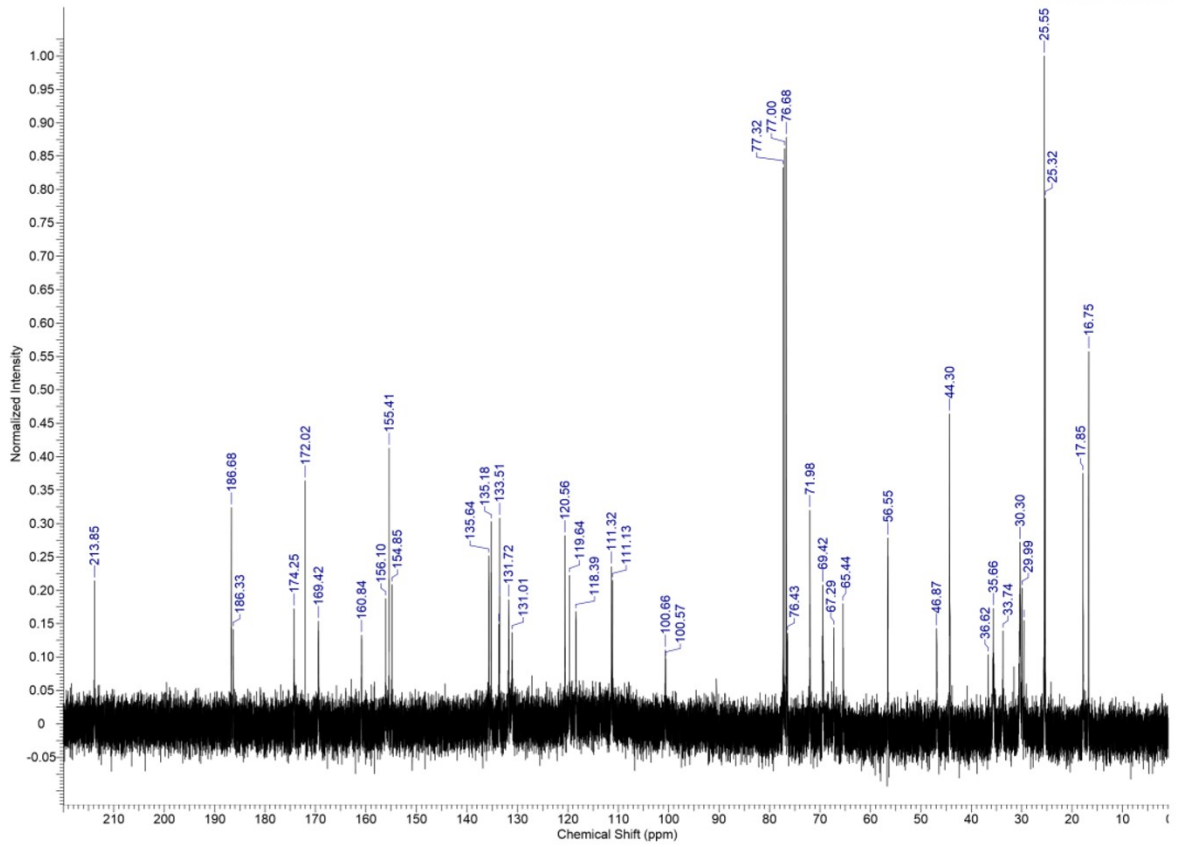
Synthesis of tetrazine-NHS ester.

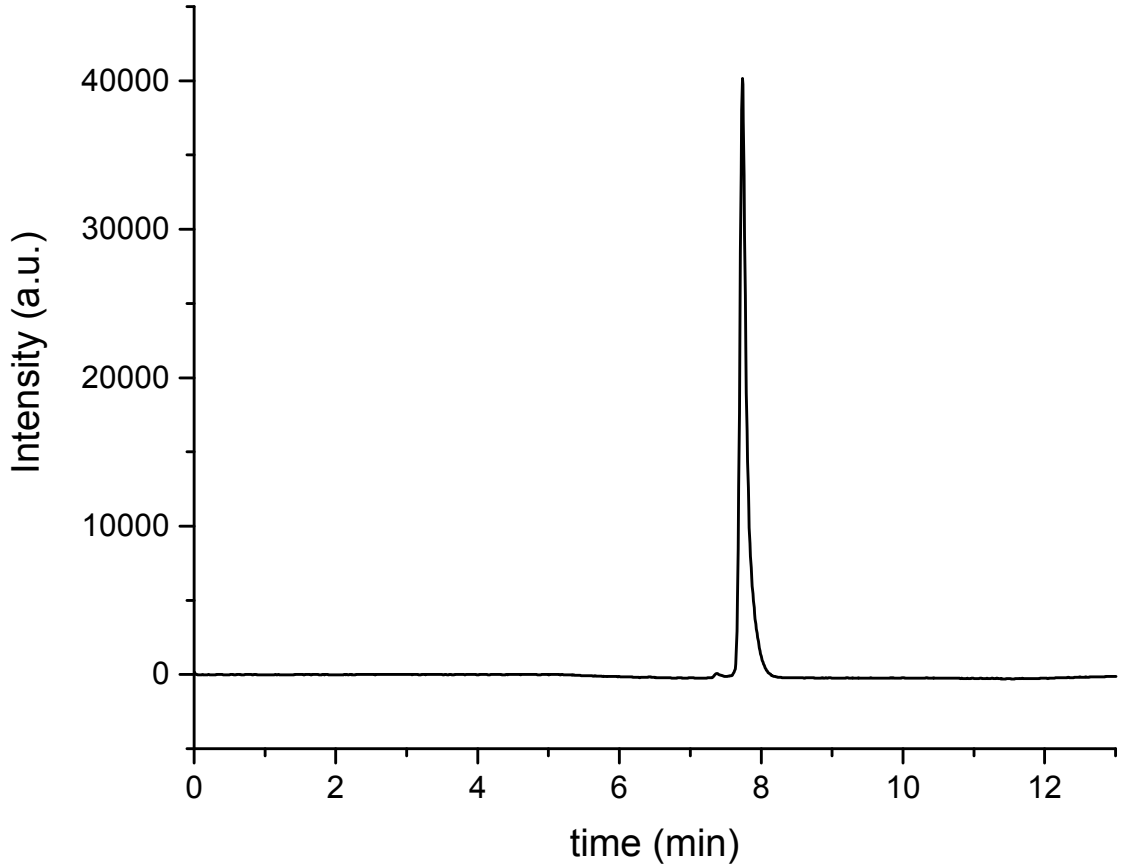


Tetrazine (52 mg, 0.17 mmol), PEG3-di-NHS ester (304 mg, 0.68 mmol), and triethylamine (46 μL, 0.34 mmol) were dissolved in CH_2Cl_2 (10 mL). The reaction mixture was stirred at RT under nitrogen atmosphere for 18 h. The title product was purified as a pink powder by preparative TLC using EtOAc as mobile phase. Yield = 83 mg (62%).

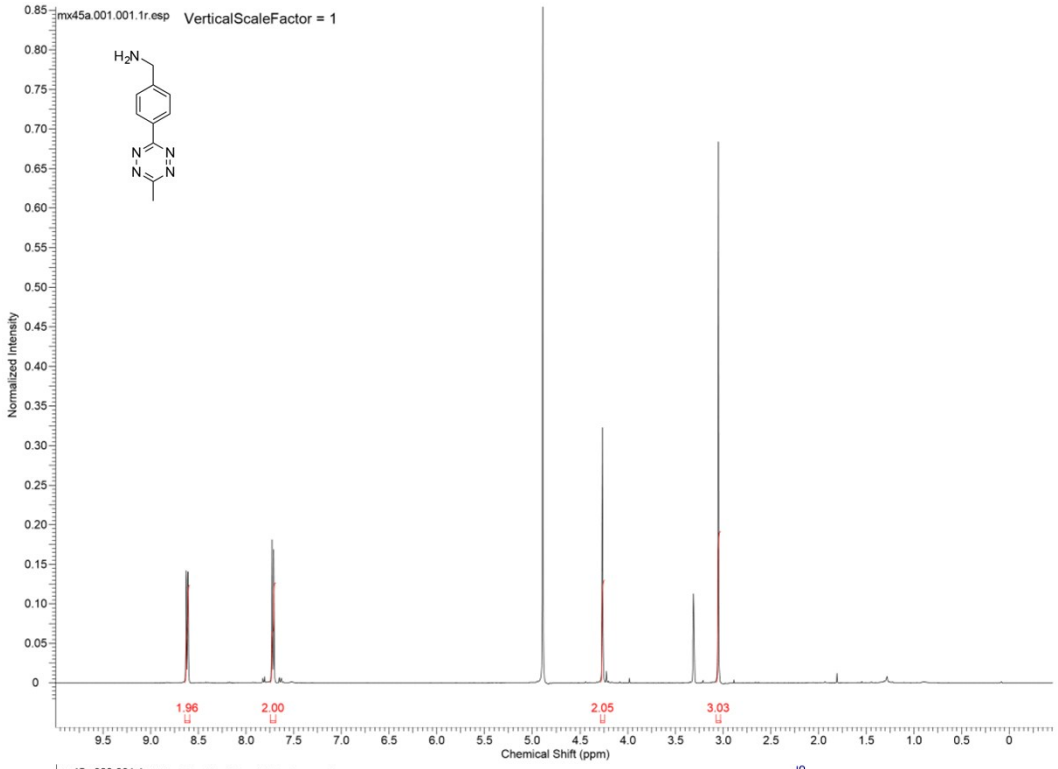
^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.52 (d, *J* = 8.2 Hz, 2H), 7.50 (d, *J* = 8.2 Hz, 2H), 7.31 (bs, 1H), 4.47 (d, *J* = 5.5 Hz, 2H), 3.80-3.73 (m, 4H), 3.63 (d, *J* = 6.8 Hz, 5H), 3.54 (d, *J* = 2.7 Hz, 4H), 3.08 (s, 3H), 2.83-2.79 (m, 7H), 2.60 (t, *J* = 6.8 Hz, 2H)
^{1}H NMR ($CDCl_3$, 100 MHz) δ 172.55, 171.71, 169.00, 167.17, 166.69, 163.90, 143.54, 130.67, 128.12, 70.61, 70.28, 70.18, 67.09, 65.64, 42.98, 36.69, 32.08, 29.64, 25.55, 25.34, 21.09
HRMS (ESI) *m/z*: calcd. for $C_{24}H_{31}N_6O_8$ $[M+1]^+$ 531.2203; found 531.2213.

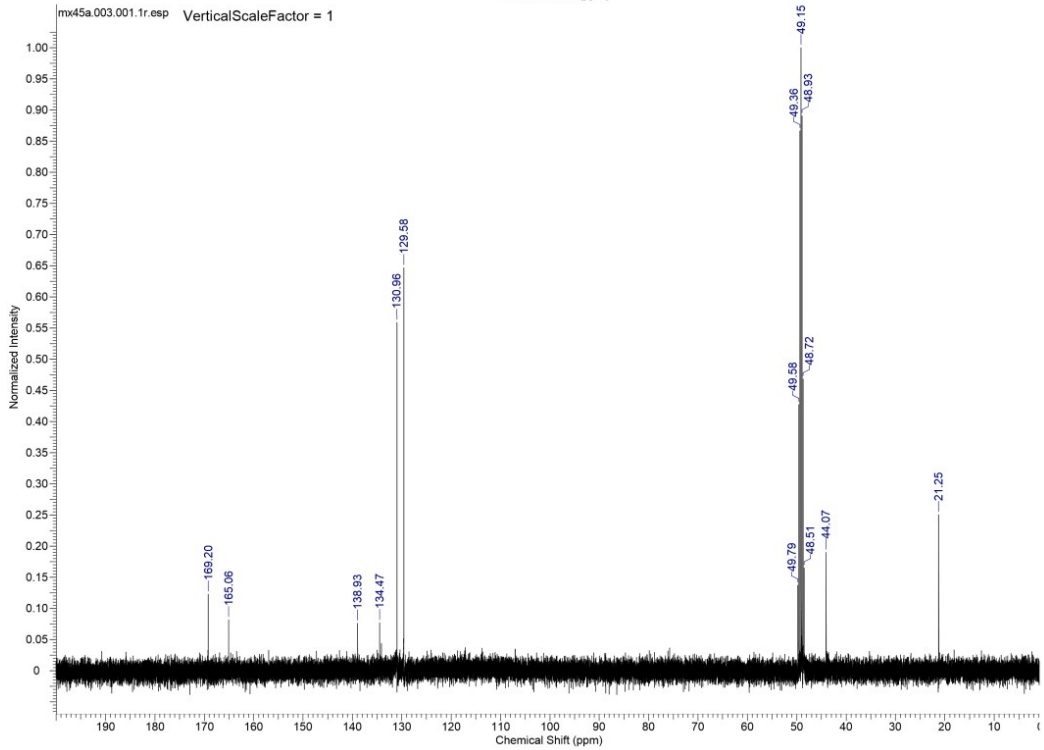

Normalized Intensity
Chemical Shift (ppm)
1.00 1.06 1.04 1.17 0.80 2.12 2.24 1.06 2.33 0.83 1.15 3.31 1.11 1.08 1.08 2.07 0.87 1.19 1.29 7.77 3.03 1.34 4.10 0.80 0.91

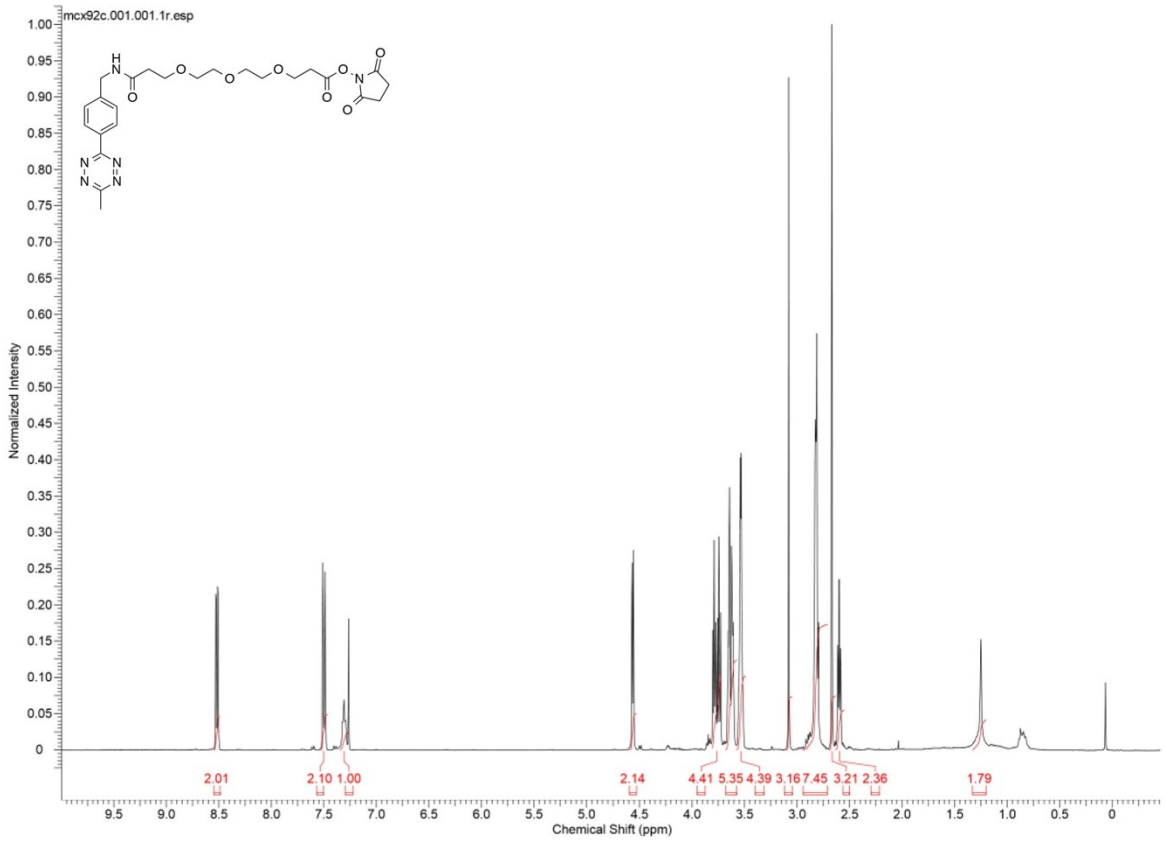

Normalized Intensity
Chemical Shift (ppm)
213.85
186.68
186.33
174.25
172.02
169.42
160.84
156.10
155.41
154.85
135.64
135.18
133.51
131.72
131.01
120.56
119.64
118.39
111.32
111.13
100.66
100.57
77.32
77.00
76.68
76.43
71.98
69.42
67.29
65.44
56.55
46.87
44.30
36.62
35.66
33.74
30.30
29.99
25.55
25.32
17.85
16.75

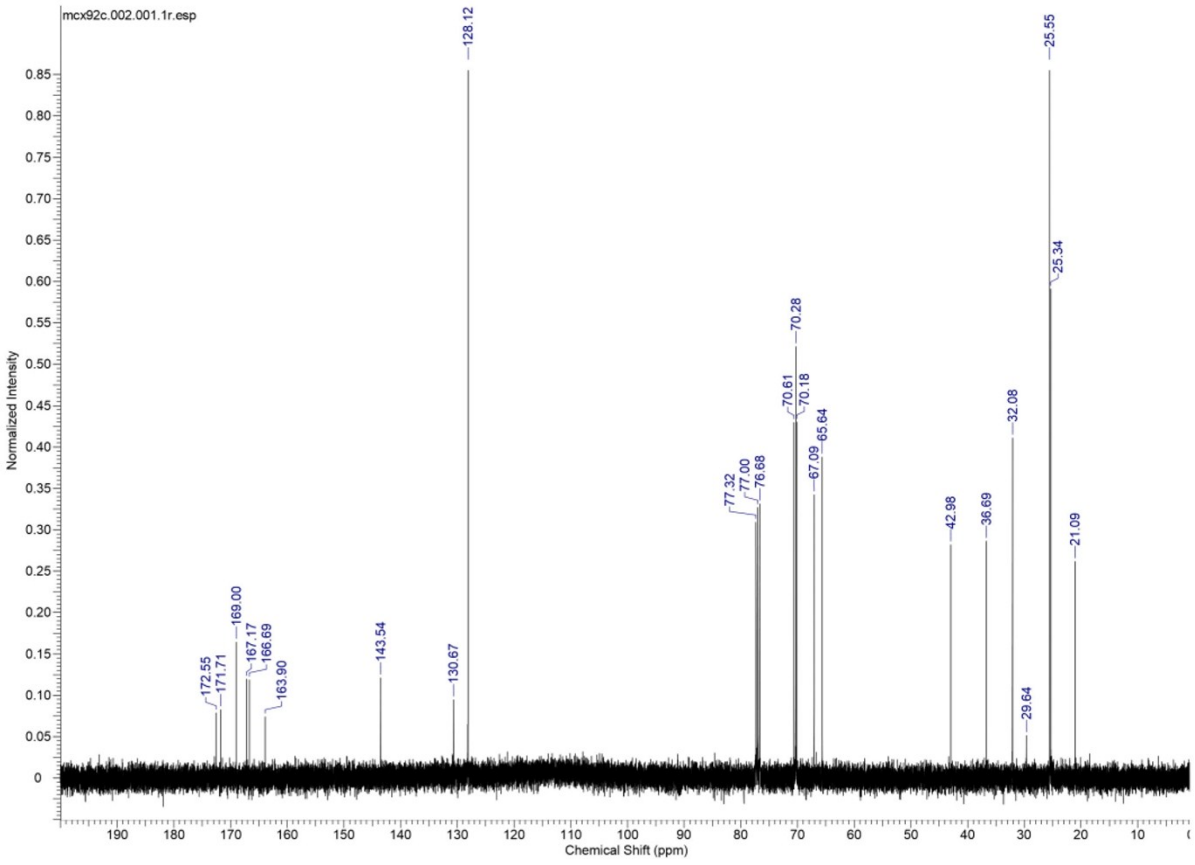


HPLC spectrum of DOX-TCO


mx45a.001.001.1r.esp
VerticalScaleFactor = 1
Normalized Intensity
Chemical Shift (ppm)
1.96
2.00
2.05
3.03


mx45a.003.001.1r.esp
VerticalScaleFactor = 1
Normalized Intensity
Chemical Shift (ppm)
169.20
165.06
138.93
134.47
130.96
129.58
49.79
49.58
49.36
49.15
48.93
48.72
48.51
44.07
21.25


mcx92c.001.001.1r.esp
Normalized Intensity
Chemical Shift (ppm)
2.01
2.10
1.00
2.14
4.41
5.35
4.39
3.16
7.45
3.21
2.36
1.79


mcx92c.002.001.1r.esp
Normalized Intensity
Chemical Shift (ppm)
128.12
25.55
25.34
70.28
70.61
70.18
65.64
67.09
77.32
77.00
76.68
42.98
36.69
32.08
21.09
29.64
172.55
171.71
169.00
167.17
166.69
163.90
143.54
130.67

References.

1. M. V. Yigit, D. Mazumdar and Y. Lu, *Bioconjugate Chem.*, 2008, **19**, 412-417.
2. M. V. Yigit, D. Mazumdar, H. K. Kim, J. H. Lee, B. Odintsov and Y. Lu, *Chembiochem*, 2007, **8**, 1675-1678.
3. M. V. Yigit, S. K. Ghosh, M. Kumar, V. Petkova, A. Kavishwar, A. Moore and Z. Medarova, *Oncogene*, 2013, **32**, 1530-1538.
4. A. Tsourkas, O. Hofstetter, H. Hofstetter, R. Weissleder and L. Josephson, *Angew. Chem. Int. Ed. Engl.*, 2004, **43**, 2395-2399.
5. L. Josephson, C. H. Tung, A. Moore and R. Weissleder, *Bioconjugate Chem.*, 1999, **10**, 186-191.
6. E. Y. Sun, L. Josephson, K. A. Kelly and R. Weissleder, *Bioconjugate Chem.*, 2006, **17**, 109-113.
7. M. Royzen, J. J. Wilson and S. J. Lippard, *J. Inorg. Biochem.*, 2013, **118**, 162-170.
8. J. Yang, M. R. Karver, W. Li, S. Sahu and N. K. Devaraj, *Angew. Chem. Int. Ed. Engl.*, 2012, **51**, 5222-5225.