Supporting Information

Biodegradable hyperbranched polyesters of trimethylolpropane with acrylate side chains enabling sustainable gel materials and nanomaterials for drug delivery applications

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Materials and Methods

Materials

Trimethylolpropane (TMP), acryloyl chloride, triethylamine (TEA), adipoyl dichloride, glutaroyl dichloride, malonyl dichloride, succinyl dichloride, suberoyl chloride, pyridine, N,N-diisopropylethylamine (DIEA), 1,8-diazabicyclo[5.4.0]-7-undecene (DBU), N,N'-dimethylaminopyridine (DMAP), bromoethane, 2,3,3-trimethylindolenine, cyclohexanone, phosphorus oxychloride, sodium acetate, 3,3'-dithiodipropionic acid, sodium chloride, 4-nitrophenyl chloroformate, glutathione (GSH), phosphate-buffered saline (PBS), dithiothreitol (DTT), paraformaldehyde (PFA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and silica gel (spherical, 100 μm) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Gibco™ Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin (PS), trypsin-EDTA, and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Deuterated chloroform and dimethyl sulfoxide solvents were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). All the other solvents used in this research were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA) and directly used without further purification.

Synthesis of TMPA

Acryloyl chloride (80 μL, 1.0 mmol) in 1 mL of anhydrous THF was added dropwise to a solution of TMP (310 μL, 2.5 mmol) and TEA (280 μL, 2.0 mmol) in anhydrous THF cooled in an ice bath. The resulting solution was stirred at room temperature for 4 h. The mixture was filtered, and the filtrate was condensed under vacuum. The crude product was purified by flash column chromatography using silica gel as the stationary phase and a mixture of ethyl acetate and hexane as the mobile phase. The final product was obtained as a colorless oil (115 mg, 61%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 6.45 (d, J = 17.3 Hz, 1H), 6.19-6.12 (m, 1H), 5.90 (d, J = 10.4 Hz, 1H), 4.28 (s, 2H), 3.63-3.55 (m, 4H), 3.09 (brs, 2H), 1.32 (q, J = 7.5 Hz, 2H), 0.89 (t, J = 7.5 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ (ppm) 167.07, 131.66, 128.01, 65.64, 64.32, 42.97, 22.54, 7.38. HRMS (ESI, m/z): calcd for $C_9H_{16}O_4$ ($[M+H]^+$) 189.1121; found 189.1123.

A general procedure for the synthesis of TMPA polymers

Dicarboxylic acid dichloride (1.0 mmol) was added slowly to a stirring solution of TMPA (1.0 mmol) in 2 mL of anhydrous DCM, followed by the dropwise addition of pyridine (2.2 mmol). The resulting solution was stirred at room temperature for 72 h. After filtration, the filtrate was diluted with DCM and washed with brine. The organic phases were combined, dried over Na_2SO_4, filtered, and condensed. Upon precipitation in cold diethyl ether, the polymer was obtained as an oil after drying under vacuum. The structural compositions of the polymers were analyzed by NMR using $CDCl_3$ as the solvent.

In the experiments screening for optimal reaction conditions, only one parameter, e.g., the ratio between the dicarboxylic acid dichloride and TMPA, the amount of pyridine, or the reaction time, was adjusted in a series of parallel reactions, while maintaining all the other conditions the same.

Synthesis of DTPC

$(COCl)_2$ (4.3 mL, 50 mmol) was added slowly to a stirring mixture of DTPA (1.05 g, 5.0 mmol) in 5 mL of anhydrous benzene. The reaction was stirred at 60 ℃ for 12 h. After condensation, 3 mL of anhydrous benzene was added, and the mixture was condensed again. Upon drying under vacuum, the product was obtained as a yellowish solid (1.24 g, quantitative yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 3.33 (t, J = 6.9 Hz, 4H), 2.96 (t, J = 6.9 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$): δ (ppm) 172.30, 46.35, 32.19.

A general procedure for the polymer crosslinking

A solution of TMPA-AA (1.0 mmol), a crosslinker (0.5 mmol), and TEA (1.0 mmol) in 0.5 mL of anhydrous DCM was stirred at room temperature for 12 h. After evaporating the solvent, the resulting gel was washed with 10% NH_4Cl and DI water and then dried under vacuum.

Synthesis of MPA-NHS

MPA (174 μL, 2.0 mmol) was added to a solution of N-hydroxysuccinimide (345 mg, 3.0 mmol) in 10 mL of anhydrous acetonitrile, followed by a dropwise addition of N,N′-dicyclohexylcarbodiimide (454 mg, 2.2 mmol) in 2 mL of anhydrous acetonitrile. The mixture was stirred for 16 h at room temperature. After condensation under reduced pressure, the residue was washed with DCM. The filtrate was

condensed, and the crude product was purified by flash column chromatography using silica gel as the stationary phase and a mixture of ethyl acetate and hexane as the mobile phase. The final product was obtained as a colorless oil (370 mg, 91%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 2.98 (t, J = 6.6 Hz, 2H), 2.90-2.85 (m, 6H), 1.85 (t, J = 8.6 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ (ppm) 168.96, 166.82, 35.68, 25.59, 19.35.

A general procedure for conjugating thiol-contained molecules to polymers

A solution of TMPA-AA, a thiol-contained compound (or a mixture of multiple thiol-contained compounds), and TEA in anhydrous DCM was stirred at room temperature for 6 h. The reaction mixture was diluted with DCM and washed with 10% NH_4Cl and DI water. The organic phases were combined, dried over Na_2SO_4, filtered, and condensed. Upon precipitation in cold diethyl ether, the modified polymer was obtained after drying under vacuum.

Simultaneous conjugation of multiple thiol-contained compounds to TMPA-AA can be conveniently achieved by a reaction mixture of TMPA-AA, multiple thiol-contained compounds, and TEA in anhydrous DCM.

Synthesis of Cy7-DTT

The starting material Cy7-Cl was synthesized following a reported procedure.[1] A solution of Cy7-Cl (118 mg, 0.2 mmol) in 1 mL of methanol was added dropwise to a solution of DTT (154 mg, 1.0 mmol) and DIEA (52 μL, 0.3 mmol) in 5 mL of methanol. The mixture was stirred at room temperature for 1 h. After condensation, the residue was washed with DI water. The crude product was purified by flash column chromatography using silica gel as the stationary phase and a mixture of DCM and methanol as the mobile phase. The final product was obtained as a green solid (118 mg, 83%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.92 (d, J = 14.0 Hz, 2H), 7.39-7.31 (m, 4H), 7.22 (t, J = 7.4 Hz, 2H), 7.08 (d, J = 7.6 Hz, 2H), 6.07 (d, J = 14.0 Hz, 2H), 4.11 (q, J = 6.3 Hz, 4H), 3.91 (t, J = 6.2 Hz, 1H), 3.64 (t, J = 6.2 Hz, 1H), 3.18-3.07 (m, 2H), 2.78-2.73 (m, 2H), 2.67-2.57 (m, 4H), 1.94 (t, J = 5.6 Hz, 2H), 1.83 (t, J = 8.2 Hz, 1H), 1.76 (s, 12H), 1.46 (t, J = 6.8 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ (ppm) 171.87, 171.73, 159.57, 159.27, 146.59, 141.84, 141.82, 141.21, 141.17, 133.73, 133.63, 128.62, 128.59, 124.96, 124.93, 122.47, 122.37, 110.17, 110.07, 100.37, 100.31, 74.56, 73.37, 73.30, 70.76, 49.40, 49.34, 42.32, 41.68, 39.44, 28.33,

28.12, 28.09, 28.07, 28.01, 26.28, 20.87, 12.27. HRMS (ESI, m/z): calcd for $C_{38}H_{49}N_2O_2S_2$ ($[M-Br]^+$) 629.3230; found 629.3203.

Synthesis of TMPA-AA-PEG-Cy7

TEA (20 μL) was added slowly to a stirring solution of TMPA-AA (200 mg), PEG-SH (100 mg), and Cy7-2-DTT (10 mg) in 1 mL of anhydrous DCM. The reaction was stirred at room temperature for 16 h in the dark. Then 0.5 mL of DI water was added, and the mixture was stirred for 30 min. The mixture was diluted with 10 mL of DCM and washed with 10% NH_4Cl and DI water. The organic phase was dried over Na_2SO_4, filtered, and condensed. Upon precipitation in cold diethyl ether, the polymer was obtained as a green oil after drying under vacuum. The structural composition of the polymer was analyzed by NMR using $CDCl_3$ as the solvent.

Polymer degradation

To examine the degradation of TMPA-AA, the polymer (20 mg) was dispersed into 10 mL of a mixed solution of DMSO and aqueous NaOH (1:9, by volume). The resulting polymer solution was incubated at 37 °C. At predetermined time points, a sample of the polymer solution was taken for GPC analysis to monitor the molecular weight of the polymer in the solution.

For the biodegradability test, the polymer (20 mg) was incubated in 10 mL of a mixed media of DMSO and PBS buffer solution (1:9, by volume) containing varied concentrations of the esterase enzyme at 37 °C. As a control experiment, the polymer was incubated in a PLE-free mixed media under the same conditions. At predetermined time points, the polymer solutions were sampled for GPC analysis to monitor the molecular weight of the polymer in the media.

Gel degradation

To examine the degradation of the polymeric gels, the gel (100 mg) was dispersed into 10 mL of aqueous NaOH and incubated at 37 °C. At predetermined time points, the solution was centrifuged, and the gel residue was weighed to monitor the weight change of the gel.

For the biodegradability test, the gel (100 mg) was incubated in 10 mL of PBS buffer solution containing varied concentrations of the esterase enzyme PLE at 37 °C. As a control experiment, the polymer was incubated in a PLE-free PBS solution under the same conditions. At predetermined time points, the solution was centrifuged, and the gel residue was weighed to monitor the weight change of the gel.

Formulation of polymeric nanoparticles

The polymeric nanomaterials were formulated via a nanoassembling procedure. A solution of EDT (1.5 μL) in 15 μL of DMSO was dropped into a solution of a polymer or a polymer mixture (10 mg) and TEA (10 μL) in 0.5 mL of DMSO upon robust stirring. The resulting solution was stirred at room temperature for 2 h and then dropped into 10 mL of deionized water or PBS buffer (pH 7.4) under robust stirring and maintained for 4 h. The polymer molecules were assembled into nanostructures spontaneously due to their amphiphilicity. Thereafter, the resulting nanoparticles were dialyzed in a Spectra/Por® dialysis tube (MWCO: 6-8 kDa) against DI water or PBS buffer (pH 7.4) for 24 h. The nanoparticle size, size distribution indicated by polydispersity index (PDI), and surface charge of the nanoparticles were measured by dynamic light scattering (DLS) using Zetasizer (Nano ZS, Malvern Instruments Ltd, Malvern, UK). The morphology of polymeric nanoparticles was observed using a Hitachi HT7800 transmission electron microscopy (TEM, Hitachi High-Technologies Corporation, Tokyo, Japan). The final nanoparticles were stored at 4 °C for future use.

Dynamic serum stability

The colloidal stability of the NPs was obtained by DLS analysis. NPs were first dispersed into PBS buffer (pH 7.4) and PBS buffer with 10% FBS (pH 7.4) and then incubated at 37 °C. The hydrodynamic sizes of the NPs were determined with DLS at predetermined time points.

Photophysical properties

The spectroscopic properties of Cy7-DTT and TMPA-AA-PEG-Cy7 were measured in 1 mm path-length quartz cuvettes at room temperature. UV-Vis absorption spectrum was recorded with a PerkinElmer Lambda 1050 UV/Vis/NIR spectrophotometer. Fluorescence emission was obtained using a Shimadzu RF-6000 spectrophotometer. All the measurements were conducted with an optical density no higher than

0.12 at excitation wavelength to avoid reabsorption. The fluorescence quantum yield of Cy7-DTT was calculated by a standard relative method using the equation below with indocyanine green (ICG, $\Phi_{FL} \approx$ 0.13 in ethanol) as a reference.[2]

$$\Phi_{FL} = \Phi_R \frac{I\ OD_R n^2 RP_R}{I_R\ OD\ n_R^2\ RP}$$

Where Φ is the quantum yield, the subscript R refers to the reference, I is the integrated emission signal, OD is the optical density at the excitation wavelength, n is the refractive index of the solvent, and RP is the relative power of the light source of the spectrofluorometer at the excitation wavelength.

Cell culture

Human umbilical vein endothelial HUVEC cells, human lung cancer A549 cells, and human pancreatic cancer BxPC-3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco™) supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. All cells were cultured at 37 °C in 100 mm culture dishes under a humidified atmosphere of 5% CO_2. Cells were sub-cultured when the cell confluence reached 70~80%.

Confocal fluorescence imaging

Fluorescence images of cells were recorded by confocal laser scanning microscopy (CLSM). Cells were seeded at a density of 200,000 cells/dish in a 35 mm^2 Petri dish with a glass window at the bottom for 24 h at 37 °C with 5% CO_2. After washing with PBS (pH 7.4), cells were incubated with Cy7-DTT or the Cy7 NPs for 3h. Cells cultured in fresh medium without any incubation were used as a control. All cells were washed with PBS three times and fixed with paraformaldehyde (4% in PBS) for 10 min at room temperature. Following the removal of paraformaldehyde, cells were further washed with PBS thrice, and the nuclei of cells were stained with Hoechst 33342 (final concentration 1μg/mL) for 10 min. After washing three more times with PBS, cells were imaged under a confocal microscope (Olympus FV3000 Laser Scanning Confocal Microscope).

Cell viability

The toxicity toward human cells (HUVEC, A549, and BxPC-3 cells) was evaluated by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded at a density of 5,000 cells/well in 96-well plates at 37 °C with 5% CO_2 for 24 h before the test. In the tests, cells were incubated with fresh medium containing NPs at varied concentrations for 24 h. In the control group, cells were cultured in a fresh medium without any treatment. After that, the medium was replaced with a solution of the MTT reagent in a fresh medium at the concentration of 1 mg/mL, and the cells were incubated for another 4 h. The culture medium was carefully removed, and 100 μL of DMSO was added to each well of the plate. The plate was gently shaken to dissolve the generated purple crystals of the MTT reagent. The optical density at 570 nm was recorded on a microplate reader (BioTek Synergy H1 multimode microplate reader).

Animal model

Animal experiments were carried out following NIH regulations and approved by the Institutional Animal Care and Use Committee of the University of North Dakota. A xenograft tumor model of A549 cancer was established in athymic BALB/c nude mice (8-10 weeks old, ~20 g, the Jackson Laboratories), as described in our previous study.[1] Briefly, A549 cells suspended in DMEM culture medium (2,000,000 cells/100 μL) were inoculated subcutaneously to mice. The tumor volume was measured by a digital caliper and calculated using the following formula: Tumor volume = 0.5 × (tumor length) × (tumor width)^2. Tumor volumes were monitored every other day, and the animals were observed for body weight change and signs of pain throughout the experiments.

In vivo biodistribution

When the size of tumors in mice reached 100 mm^3, mice were randomly assigned into 3 groups (n = 5 for each group), including 1 control group and 2 experimental groups. All mice were individually marked and weighed prior to administration. They were then anesthetized by isoflurane and intravenously administrated with sterilized saline (the control group), nano-Cy7 NPs, or nano-Cy7-RGD NPs in sterilized saline at a dose of 1 mg/kg equivalent to Cy7. After 6 h postinjection, the whole mice were imaged under anesthesia by a noninvasive Lago X whole-body imaging system (Spectral Instruments

Imaging Inc.). All images were collected under identical system settings. Later, the mice were all sacrificed, and the main organs (heart, liver, spleen, lung, and kidney) and tumors were excised for ex vivo imaging. The fluorescence emission of the collected tissues was recorded with the Lago X imaging system.

In vivo biocompatibility

The in vivo biocompatibility of the new polymers was investigated in BALB/c mice. Mice were randomly assigned into 3 groups (n = 5 for each group), including 1 control group and 2 experimental groups. Each mouse was separately marked and weighed prior to treatment. In the control group, mice were intravenously administrated with sterilized saline. In the experimental groups, mice were intravenously injected with polymers at contractions of 100 mg/kg and 300 mg/kg body weight. The injections were administered to mice through the tail vein two times per week for a period of 4 weeks. The body weight and health conditions of mice were monitored every day during the entire experiment process. At the end of the experiment, all mice were sacrificed to harvest the main organs (heart, liver, spleen, lung, and kidney) for further histological analysis.

Histological analysis

The collected organ tissues (heart, liver, spleen, lung, and kidney) were fixed by 4% paraformaldehyde. The fixed organs were embedded in optimal cutting temperature (OCT) gel, sectioned into ~5 μm, stained with hematoxylin and eosin (H&E), and analyzed under a light microscope. Histology was conducted in a blind fashion by professional personnel at the University of North Dakota.

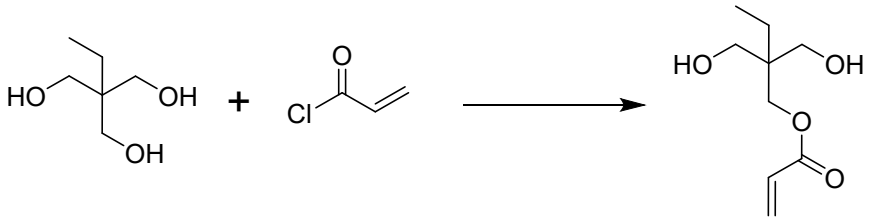

Figure S1. Synthesis of monomer TMPA.

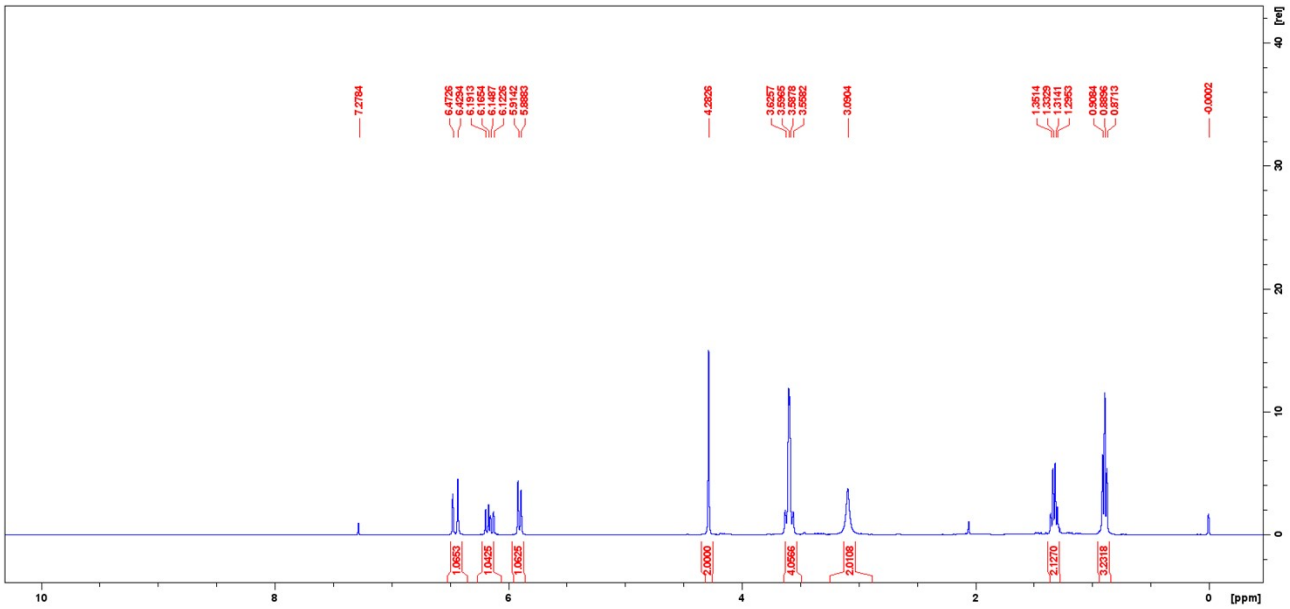

Figure S2. ^{1}H NMR spectrum of TMPA.

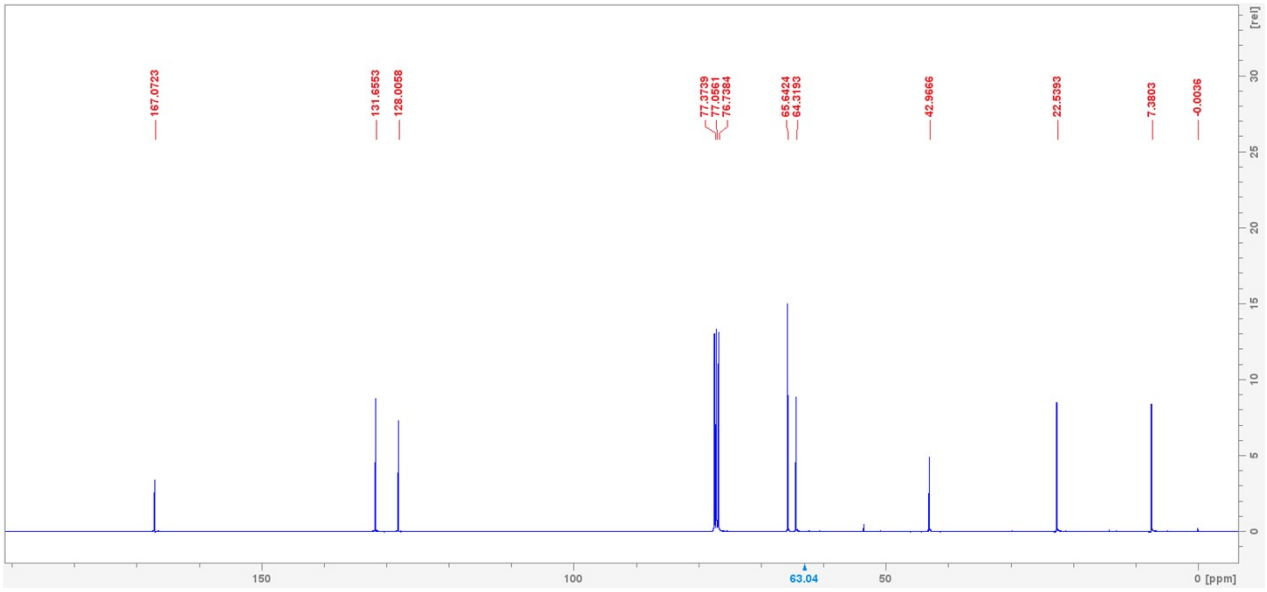

Figure S3. ^{13}C NMR spectrum of TMPA.

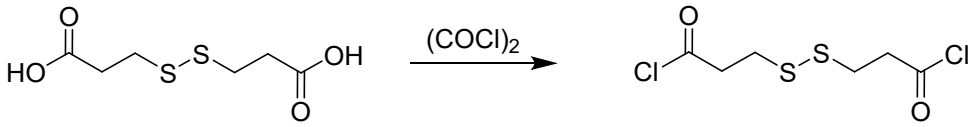

Figure S4. Synthesis of monomer DTPC.

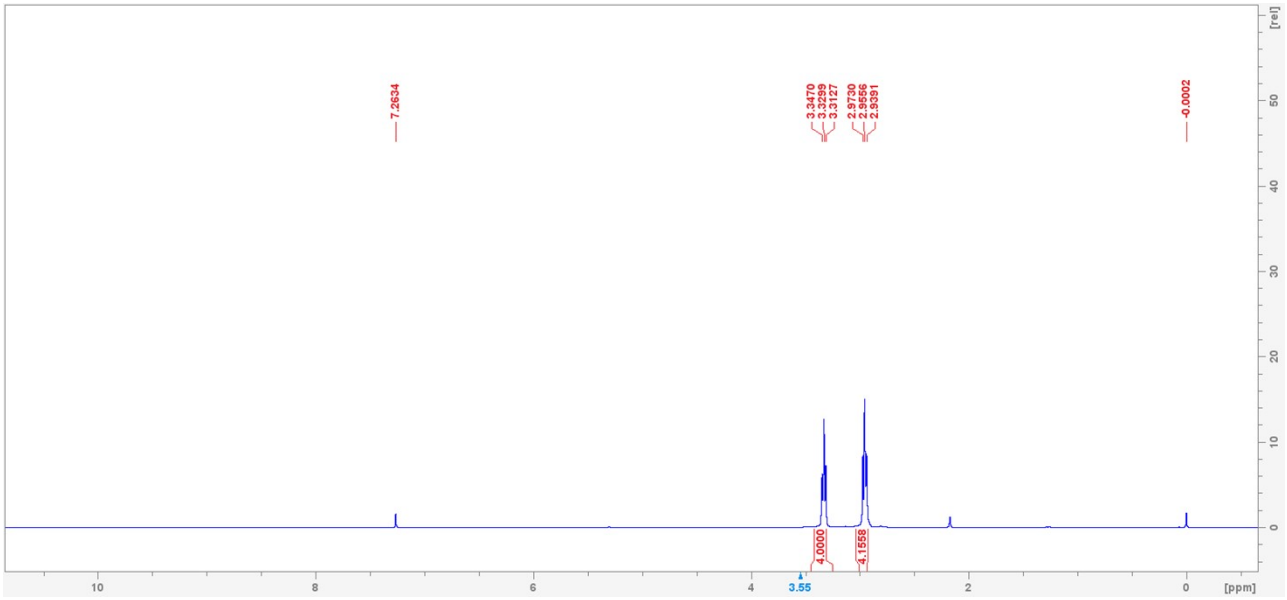

Figure S5. ^{1}H NMR spectrum of DTPC.

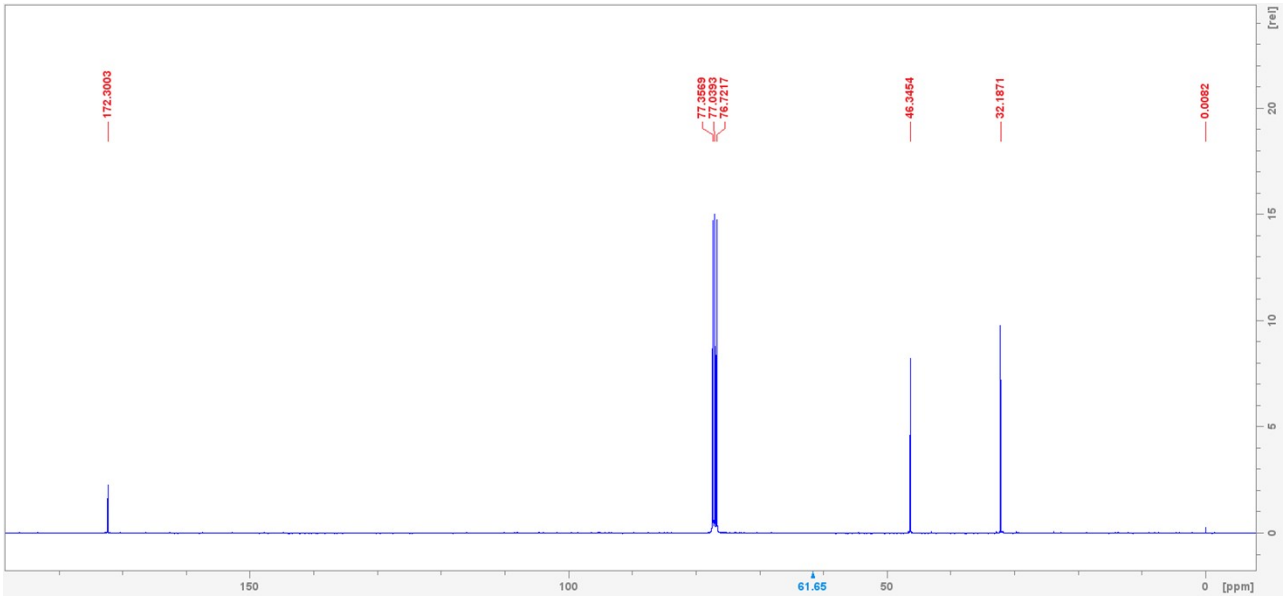

Figure S6. ^{13}C NMR spectrum of DTPC.

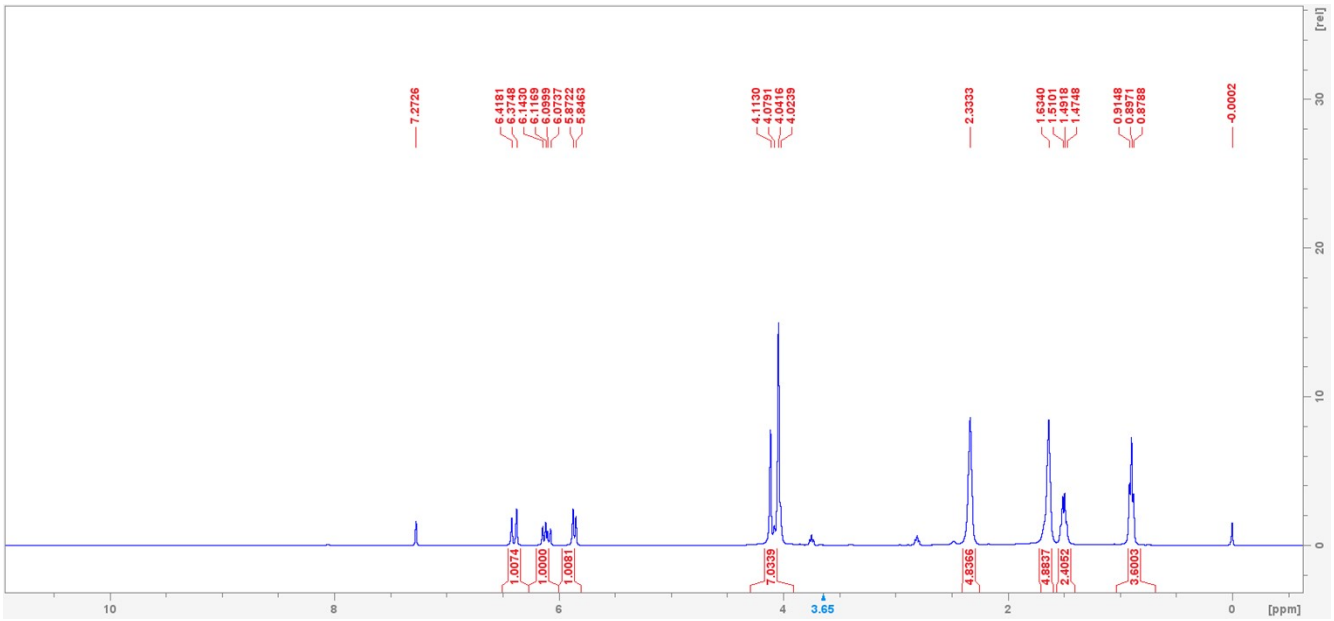

Figure S7. [1]H NMR spectrum of TMPA-AA.

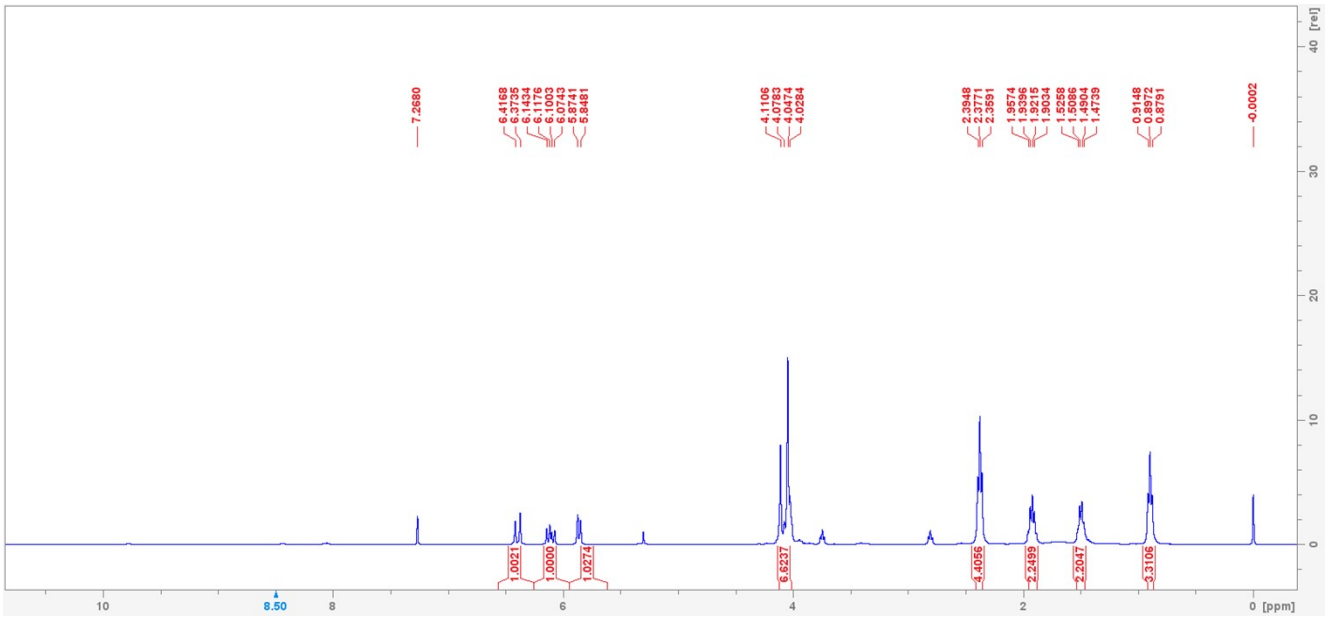

Figure S8. [1]H NMR spectrum of TMPA-GA.

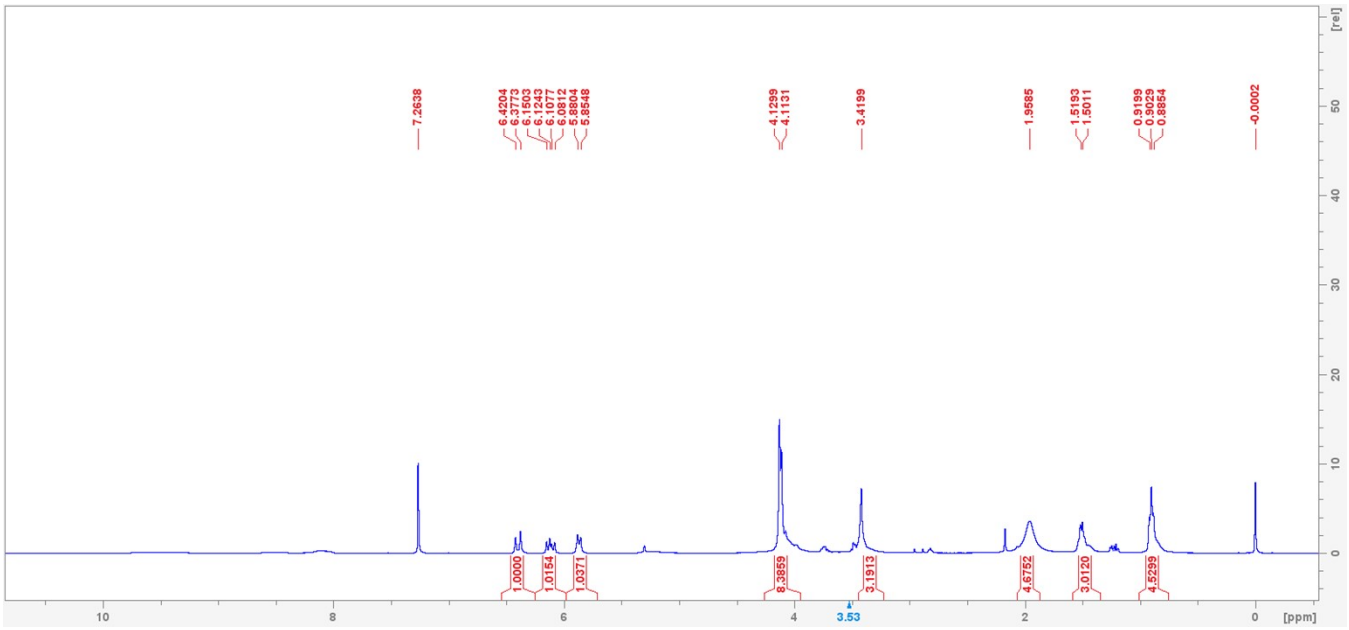

Figure S9. ^{1}H NMR spectrum of TMPA-MA.

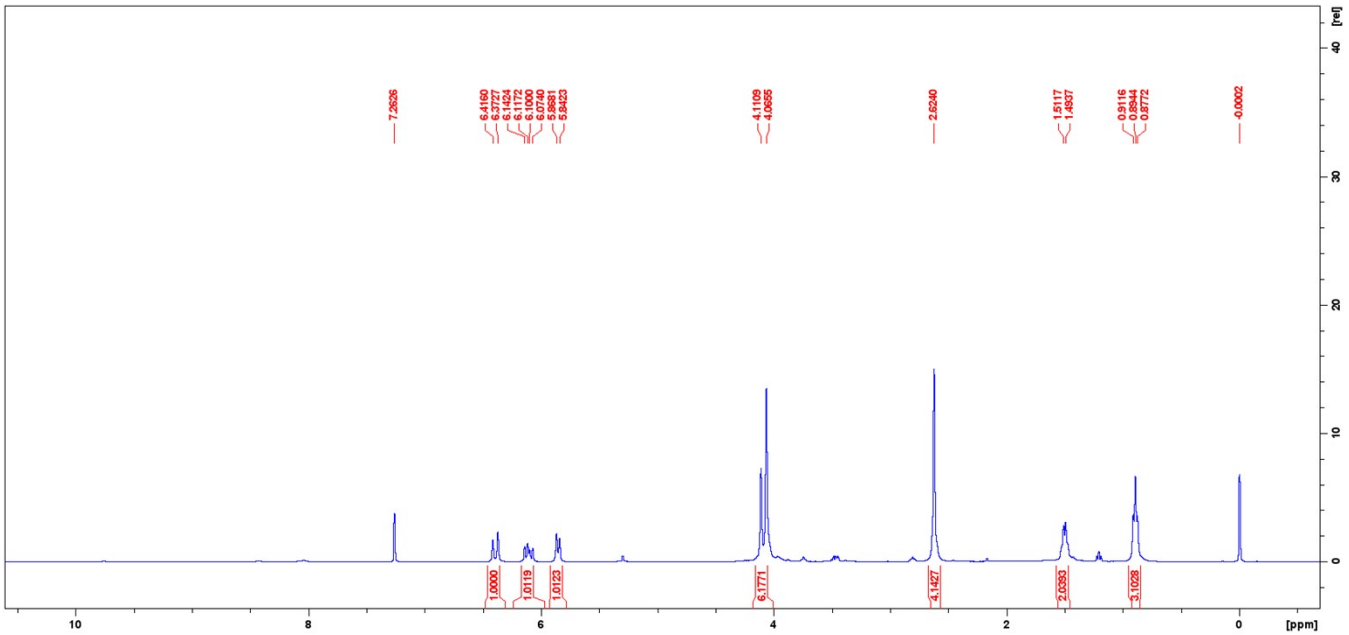

Figure S10. ^{1}H NMR spectrum of TMPA-SA.

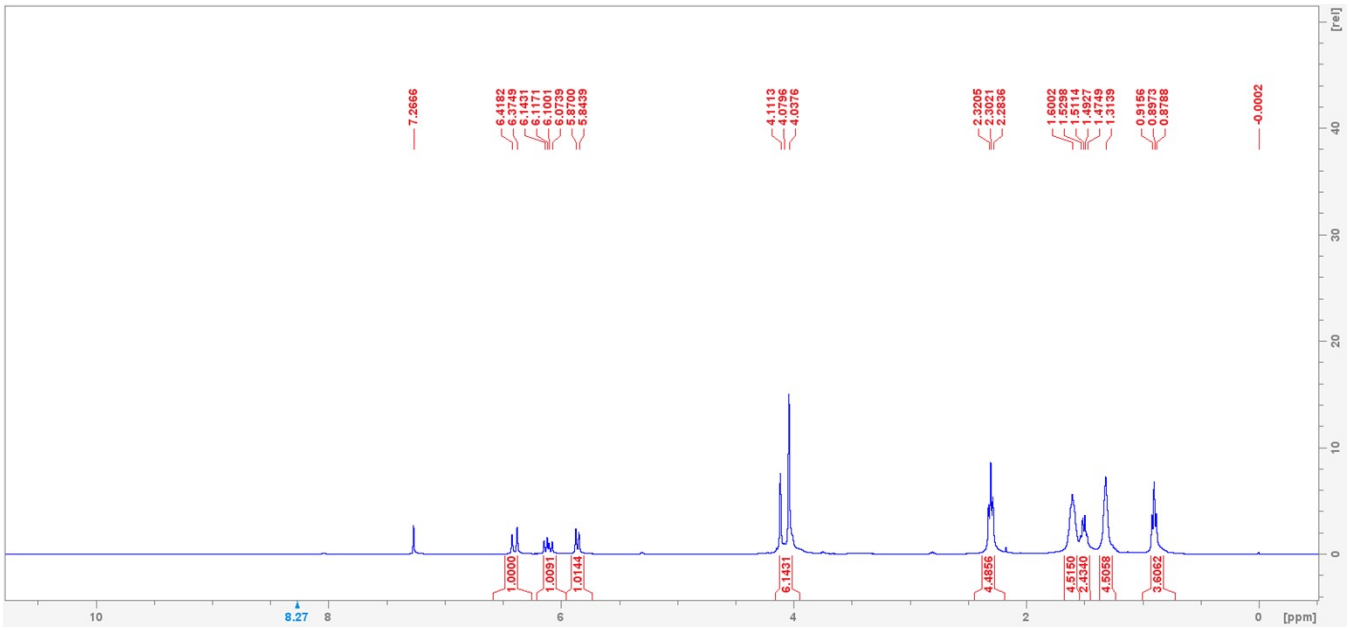

Figure S11. ^{1}H NMR spectrum of TMPA-SbA.

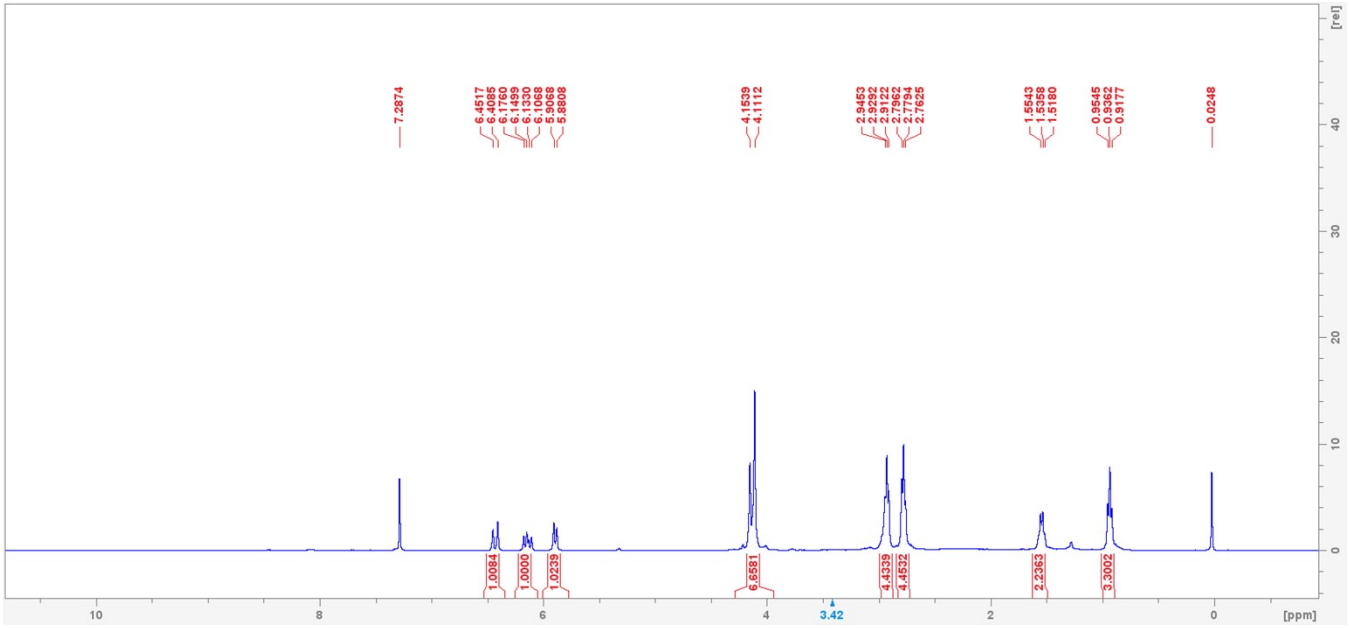

Figure S12. ^{1}H NMR spectrum of TMPA-DTPA.

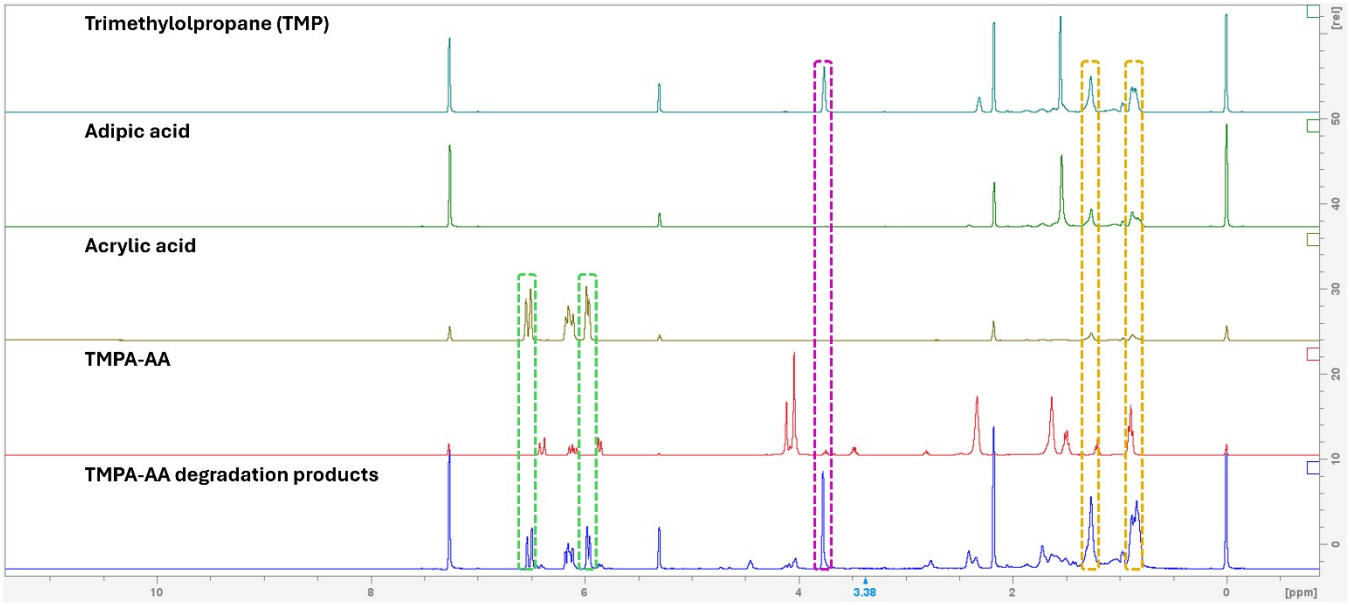

Figure S13. Comparison of ^{1}H NMR spectra of TMP, adipic acid, acrylic acid, TMPA-AA, and TMPA-AA degradation products.

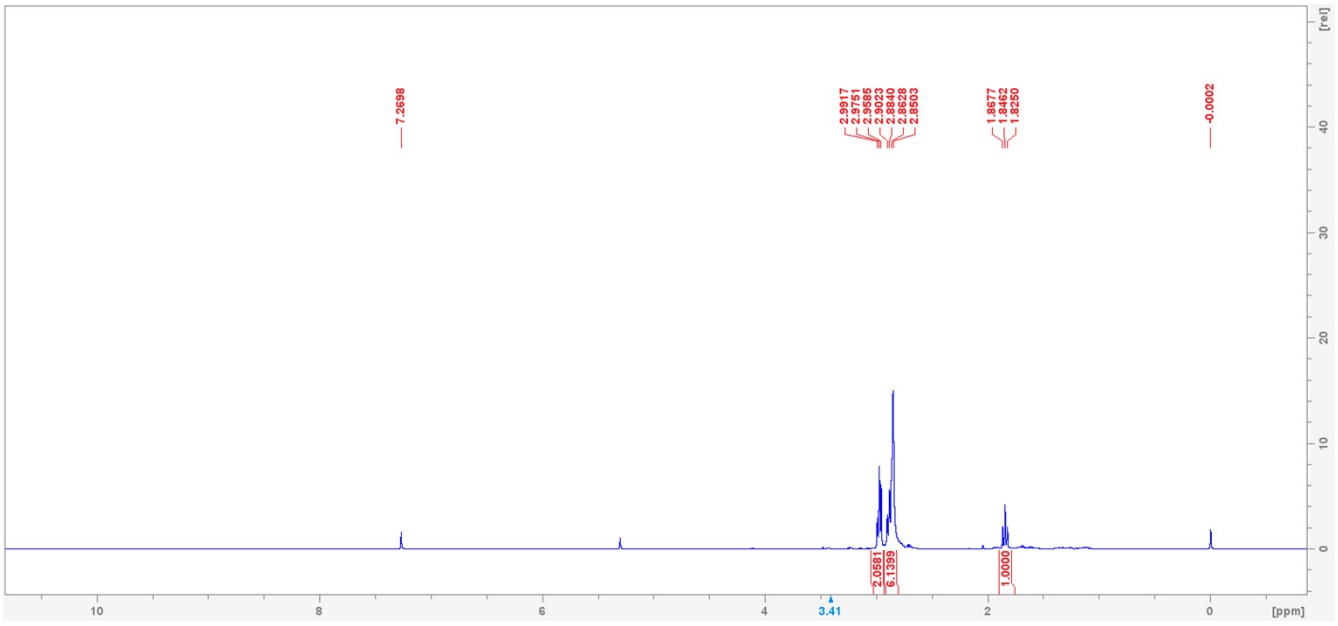

Figure S14. ^{1}H NMR spectrum of MPA-NHS.

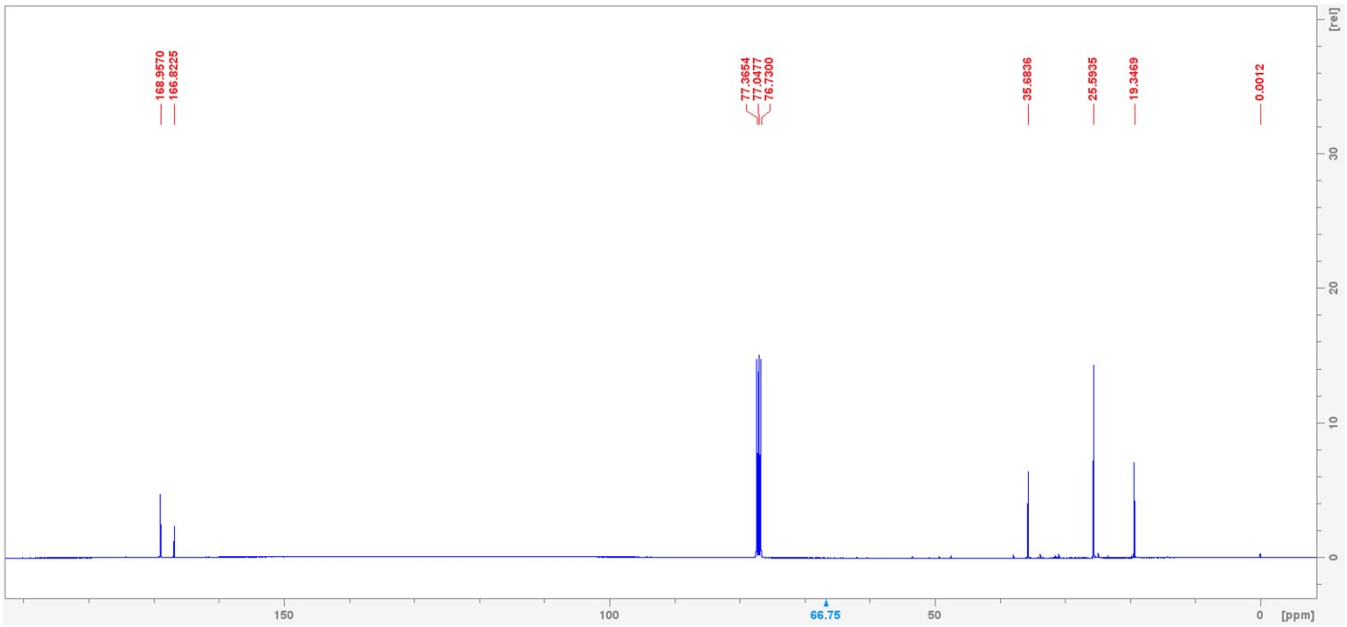

Figure S15. ^{13}C NMR spectrum of MPA-NHS.

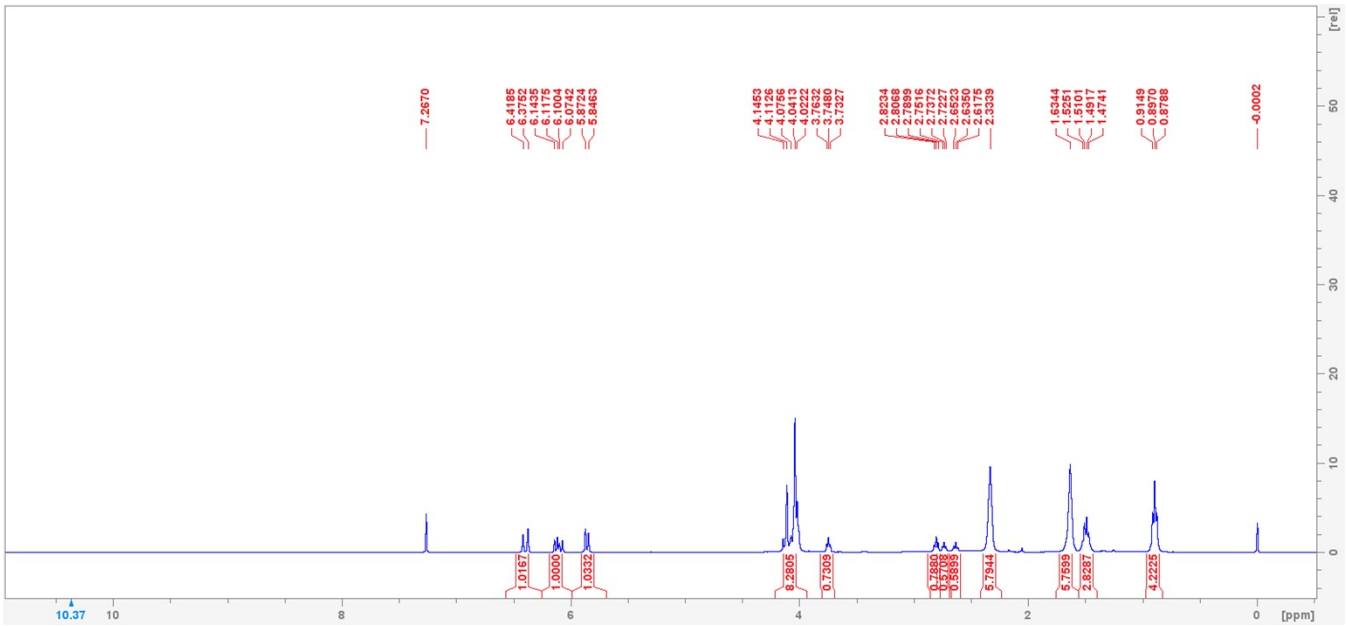

Figure S16. ^{1}H NMR spectrum of TMPA-AA-BME.

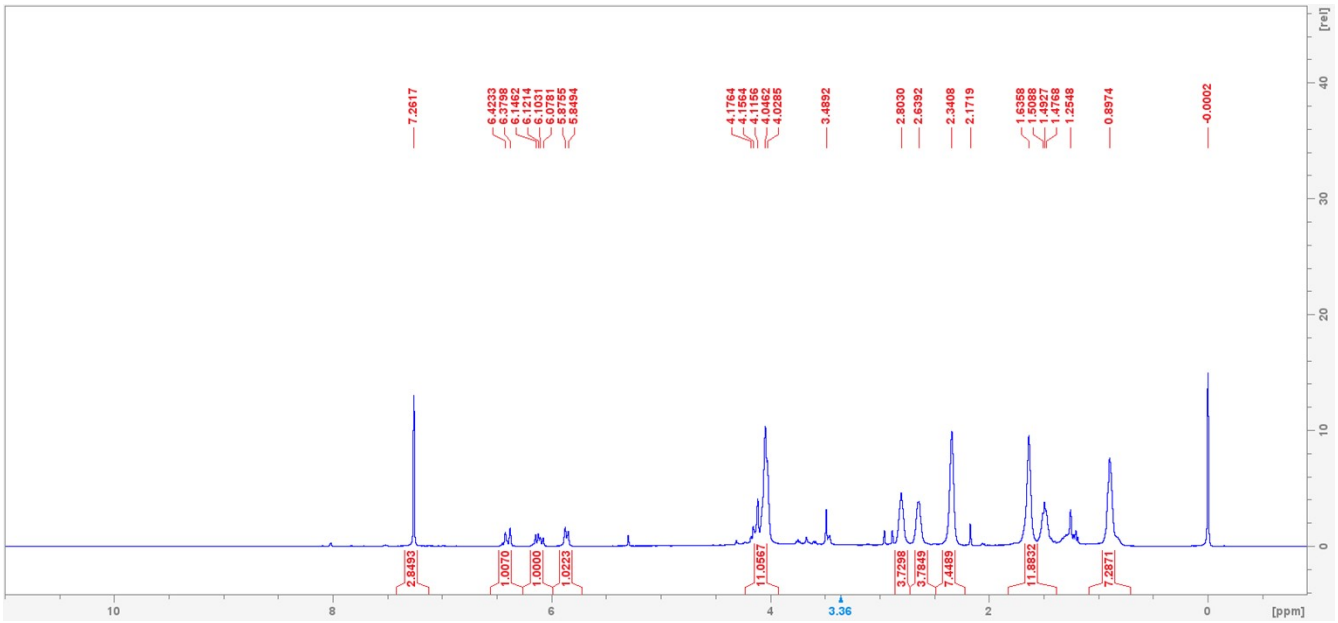

Figure S17. ^{1}H NMR spectrum of TMPA-AA-MPA.

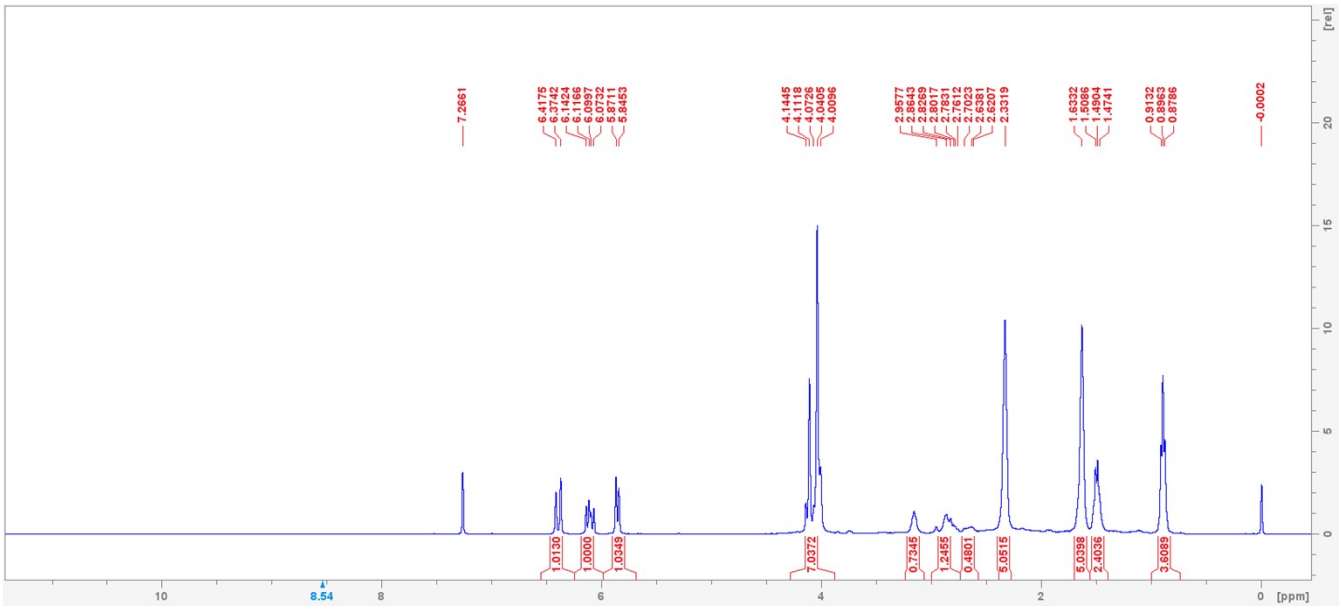

Figure S18. ^{1}H NMR spectrum of TMPA-AA-MPA-NHS.

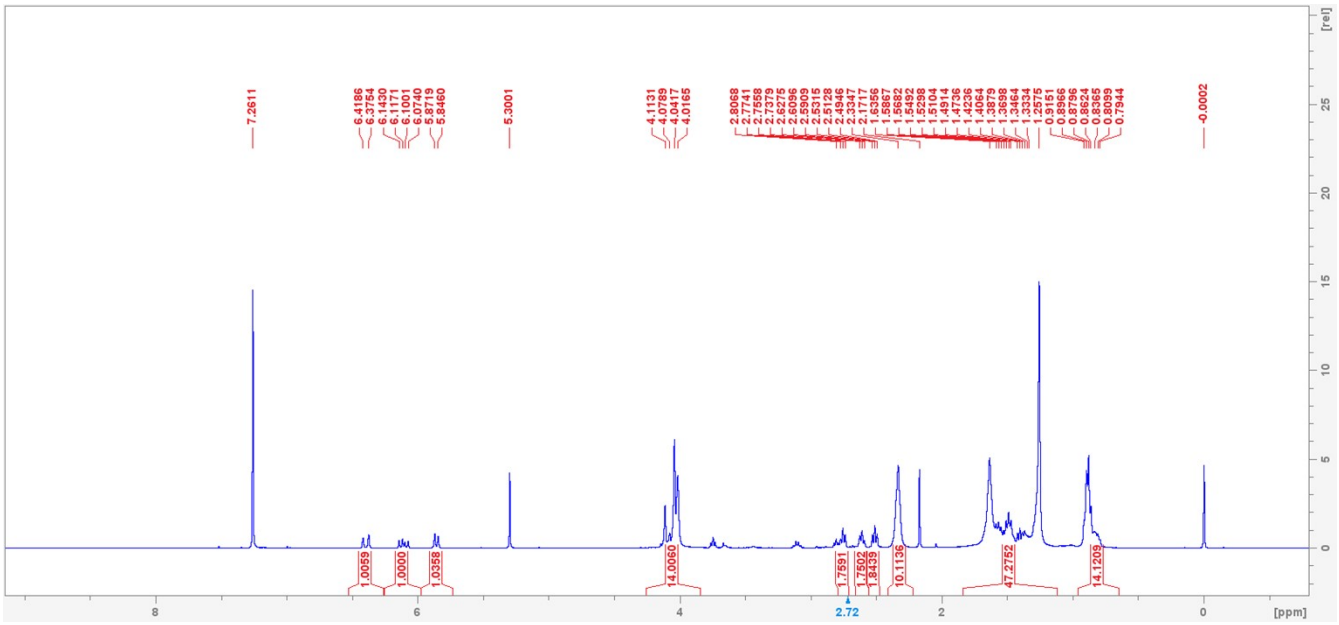

Figure S19. ^{1}H NMR spectrum of TMPA-AA-DDT.

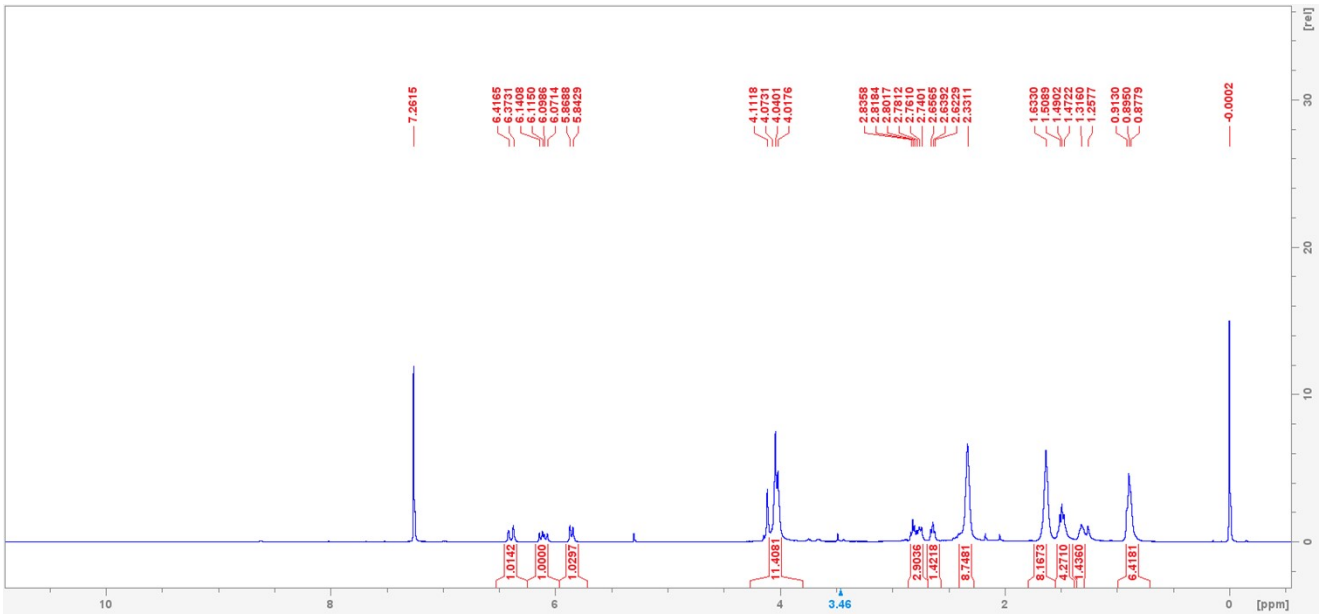

Figure S20. ^{1}H NMR spectrum of TMPA-AA-PFDT.

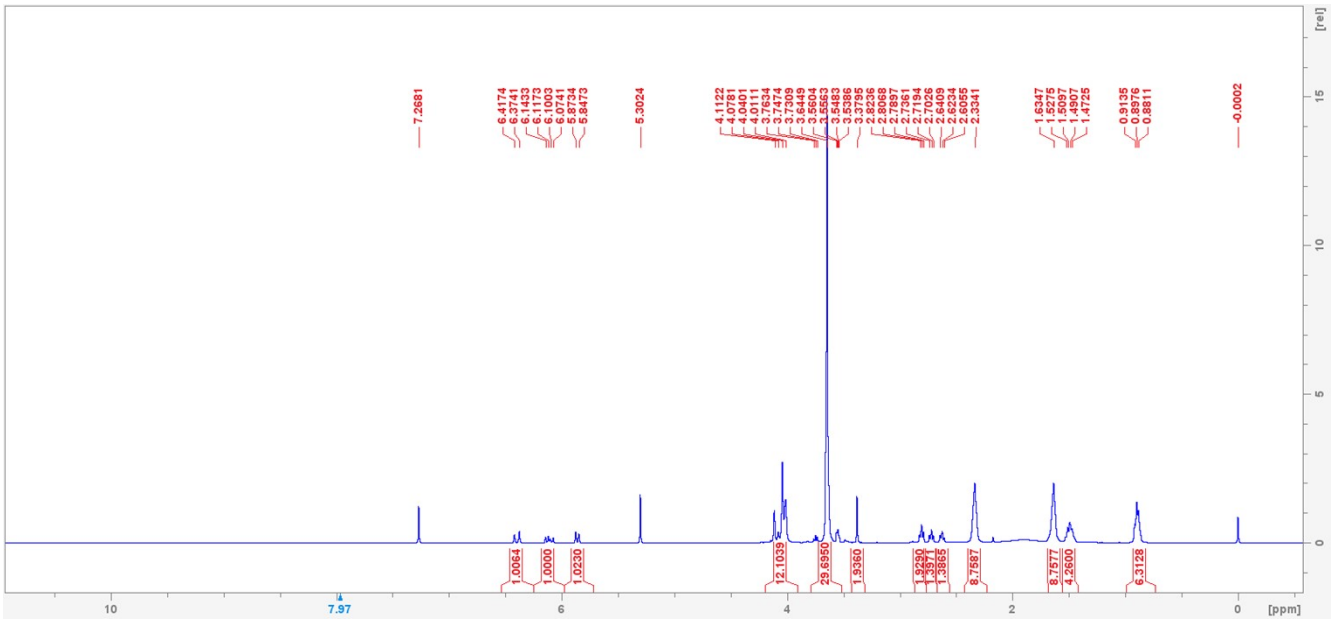

Figure S21. [1]H NMR spectrum of TMPA-AA-PEG.

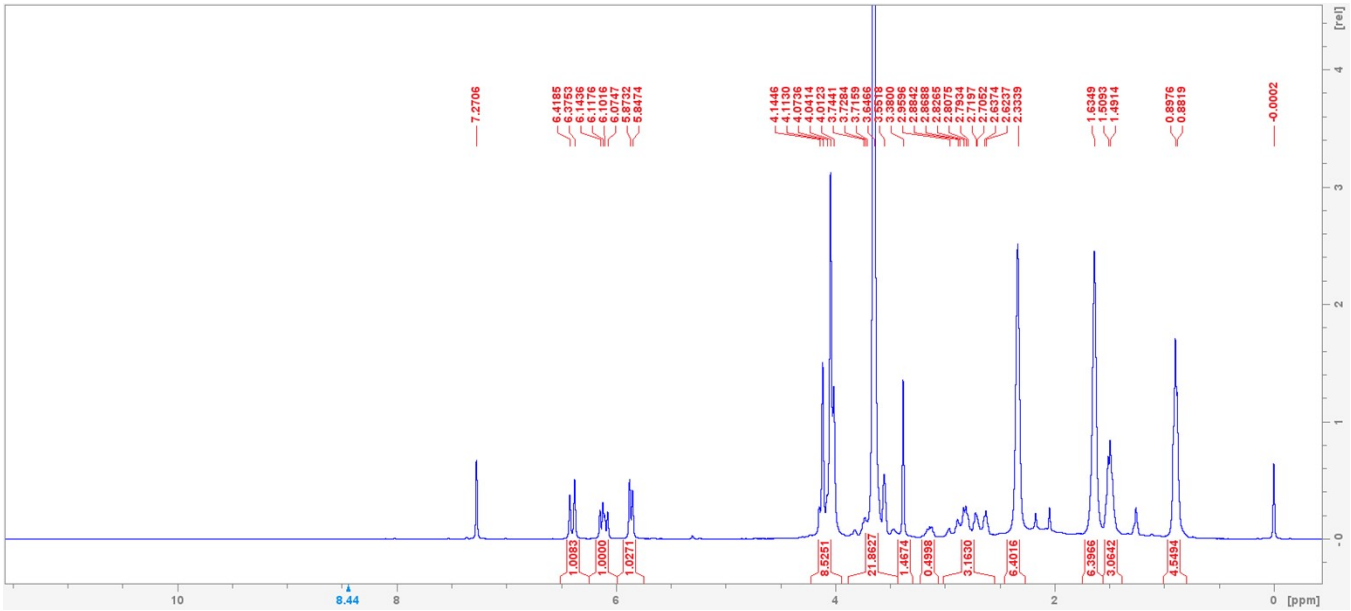

Figure S22. [1]H NMR spectrum of TMPA-AA-PEG-NHS.

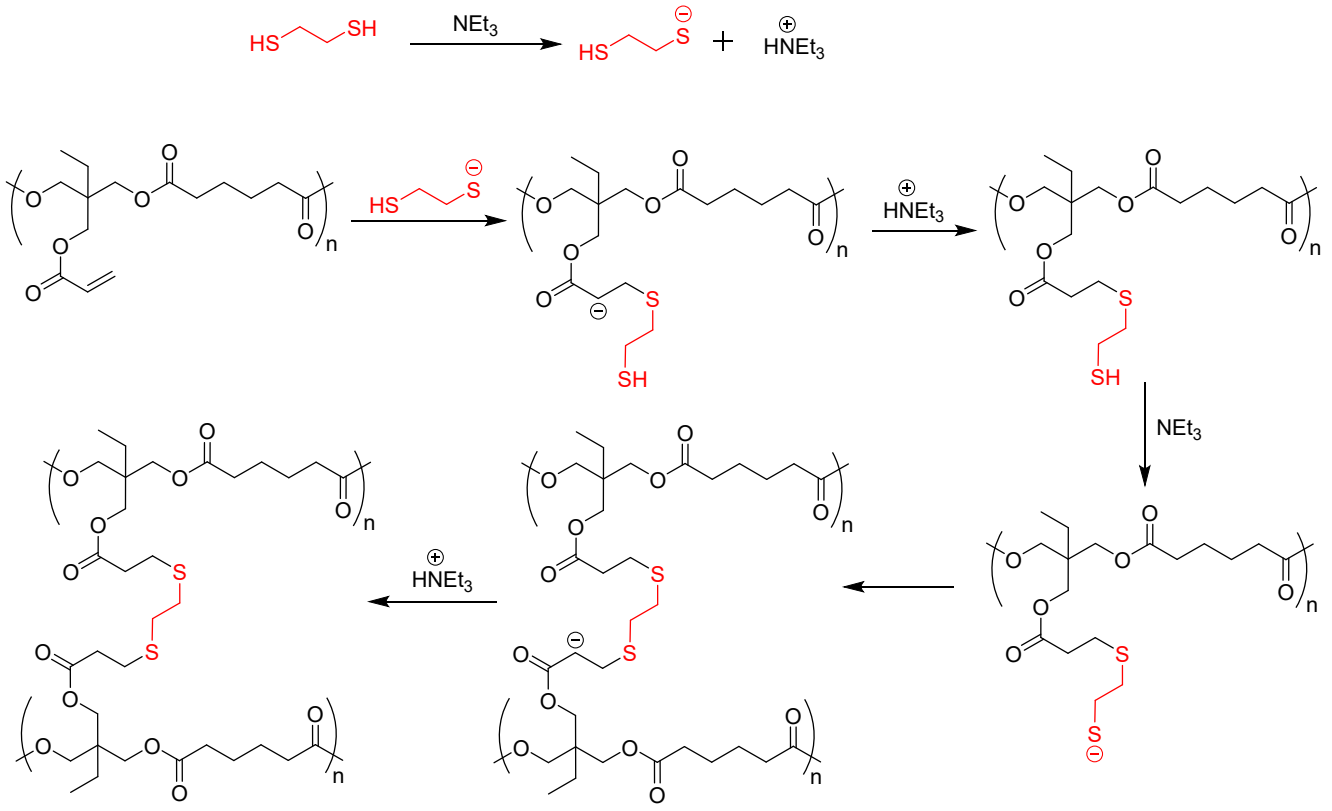

Figure S23. The mechanism for the polymer crosslinking through Michael addition.

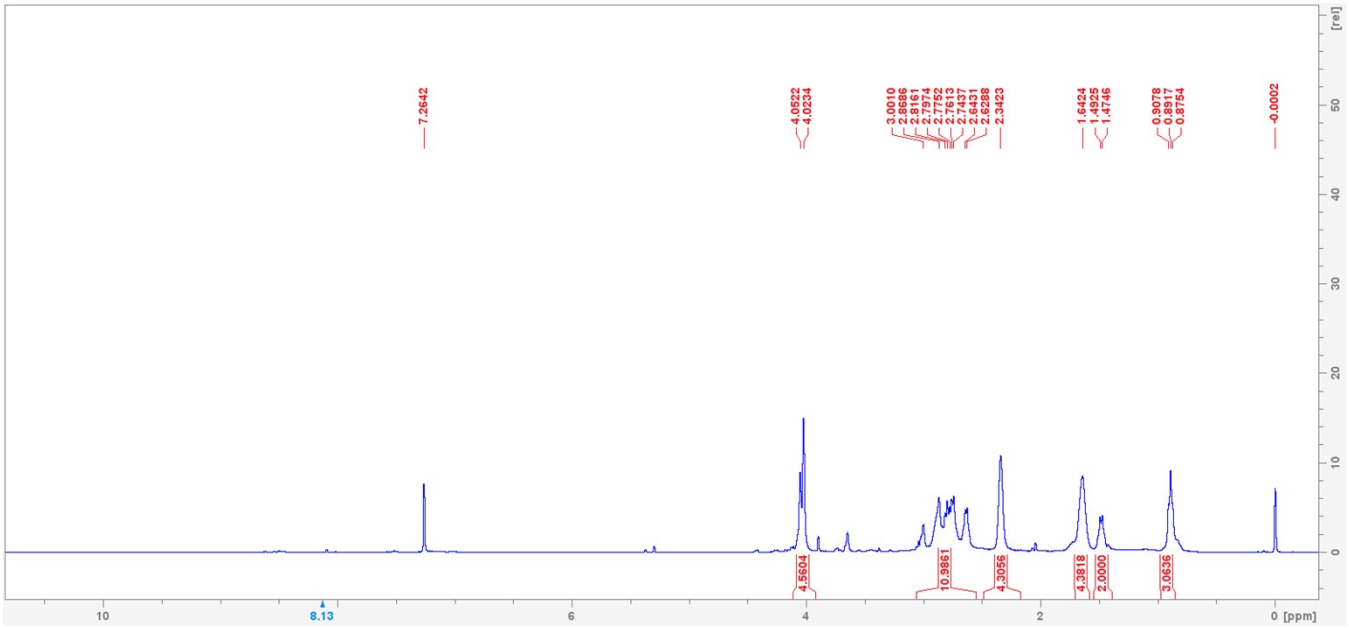

Figure S24. ^{1}H NMR spectrum of TMPA-AA-EDT.

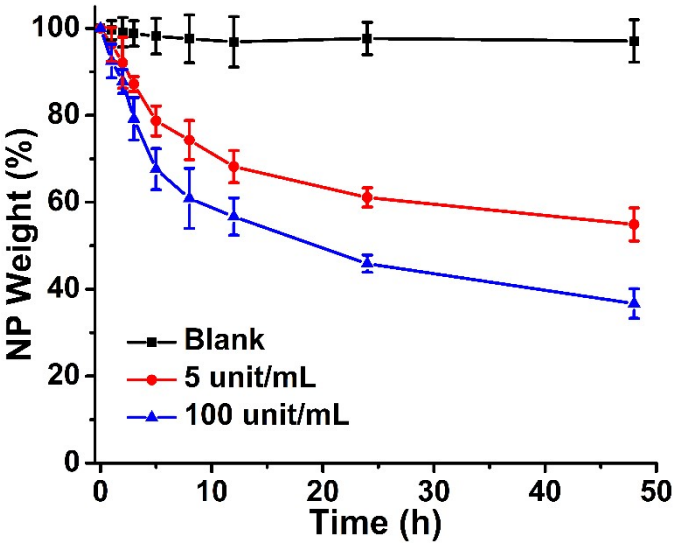

Figure S25. The degradation of TMPA-AA-PEG NPs upon incubation in PBS buffer containing the PLE enzyme (0, 5, and 100 units/mL).

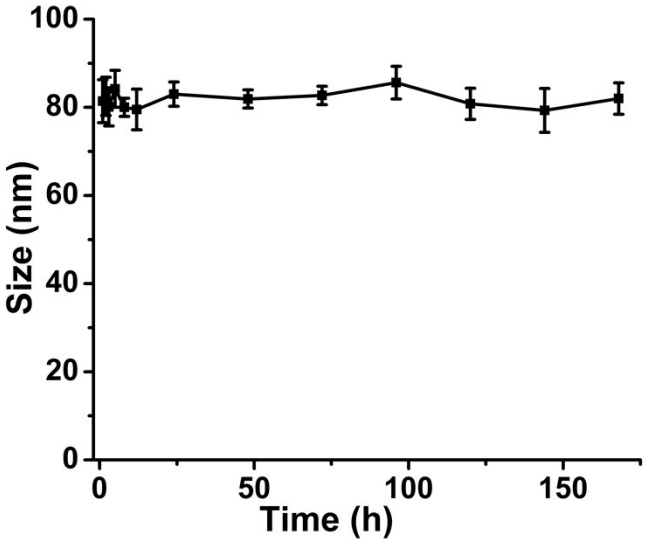

Figure S26. The hydrodynamic size of TMPA-AA-PEG NPs in deionized water over a week.

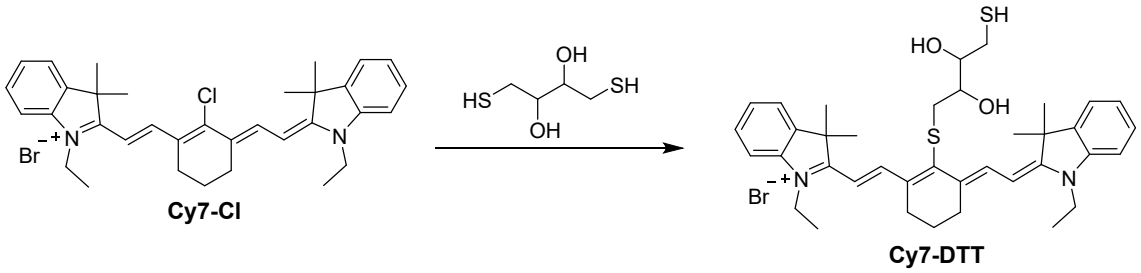

Figure S27. Synthesis of NIR fluorescent dye Cy7-DTT.

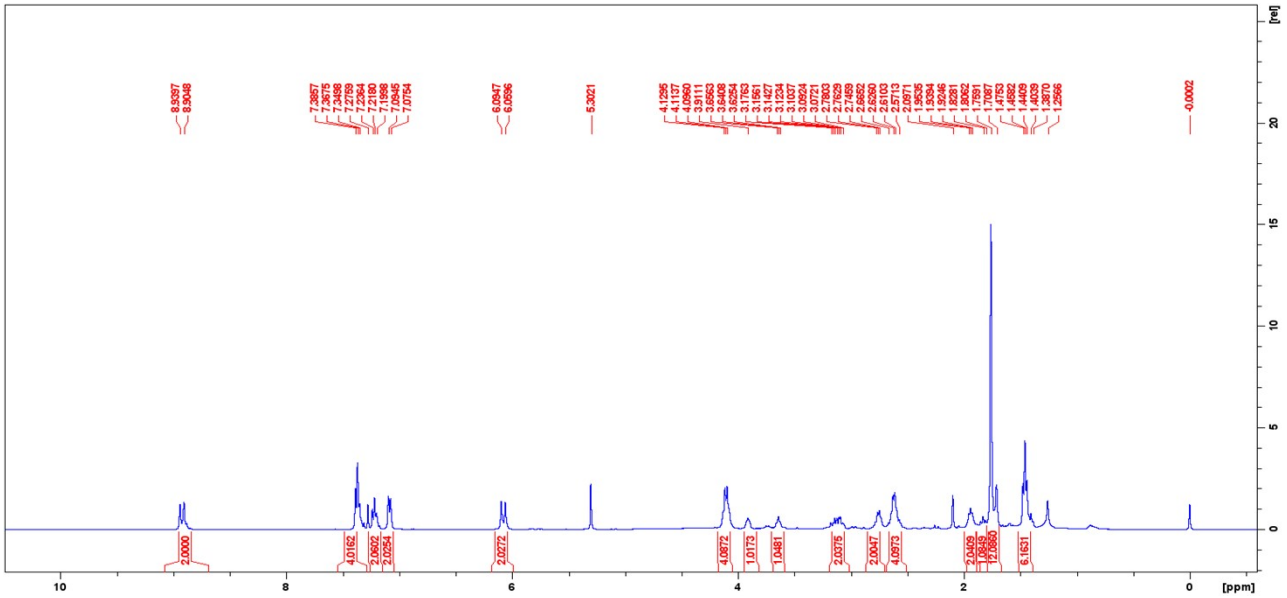

Figure S28. ^{1}H NMR spectrum of Cy7-DTT.

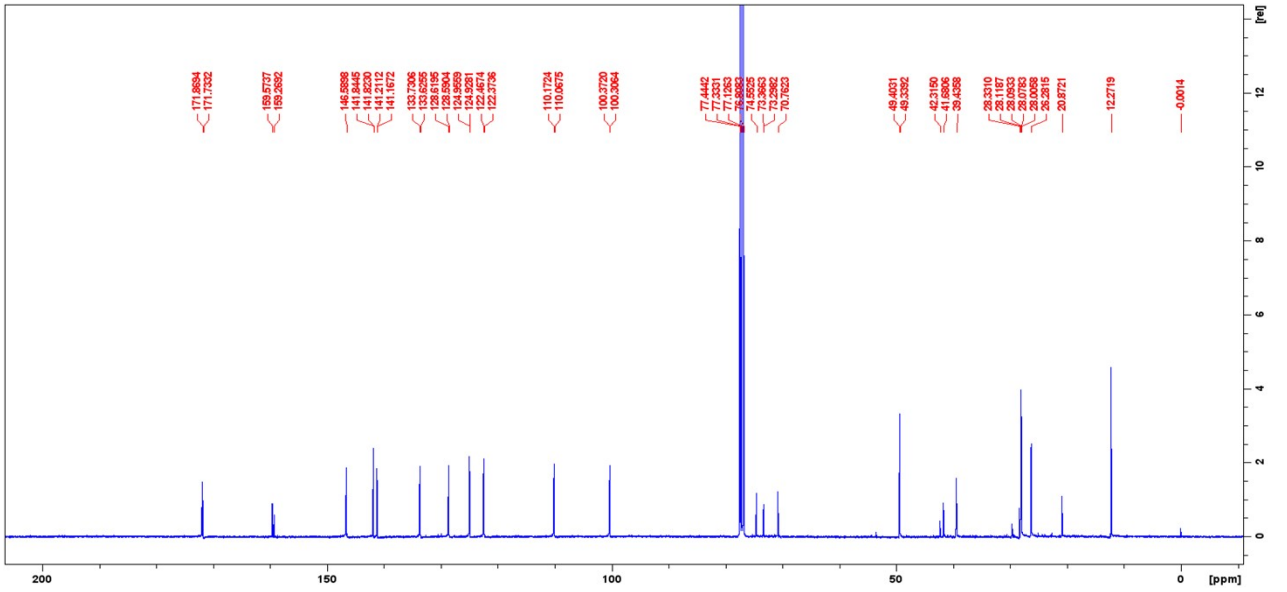

Figure S29. ^{13}C NMR spectrum of Cy7-DTT.

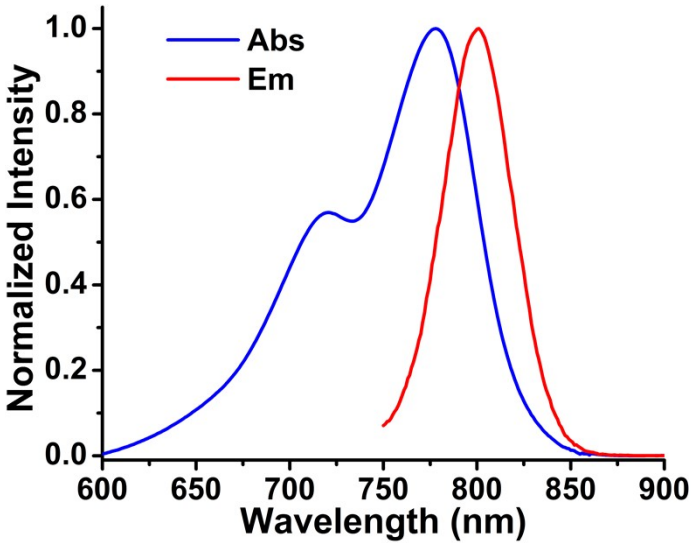

Figure S30. UV-vis absorption (blue) and fluorescence emission (red) spectra of Cy7-DTT.

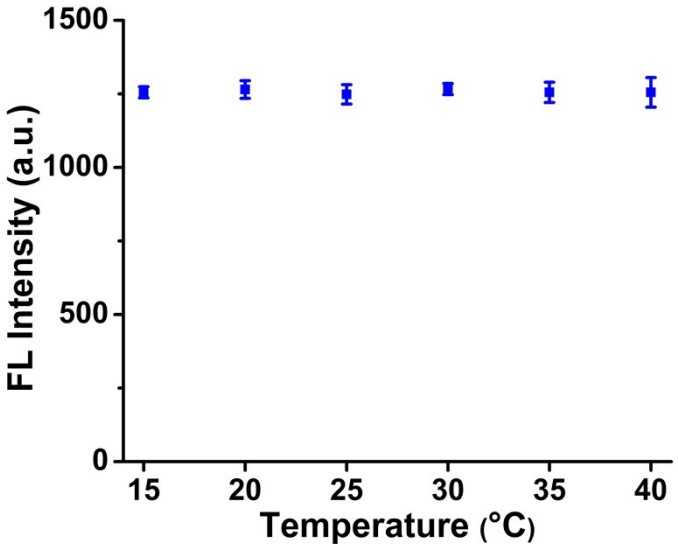

Figure S31. The fluorescence emission intensity of Cy7-DTT at different temperatures.

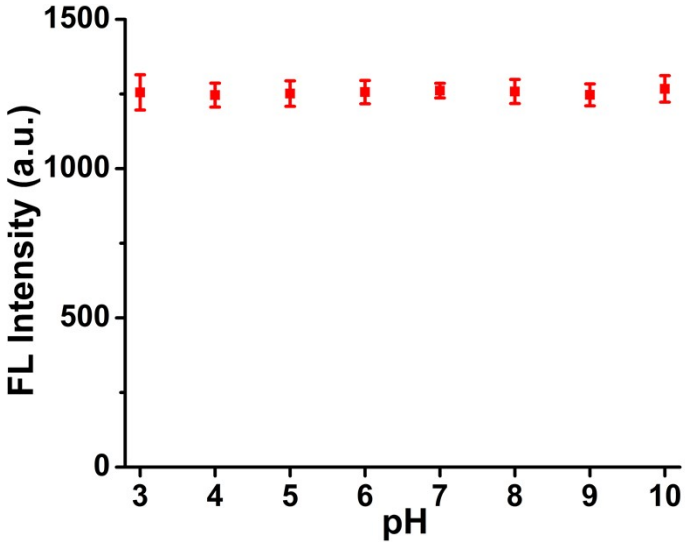

Figure S32. The fluorescence emission intensity of Cy7-DTT at varied pH.

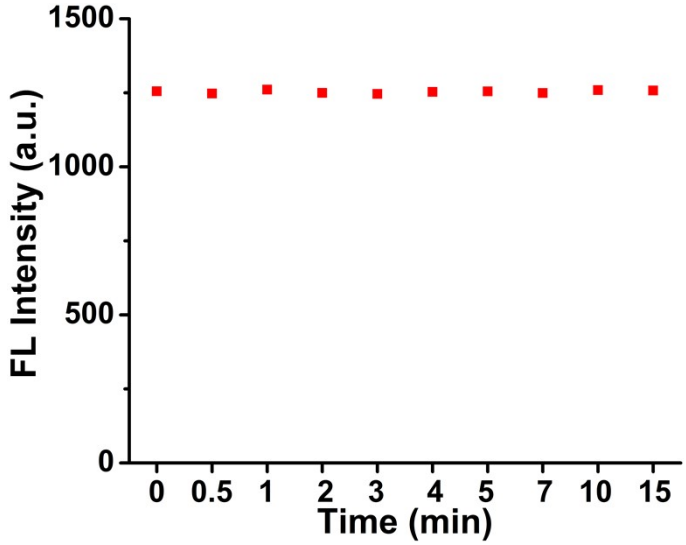

Figure S33. The Fluorescence emission intensity of Cy7-DTT after consecutive repeating measurements.

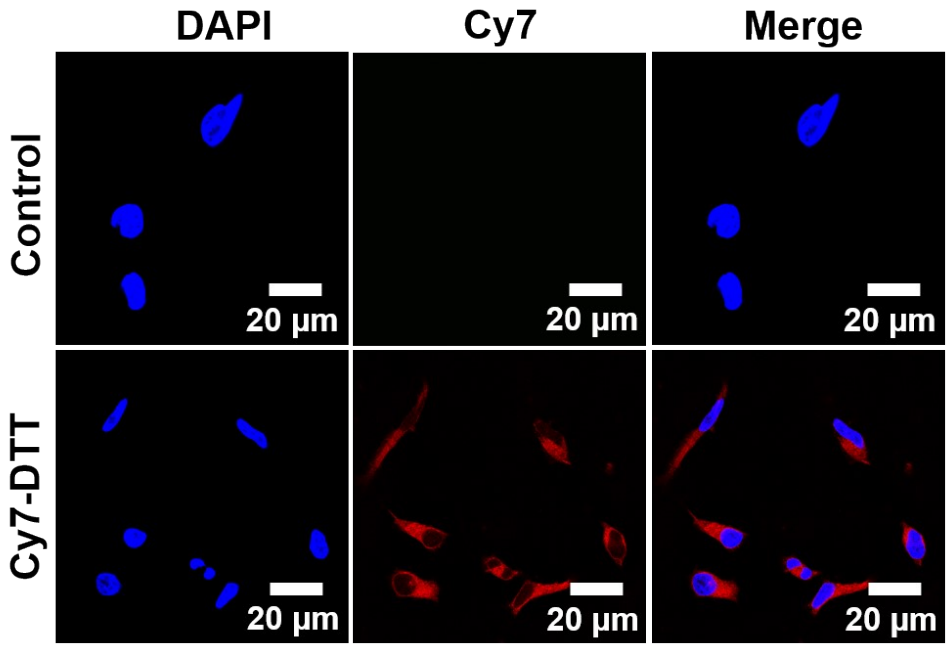

Figure S34. Confocal fluorescence images of Cy7-DTT in A549 cells after incubation for 3 h. Cell nuclei were stained with Hoechst 33342. The blue and red fluorescence are from Hoechst 33342 and Cy7-DTT, respectively.

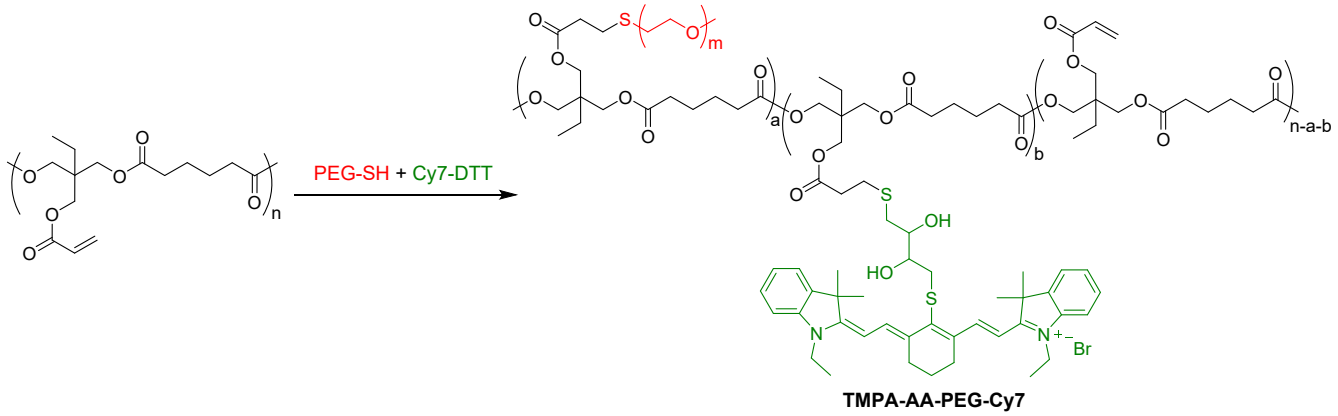

Figure S35. Synthesis of Cy7-labeled polymer TMPA-AA-PEG-Cy7.

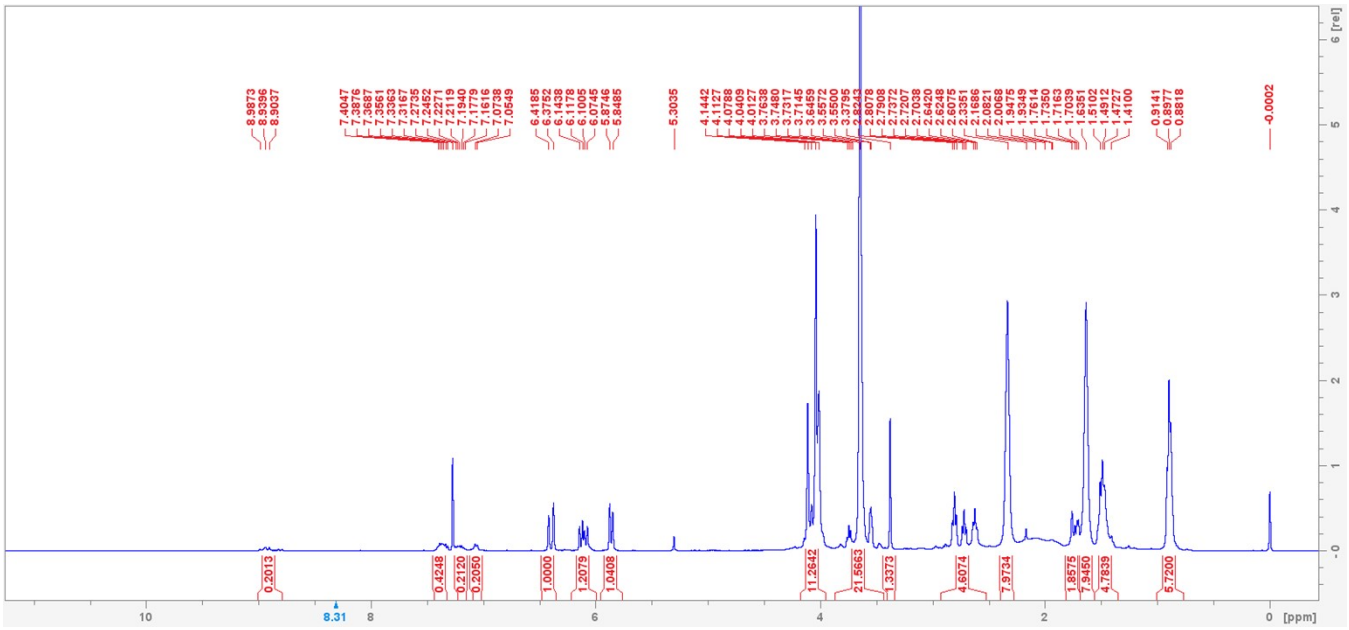

Figure S36. ^{1}H NMR spectrum of TMPA-AA-PEG-Cy7.

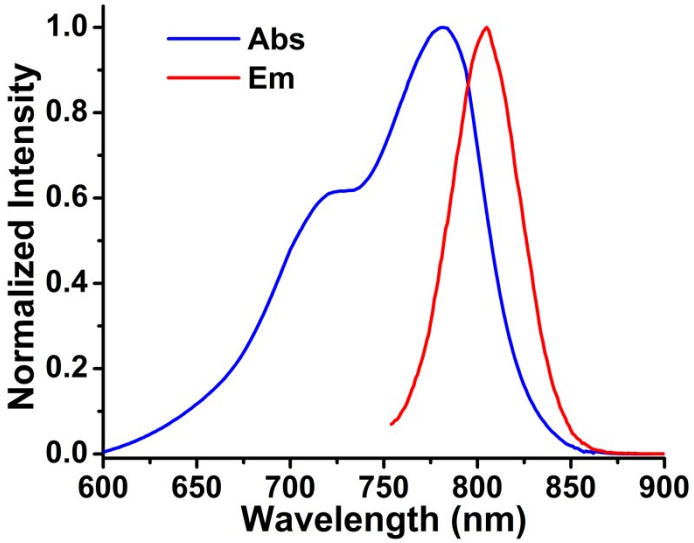

Figure S37. UV-vis absorption (blue) and fluorescence emission (red) spectra of TMPA-AA-PEG-Cy7.

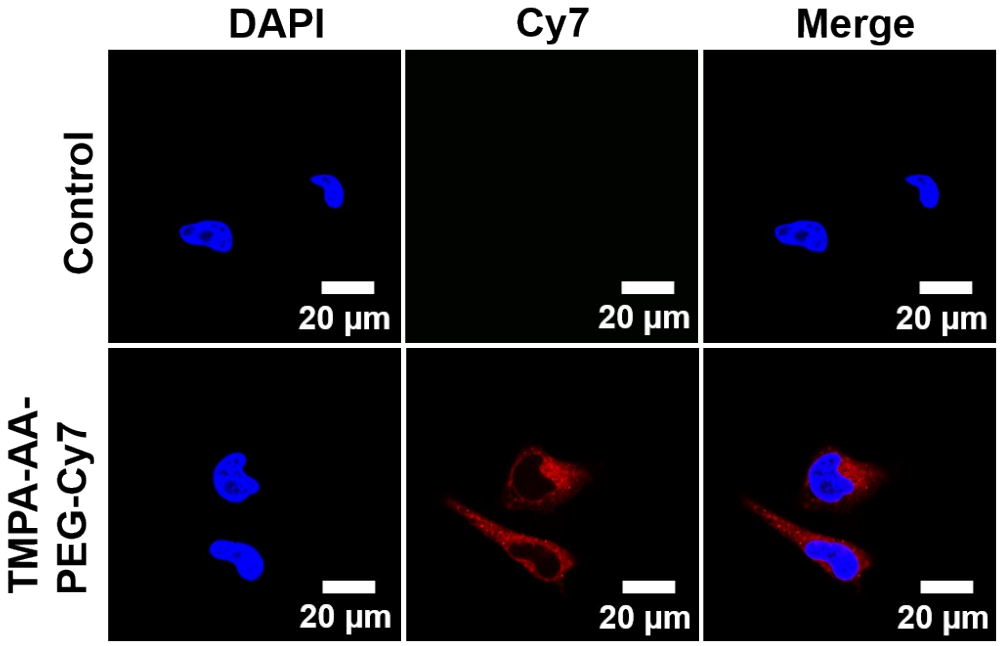

Figure S38. Confocal fluorescence images of TMPA-AA-PEG-Cy7 in A549 cells after incubation for 3 h. Cell nuclei were stained with Hoechst 33342. The blue and red fluorescence are from Hoechst 33342 and Cy7, respectively.

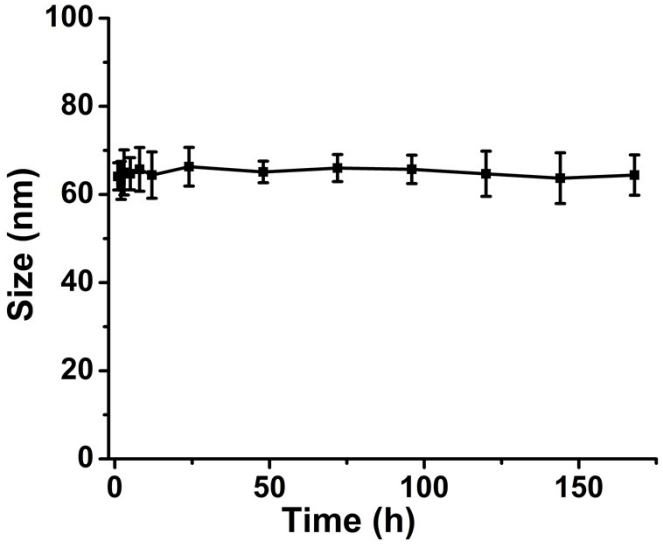

Figure S39. The hydrodynamic size of nano-Cy7 NPs in deionized water over a week.

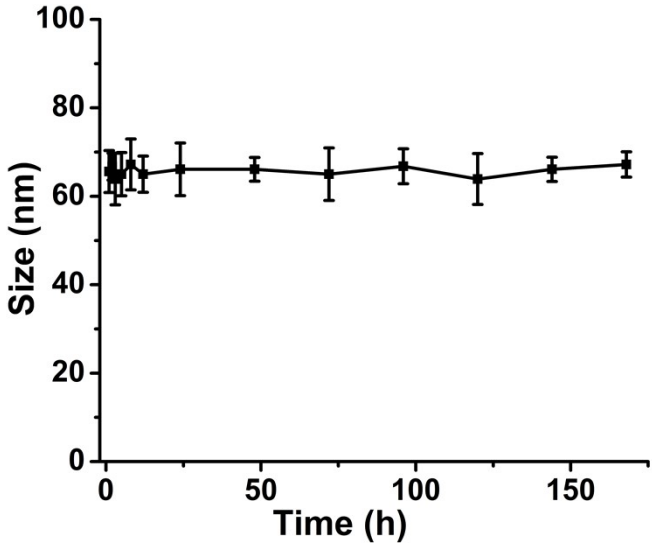

Figure S40. The hydrodynamic size of nano-Cy7-RGD NPs in deionized water over a week.

References

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2. Rurack, K.; Spieles, M. Fluorescence Quantum Yields of a Series of Red and Near-Infrared Dyes Emitting at 600-1000 nm. *Anal. Chem*. **2011**, *83*, 1232-1242.