Supplementary Information

A Facile Approach to Hydrophilic Reverse Zwitterionic Choline Phosphate Polymers

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Contents

I. General Methods and Materials

Starting materials, reagents and solvents were purchased from commercial suppliers and used without further purification unless otherwise stated. Ethyl α-bromoisobutyrate (EBiB), copper(I) bromide, 2,2′-bipyridine (bpy), 4-(phenylcarbonothioylthio)pentanoic acid, 4,4′-azobis(4-cyanovaleric acid) (ACVA), 2-methacryloyloxyethyl phosphoryl choline (MPC), *n*-butanol (anhydrous), anhydrous acetonitrile (ACN), toluene and trimethylamine were purchased from Sigma Aldrich. Toluene was distilled over sodium/benzopenone, and trimethylamine over calcium hydride. 2-Chloro-2-oxo-1,3,2-dioxaphospholane (COP, **7**) was purchase from Alfa Aesar and freshly distilled from a Kugelrohr distillation apparatus at 160 °C. Dialysis membranes (MWCO 3,500) were purchased from Fisher Scientific and hydrated in water prior to use. The human ovarian adenocarcinoma (SKOV3) and mouse fibroblast (NIH 3T3) cells were purchased from American Type Culture Collection (ATCC), whereas DMEM and RPMI cell culture media, phosphate buffered saline (PBS), CellTrace™ CFSE stain, TrypLE™ Express, Vybrant® Phagocytosis Assay kit were purchased from Life Technologies. Fetal bovine serum (FBS) was purchased from Atlanta biologicals. Pharm Lyse™ and Cytometric Bead Array Mouse Th1/Th2/Th17 Kit was purchased from BD Biosciences. Anti-mouse CD3e and CD28, Concanavalin A and Lipopolysaccharide from *Salmonella typhosa* were purchased from Sigma-Aldrich. APC/Cy7 labelled anti-mouse CD3, CD19b and F4/80 and FITC Annexin V apoptosis detection kit were purchased from BioLegend. Cell viability was measured using CellTiter-Glo luminescent cell viability assays (Promega). Monomer syntheses were conducted in flame-dried flasks under an inert atmosphere of nitrogen. Proton nuclear magnetic resonance spectra (^{1}H NMR) were recorded on a Varian-700 (700 MHz), Bruker Avance-400 (400 MHz) or Bruker Avance-300 instrument (300 MHz). Chemical shifts are reported in ppm relative to the residual H_2O signal in MeOD-d_4 or $CH_3C(=O)CH_3$ signal in acetone-d_6. Data is reported as follows: chemical shift, multiplicity (s=singlet, d = doublet, t = triplet, q = quartet, br

= broad, m = multiplet), coupling constant (Hz), and integration. ^{13}C NMR spectra were recorded on either a Varian-700 (176 MHz), a Bruker Avance-400 (100 MHz) or a Bruker Avance-300 instrument (75 MHz). ^{31}P NMR spectra were recorded on a Bruker Avance-400 (162 MHz) or a Bruker Avance-300 instrument (121 MHz). High resolution mass spectral data were obtained on a Bruker MicrOTOF ESI-TOF Mass Spectrometer. GPC elution was accomplished using 1,1,1-trifluoroethanol (TFE) (with 0.02 M sodium trifluoroacetate) as the mobile phase, and poly(methyl methacrylate) (PMMA) calibration standards, operating at 0.75 mL/min at 40 °C with three Agilent PL HFIPgel columns (300 × 7.5 mm) and equipped with RI and UV/Vis detection. Dynamic light scattering (DLS) was performed on a Malvern Zetasizer Nano-ZS. All the samples were filtered through a 0.4 μm filter before GPC and DLS analysis.

II. Experimental Procedures

1. MBP Monomer 5. To a solution of anhydrous *n*-butanol (4.10 mL, 44.6 mmol) in 45 mL anhydrous toluene (1.0 M) was added freshly distilled trimethylamine (6.20 mL, 44.6 mmol) at –20 °C. COP (**7**) was slowly added over 30 min, and the mixture was stirred for 30 min. The cooling bath was removed and mixture was allowed to warm to room temperature and stirred for another 2 hours. The white suspension was filtered through a pad of Celite under nitrogen atmosphere, and the colorless filtrate was concentrated by rotary evaporation, and dried *in vacuo* to give **8** as a light yellow oil in nearly quantitative conversion and used without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.51 – 4.25 (4H, m), 4.10 (2H, dq, J = 11.0, 6.7 Hz), 1.66 (2H, dt, J = 14.9, 6.6 Hz), 1.39 (2H, ddq, J = 14.5, 7.3, 3.0 Hz), 0.91 (3H, dd, J = 9.4, 5.4 Hz); DEPT135 NMR (75 MHz, $CDCl_3$) δ 68.87 (CH_2, d, $^{2}J_{CP}$ = 6 Hz), 65.97 (CH_2, d, $^{2}J_{CP}$ = 3 Hz), 32.28 (CH_2, d, $^{3}J_{CP}$ = 6 Hz), 18.58 (CH_2), 13.55 (CH_3); ^{31}P (121 MHz, $CDCl_3$) δ 17.74.

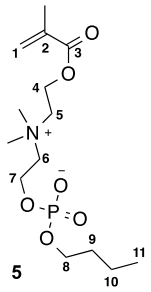
To a pressure flask containing MeHQ (44.20 mg, 2000 ppm) was added **8** in anhydrous acetonitrile (22.1 mL, 2.0 M), followed by *N,N*-dimethylaminoethyl methacrylate (DMEMA, **9,** 7.4 mL) at room temperature. The flask was quickly sealed under N_2, and the mixture stirred at 80 °C. After 5 days, the mixture was cooled to room temperature and diethyl ether was added until no more white precipitates formed. The suspension settled to give an oil layer at the bottom of the flask. The top layer was decanted, and the oily residue was rinsed several times with diethyl ether to remove unreacted starting material. The residue was carefully dried *in vacuo* to yield a viscous yellow oil as the product (**5**, 11.13 g, 75% yield over two steps). ^{1}H and ^{13}C NMR spectra data in Part III; ^{31}P (162 MHz, acetone-d_6) δ –0.62; HRMS-ESI calculated mass for $C_{14}H_{29}NO_6P$ $[M+H]^+$: 338.1740, found: 338.1724.

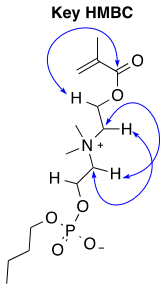
2. General procedures for the preparation of polymers 1 and 6 by ATRP. MPC or MBP, 2,2'-bipyridine (bpy), and ethyl α-bromoisobutyrate (EBiB) were dissolved in methanol as a 1.0 M solution. The solution was degassed with nitrogen for 30 min. Copper (I) bromide (CuBr) was quickly added to the solution, and the mixture degassed for 10 min. The molar ratio of CuBr:bpy:EBiB was 1:2:1. The mixture was stirred for 10-24 h at room temperature, then precipitated in diethyl ether. The residue was dried *in vacuo*, dissolved in water, and subjected to dialysis against water using a 3.5K MWCO membrane. The aqueous solution was dried by lyophilization to give zwitterionic polymers **1** or **6** as white solids. For high molecular weight targets (i.e., Entry 4 in Table 1), stock solutions were used instead. EBiB in methanol (0.1 M) was prepared, and CuBr and bpy solutions were degassed in separate vials for 20 min, and dissolved in degassed methanol to form a 0.1 M [Cu] stock solution. The solution of monomers and EBiB in methanol (1.0 M) was degased for 30 min, and the prepared [Cu] solution was added. polyMPC **1**: ^{1}H NMR (400 MHz, MeOD-d_4) δ 4.47 – 3.92 (6H, br), 3.74 (2H, br), 3.30 (9H, s, br), 1.87 (2H, br), 1.03 (3H, br); polyMBP **6**:^{1}H NMR (700 MHz, MeOD-d_4) δ 4.75 – 3.62 (10H, br), 3.36 (6H, br), 1.97 (2H, br), 1.66 (2H, br), 1.47 (2H, br), 1.40 – 0.86 (6H, br).

3. General Procedures for RAFT Polymerizations to afford 1, 6, 12, 13 and polyMPC starting material for chain extension (1). MPC or MBP monomers, 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (CTA), and 4,4′-azobis(4-cyanovaleric acid) (ACVA) were dissolved in methanol (1.0 M) at a CTA:ACVA molar ration of 3:1. The mixture was degassed with nitrogen for 30 min, and placed in a preheated oil bath at 70 °C. Propagation was terminated by placing the reaction flask in liquid nitrogen, and allowing the mixture to warm while open to air. The mixture was precipitated in diethyl ether. The residue was dried *in vacuo*, dissolved in water, and subjected to dialysis using a 3.5K MWCO membrane against water. Lyophilization

of the aqueous solution gave pink solids for polyMPC **1** and polyMBP **6**. For the synthesis of poly(MPC-*b*-MBP) **12** and poly(MPC-*b*-HEMA) **13** and polyMPC homopolymer for chain extension, homopolymerization of MPC was quenched after 6 h at 70 °C to give 63% monomer conversion (as characterized by ^{1}H NMR spectroscopy). The MPC homopolymer was purified by precipitation and dialysis to afford the macroCTA which was used in the MBP/HEMA/MPC polymerization step following the same RAFT polymerization procedure to afford the polymers **12** and **13** and chain extended structures.

III. NMR assignment of MBP 5 and HMBC correlations in acetone-d_6 (Varian-700 NMR)





Entry	^{1}H NMR δ	^{13}C NMR δ	Entry	^{1}H NMR δ	^{13}C NMR δ
1	6.16 (s), 5.69 (s)	126.76	**7**	4.42 – 4.26 (m)	59.69 (d, $^2J_{CP}$= 4.8 Hz)
2	-	136.77	**8**	3.80 (dd, *J* = 13.1, 6.5 Hz)	65.34 (d, $^2J_{CP}$ = 11.6 Hz)
2-CH$_3$	1.94 (s)	18.39	**9**	1.59 – 1.51 (m)	33.80 (d, $^3J_{CP}$ = 7.4 Hz)
3	-	166.87	**10**	1.37 (td, *J* = 14.9, 7.4 Hz)	19.75
4	4.82 – 4.62 (m)	59.34	**11**	0.89 (t, *J* = 7.4 Hz)	14.13
5	4.20 – 4.10 (m)	64.36	**N-CH$_3$**	3.51 (s)	52.68
6	4.02 – 3.95 (m)	65.67			

IV. Kinetics: Homopolymerization of MBP 5 by ATRP and RAFT

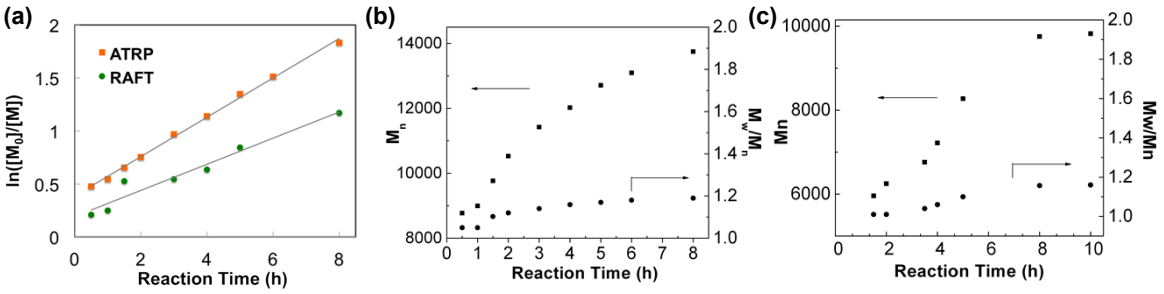


Fig. S1 (a) Kinetics plots of ATRP and RAFT. (b) Evolution of number molecular weight (M_n) and PDI vs. time in ATRP. (c) Evolution of M_n and PDI vs. time in RAFT.

V. TFE GPC traces of macroCTA 1 and Poly(MPC-*b*-MBP) 12a-c.

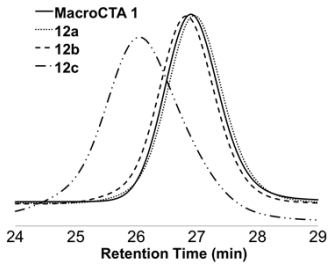


Fig. S2 TFE GPC traces of macroCTA **1** and diblock copolymers **12a-c**.

VI. Size distribution by volume of 12a in aqueous NaCl

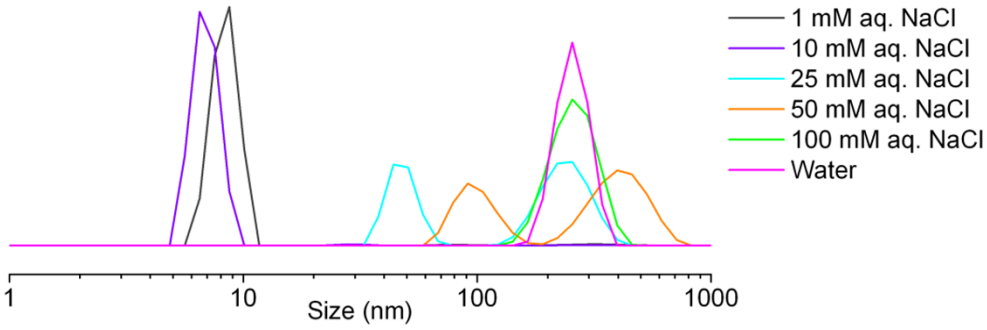


Fig. S3 Size distribution by volume of **12a** in aqueous NaCl solutions with various concentrations.

VII. Bioassays

Table S1 Characterizations of **1** and **6** used in bioassays

sample	target M_w (kDa)	%conv.[b]	TFE GPC		% Yield
			M_n	PDI	
PMBP10K	10.0	>95	8.5	1.24	64
PMBP20K	20.0	>95	22.8	1.44	74
PMBP32K	40.0	81	27.3	1.37	70
PMPC10K	10.0	92	24.8	1.12	94
PMPC20K	20.0	>95	27.2	1.11	98
PMPC32K	40.0	79	34.9	1.19	70

[a] Reaction conditions: CuBr:bpy:EBiB (1:2:1), MeOH (1.0 M), rt, 10-24 hr. [b] by ^{1}H NMR.

Cell culture. SKOV3 human cancer and NIH 3T3 mouse fibroblast cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS). Cells were grown in 5% CO_2 incubators at 37 ˚C.

Cytotoxicity assay. For *in vitro* cytotoxicity assays cells were seeded into 96 well plates, and after reaching about 40% cell density were incubated for 72 hours with varying concentrations of polymer. Cell viability post-exposure was measured using CellTiter-Glo luminescent cell viability assays (Promega) according to the manufacturer instructions on a FLUOstar OPTIMA plate reader (BMG LABTECH). The percentage polymer mediated toxicity was calculated with respect to untreated cells, and graphed to give dose response curves.

Hemolysis assay. Human blood (4 mL) from healthy donors was collected in EDTA containing tubes (BD Biosciences), and then centrifuged at 2000 rpm for 5 minutes at 4 ºC. The supernatant was discarded, and red blood cells (RBCs) were then washed three times with 40 mL of ice cold phosphate buffered saline (PBS) and finally the RBC concentration was adjusted to 5 x 10^8 cells/mL in PBS. RBC solutions were then mixed in a 1:1 ratio (v/v) with PMPC or PMBP solutions to yield polymer concentrations of 0.1, 1, and 10 mg/mL. The solutions were mixed and placed in the water bath at 37 ºC for 1 hr. After 1 hour, the solutions were centrifuged in a bench top centrifuge at maximum speed for 5 minutes. The supernatant was collected and absorbance measured at 560 nm. RBCs incubated with Triton X-100 (w/v) or PBS served as positive and negative controls for hemolysis. Percent hemolysis of the polymer incubated samples was calculated relative to positive controls.

Phase contrast microscopy. RBCs incubated at 37 ºC for 1 hour in 10 mg/mL polymer solution (see hemolysis assay) were gauged for aggregation using a 10X objective on an optical microscope.

Scanning electron microscopy of RBCs. RBCs (5 x 10^8 cells/mL in PBS) were incubated with PMBP (final concentration 10 mg/mL) in a water bath at 37 ºC for 1 hour, then prepared for SEM characterization using the method described by Brooks.[38] RBCs were fixed overnight at 4 ºC in 2.5% glutaraldehyde in PBS. Post fixation the solutions were centrifuged at 500 rpm for 10 minutes at 4 ºC. The supernatants were discarded, the RBCs washed once in PBS, then deposited onto a silicon wafer by drop casting. The RBCs were sequentially dehydrated by immersing the wafer in 70, 85, 95 and 100 % ethanol for 10 minutes each. The RBCs were then dried overnight, coated with gold and then imaged with an FEI Magellan 400 high-resolution scanning electron microscope.

Splenocyte isolation. 8-12 weeks old wild type male BALB/c mice were maintained and euthanized according to protocols approved by the Institution Animal Care and Use Committee (IACUC) of the University of Massachusetts. Spleens were aseptically isolated and collected in ice cold RPMI medium containing 10% FBS. The spleens were then gently mashed on a 70 µm cell strainer, and the cells collected in 1:1 RPMI: DMEM splenocytes culture media containing 10% FBS. The cells were then centrifuged at 4 ºC for 5 minutes at 300g, followed by incubation of the cell pellet with 1x BD Pharm Lyse™ for 5 minutes at 4 ºC to lyse Red Blood Cells. The cell suspension was then centrifuged at 4 ºC for 5 minutes at 300g and the cell pellet resuspended in splenocytes culture media containing anti-mouse CD28 antibody at 2 µg/mL. Splenocytes were then seeded at 1.5 x 10^6 cells/well in a 24 well plate pre-coated with anti-mouse CD3e at 10 µg/mL and grown in 5% CO_2 incubators at 37 ˚C.

Splenocyte viability assay. Isolated splenocytes seeded into 24 well plates at 1.5 x 10^6 cells/ were incubated with 1 mg/mL 32 kDa PMPC or PMBP for 72 hours. Cells were harvested and the viability gauged using the FITC Annexin V apoptosis detection kit (BioLegend) according to manufacturer's instructions on a BD LSRII flow cytometer. The percentage polymer mediated toxicity was calculated with respect to untreated cells.

Macrophage phagocytosis. Splenic macrophages were enriched by incubating freshly isolated splenocytes on 75-cm^2 flasks for 3 hours in a 5% CO_2 incubator at 37 ˚C. Non-adherent cells were poured off followed by gently washing the adherent cells with PBS. Attached cells were then harvested using TrypLE™ Express (Life Technologies), seeded into 24 well plates at 1.5 x 10^6 cells/well and co-incubated over night with 1 mg/mL 32 kDa PMPC or PMBP. Macrophage phagocytic activity was then gauged using the Vybrant® Phagocytosis Assay kit (Life Technologies), and by gating on Fluorescein positive and APC/Cy7 labelled anti-mouse F4/80 antibody positive macrophages on a flow cytometer. Phagocytic activity was calculated with respect to non-polymer exposed cells.

T- and B-cell proliferation assay. Splenocytes were stained with 5 µM CellTrace™ CFSE stain (Life Technologies) according to manufacturer's instructions, seeded at 1.5 x 10^6 cells/well of a 24 well plate, and then incubated with 32 kDa PMPC or PMBP for 72 hours in the presence or absence of 2.5 µg/mL Concanavalin A (Con A, Sigma-Aldrich) to stimulate T-cell proliferation, or 20 µg/mL Lipopolysaccharide from *Salmonella typhosa* (LPS, Sigma-Aldrich) to stimulate B-cell proliferation. Cells were then harvested, stained with APC/Cy7 labelled anti-mouse CD3 to detect T-cells or with APC/Cy7 (BioLegend) labelled

anti-mouse CD19b (BioLegend) to detect B-cells respectively, and intracellular CFSE intensity measured by flow cytometry. Splenocyte proliferation was expressed relative to only Con A and or LPS stimulated control cells.

Cytokine expression. Freshly isolated splenocytes seeded in anti-mouse CD3e antibody coated 24 wells plates at 1.5 x 10^6 cells/well were incubated with 1 mg/mL 32 kDa PMPC or PMBP for 72 hours in a 5% CO_2 incubator at 37 ˚C. After the incubation period, supernatants from individual sample wells were obtained, and cytokine expression analyzed using the BD Cytometric Bead Array Mouse Th1/Th2/Th17 Kit (BD Biosciences) according to manufacturer's instructions.

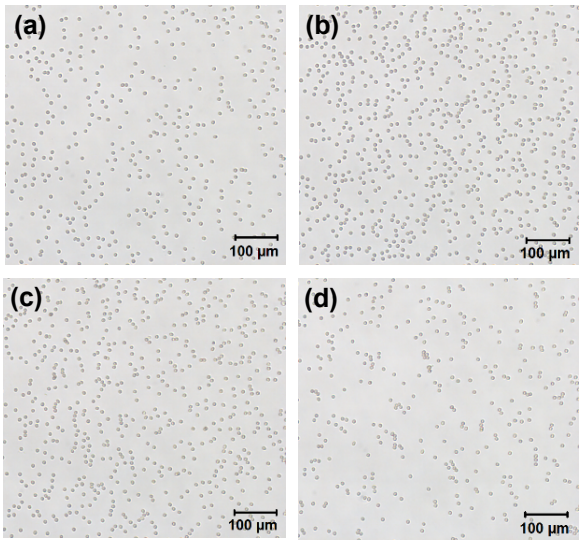


Fig. S4. 1 hour incubation of 10 mg/mL polyMBP (**6**) and polyMPC (**1**) with red blood cells. Phase contrast microscopy image of RBCs incubated with polyMBP of MW 10 kDa (a) and 20 kDa (b); and polyMPC MW 32 kDa (c); and PBS (d) for 1 hour. Scale bar indicates 100 µm.

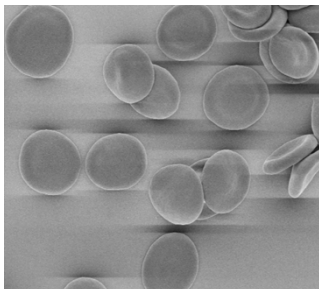


Fig. S5 SEM image of RBCs after incubation in PBS for 1 hour.

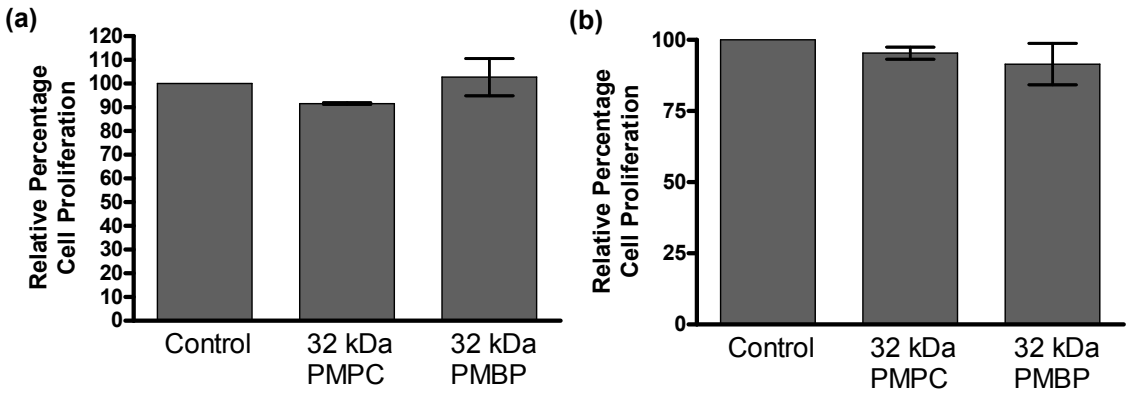


Fig. S6 Effect of 72 hour *in vitro* exposure to 1 mg/mL 32 kDa PMPC or PMBP on (a) 2.5 µg/mL Concanavlin A stimulated T-cell proliferation, and (b) 20 µg/mL Lipopolysacharide stimulated B-cell proliferation. Measurements reflect values from 3 independent experiments with readings from 10,000 cells for each experiment. Error bars indicate ± S.D.

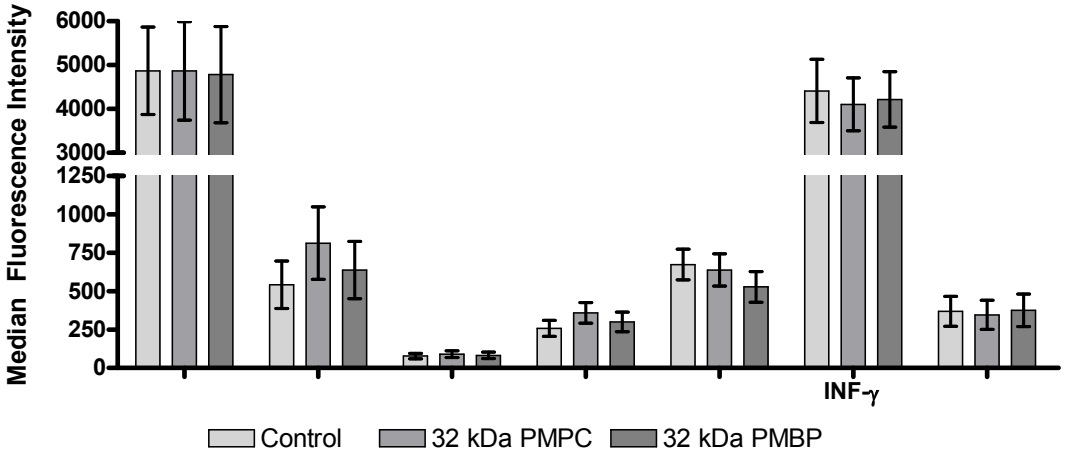
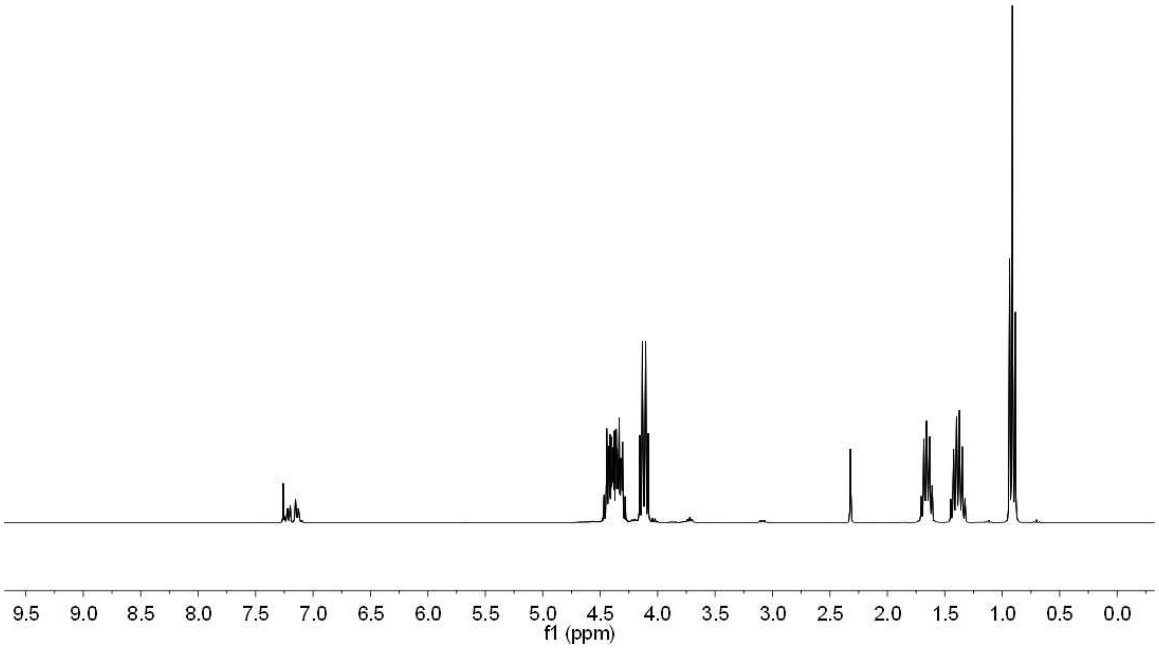


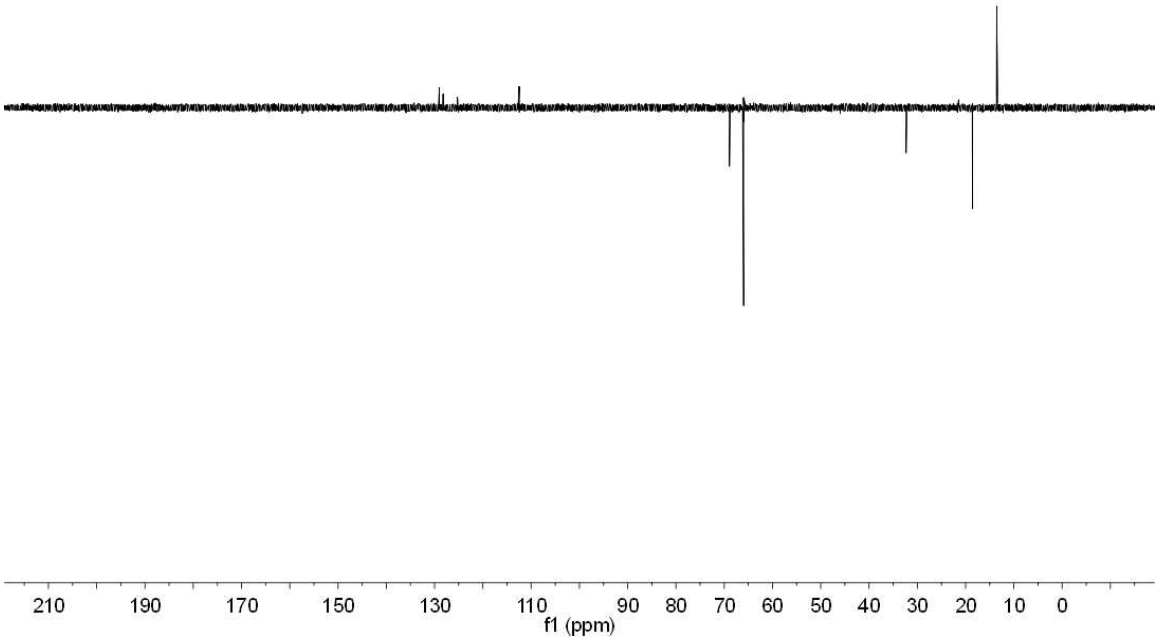
Fig. S7 Effect of 72 hour *in vitro* exposure to 1 mg/mL 32 kDa PMPC or PMBP on cytokine expression. Error bars represent ± S.D.

VI. NMR Spectra

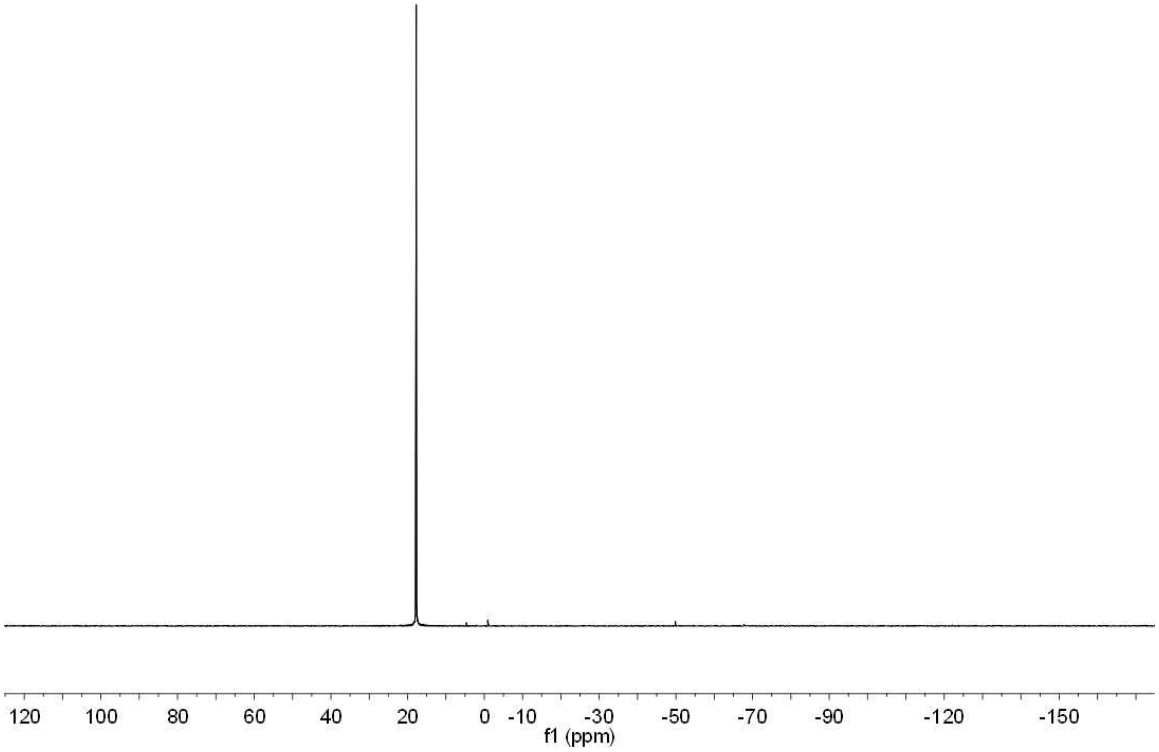
Compound 7: ^{1}H NMR



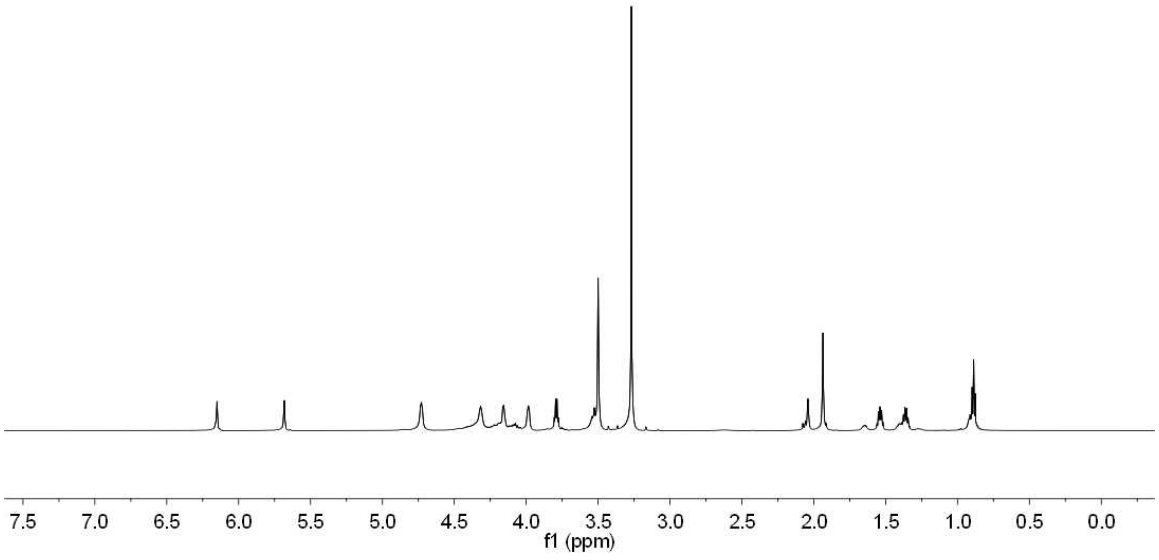
Compound 7: DEPT135



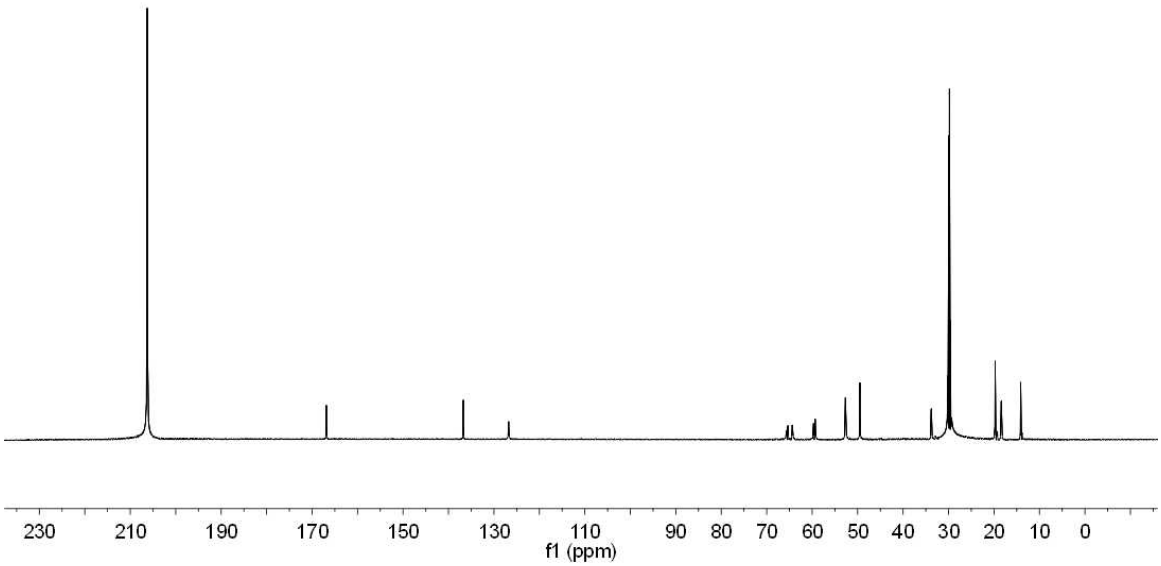
Compound 7: ^{31}P NMR



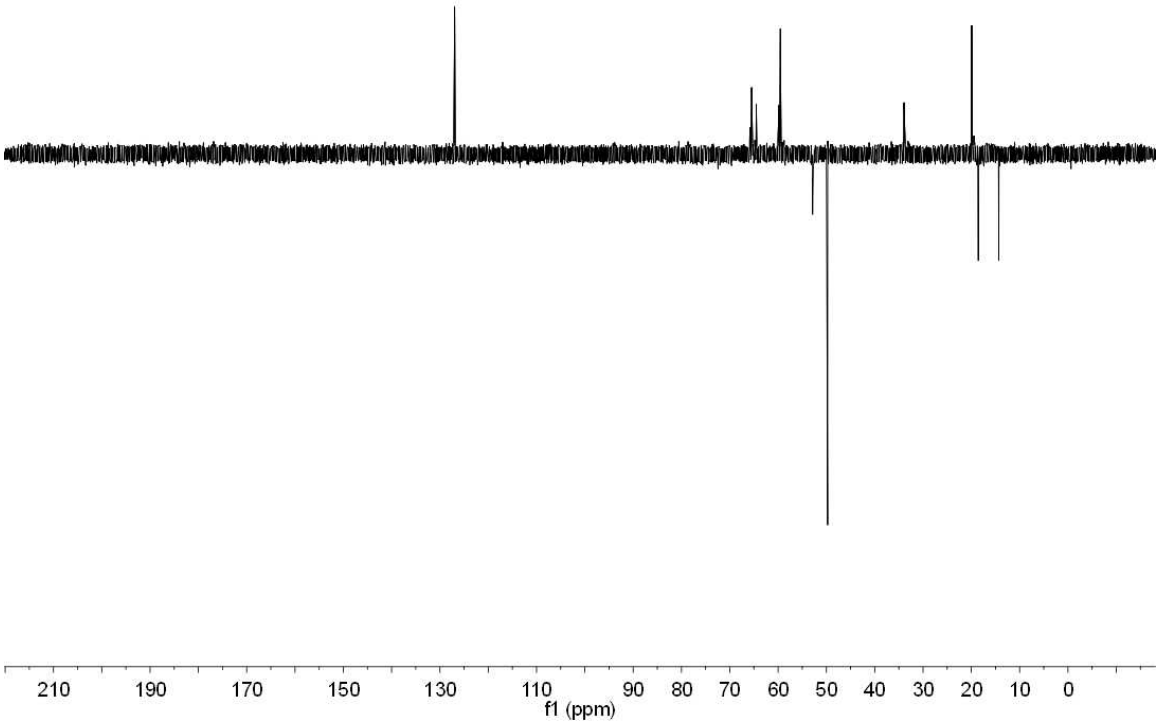
MBP (5): ^{1}H MMR



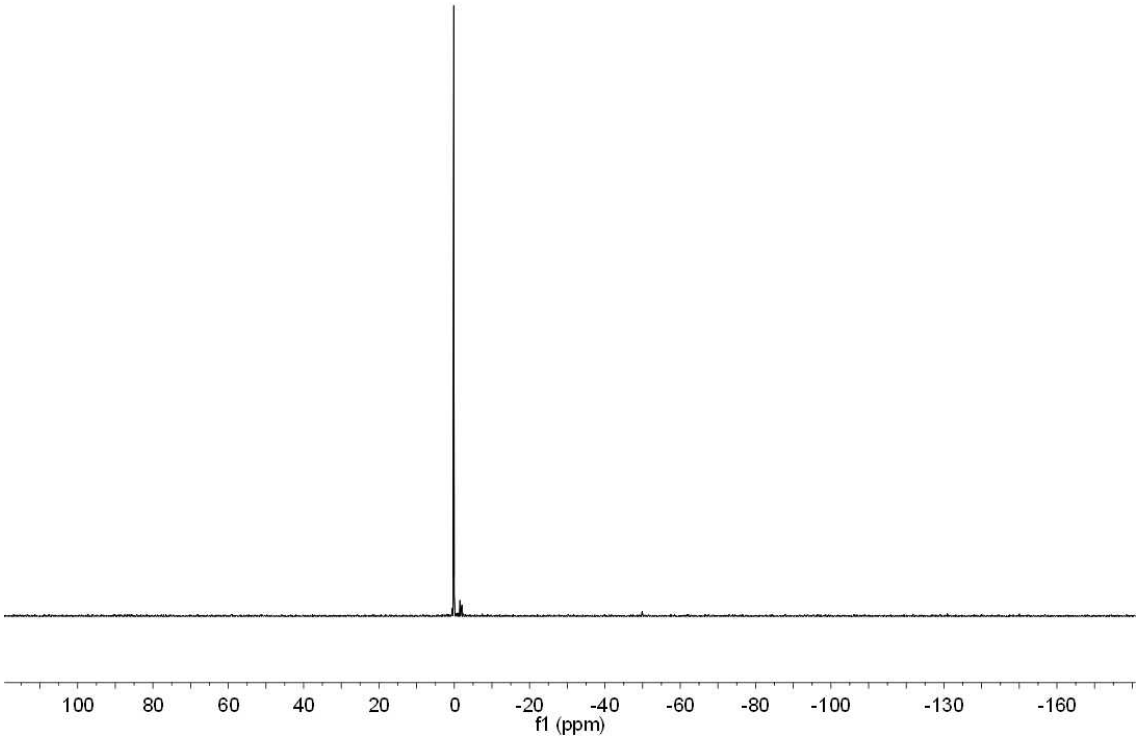
MBP 5: ^{13}C NMR



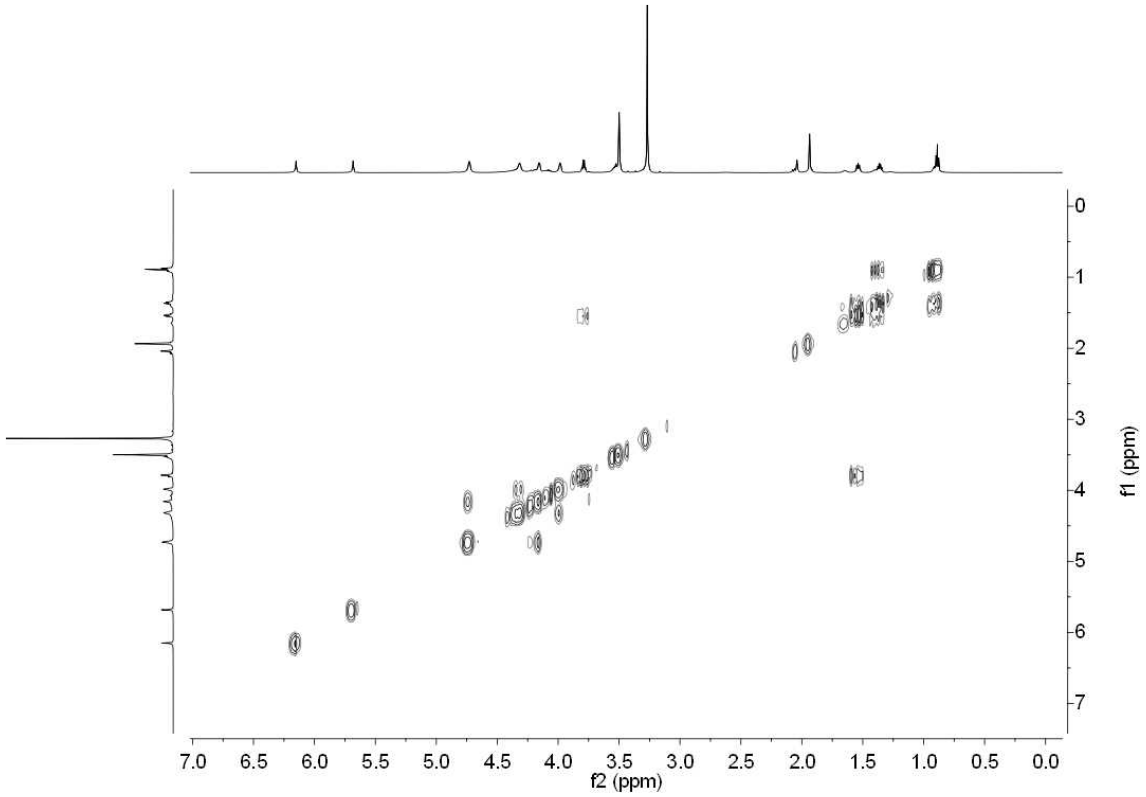
MBP 5: DEPT135



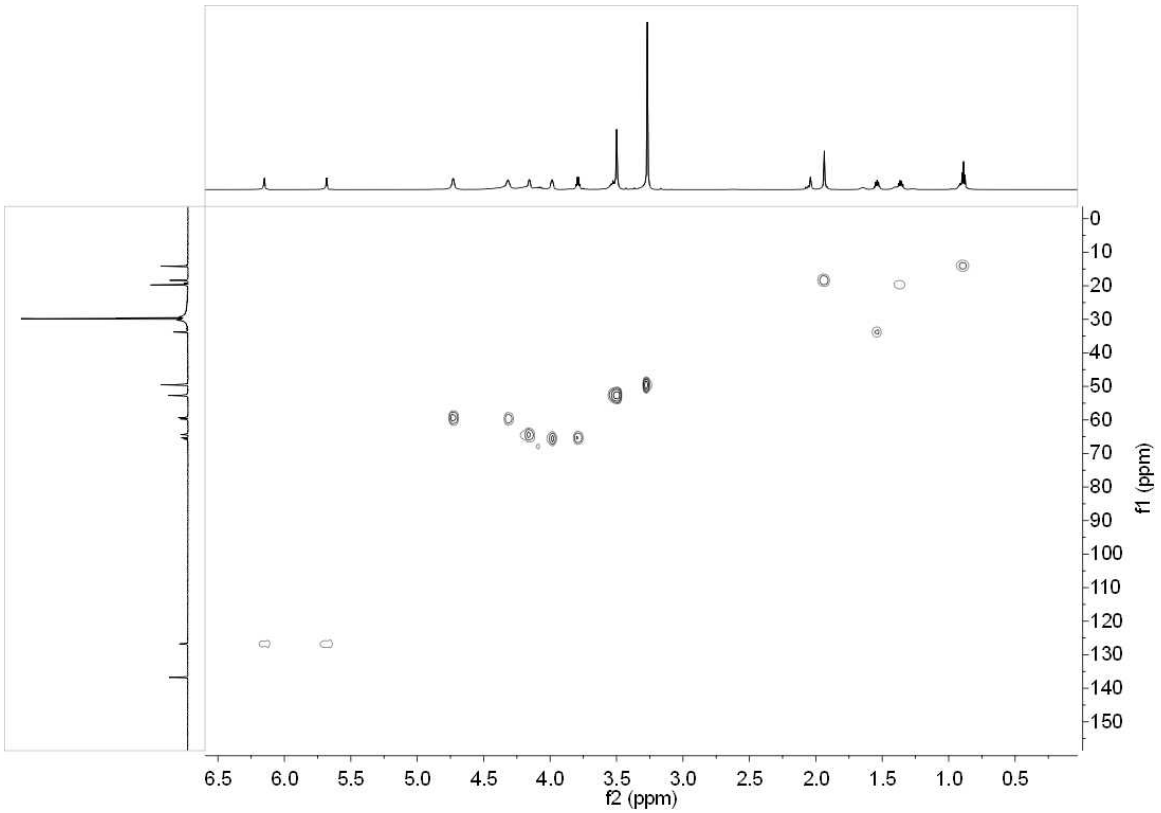
MBP 5: ^{31}P NMR



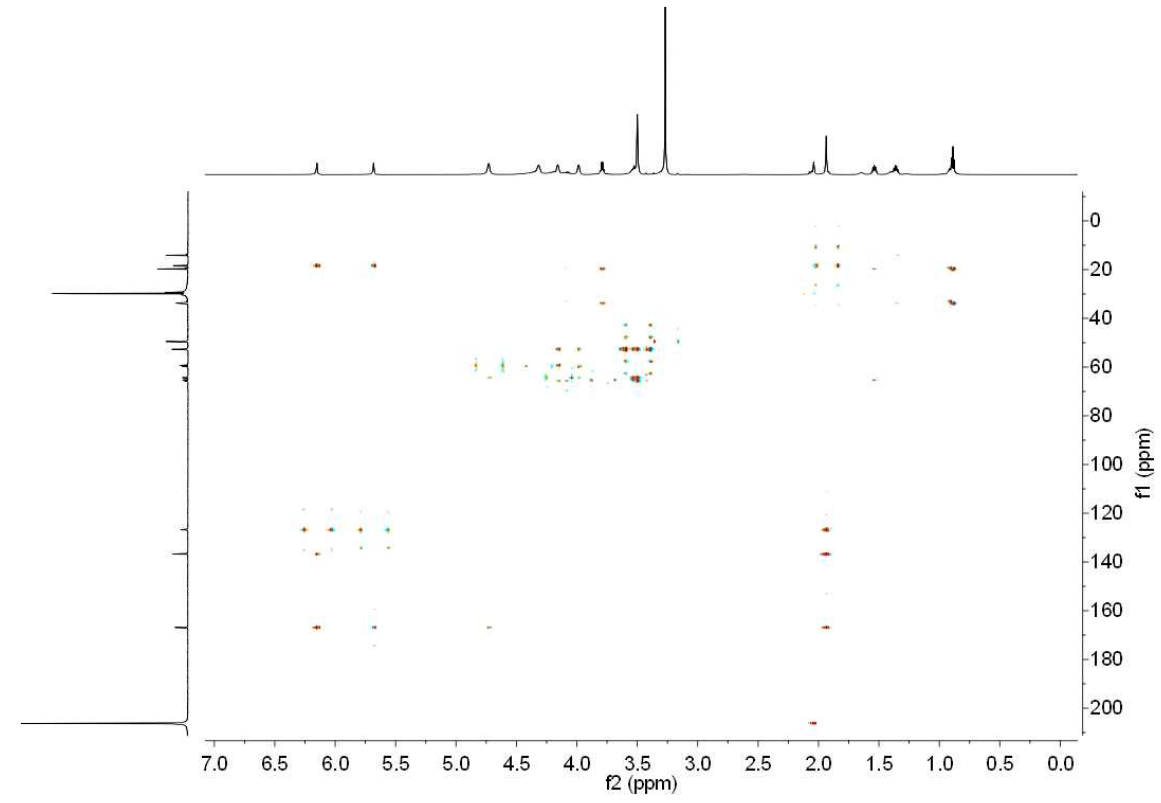
MBP (5): COSY



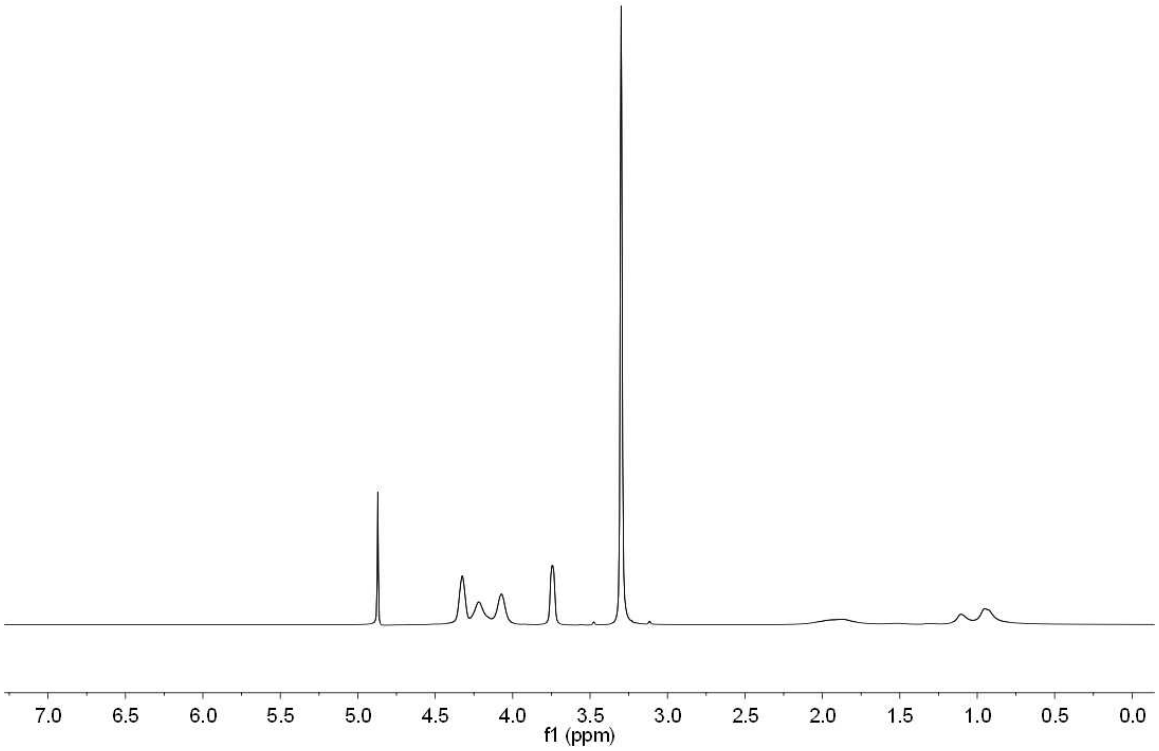
MBP (5): HSQC



MBP (5): HMBC



polyMPC (1): ^{1}H MMR



polyMBP (6): ^{1}H MMR