

Magnesium catalyzed polymerization of end functionalized poly(propylene maleate) and poly(propylene fumarate) for 3D printing of bioactive scaffolds*

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Materials

All reagents were purchased from Millipore-Sigma except for 2,6-*di-tert*-4-methylphenol, which was purchased from Acros. $\text{Mg}(\text{BHT})_2(\text{THF})_2$ was synthesized as previously reported.¹ All solvents were purchased from Fisher and dried using an Innovative Technology Inc. Pure Solv MD-3 solvent purification system. Benzyl alcohol, propargyl alcohol, 4-hydroxybutan-2-one and propylene oxide were dried over calcium hydride for 24 h before vacuum distillation. Maleic anhydride was dried *in vacuo* over P_2O_5 for one week. All other reagents were used as received.

Instrumental Methods

Proton (^1H) NMR spectra were recorded using a Varian Mercury 300 spectrometer. Carbon (^{13}C) NMR spectra were recorded using a Varian NMRS 500 spectrometer. All chemical shifts were recorded in parts per million (ppm) relative to the reference peak of chloroform solvent at $\delta = 7.26$ and 77.16 ppm for ^1H and ^{13}C NMR spectra, respectively. Molecular masses were determined through size exclusion chromatography (SEC) using a Tosoh EcoSEC HLC-8320GPC on TSKgel GMH_{HR} -M columns in series with refractive index (RI) detection. Molecular masses were calculated using a calibration curve determined from poly(styrene) standards with tetrahydrofuran (THF) as the eluent flowing at 1.0 mL min^{-1} and a sample concentration of 10.0 mg mL^{-1} . MALDI-ToF mass spectra were recorded on a Bruker Ultra-Flex III MALDI-ToF/ToF mass spectrometer equipped with a Nd:YAG laser emitting at 355 nm. The instrument was operated in positive ion mode. All samples were dissolved in THF at a final concentration of 10 mg mL^{-1} . *Trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene] malononitrile (DCTB) (20 mg mL^{-1})

served as a matrix and sodium trifluoroacetate (NaTFA) (10 mg mL^{-1}) as cationizing agent were prepared and mixed in the ratio 10 : 1. Matrix and sample solutions were applied onto the MALDI-ToF target plate by the sandwich method. FlexAnalysis software was used to analyze MALDI-ToF data. PPF films were printed using an Envisiontec™ Micro Plus Advantage® continuous digital light processing (cDLP) printer. Fluorescence microscopy was conducted on an Olympus IX81 Fluorescence Microscope with FITC and TRITC filters.

General synthesis of end-functionalized poly(propylene maleate) (PPM)

Using standard Schlenk line techniques, an ampoule was filled with $\text{Mg(BHT)}_2(\text{THF})_2$ (586.8 mg, 0.97 mmol), benzyl alcohol (0.1 mL, 0.97 mmol), propylene oxide (3.38 mL, 48.3 mmol) and maleic anhydride (4.74 g, 48.3 mmol). The solution was dissolved into toluene to a total monomer concentration of 2 M. The ampoule was sealed and heated at 80°C for a defined time period. The resultant polymer was recovered by precipitation in excess diethyl ether.

Benzyl alcohol initiated PPM:

^1H NMR (300 MHz, 303 K, $\text{DMSO}-d_6$): δ = 7.37 (m, C_6H_5), 6.52-6.44 (m, OC(=O)H=CH(=O)O), 5.16-5.10 (m, $\text{CH}_2\text{CH}(\text{CH}_3)\text{O}$ and $(\text{C}_6\text{H}_5)\text{CH}_2\text{O}$), 4.21-4.14 (m, $\text{CH}_2\text{CH}(\text{CH}_3)\text{O}$), and 1.30-1.15 (m, $\text{CH}_2\text{CH}(\text{CH}_3)\text{O}$) ppm. ^{13}C NMR (125 MHz, 298 K, $\text{DMSO}-d_6$): δ = 164.60 and 164.30 ($\text{MAN}^*\text{-PO}$, OCOCH_2), 130.58 and 130.14 ($\text{MAN}^*\text{-PO}$, $\text{O(O)C}^*\text{CH=CH}$), 130.00 and 129.51 ($\text{MAN}^*\text{-PO}$, $\text{O(O)C}^*\text{CH=CH}$), 128.40 and 128.19 (aromatic Cs), 68.76 ($\text{MAN}^*\text{-PO}$, $\text{OCH}(\text{CH}_3)\text{CH}_2$), 66.98 ($\text{MAN}^*\text{-PO}$, $\text{OCH}(\text{CH}_3)\text{CH}_2$), 65.80 ($(\text{C}_6\text{H}_5)\text{CH}_2\text{O}$) and 15.73 (PO , $\text{CH}_2\text{CH}(\text{CH}_3)\text{O}$) ppm. SEC (THF): M_n = 3.7 kDa, M_w = 4.6 kDa, \mathcal{D}_M = 1.3. Yield = 84%.

Propargyl alcohol initiated PPM:

^1H NMR (300 MHz, 303 K, $\text{DMSO}-d_6$): δ = 6.54-6.27 (m, OC(=O)H=CH(=O)O), 5.39-5.21 (m, $\text{CH}_2\text{CH}(\text{CH}_3)\text{O}$), 4.88 (s, $\text{HC}\equiv\text{CCH}_2\text{O}$), 4.78 (s, $\text{HC}\equiv\text{CCH}_2\text{O}$), 4.38-4.21 (m, $\text{CH}_2\text{CH}(\text{CH}_3)\text{O}$), 2.27 (s, $\text{HC}\equiv\text{C}$) and 1.38-1.19 (m, $\text{CH}_2\text{CH}(\text{CH}_3)\text{O}$) ppm. ^{13}C NMR (125 MHz, 298 K, $\text{DMSO}-d_6$): δ = 164.60 and 164.29 ($\text{MAN}^*\text{-PO}$, OCOCH_2), 130.58 and 130.14 ($\text{MAN}^*\text{-PO}$, $\text{O(O)C}^*\text{CH=CH}$), 130.00 and 129.51 ($\text{MAN}^*\text{-PO}$, $\text{O(O)C}^*\text{CH=CH}$), 83.56 ($\text{HC}\equiv\text{CCH}_2$), 68.90 ($\text{HC}\equiv\text{CCH}_2$), 68.76 ($\text{MAN}^*\text{-PO}$, $\text{OCH}(\text{CH}_3)\text{CH}_2$), 66.98 ($\text{MAN}^*\text{-PO}$, $\text{OCH}(\text{CH}_3)\text{CH}_2$), 65.83 ($\text{HC}\equiv\text{CCH}_2\text{O}$) and 15.72 ($\text{CH}_2\text{CH}(\text{CH}_3)\text{O}$) ppm. SEC (THF): M_n = 3.4 kDa, M_w = 3.5 kDa, \mathcal{D}_M = 1.1. Yield = 83%.

4-hydroxybutan-2-one initiated PPM:

^1H NMR (300 MHz, 303 K, CDCl_3): δ = 6.42-6.28 (m, OC(=O)H=CH(=O)O), 5.33-5.25 (m, $\text{CH}_2\text{CH}(\text{CH}_3)\text{O}$), 4.32-4.18 (m, $\text{CH}_2\text{CH}(\text{CH}_3)\text{O}$), 4.08 (m, $\text{CH}_2\text{CH}_2\text{O}$), 3.64 and 3.53 (m, $\text{CH}_3\text{C(=O)CH}_2\text{CH}_2$), and 1.38-1.13 (m, $\text{CH}_2\text{CH}(\text{CH}_3)\text{O}$ and $\text{CH}_3\text{C(=O)CH}_2$) ppm. ^{13}C NMR (125 MHz, 303 K, $\text{DMSO}-d_6$): δ = 165.42 ($\text{CH}_3\text{C(=O)CH}_2$), 164.60 and 164.30 ($\text{MAN}^*\text{-PO}$, OCOCH_2), 130.55 and 130.16 ($\text{MAN}^*\text{-PO}$, $\text{O(O)C}^*\text{CH=CH}$), 129.81 and 129.84 ($\text{MAN}^*\text{-PO}$, $\text{O(O)C}^*\text{CH=CH}$), 128.15, 68.77 ($\text{MAN}^*\text{-PO}$, $\text{OCH}(\text{CH}_3)\text{CH}_2$), 66.97 ($\text{MAN}^*\text{-PO}$, $\text{OCH}(\text{CH}_3)\text{CH}_2$), 65.81 ($\text{CH}_2\text{CH}_2\text{O}$), 30.63 ($\text{C(=O)CH}_2\text{CH}_2$), 25.08 ($\text{CH}_3\text{C(=O)CH}_2$) and 15.74 ($\text{CH}_2\text{CH}(\text{CH}_3)\text{O}$) ppm. SEC (THF): M_n = 1.5 kDa, M_w = 2.0 kDa, \mathcal{D}_M = 1.3. Yield = 88%.

General procedure for the isomerization of PPM

End-functionalized poly(propylene fumarate) (1.0 g, 12 mol. eq. olefin) was dissolved into chloroform (50 mL). Diethylamine (0.01 mL, 0.15 mol. eq. olefin) was added to the solution and refluxed for 24 h under a nitrogen atmosphere. After cooling to room temperature, the organic solution was washed with phosphate buffer solution (150 mL, pH = 6) and the polymer was recovered through precipitation from hexanes.

Propargyl alcohol initiated PPF:

^1H NMR (300 MHz, 303 K, DMSO- d_6): δ = 6.84-6.64 (m, OC(=O)H=CH(=O)O), 5.27-5.07 (m, CH₂CH(CH₃)O), 4.85 (s, HC≡CCH₂O), 4.78 (s, HC≡CCH₂O), 4.44-4.14 (m, CH₂CH(CH₃)O), 2.32 (s, HC≡C) and 1.38-1.10 (m, CH₂CH(CH₃)O) ppm. Yield = 98%.

Subtractive method of surface concentration determination

PPF thin films (1 cm × 1 cm) were immersed in a dye solution (0.5 μM Chromeo® 546-azide dye, 2.5 mg CuSO₄, 3.2 mg sodium ascorbate in a 50 %v/v solution of EtOH/H₂O,) for 1 h, and the concentration of the solution was determined using fluorescence spectroscopy. The unused solution was used as a standard to measure dye attachment to films.

Copper-mediated azide-alkyne cycloaddition of Megastokes® 673-azide dye to propargyl functionalized PPF

A Megastokes® 673-azide solution (1 mM dye in a 50 % v/v solution of 50 : 50 isopropyl alcohol : H₂O, 0.5 mg CuSO₄, 1.5 mg sodium ascorbate) was pipetted onto a PPF film and allowed to remain for 1 h, before being washed with isopropyl alcohol and H₂O to remove any non-tethered dye and catalyst.

Synthesis of N₃-GRGDS peptide

GRGDS was synthesized by microwave-assisted solid phase peptide synthesis (SPSS) on a CEM Liberty1 peptide synthesizer using standard Fmoc chemistry conditions (0.25 mmol scale). 6-Bromohexanoic acid (1 mmol) was added along with the GRGDS Wang resin (0.25 mmol), diisopropylcarbodiimide (DIC, 1.1 mmol) and hydroxybenzotriazole (HOBt, 1.1 mmol) and allowed to react for 2 hours. The Br-functionalized peptide was then cleaved from the resin using 15 mL of a solution of trifluoroacetic acid, triisopropylsilane, and water (95 : 2.5 : 2.5 vol.%). Following three trituration cycles in diethyl ether, the resulting white solid was dried overnight under vacuum. The solid was then redissolved in a 10% ethanol solution in water. Coupling of the azide group was performed after the microwave synthesis. NaN₃ (1.25 mmol) and 18-Crown-6 (0.0625 mmol) was added to the Wang resin and allowed to react for 12 h to yield N₃-GRGDS (ESI m/z: [M + H]⁺ Calcd for C₂₃H₄₀N₁₁O₁₀ 630.29; Found 630.157).

Seeding of MC3T3-E1 onto poly(propylene fumarate) discs

Propargyl alcohol-initiated poly(propylene fumarate) disks (ø = 6 mm) were washed with chloroform, acetone, and ethanol for 1 h each, followed by soaking in 1 × PBS for 12 hours. Afterwards, films were sterilized by soaking in 70% EtOH for 1 h, followed by 15 min exposure to UV light. Prior to cell seeding, the films were submerged in Alpha-MEM for 2 h prior to cell seeding. Mouse calvarial stem cells (MC3T3-E1) were cultured in Alpha-MEM media supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg.mL⁻¹ streptomycin and passaged every 3 days. MC3T3s at passage 8 were seeded at 250 cells.mm⁻² and all subsequent experiments were performed 48 h after cell seeding.

MC3T3 cell viability on poly(propylene fumarate) discs

Cell viability was evaluated using a LIVE/DEAD viability cytotoxicity kit at 48 h. Briefly, 5 μL of the 4 mM Calcein-AM stock solution and 10 μL of the 2 mM ethidium homodimer-1 (EthD-1) stock solution were added to 10 mL of PBS to prepare the Live/Dead staining solution. Samples were washed thrice with 1 × PBS (1 mL). The stock solution (200 μL) was added to each sample and incubated for 15 minutes. The

staining solution was then removed, and samples were viewed under a IX81 fluorescence microscope using FITC and TRITC emission filters. For analysis, 10 random areas were chosen per film and each film was performed in triplicate. The values were normalized to cell viability calculated on a glass slide.

MC3T3 cell spreading on poly(propylene fumarate) discs

Cell spreading was evaluated by staining of cytoskeletal actin 48 h after cell seeding. Samples were prefixed in a 3.7% paraformaldehyde in CS buffer solution for 1 hour, washed thrice with 1 × PBS and stored at -80° C until staining. To stain, samples were incubated in a 0.5% v/v Triton X-100 in CS buffer solution for 10 minutes and washed thrice with 1× PBS. Next, samples were incubated in a 0.1 wt.% NaBH₄ solution in 1 × PBS solution for 10 minutes and washed thrice with 1 × PBS. Rhodamine phalloidin (1:40 v/v in 1 × PBS) was then added to the samples and incubated for 1 hour. After washing thrice with 1 × PBS, DAPI solution (6 µL of a 5 µg.mL⁻¹ DAPI in 10 mL PBS) was added to the samples and incubated for 20 minutes. After washing 3x with 1 × PBS, samples were mounted using fluorescence mounting media and imaged using a fluorescence microscope.

Table S1 Conditions and yields of PPF polymers produced using Mg(BHT)₂(THF)₂ as a catalyst with varying alcohol initiators and targeted DPs.

| Entry | Initiator (I) | DP | [MAn] : [PO] : [I] : [cat.] | Time (h) | MAn Conversion (%) | Yield (g) | Yield (%) |
|-------|-------------------|-----|--------------------------------|----------|--------------------------|-----------|-----------|
| 1 | Benzyl alcohol | 10 | 10 : 10 : 1 : 1 | 18 | 75 | 7.8 | 89 |
| 2 | Benzyl alcohol | 25 | 25 : 25 : 1 : 1 | 24 | 85 | 7.6 | 86 |
| 3 | Benzyl alcohol | 50 | 50 : 50 : 1 : 1 | 48 | 81 | 7.1 | 81 |
| 4 | Benzyl alcohol | 100 | 100 : 100 : 1 : 1 | 72 | 85 | 6.9 | 78 |
| 5 | Propargyl alcohol | 10 | 10 : 10 : 1 : 1 | 18 | 97 | 4.1 | 92 |
| 6 | Propargyl alcohol | 25 | 25 : 25 : 1 : 1 | 24 | 96 | 3.8 | 85 |
| 7 | Propargyl alcohol | 50 | 50 : 50 : 1 : 1 | 48 | 91 | 3.5 | 77 |
| 8 | Propargyl alcohol | 100 | 100 : 100 : 1 : 1 | 72 | 93 | 3.5 | 76 |
| 9 | 4HB ^a | 10 | 10 : 10 : 1 : 1 | 18 | 89 | 6.6 | 92 |
| 10 | 4HB ^a | 25 | 25 : 25 : 1 : 1 | 24 | 90 | 6.8 | 95 |
| 11 | 4HB ^a | 50 | 50 : 50 : 1 : 1 | 48 | 86 | 6.0 | 83 |
| 12 | 4HB ^a | 100 | 100 : 100 : 1 : 1 | 72 | 89 | 5.8 | 80 |

^a4-Hydroxy-2-butanone.

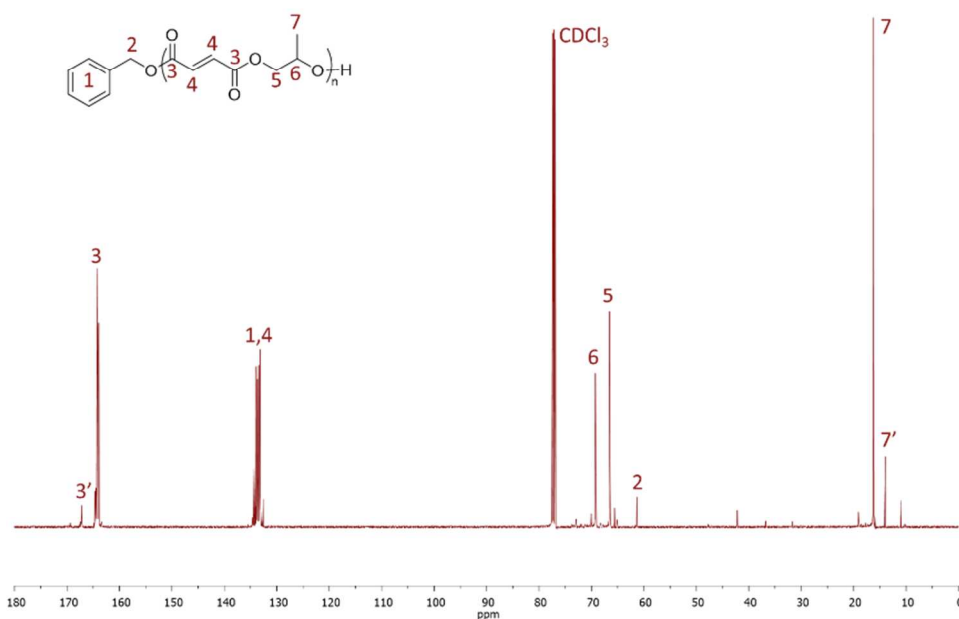


Figure S1 ^{13}C NMR spectra for DP 10 benzyl alcohol initiated PPM (Table 1, Entry 1) (125 MHz, CDCl_3 , 303 K).

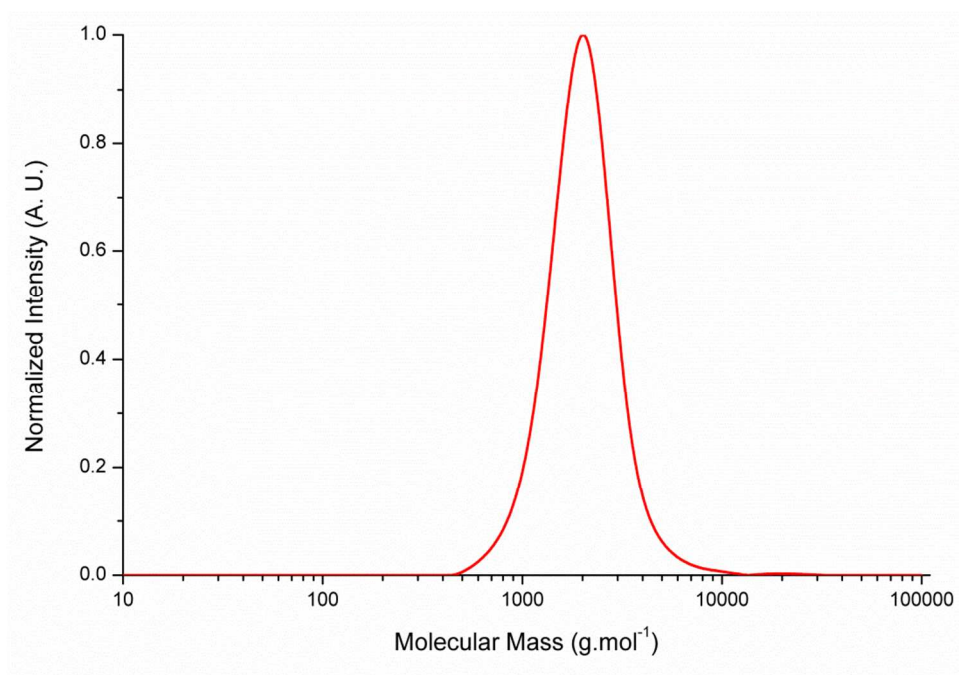


Figure S2 SEC chromatogram for DP 25 benzyl alcohol initiated PPM (Table 1, Entry 2). The molecular mass determined against poly(styrene) standards.

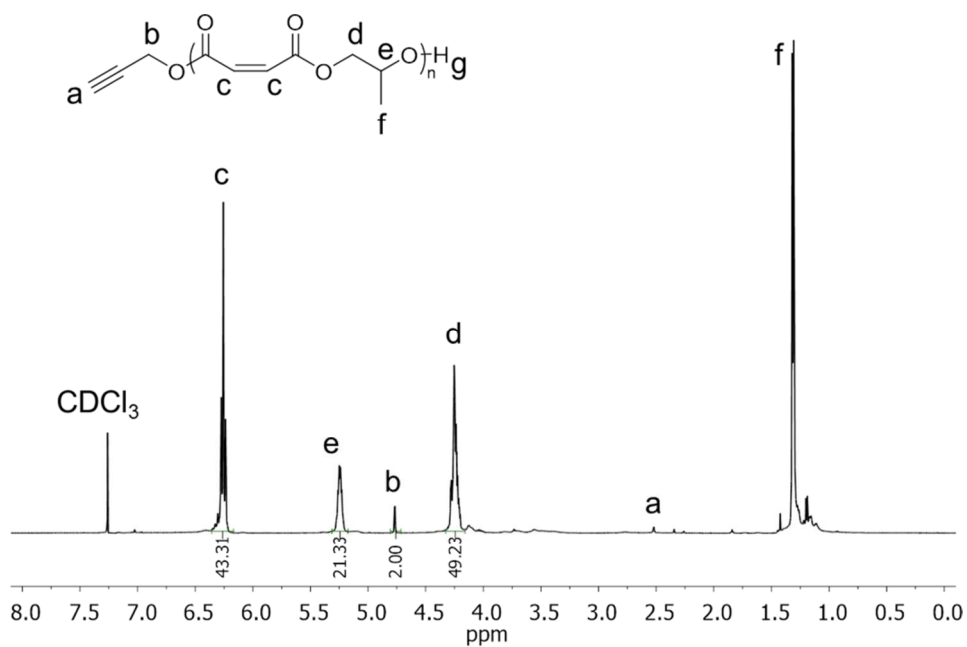


Figure S3 ¹H NMR spectra for DP 25 propargyl alcohol initiated PPM (Table 1, Entry 6) (500 MHz, CDCl₃, 303 K).

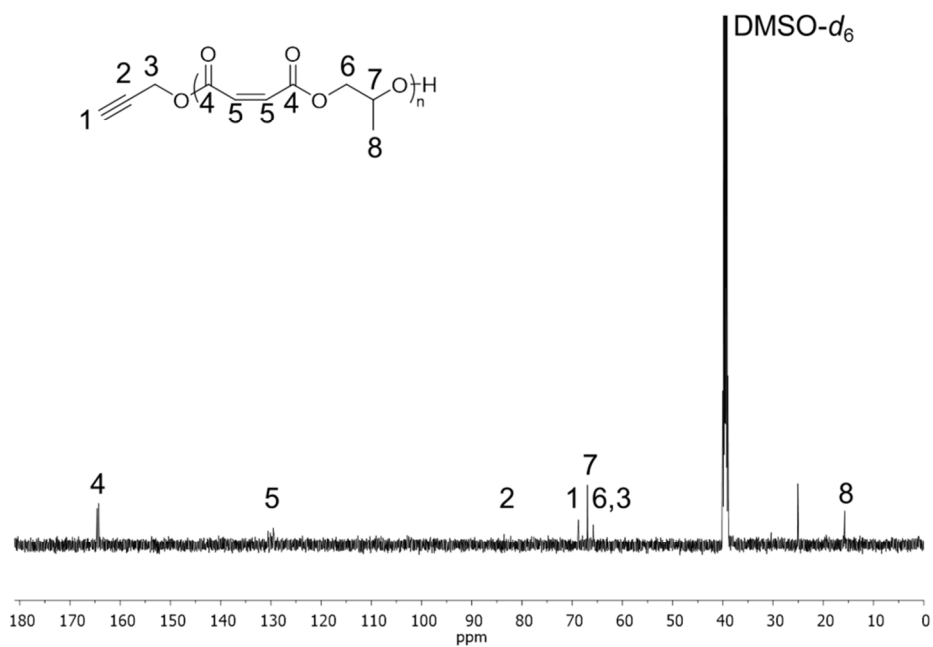


Figure S4 ¹³C NMR spectra for DP 25 propargyl alcohol initiated PPM (Table 1, Entry 6) (125 MHz, DMSO-*d*₃, 303 K).

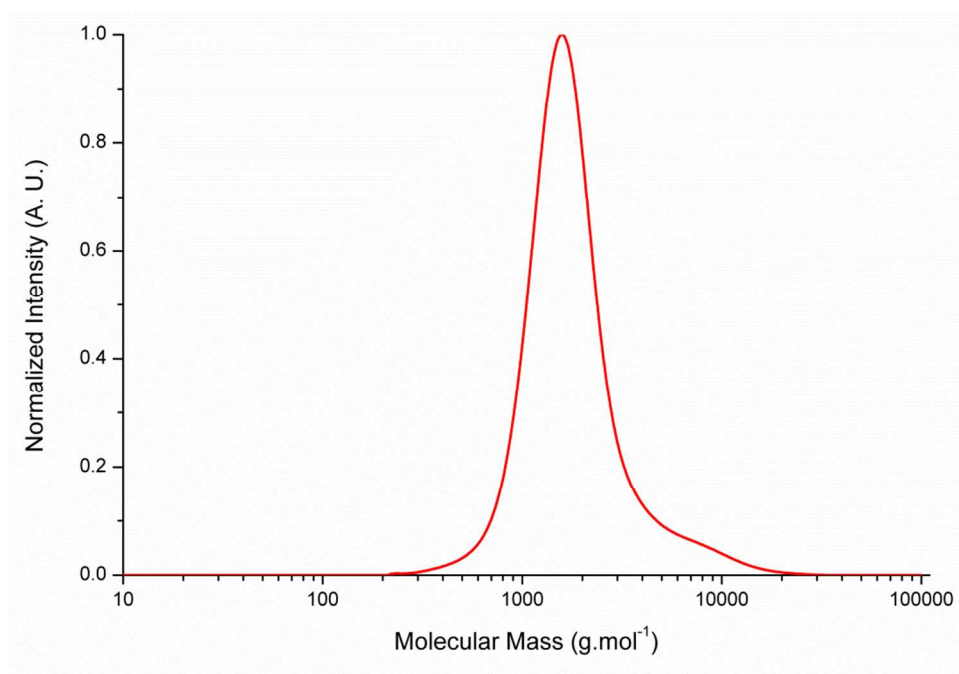


Figure S5 SEC chromatogram for DP 25 propargyl alcohol initiated PPM (Table 1, Entry 6). Molecular mass determined against poly(styrene) standards.

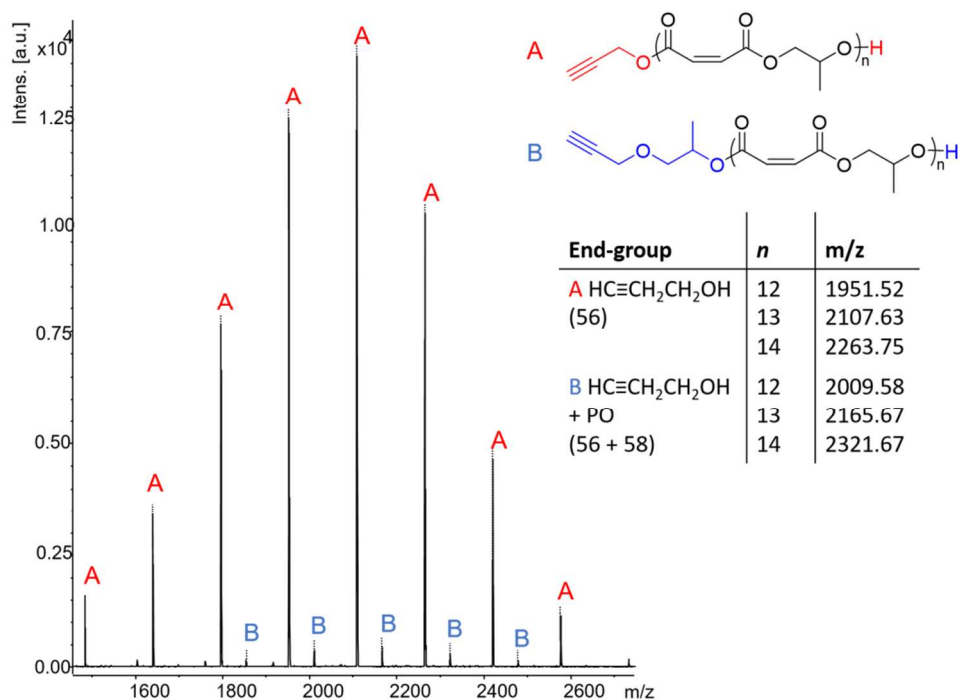


Figure S6 MALDI-ToF mass spectrum for DP 25 propargyl alcohol initiated PPM (Table 1, Entry6).

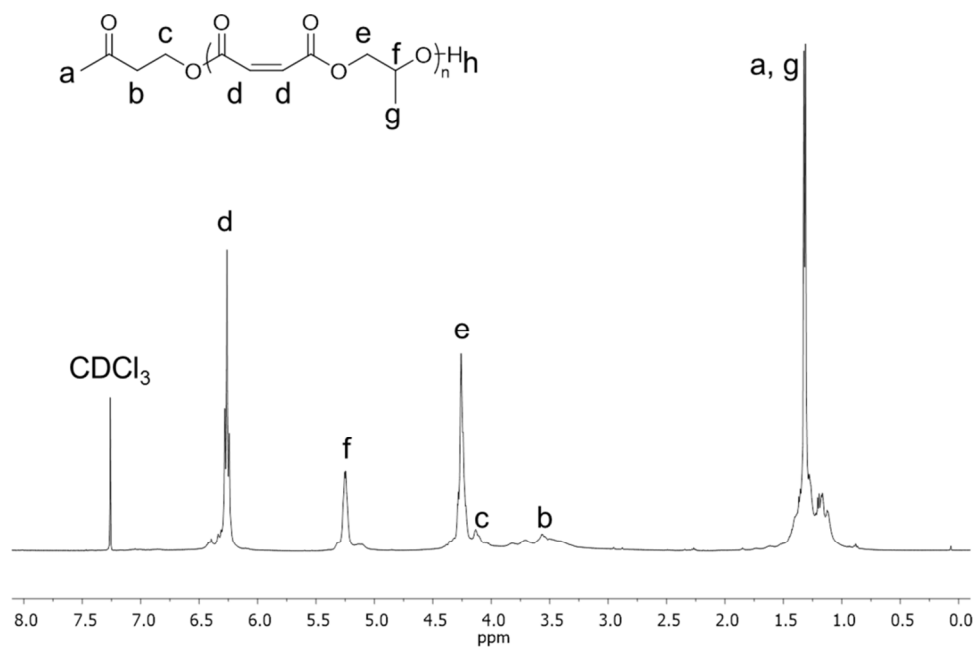


Figure S7 ^1H NMR spectra for DP 25 4-hydroxybutan-2-one initiated PPM (Table 1, Entry 10) (300 MHz, CDCl₃, 303 K).

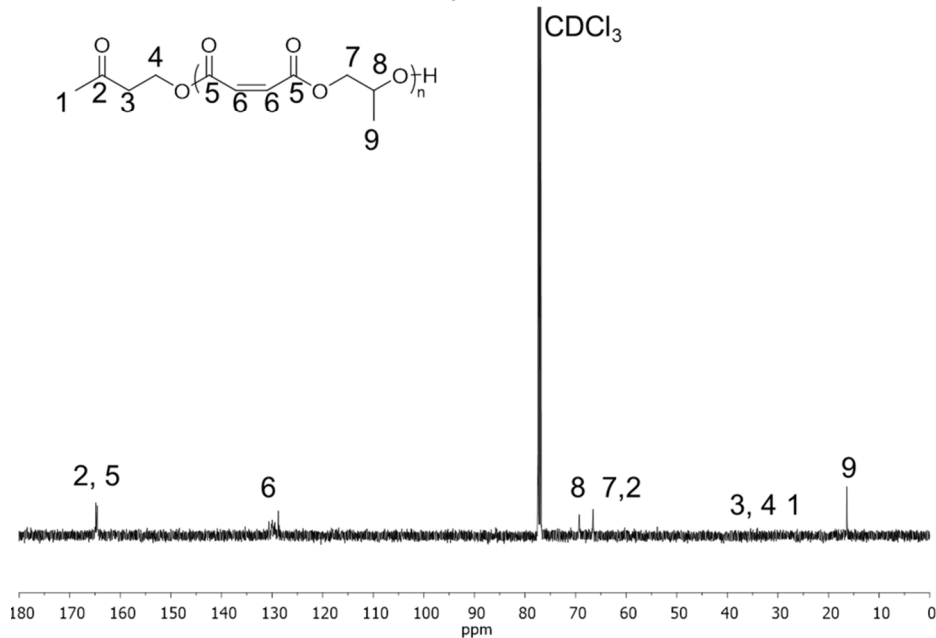


Figure S8 ^{13}C NMR spectra for DP 25 4-hydroxybutan-2-one initiated PPM (Table 1, Entry 10) (125 MHz, CDCl₃, 303 K).

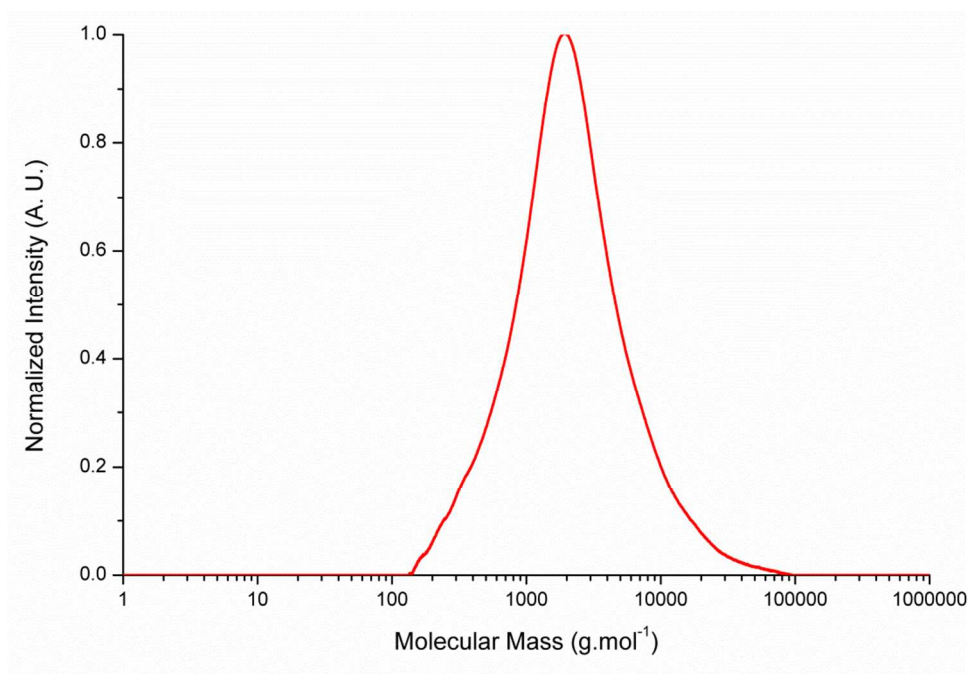


Figure S9 SEC chromatogram for DP 25 4-hydroxybutan-2-one initiated PPM (Table 1, Entry 10). The molecular mass was determined against poly(styrene) standards.

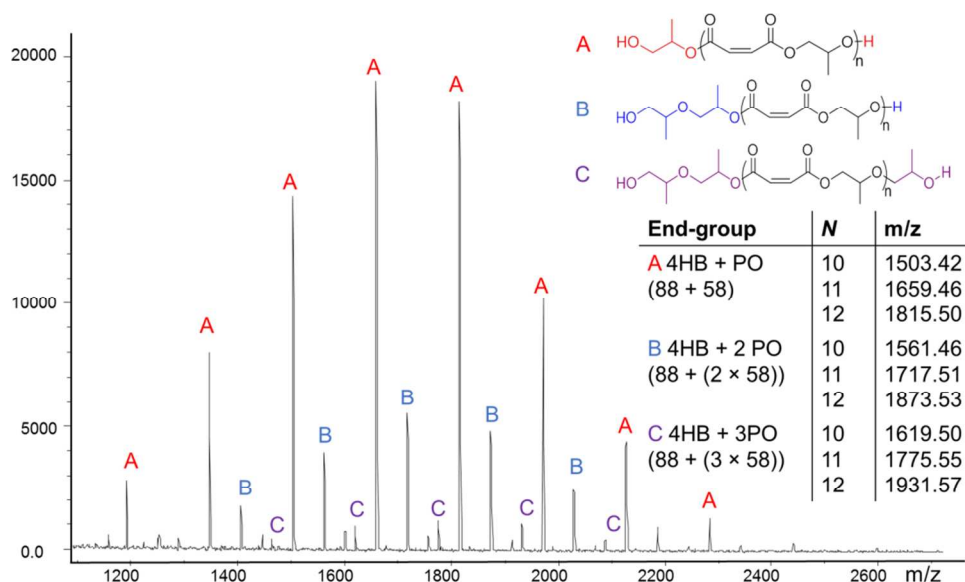


Figure S10 MALDI-ToF mass spectrum plot for DP 25 4-hydroxybutan-2-one initiated PPM (Table 1, Entry 10). End-group cleavage as a consequence of ionization-induced acrolein formation.

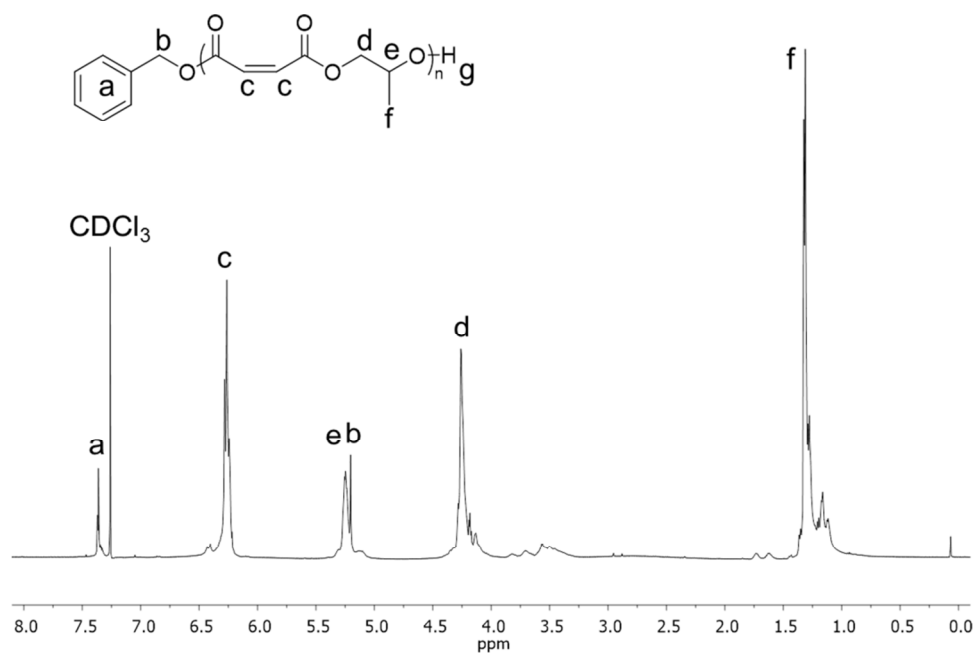


Figure S11 ^1H NMR spectra for DP 25 benzyl alcohol initiated PPM polymerized in hexanes at 60 °C (Table 2, Entry 2) (300 MHz, CDCl_3 , 303 K).

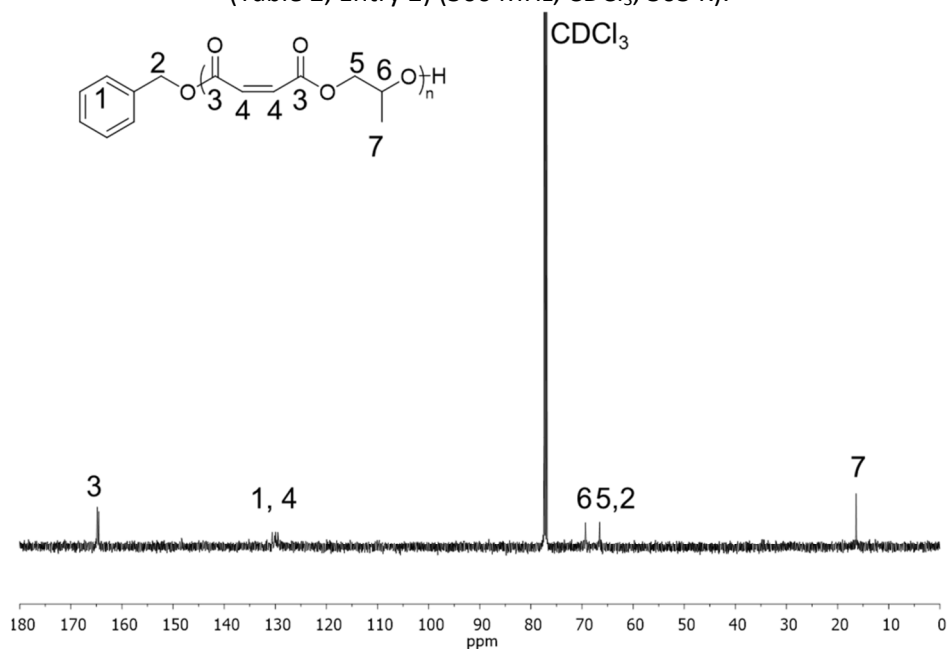


Figure S12 ^{13}C NMR spectra for DP 25 benzyl alcohol initiated PPM polymerized in hexanes at 60 °C (Table 2, Entry 2) (125 MHz, CDCl_3 , 303 K).

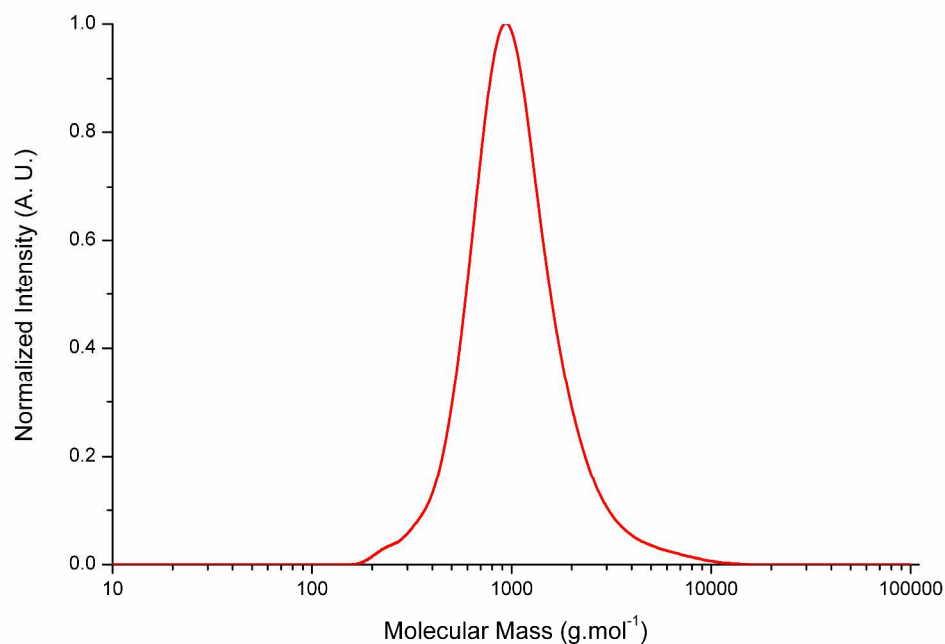


Figure S13 SEC chromatogram for DP 25 benzyl alcohol initiated PPM polymerized in hexanes at 45 °C (Table 2, Entry 2). Molecular mass determined against poly(styrene) standards.

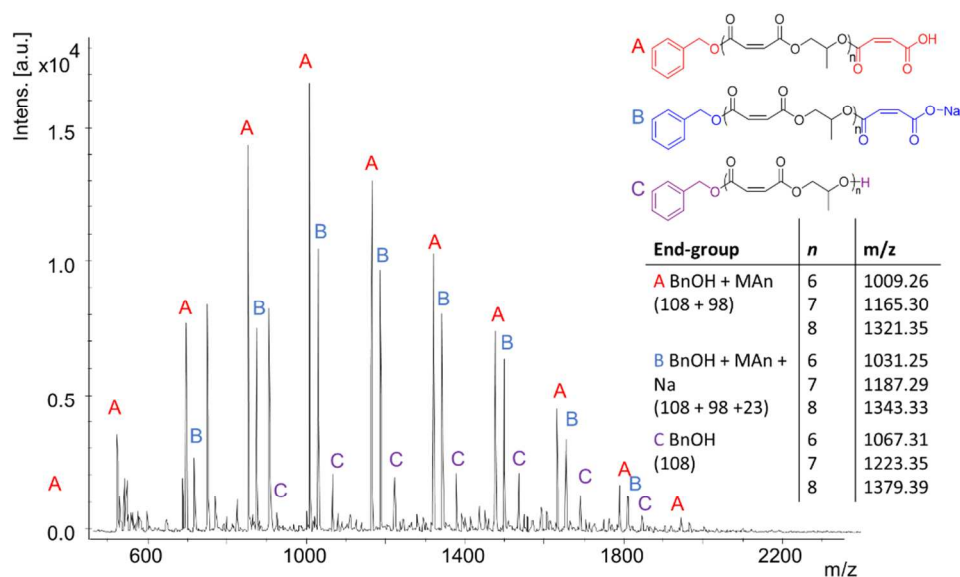


Figure S14 MALDI-ToF mass spectrum for DP 10 benzyl alcohol initiated PPM polymerized in hexanes at 45 °C (Table 2, Entry 1).

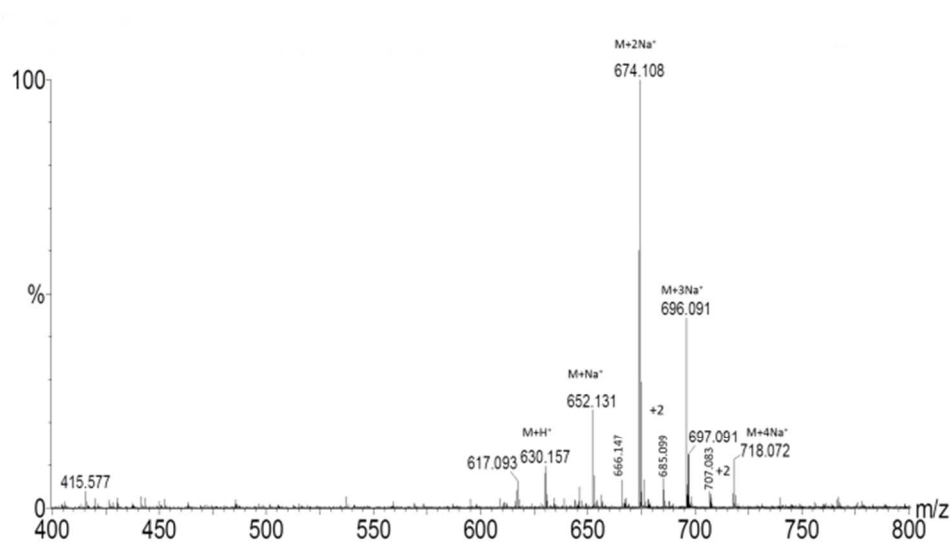


Figure S15 ESI spectrum for azide-functionalized GRGDS (N_3 -GRGDS) polypeptide sequence.

References

1. Wilson, J. A.; Hopkins, S. A.; Wright, P. M.; Dove, A. P., *Polym. Chem.*, **2014**, 5, 2691-2694.