Block Copolymer Synthesis using Free-Radical Polymerization and Thiol-Maleimide 'Click' Conjugation

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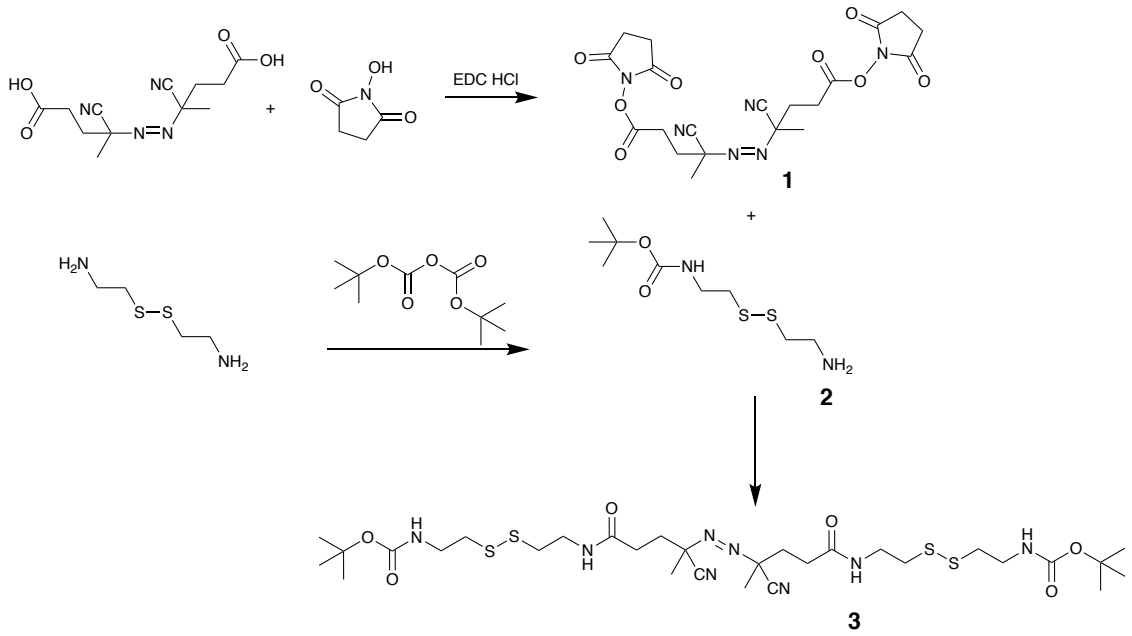
Supporting Information

Experimental

1. Materials and Methods

Unless otherwise stated, all materials were purchased from Sigma-Aldrich / Millipore-Sigma (Oakville, ON) and used as received. Poly(L-lactide) N-2-hydroxyethylmaleimide terminate (M_n 5000) was purchased from Sigma Millipore, actual M_n by ^{1}H NMR in $CDCl_3$ was calculated to be 6200 g/mol as per end group analysis. Methacrylic acid (MAA) was purified over inhibitor remover to remove hydroquinone and monomethyl ether hydroquinone. Di-tert-butyl dicarbonate was purchased from TCI America. DTNB (Ellman's Reagent) (5,5-dithio-bis-(2-nitrobenzoic acid) was purchased from ThermoFisher Scientific. 3-acrylamidophenylboronic acid (PBA) was purified by recrystallization in purified water. 1,4-Dioxane, tetrahydrofuran, diethyl ether, dichloromethane and acetone were purchased from Caledon Laboratories (Caledon, ON) and used as received. $CDCl_3$ and DMSO-d_6 was purchased from Cambridge Isotope Laboratories (Andover, MA) and used as received. Purified water with a resistivity of 18.2 MΩ cm was prepared using a Milli-pore Barnstead water purification system (Graham, NC). Phosphate-buffered saline (1x PBS) was purchased from BioShop (Burlington, ON). Regenerated cellulose dialysis membranes with molecular weight cutoff (MWCO) values of 3.5 kDa were purchased from Spectrum Laboratories (Rancho Dominguez, CA). ^{1}H NMR and ^{13}C NMR were recorded at room temperature on a Bruker AV 600 spectrometer (at 600 MHz for ^{1}H) using deuterated solvent. UV-Vis spectra were measured on an Agilent Technologies Cary 60 UV-Vis spectrophotometer. DLS measurements were run on Brookhaven Instruments Nanobrook 90Plus PALS particle sizer, Angle: 90°, Temperature: 25°C Count duration: 120s/cycle and 5 cycles. The micelle sample was filtered using 450nm nylon filter prior to analysis.

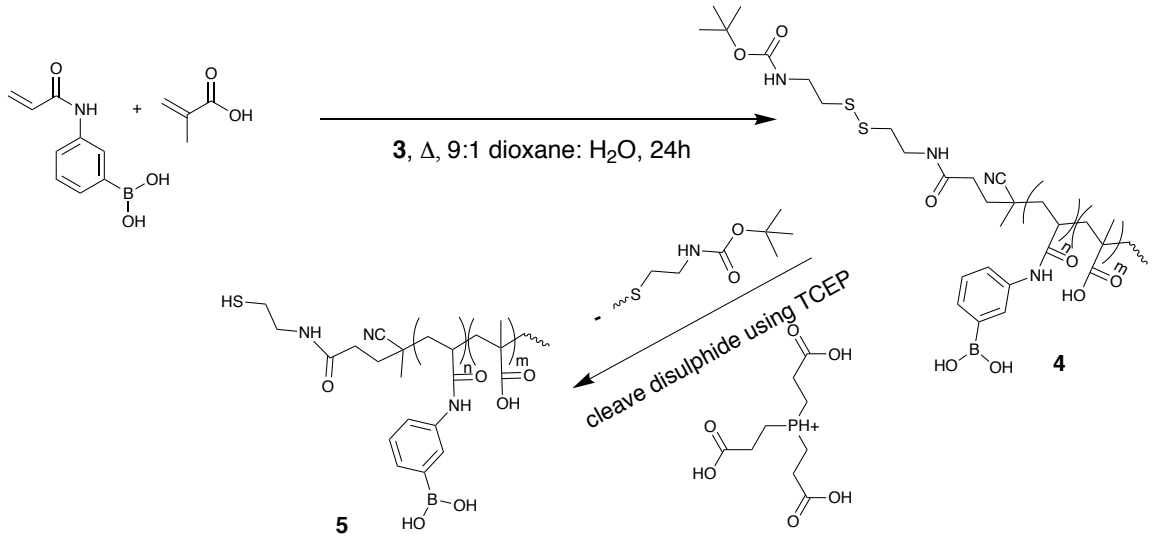
2. Synthesis of 3, Disulphide Radical Initiator Synthesis



Scheme 1: Synthesis of 3, functional radical initiator

An N-hydroxysuccinimide (NHS) functional radical initiator, **1**, was made from 4,4-Azobis(4-cyanovaleric acid) (ACVA) and NHS using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as a coupling reagent, following a procedure from Wang et al.[1] N-tert-Butoxycarbonyl cysteamine, **2**, was made by reacting cystamine with di-tert-butyl dicarbonate following the procedure from Suga et al.[2] Synthesis of the disulphide functional radical initiator, **3**, was adapted from Fan et al.[3] Briefly, 0.2 g (0.42 mM) of **1** was mixed with 3 mL of DCM in a round bottom flask and stirred for 20 min, and cooled to 0°C (cloudy mixture). 0.24 g (0.95 mM) of **2** was dissolved into 3 mL of DCM with 300μL of triethylamine, this mixture was then added to the cooled solution of **1**. After 5 minutes, the mixture became transparent. The reaction was allowed proceed, stirring at 0°C, and slowly warmed to room temperature over 12 hours. The reaction mixture was transferred to a separatory funnel and washed with 10 mL of 0.1M sodium bicarbonate. The aqueous portion was discarded, then the organic layer was washed with 2 x 10 mL of brine. The organic layer was dried over anhydrous magnesium sulphate, gravity filtered, and the solvent removed using a rotary evaporator. The solvent evaporated producing a white foam which then collapsed into a sticky oil. **3** was further purified by column chromatography using a 50:50 mixture of acetone and hexane as the eluent. Following removal of the solvent, 0.11 g (34% yield) of initiator was obtained as a sticky viscous oil. **3** was characterized by ^{1}H NMR in $CDCl_3$. To utilize the initiator, a stock solution was made in 1,4-dioxane.

3. Polymerization of MAA and PBA with 3



Scheme 2: Polymerization of MAA and PBA and disulphide end group cleavage

95.6mg of **3** dissolved in 1,4-dioxane was added to a round bottom flask, followed by 405.5 mg of PBA and 745.6 mg of MAA. 12 mL of 9:1, 1,4-dioxane:purified water was added to make a ~10 weight % solution. A stir bar was added and the mixture was agitated until homogenous. The reaction mixture was covered with a septum, sealed with parafilm and bubbled with nitrogen for 90 minutes to remove any dissolved oxygen. The reaction was placed in an 80°C oil bath for 24 hours with stirring.

After 24 hours, the reaction was removed from the heat and allowed to cool to room temperature. The septum was removed to expose the reaction to air/oxygen to quench the reaction with continue stirring for 5 min. The dioxane/water was removed in vacuo and the polymer was dissolved in 8 mL of THF. The mixture was added drop wise slowly into 400 mL of cold stirring diethyl ether, precipitating the polymer as a fluffy white powder. The polymer, **4**, was isolated by vacuum filtration over Whatman 1 filter paper. The polymer was dissolved again in THF and the precipitation/isolation was repeated an additional two times into fresh ether.

After the last precipitation, the polymer was left to dry on the filter paper under vacuum. The mass of **4** obtained was 1.1700 g. ^{1}H NMR and ^{13}C NMR in DMSO-d_6, Figure 1, demonstrated the successful synthesis of **4** and the precipitation steps were successful at removing any unreacted monomer, necessary to move onto the next step of the reaction. **4** contained trace amounts of solvent and was used as-is for the next step.

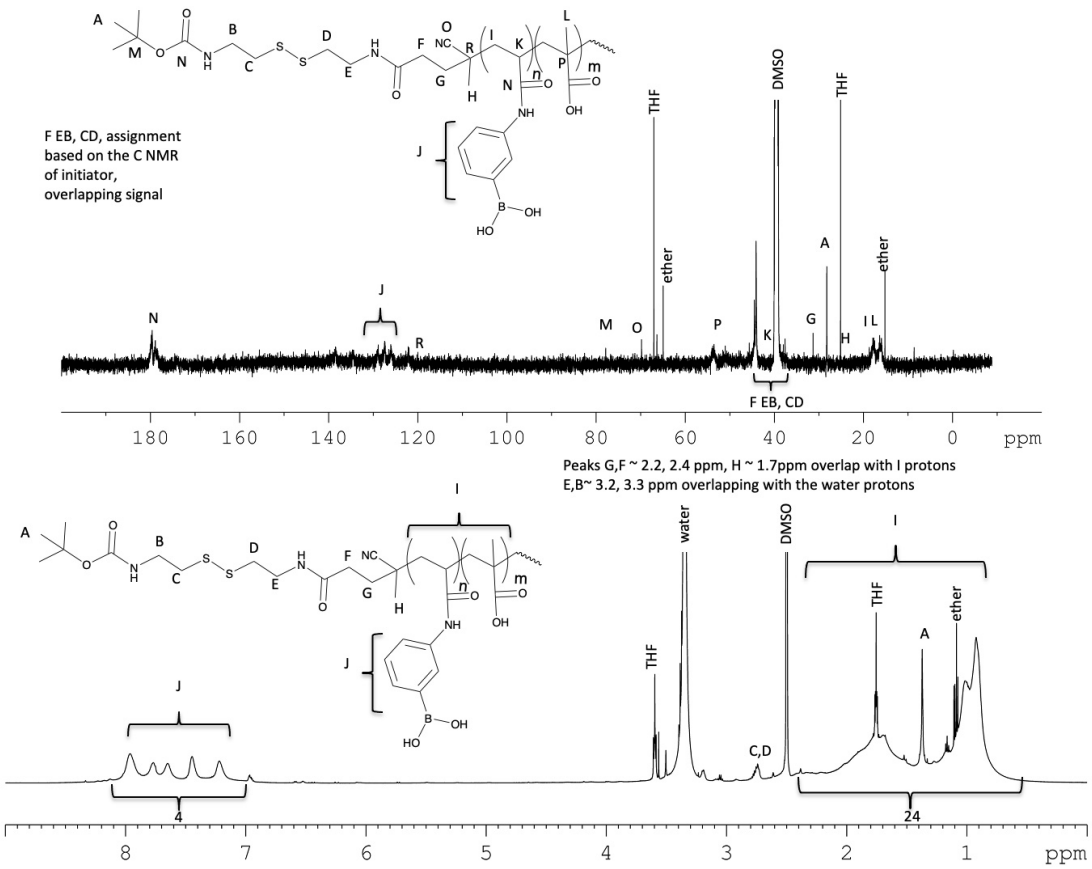


Figure 1: ^{1}H NMR (bottom) and ^{13}C NMR (top) of 4 in DMSO-d_6, tBoc terminated p-MAA-co-PBA

4. Synthesis of 5, thiol terminated p-MAA-co-PBA

4 was dissolved in 6 mL of 1:1 acetone: deionized (DI) water. An excess of Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 60 mg) was first dissolved in 2 mL of 1:1 acetone:DI water and then added to the polymer mixture and allowed to stir at room temperature for 5 hours. The mixture was transferred to 3.5K MWCO RC dialysis tubing and dialyzed sequentially against 1L 1:1 acetone/DI water for 6 hours and 4L of DI water for 12 hours. Some polymer precipitated once all the acetone is removed. The polymer suspension was transferred to a vial, frozen and lyophilized to yield 0.8900 g of the thiol terminated hydrophilic block, **5**. **5** was Characterized by ^{1}H NMR and ^{13}C NMR in DMSO-d_6 (Figure 2), which validated the absence of the tBoc end group. A small amount of polymer was taken for use in an Ellman's test to determine the amount of thiol and consequently, the molecular weight of the hydrophilic block. The rest of the polymer was used immediately for the next step of reaction.

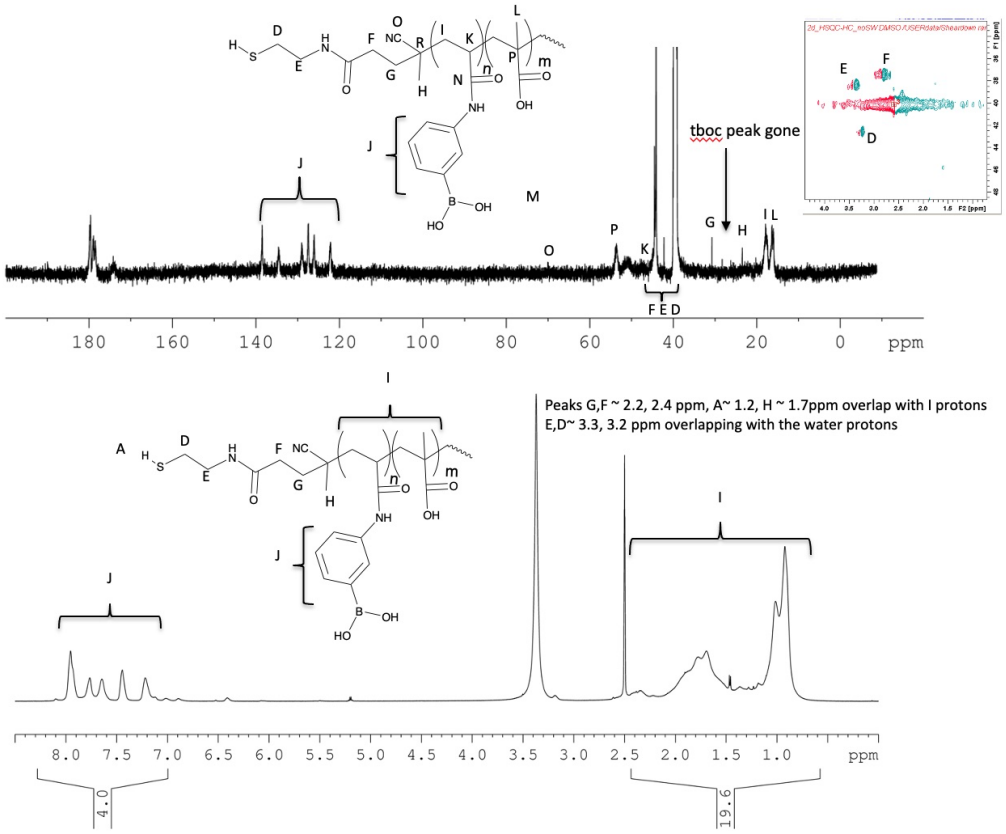


Figure 2: ^{1}H NMR (bottom) and ^{13}C NMR (top) of 5 in DMSO-d$_6$, thiol terminated p-MAA-co-PBA

5. Ellman's Test, Polymer Molecular weight

To determine the molecular weight of the hydrophilic block, Ellman's reagent was used to quantify the amount of end groups, thiols, terminating polymer **5**. The procedure was adapted from Thermo Scientific.[4] 0.1M Sodium phosphate at pH 8 containing 1 mM of EDTA (reaction buffer) was freshly prepared. A 4 mg/mL solution of Ellman's reagent was prepared in reaction buffer. **5** was dissolved in a vial with 2 mL of reaction buffer and 150 uL of 1 M NaOH was added to aid in dissolution and adjust the pH to 7.5. 3 concentrations of polymer **5** were used for the Ellman's test: 11.77 mg/mL, 5.88 mg/mL and 2.94 mg/mL. For each concentration: to a test tube 2.5 mL of reaction buffer, 50 uL of Ellman's reagent solution and 250 uL of polymer solution were added, then the solution was mixed. After 30 min, the mixtures were transferred to a cuvette (path length 12.5 mm) and the UV absorbance was measured at 412 nm, using reaction buffer as a blank. The following equation calculate the concentration:

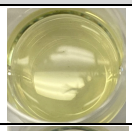
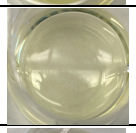
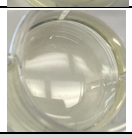
$$c = \frac{A}{bE}$$

Where E = 14,150 $M^{-1}cm^{-1}$ and b=1.25 cm.

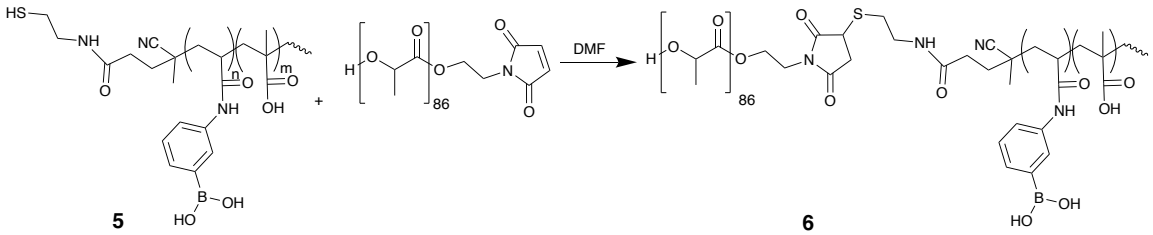
Using the absorbance values, the number of moles of polymer can calculated from the 2.8 mL test solution. This is equivalent to the number of moles in the original 250 uL polymer solution used. Have both the mass used to make the polymer solution and the number of moles, the molecular weight was calculated. The average molecular weight was determined from the different concentrations tested. The results are summarized in Table 1.

Results Ellman's test

Table 1: Ellman's Test Results

Concentration (mg/mL)	Absorbance	Calculated Molecular Weight (g/mol)	Image the of solution
11.767	1.19	15,600	
5.88	0.58	16,000	
2.94	0.28	16,300	
Average:	16,000 ± 300		

6. Synthesis of Polymer 6, Thiol-Maleimide Click Reaction



Scheme 3: Click ligation of Mal-PLA and p-MAA-co-PBA

0.8400 g of **5** and 1.297 g (excess) poly(L-lactide) N-2-hydroxyethylmaleimide terminated (PLA, 6,200 g/mol, ^{1}H NMR and ^{13}C NMR in Figure 3) were weighed into a round bottom flask. 10 mL of DMF and a stir bar were added, then the mixture was stirred to dissolve the polymers. While the mixture was stirring to dissolve, it was sealed with a septum and

parafilm. Nitrogen was bubbled through the mixture for 1 h to remove any dissolved oxygen, which could cause thiol oxidation.

The reaction was left to stir for 4 days at room temperature. The polymer mixture was then precipitated into 350 mL of cold diethyl ether and allowed it to stir for 3 minutes. The mixture was vacuum filter over Whatman 1 filter paper. The polymer was removed from the filtration apparatus, 50 mL of cold DCM was added, and the suspension stirred for a few minutes. The mixture was then vacuum filtered over a 90 mm Buchner funnel with Whatman 1 filter paper and the polymer was rinsed with 2 x 15 mL of DCM and allowed to dry on the filter paper with the vacuum running and subsequently placed it in the vacuum oven at 50°C for 12 hours to dry. 0.896 g of polymer **6** was obtained. The polymer was characterized by ^{1}H NMR in DMSO-d_6, demonstrating pure block co-polymer (no unreacted maleimide PLA), however, trace amounts of DMF were still present despite the rinsing and drying steps.

If desired, a quick round of dialysis could remove the trace DMF. The polymer was dissolved in 10 mL 1:1 acetone: DI water and placed into 3.5 MWCO dialysis tubing and dialyzed against 4 L of DI water for several hours to remove any trace DMF solvent. The polymer was then placed in a vial, frozen and lyophilized to yield 0.742 g of pure **6.** ^{1}H NMR and ^{13}C NMR in DMSO-d_6 was used to characterize the polymer (Figure 4). The known PLA molecular weight (6,200 g/mol) was used as an internal standard to calculate the average polymer molecular weight, $M_{n,}$ of 19 300 g/mol. The hydrophilic portion accounted for ~13, 100 g/mol with an MAA: PBA of 4:1.

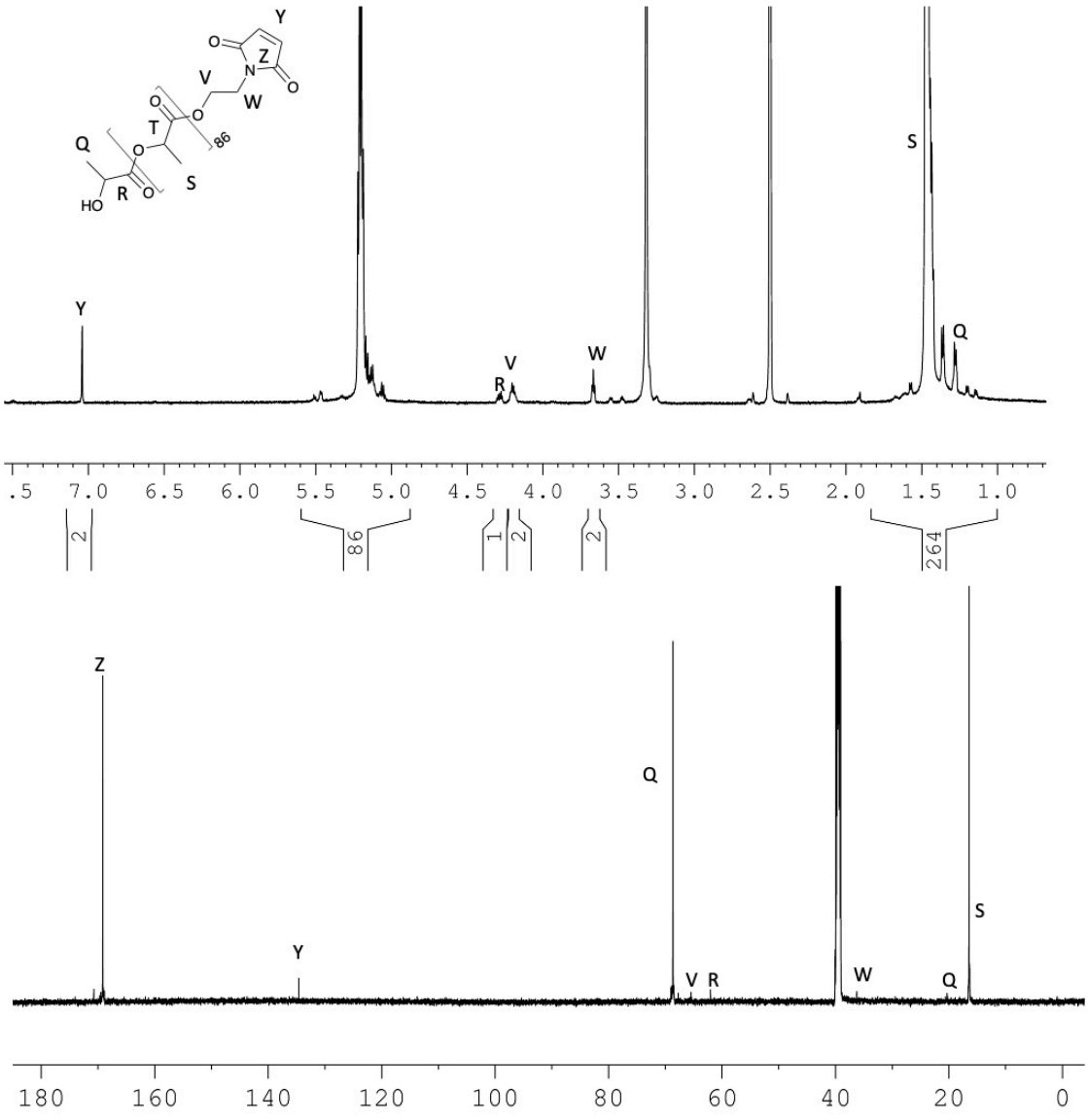


Figure 3: maleimide functional PLA ^{1}H NMR (top) and ^{13}C NMR (bottom) in DMSO-d$_6$

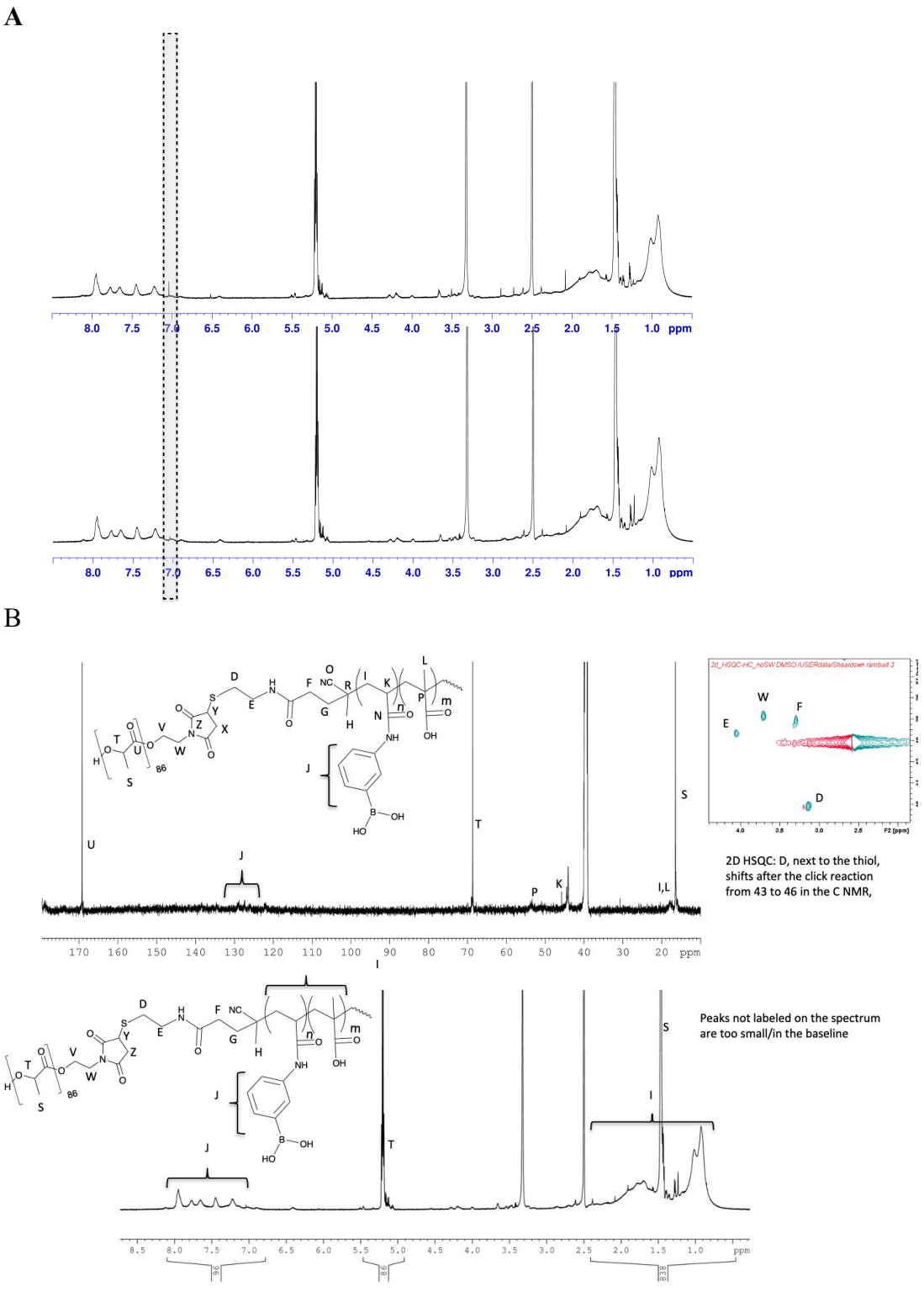


Figure 4: ^{1}H NMR of 6, p-PLA-b-(MAA-co-PBA) in DMSO-d_6 A – zoomed in after precipitaing in ether vs after rinsing with DCM removing excess PLA and B – the final polymer ^{1}H NMR (bottom) and ^{13}C NMR (top)

7. Micelle Formation

To form micelles of polymer **6**, the solvent evaporation method previously described was utilized[5]. Briefly, 20 mg of **6** was added to a vial with 2 mL of acetone. The mixture was gently heated to 50°C for 2 min intervals and 5 drops of DI water were added and gently swirled until the polymer was dissolved. The mixture was then added dropwise to 4 mL of stirring PBS at pH 7.4. The mixture was left stirring loosely covered for 1 day to evaporate the acetone. The pH of the mixture was adjusted to 6.2 with 1M NaOH and the final volume was adjusted to 4 mL, producing a micelle solution of polymer **6** at a concentration of 5 mg/mL. The particle size was 85 nm as determined by DLS. The procedure was repeated first dissolving 2 mg of cyclosporine A into the 2 mL of acetone prior to solubilizing polymer **6,** leading to the final solution containing hydrophobic drug encapsulated in the assembled polymers core.

8. References

1. Wang, H.; Chen, Z.; Xin, L.; Cui, J.; Zhao, S.; Yan, Y., Synthesis of pyrene-capped polystyrene by free radical polymerization and its application in direct exfoliation of graphite into graphene nanosheets. *Journal of Polymer Science Part A: Polymer Chemistry* **2015,** *53* (18), 2175-2185.
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3. Fan, W.; Shi, W.; Zhang, W.; Jia, Y.; Zhou, Z.; Brusnahan, S. K.; Garrison, J. C., Cathepsin S-cleavable, multi-block HPMA copolymers for improved SPECT/CT imaging of pancreatic cancer. *Biomaterials* **2016,** *103*, 101-115.
4. ThermoScientific, Ellman's Reagent User Guide, (accessed July, 2020).
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