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Selective Binding of pVTK Peptide- and Bisphosphonate-Functionalized Micelles to Prostate Cancer Cells, Osteoblasts, and Osteoclasts

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Materials

Cabazitaxel (CTX) was purchased from Selleckchem (Houston, US). Imidazole 4-carboxylic acid, tert-butyl-3-bromopropionate, potassium hydroxide (KOH), phosphorus pentachloride (PCl_5), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC.HCl), 2-(N-morpholino)ethanesulfonic acid (MES), 4-dimethylaminopyridine (DMAP), phosphonic acid (H_3PO_3), anhydrous tetrahydrofuran (THF, >99.9 %), methyl methacrylate (MMA, 99 %), *tert*-butyl acrylate (*t*BA, 98%), N, N, N', N'', N''-pentamethyldiethylenetriamine (PMDETA, 99%), anhydrous 1,4-Dioxane, triethylamine (TEA, ≥99%), 4-pentynoic acid (99%), sodium hydride (NaH, 60% in mineral oil), Poly(ethylene glycol) monomethylether (PEG, M_n: 5000 g/mol), Copper (I) bromide (CuBr, 99.9%), hydroxybenzotriazole (HOBt), anhydrous dichloromethane (DCM, >99.9%), anhydrous N-N-dimethylformamide (DMF, >99.9%), resazurin sodium salt, hydroxyapatite (HA, reagent grade powder), Trizma® Pre-set crystals (pH: 7.5) were all purchased from Sigma-Aldrich (St. Louis, MO,). MMA, *t*BA and PMDETA were purified by passing through a basic alumina column before use. The NaH was washed twice with distilled hexane before use. *N*, *N*-diisopropylethylamine (DIPEA), phosphonic oxychloride ($POCl_2$), and chlorobenzene were purchased from Alfa Aesar (Haverhill, MA). Sodium azide (NaN_3) and trifluoroacetic acid (TFA, 99%) were purchased from Acros Chemicals (New Jersey, NJ). The pVTK-NH_2 peptide was synthesized by the University of Michigan Peptide Core.

Instruments

^{1}H NMR and ^{13}C NMR spectra of the polymer blocks, the acid-labile linker, and the formulated micelles were done in 5–10 % (w/w) solutions in $CDCl_3$, DMSO-d6 or D_2O and recorded on 500 or700 MHz Varian Mercury system (Palo Alto, CA) at room temperature. NMR spectra were referenced using Me_4Si (0 ppm), residual $CHCl_3$ (δ ^{1}H-NMR 7.26 ppm, ^{13}C-NMR 77.0 ppm, CD_3SOCD_3 (δ ^{1}H-NMR 2.49 ppm, ^{13}C-NMR 39.5 ppm) and D_2O (δ ^{1}H-NMR 4.56 ppm).

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Gel permeation chromatography (GPC) measurements were done on a Viscotek GPCmax Autosampler system consisting of a pump and Water 2414 refractive index (RI) detector. The molecular weight and molecular weight distribution of polymers were determined based on their elution volume on a Styragel HR 4E column compared to a series of poly(methyl methacrylate) standards using THF as a mobile phase at a flow rate of 1 ml/min at 35 °C. Data was analyzed using Viscotek OmniSEC Omni-01 software. The azide functional group transformation was determined by Perkin-Elmer FT-IR Spectrum 4100 type A. The 90Plus particle size analyzer (Brookhaven Instruments Corporation, Holtsville, NY) was used to measure micelles size and surface charge.

Synthesis of pVTK-, BP-, and FITC-functionalized polymers

Amphiphilic triblock PMMA-*b*-PAA-*b*-PEG copolymer (5) was synthesized and used for formulation of non-targeted micelles following established protocols[1]. Synthesis of PMMA-*b*-PAA-*b*-PEG-pVTK (8), PMMA-*b*-PAA-*b*-PEG-BP (17), and PMMA-*b*-PAA-*b*-PEG-FITC (20) polymers was carried out as described in the following protocols shown in Figure 2.

Synthesis of PMMA-b-PAA-b-PEG-pVTK polymer (8)

The strategy is to synthesize the *PMMA-b-PtBA-b-PEG-NHS* (6) polymer with an activated NHS group at one end of the PEG block, which is used for covalent coupling with pVTK peptide. Briefly, the synthesis starts by adding PMMA-*b*-P*t*BA-N_3 polymer (3, 60 mg, 5.12 x 10^{-2} mmol), CuBr (11 mg, 7.68 x 10^{-2} mol), PMDETA (16 µL, 1.45 x 10^{-7} mmol), commercially available Alkyne-PEG-NHS (40 mg, 8.04 x 10^{-3} mmol) and DMF (3 mL) in a schlenck tube (25 mL) under argon. The mixture was degassed by three freeze–pump–thaw cycles and stirred at room temperature for 2 days. The reaction mixture was diluted with THF and passed through a neutral alumina column to remove the copper complex followed by evaporating the solvent before precipitating the polymer in cold diethyl ether. The precipitated polymer was collected and dried under air before dissolving it in 1 mL of DCM followed by the addition of hexane (20 mL) to precipitate the PMMA-*b*-P*t*BA-*b*-PEG-NHS polymer (6, 70 mg, 70% yield). ^{1}H NMR spectrum of PMMA-*b*-P*t*BA-*b*-PEG-NHS polymer (500 MHz, $CDCl_3$) is shown in Supplementary Data, Figure S12.

PMMA-*b*-P*t*BA-*b*-PEG-NHS polymer (6, 27 mg, 2.16 x 10^{-3} mmol) was dissolved in DMF (1.5 mL) followed by addition of EDC (1.24 mg, 6.48 x 10^{-3} mmol), HOBt (0.87 mg, 6.48 x 10^{-3} mmol), and DIPEA (2.25 µL, 1.29 x 10^{-2} mmol) in a round bottom flask and stirred for 15 min. The pVTK peptide (5.45 mg, 2.59 x 10^{-3} mmol) was dissolved in DMSO (1 mL) and added slowly to the polymer solution and stirred at room temperature for 2 h before heating the reaction mixture to 35 °C for 2 days. After the given time, the mixture was cooled down to room temperature and diluted with DI water (2 mL) before transferring into a dialysis cassette (MWCO 7 kDa) and dialyzed against deionized water for 24 h followed by lyophilization to obtain PMMA-*b*-P*t*BA-*b*-PEG-pVTK copolymer (7, 28 mg, 90% yield). Copolymer 7 (22 mg, 1.51 x 10^{-3} mmol) was dissolved in DCM:TFA (1.5:1, 3 mL) and stirred at 0 °C for 2 h then temperature raised to room temperature for additional 2 hours. The solvents were removed by rotary evaporation (30 °C) followed by the addition of DCM (3 x 2 mL) and evaporated to ensure removal of TFA. The residue was dissolved in double distilled water/THF mixture before transferring into a dialysis cassette (MWCO 7 kDa) and dialyzed against deionized water for 24 h followed by lyophilization to obtain PMMA-*b*-PAA-*b*-PEG-pVTK copolymer (8, 18 mg, 90% yield). The ^{1}H NMR spectra of copolymer 7 and 8 (500 MHz, $CDCl_3$) are shown in Supplementary Data, Figure S14 & S17 respectively.

Synthesis of PMMA-b-PAA-b-PEG-BP polymer (17)

The strategy is to synthesize ZA analogue and covalently couple to primary amine of the PEG block (15) before "click" coupling to the PMMA-*b*-P*t*BA-N_3 (3) copolymer followed by hydrolysis of the tertiary butyl group to yield PMMA-*b*-PAA-*b*-PEG-BP polymer (17, Figure 2). Synthesis of the imidazole-4-carboxylic acid ester (10) as a precursor of Zoledronic acid (ZA) analogue started with *N*-alkylation of imidazole 4-carboxylic acid following a published method with some modifications[2]. Briefly, commercially available imidazole 4-carboxylic acid (0.25 g, 2.23 mmol) was dissolved in DI water (3 mL) followed by the addition of KOH (0.375 g, 6.69 mmol) and stirred for 3 h at room temperature before evaporating the solvents on rotary evaporator. The residue was dissolved in 4:1 DMSO:1,4 Dioxane mixture (5 mL) and added DIPEA (1.16 mL, 6.69 mmol), *tert*-butylbromo-

propionate (0.74 mL, 4.46 mmol) and stirred at 50 °C for 24 h. The reaction mixture was cooled down to 0 °C, neutralized with 1N HCl, and extracted twice with 8:2 chloroform:*iso*-propanol solvent mixture (100 mL). The combined organic layers were washed with brine (25 mL) and dried over Na_2SO_4. The organic solvents were evaporated and followed by air drying to obtain 1-(3-(tert-butoxy)-3-oxopropyl)-1H-imidazole-4-carboxylic acid solid (9). This dry material was used for further reaction without purification. However, a portion of the crude material (20 mg) was dissolved in a 7:3 mixture of chloroform:*iso*-propanol and purified by preparative column chromatography using the same solvent system, which yielded 12 mg (70% yield) of compound 9, (Supplementary Data, Figure S21). Compound 9 (20 mg, 0.083 mmol) was dissolved in DMSO (1.5 mL) followed by the addition of PCl_5 (86 mg, 0.416 mmol) and stirred at room temperature for 4 h to yield compound 10 (Supplementary Data, Figure S24). The reaction mixture was purified by preparative column chromatography using a 7:3 mixture of chloroform:*iso*-propanol and obtained ~70% yield.

Commercially available BocHN-PEG-NH_2 polymer (5 kDa, 500 mg, 0.1 mmol) and 4-pentynoic acid (49 mg, 0.5 mmol) were dissolved in DMF (7 mL) followed by the addition of DIPEA (87 µL, 0.5 mmol), EDC (38 mg, 0.2 mmol) and stirred overnight at room temperature. The reaction mixture was precipitated in DCM/diethyl ether then again in THF/Hexane to obtain the Alk-PEG-NHBoc polymer (11, 430 mg, 86% yield, 92% functionalization efficiency, Supplementary Data, Figure S26). Compound 11 (300 mg, 0.0059 mmol) was dissolved in DCM (12 mL) followed by the addition of TFA (6 mL) at 0 °C and stirred overnight at room temperature. The solvents were evaporated by rotary evaporation (40 °C), the residue was transferred into a dialysis bag (MWCO 1 kDa), dialyzed for 1 day against DI water, and lyophilized to obtain the Alk-PEG-NH_2 polymer (12, 260 mg, 94% yield, Supplementary Data, Figure S29).

Compound 10 (26 mg, 0.101 mmol) was dissolved in DMSO (1.5 mL) and treated with DIPEA (17.5 µL 0.101 mmol) and stirred for 15min, before slowly adding a solution of the Alk-PEG-NH_2 polymer (12, 0.1g, 0.0202 mmol) in DMF (2 mL). The reaction mixture was stirred at room temperature for 4 h, then raised the temperature to 40 °C and stirred for 2 days. The reaction mixture was cooled down to room temperature, diluted with 2 mL of water, transferred into a dialysis cassette (MWCO 3.5 kDa), and dialyzed against deionized water for 24 h before lyophilization. The dried polymer was dissolved in DCM and precipitated in hexane to obtain the Alk-PEG-NH-Imidazole ester polymer (13, 86 mg, 83% yield, Supplementary Data, Figure S32). Compound 13 (80 mg, 0.154 mmol) was dissolved in DI H_2O (3 mL), adjusted the pH to 3 by 1N HCl solution at 0 °C and stirred for 1 h. The temperature raised to room temperature before transferring into a dialysis bag (MWCO 1 kDa), dialyzed against DI water for 24 h, followed by lyophilization. The dried polymer was dissolved in DCM and precipitated in hexane to obtain pure Alk-PEG-Imidazole acid polymer (14, 70 mg, 90% yield, Supplementary Data, Figure S35). The polymer 14 was phosphorylated following a published protocol with minor modification[3]. Specifically, compound 14 (65 mg, 0.0127 mmol) was dissolved in chlorobenzene (3 mL) followed by the addition of H_3PO_3 (2.8 mg, 0.0344 mmol) at room temperature and raised the temperature to 80 °C, then $POCl_3$ (4.7 mg, 0.0306 mmol) was added and stirred for 2.5 h. The reaction mixture was cooled down to 60 °C and added water (4 mL), water and organic layers were separated, collected the water layer, and refluxed for 18 h. The solution was cooled down to room temperature before adding 3 mL of methanol, stirred for 3 h, transferred the mixture into a dialysis bag (MWCO 1 kDa), dialyzed against deionized water for 24 h and lyophilized. The dried polymer was dissolved in DCM and precipitated in hexane to obtain 38 mg (58% yield) of pure Alk-PEG-Imidazole diphosphate polymer (15, Supplementary Data, Figure S37).

Click coupling of compound 15 (7.3 mg, 0.00139 mmol, 1.1 equivalent) and PMMA-*b*-P*t*BA-N_3 polymer (3, 10 mg, 0.00127 mmol, 1 equivalent) was carried out by mixing CuBr (0.3 mg, 0.00278 mmol, 2 equivalent), PMDETA (0.5 µL, 0.00278 mmol, 2 equivalent), and DMF (2 mL), in a schlenk tube followed by degassed the reaction mixture three freeze-pump-thaw cycles and stirred for 48 h at room temperature. The reaction mixture was transferred into a dialysis cassette (MWCO 7 kDa) and dialyzed against DI water for 48 h before lyophilization. The dried polymer was dissolved in DCM (1 mL) and precipitated in hexane to obtain pure *PMMA-b-PtBA-b-PEG-Imidazole-diphosphate* polymer (16, 15 mg, 94% yield, Supplementary Data, Figure S39). Compound 16 (20 mg, 0.00139 mmol) was dissolved in DCM (2 mL) before adding TFA (1.5 mL) at 0 °C and stirred the reaction mixture overnight

at room temperature. The solvents were evaporated by rotary evaporation (40 °C) before transferring the residue into a dialysis bag (MWCO 1 kDa), dialyzed for 1 day against DI water, and lyophilized the solution to obtain *PMMA-b-PAA-b-PEG-Imidazole-diphosphate* polymer (17, 18 mg, 94% yield, Supplementary Data, Figure S41).

Synthesis of PMMA-b-PAA-b-PEG-FITC polymer (20)

Commercially available 5 kDa Alkyne-PEG-NH_2 polymer (20 mg, 4 x 10^{-3} mmol) was dissolved in DI water (2 mL) followed by the addition of FITC (1.9 mg, 4.8 x 10^{-3} mmol) in acetone (0.5 mL) and stirred at room temperature for 24 h. The reaction mixture was diluted with 3 mL of water before transferring into a dialysis bag (MWCO 1 kDa), dialyzed against DI water for 24 h followed by lyophilization to obtain Alkyne-PEG-NH-FITC (18, 20 mg, 90% yield, Supplementary Data, Figure S42). Alkyne-PEG-NH-FITC (216 mg, 4.02 x 10^{-2} mmol, 1.1 equivalent), PMMA-*b*-P*t*BA-N_3 (3, 300 mg, 3.65 x 10^{-2} mmol, 1 equivalent), CuBr (6.2 mg, 4.39 x 10^{-2} mmol, 1.2 equivalent), PMDETA (9.1 µL, 4.39 x 10^{-2} mmol, 1.2 equivalent), and 5 mL of DMF were introduced into a schlenk tube followed by degassing the reaction mixture by three freeze-pump-thaw cycles and stirred at room temperature for 48 h. The reaction mixture was diluted with THF, transferred in to a dialysis bag (MWCO 1 kDa), dialyzed against DI water for 2 days and lyophilized. The dry polymer was dissolved in DCM and precipitated in hexane to obtain PMMA-*b*-PtBA-*b*-PEG-FITC polymer (19, Supplementary Data, Figure S44 & S45). Polymer 19 (20 mg, 0.00139 mmol) was dissolved in DCM (2 mL) followed by the addition of TFA (1.5 mL) at 0°C and stirred overnight at room temperature. The solvents were evaporated by air circulation and the residue was transferred into a dialysis bag (MWCO 1 kDa) and dialyzed for 2 days against DI water before lyophilizing to obtain PMMA-*b*-PAA-*b*-PEG-FITC polymer (20, 18 mg, 94% yield, Supplementary Data, Figure S46).

Spectral Data:

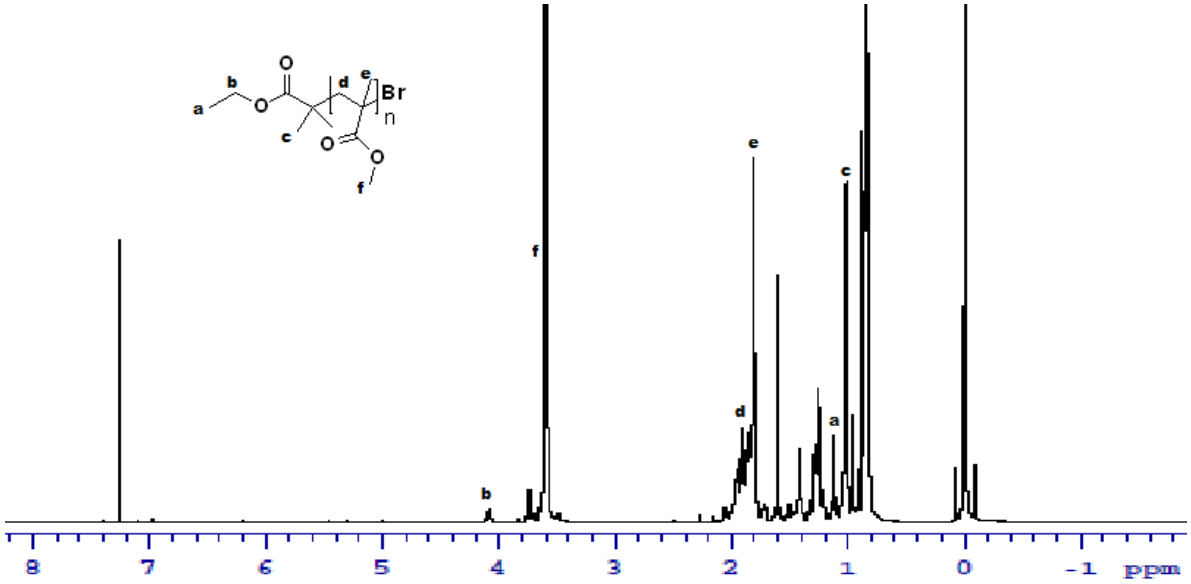

Figure S1 ^1H NMR of compound 1 in $CDCl_3$

Data: Ref: 1; http://pubs.acs.org/doi/abs/10.1021/acs.molpharmaceut.6b00147

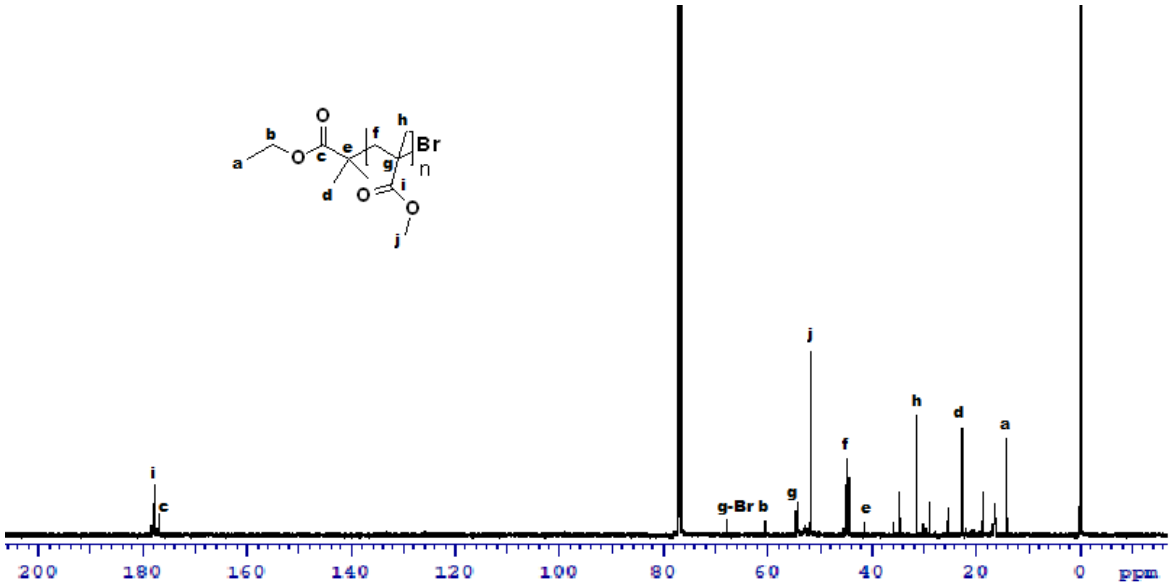

Figure S2: 13C NMR of compound 1 in CDCl3

Data: (Ref: 1)

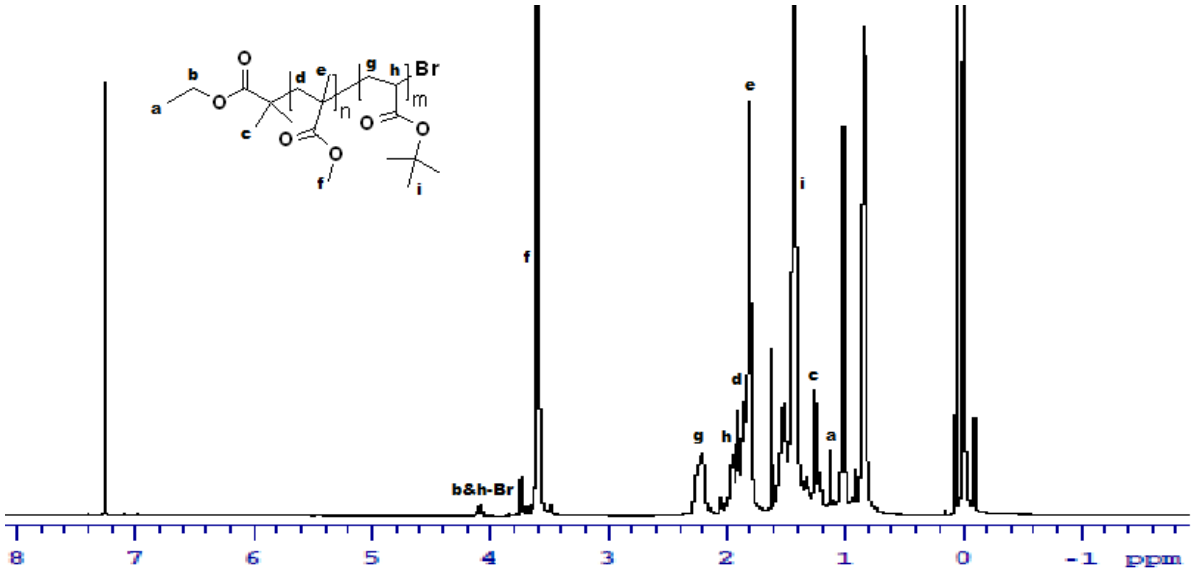

Figure S3 ^{1}H NMR of compound 2 in $CDCl_3$;

Data: (Ref: 1)

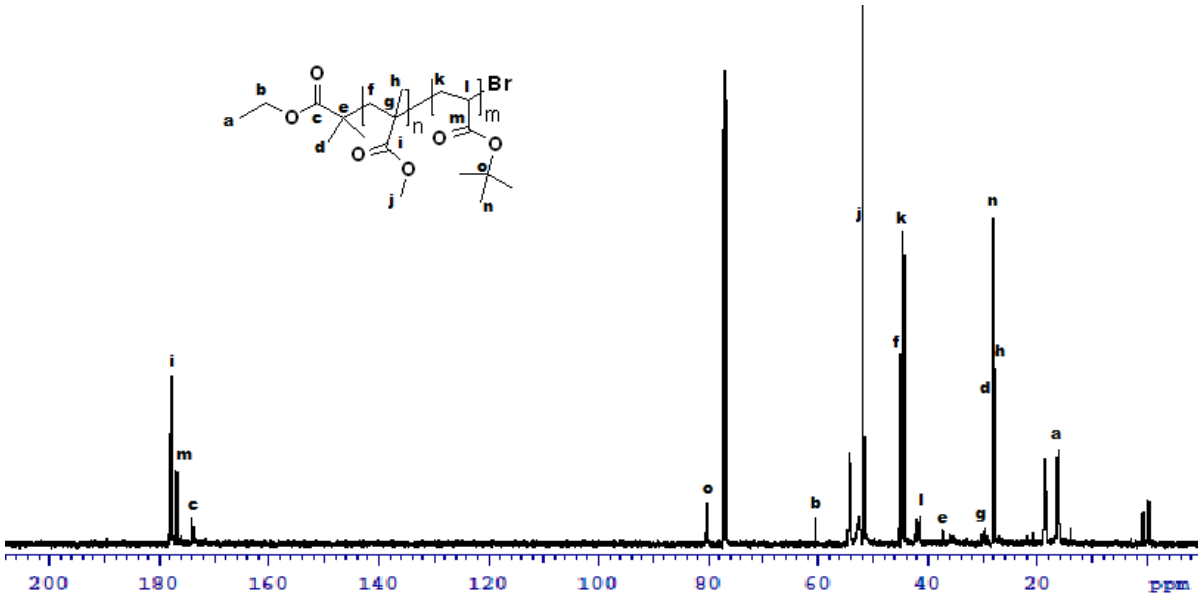

Figure S4 ^{13}C NMR of compound 2 in $CDCl_3$

Data: (Ref: 1)

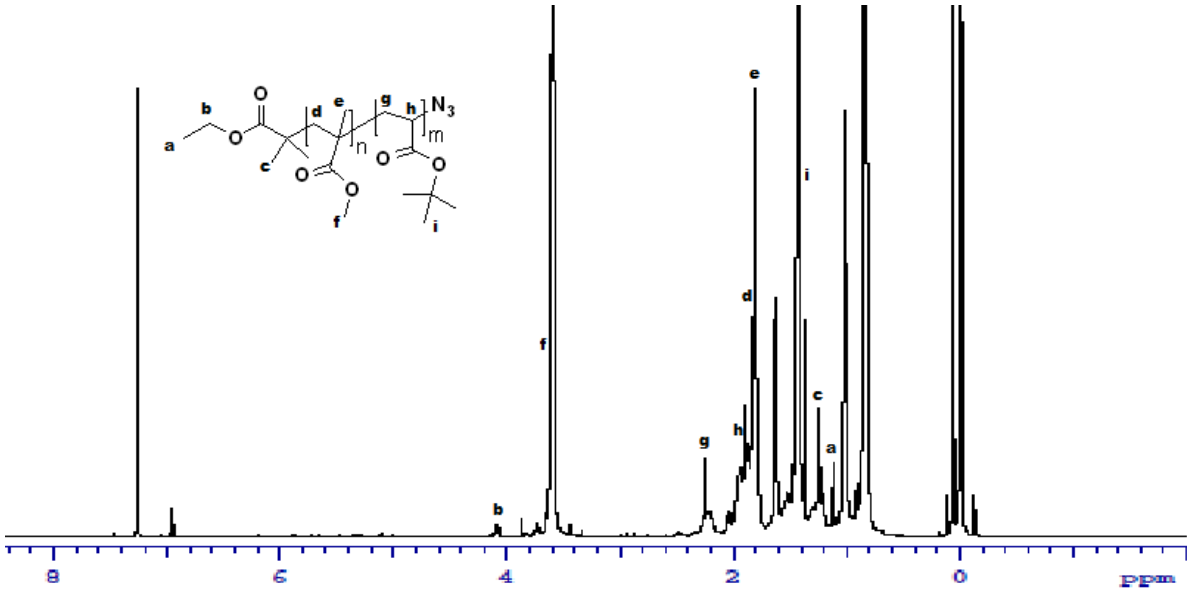

Figure S5 ^1H NMR of compound 3 in $CDCl_3$

Data: (Ref: 1)

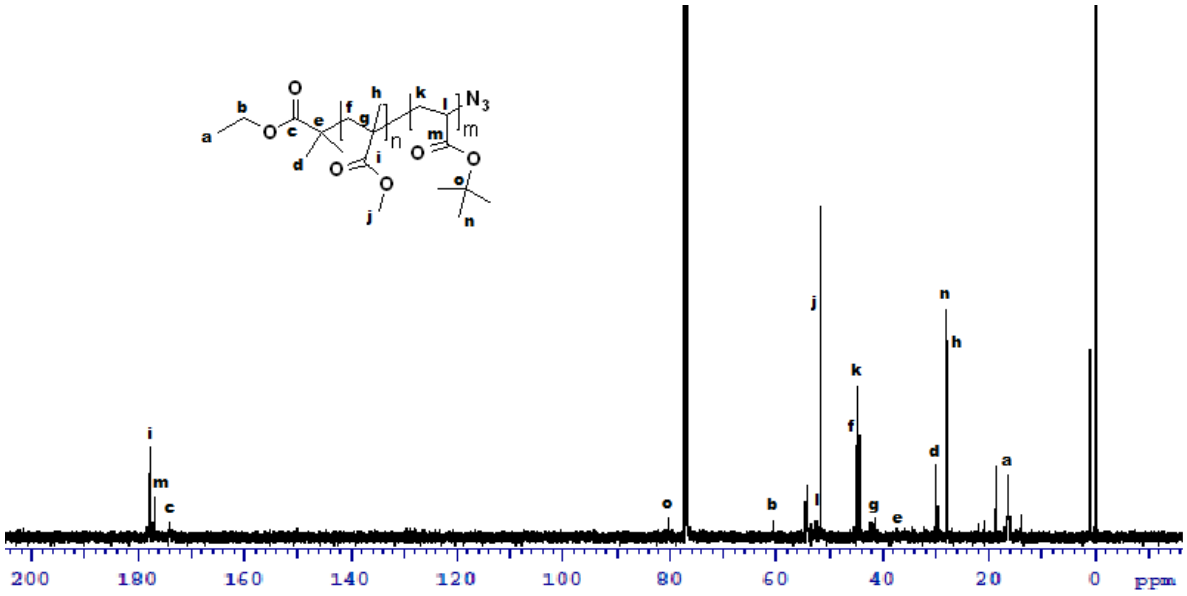

Figure S6 ^{13}C NMR of compound 3 in $CDCl_3$

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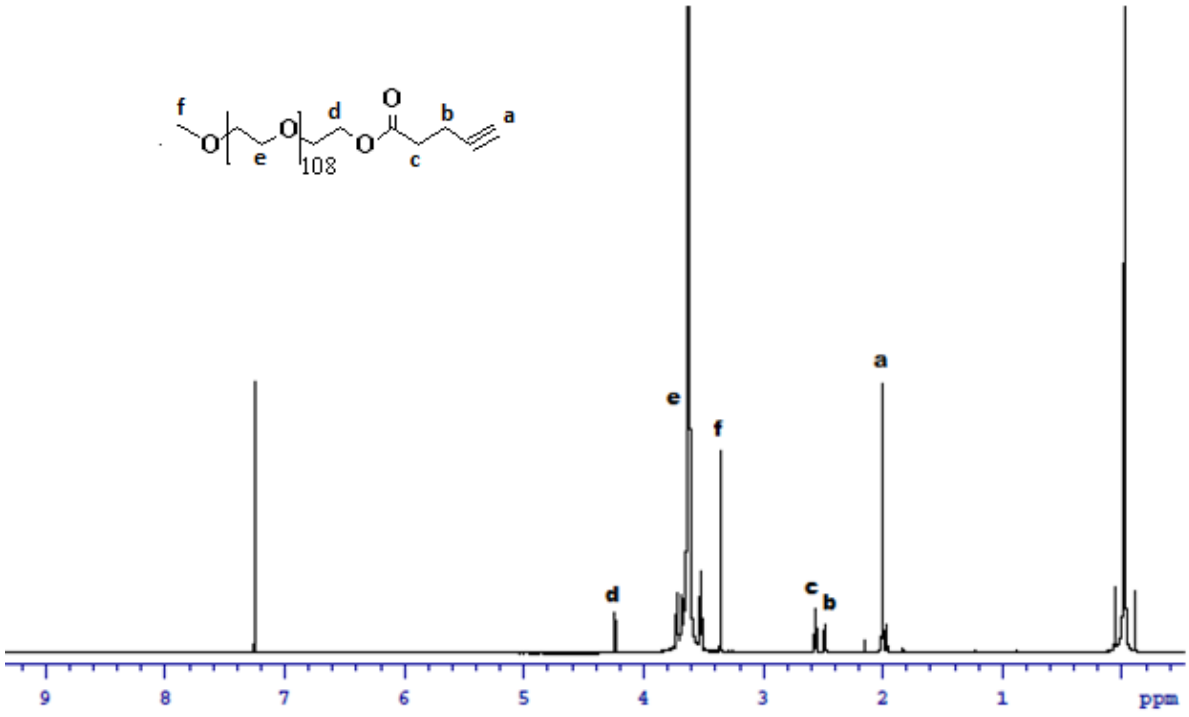

Figure S7 ^{1}H NMR of compound 3a in $CDCl_3$

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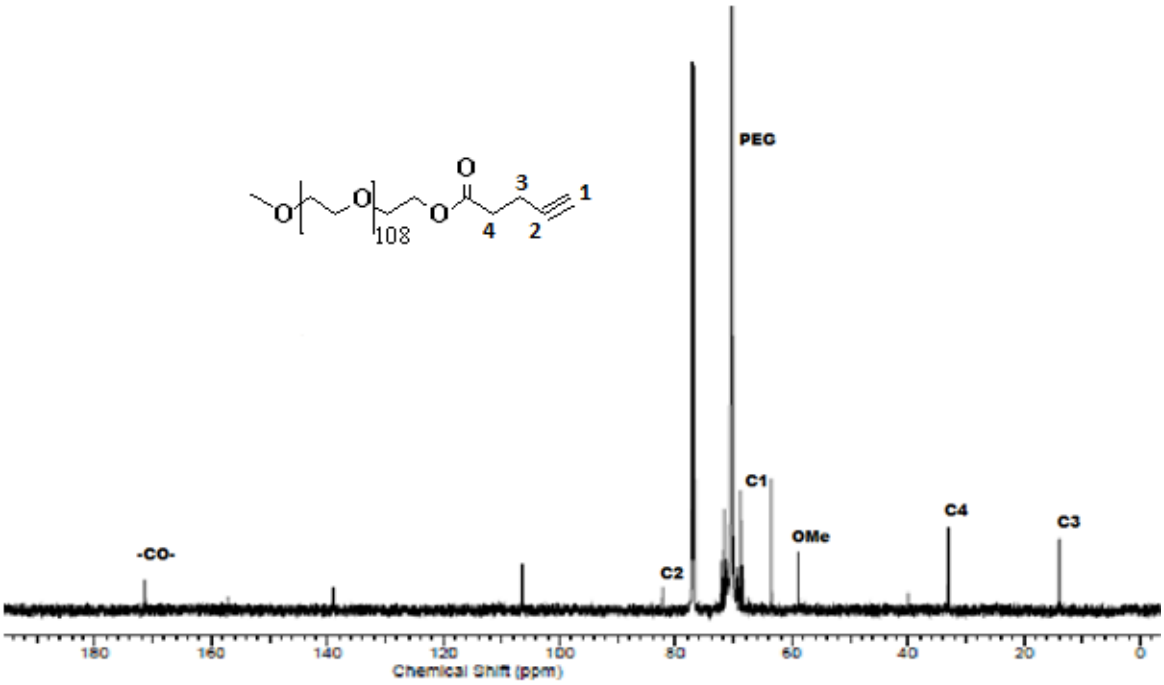

Figure S8 ^{13}C NMR of compound 3a in $CDCl_3$

Data: (Ref: 1)

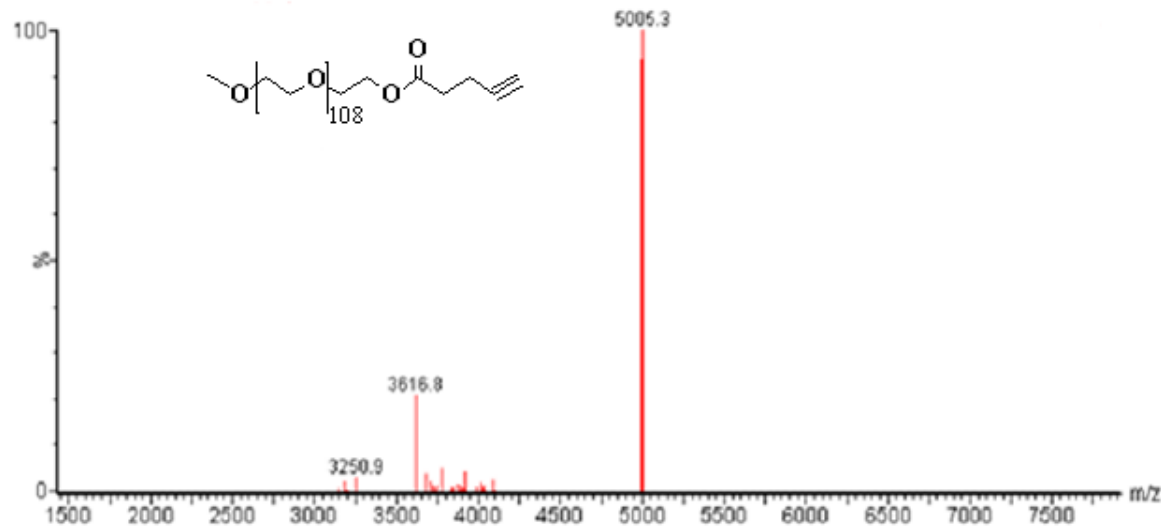

Figure S9 MALDI-TOF of Compound 3a (Alkyne-PEG)

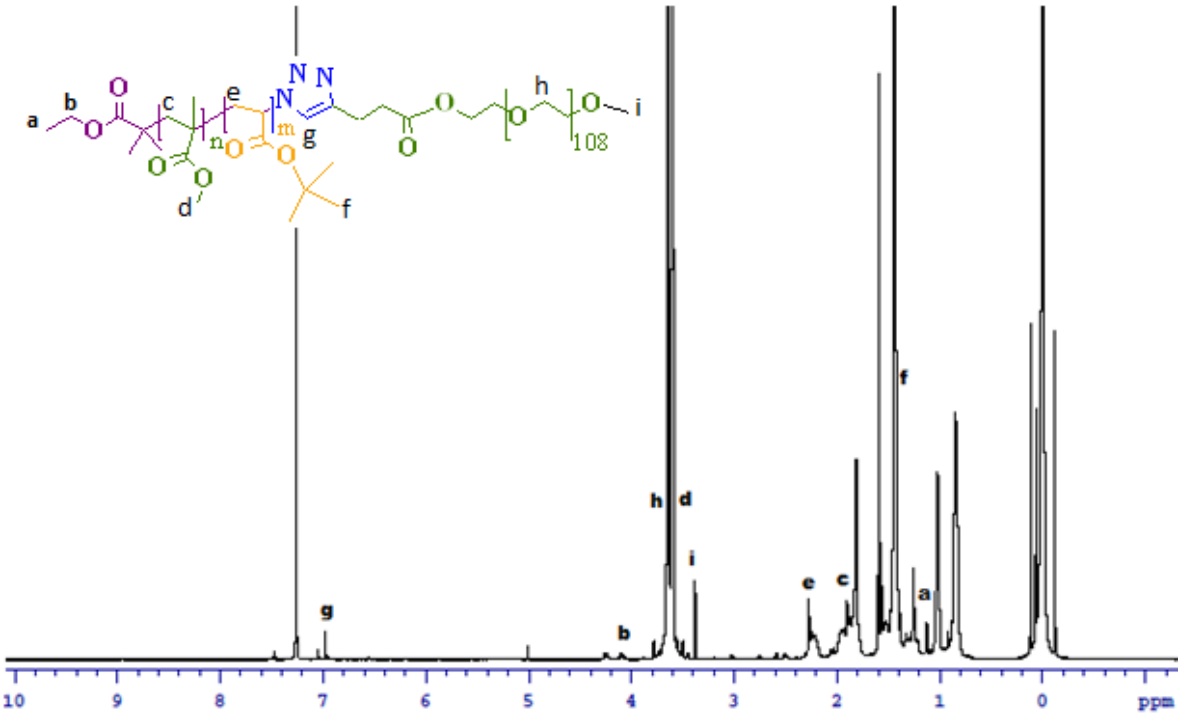

Figure S10 ^{1}H NMR of Compound 4 (PMMA-b-PtBA-PEG) in $CDCl_3$

Data: (Ref: 1)

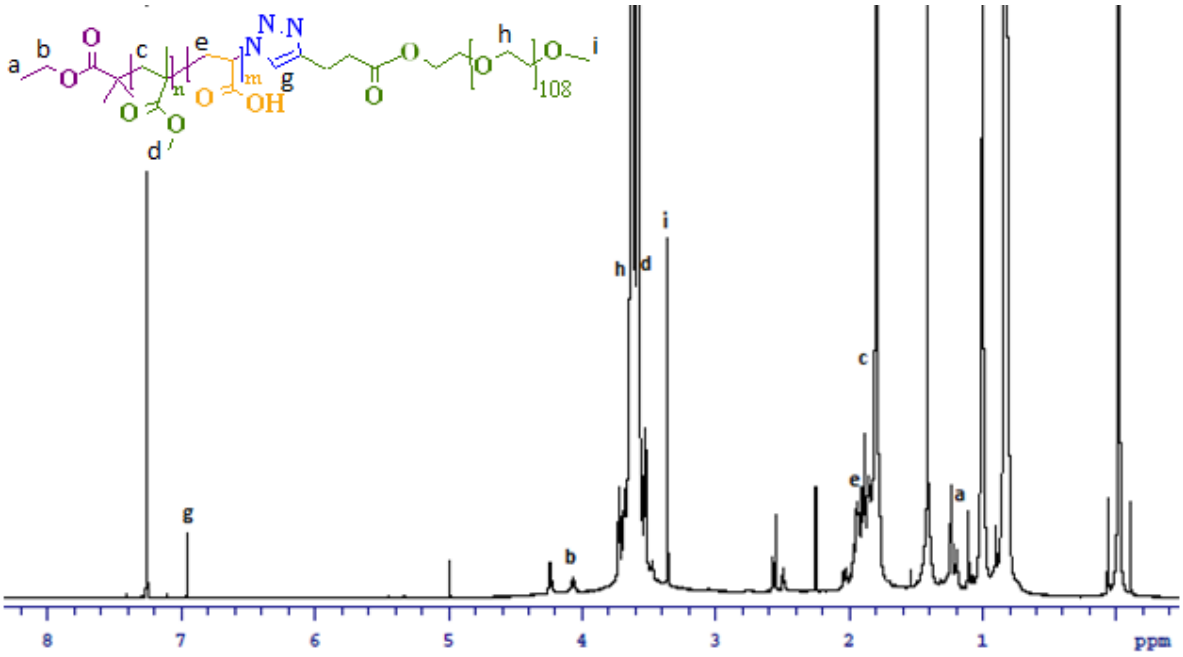

Figure S11 ^{1}H NMR of Compound 5 (PMMA-b-PAA-PEG) in $CDCl_3$

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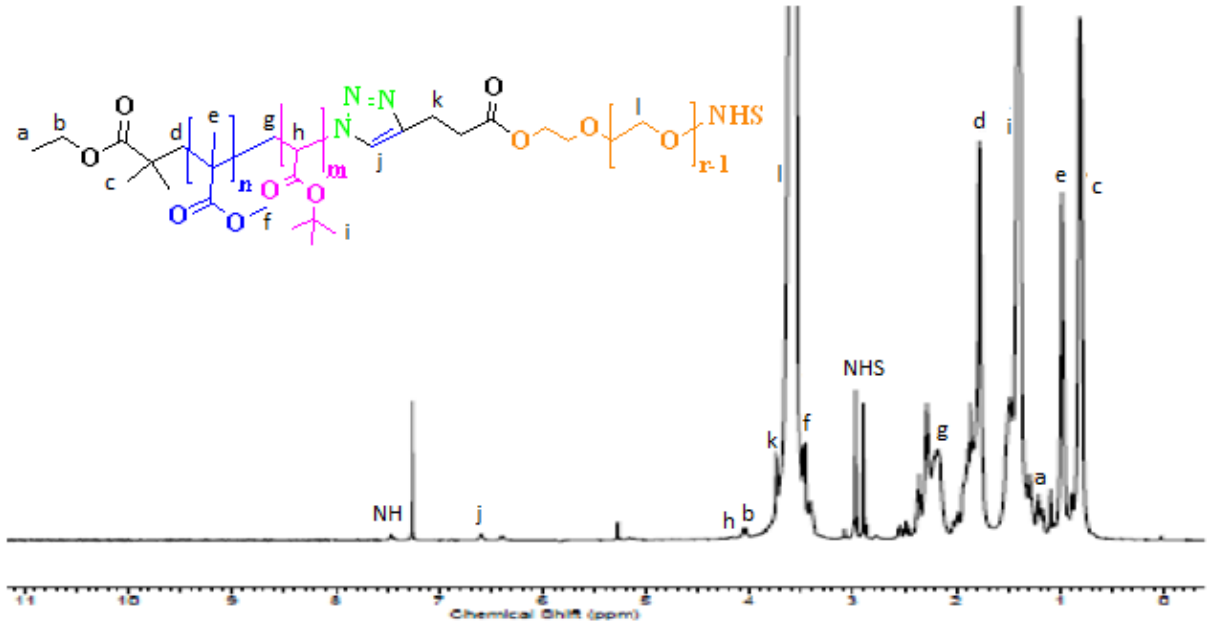

Figure S12: ^{1}H NMR spectrum of compound 6 in $CDCl_3$

In ^{1}H NMR, the characteristic proton of the triazole ring was observed at 6.62 ppm and PMMA methyl ester peak/PEG ethylene dioxide protons were observed at 3.50-3.60 ppm.

^{1}H NMR (500 MHz, $CDCl_3$): δ 0.80 (s, 50H, H_c), 0.88 (s, 3H), 0.98 (s, 26H, H_e), 1.08 (s, 3H), 1.14-1.24 (t, 6H, including H_a), 1.29 (s, 8H), 1.39 (bs, 199H, Hi), 1.77 (bs, 35H, H_d), 1.82-2.04 (m, 27H), 2.18 (bs, 20H, H_g), 2.28 (bs, 15H, Hg), 2.36 (t, 6H, J = 7.0 Hz), 2.48 (t, 2H, J = 2.0 Hz), 2.54 (t, 2H, J = 2.0 Hz), 2.90 (s, 3H), 2.99 (s, 4H), 3.34-3.50 (m, 14H), 3.55 (s, 96H, H_f), 3.60 (s, 450H, PEG protons, H_l), 3.74 (t, 8H, J = 5.0 Hz, H_k), 4.04 (t, 2H, J = 6.0 Hz, H_b), 4.19 (t, 1H, J = 5.0 Hz, H_h), 7.46 (bs, 1H, H_j).

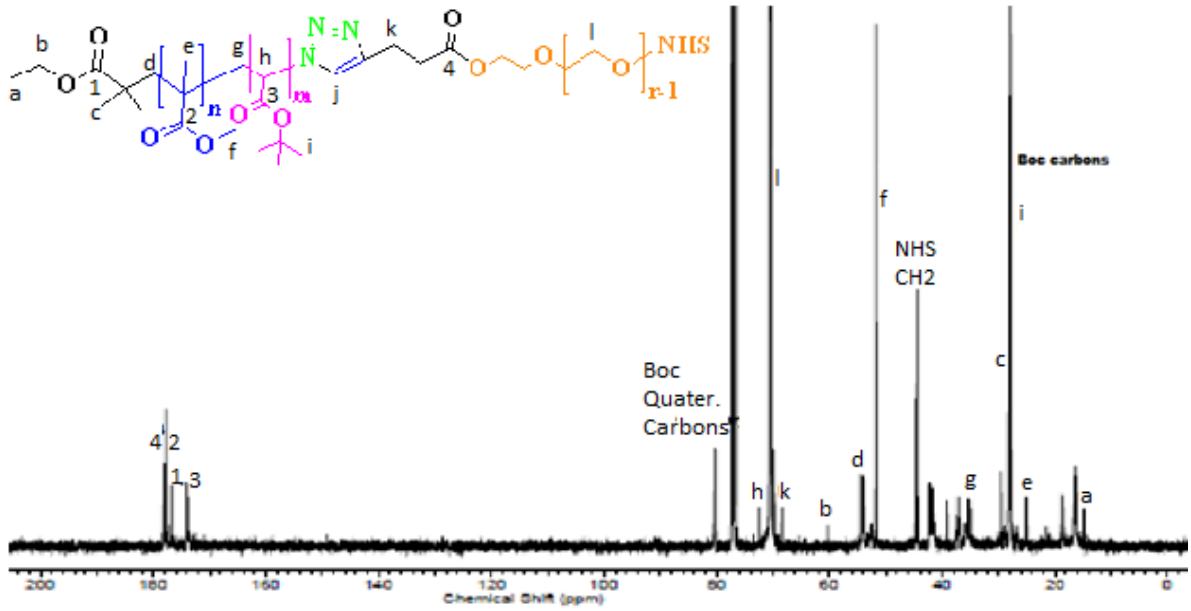

Figure S13: ^{13}C NMR spectrum of compound 6 in $CDCl_3$

^{13}C NMR (125 MHz, $CDCl_3$): δ 14.73 (C_a), 16,35, 18.64, 24.99 (C_c), 27.85, 27.94, 28.01 (all 3 belongs to Boc carbons, C_i), 29.59 (C_e), 35.05, 35.27 (C_g), 35.59, 37.11, 39.10, 3917, 41.29, 41.76, 42.22, 44.44, 44.79, 51.69 (C_f), 52.56, 54.11, 54.31 (C_d), 60.38 (C_b), 68.44 (C_k), 69.71, 69.78, 69.87, 70.00, 70.10 (C_l), 70.33, 70.45, 72.46 (Ch), 80.23 (Boc quaternary carbon), 173.85 (C_3), 174.07, 176.85 (C_1), 177.68 (C_2), 177.97 (C_4).

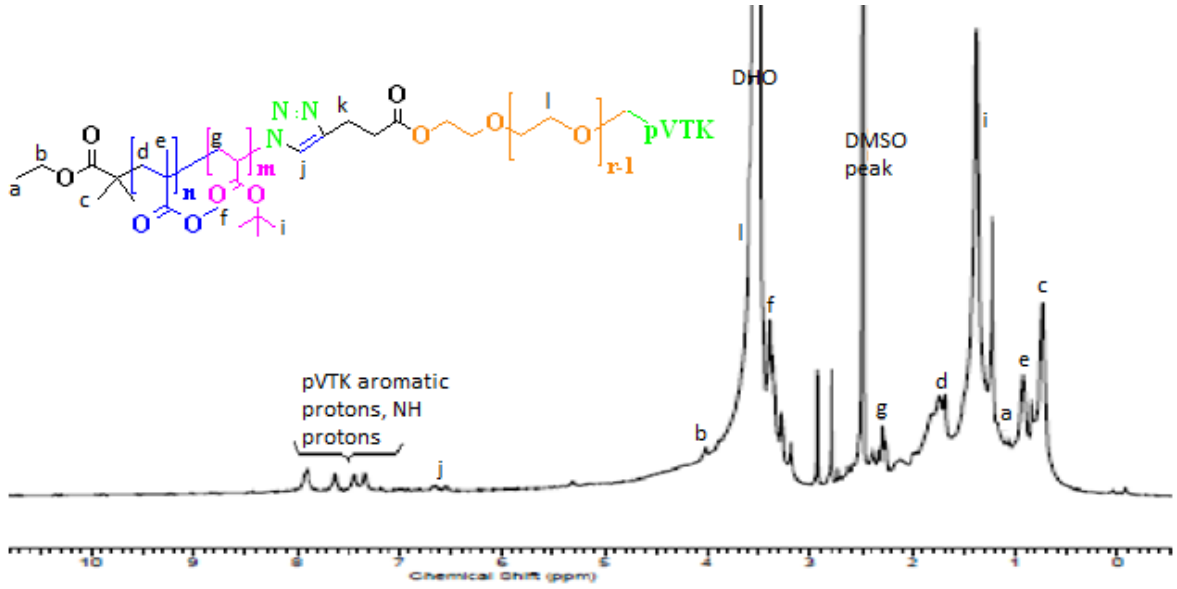

Figure S14: ^{1}H NMR spectrum of compound 7 in DMSO-d6

In ^{1}H NMR, the characteristic aromatic protons of histidine and tyrosine were observed at 7.00-8.00 ppm. PMMA methyl ester peak and PEG`s ethylene oxide protons were observed at 3.50-3.60 ppm.

^{1}H NMR (700 MHz, CD_3SOCD_3): δ 0.73 (s, 50H, H_c), 0.84 (s, 12H, valine CH_3), 0.93 (s, 26H, H_e), 1.06 (s, 3H), 1.22 (s, 16H, including H_a), 1.29 (s, 8H), 1.38 (bs, 200H, Hi), 1.60-2.04 (m, 75H, including H_d), 2.04-2.20 (m, 20H), 2.22-2.30 (m, 20H, H_g), 2.30-2.34 (m, 12H), 2.34-2.44 (m, 6H), 2.58 (bs, 2H), 2.72 (m, 2H), 2.79 (s, 3H), 2.93 (s, 4H), 3.14-3.24 (m, 14H), 3.35-3.45 (m, 100H, H_f), 3.45-3.75 (s, 450H, PEG protons, H_l), 3.78-3.84 (m, 8H, H_k), 4.02 (t, 2H, J = 5.0 Hz, H_b), 4.19 (t, 1H, H_h), 4.74 (s, 1H), 5.03 (s, 1H), 5.31 (s, 1H), 5.54 (s, 2H), 5.63 (s, 4H), 6.46-8.50 (m, 14H, including H_j).

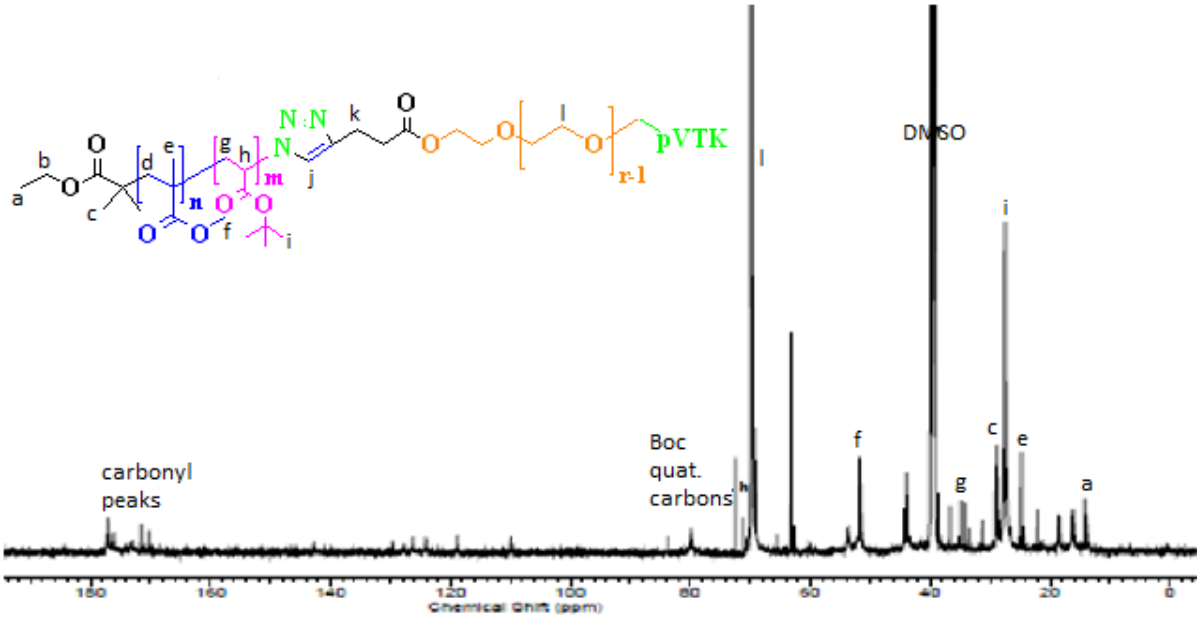

Figure S15: ^{13}C NMR spectrum of compound 7 in DMSO-d6

^{13}C NMR (175 MHz, CD_3SOCD_3): δ 13.92, 14.17 (C_a), 16.06, 16.24, 18.43, 22.07, 24.43, 24.86 (C_c), 25.08, 26.58, 27.46, 28.44, 28.55, 28.68, 28.83 (all 6 belongs to Boc carbons, C_i), 29.01, 29.46 (C_e), 31.27, 33.46, 33.57, 34.06, 35.08 (C_g), 36.60, 38.56, 40.81, 41.47, 43.87, 44.25, 51.70 (C_f), 53.76 (C_d), 60.08 (C_b), 62.62, 63.06, 64.12, 65.46, 66.94, 69.10 (C_k), 69.27, 69.42, 69.59, 69.65, 69.77, 70.46, 71.20 (PEG carbons, C_l), 72.47 (Ch), 79.92 (Boc quaternary carbon), 83.72, 109.90, 118.90, 124.06, 126.32, 127.66, 129.61, 142.84, 169.99, 170.28, 171.01, 171.58(C_3), 172.93, 173.21, 173.77, 176.13 (C_1), 177.09 (C_2), 177.33 (C_4).

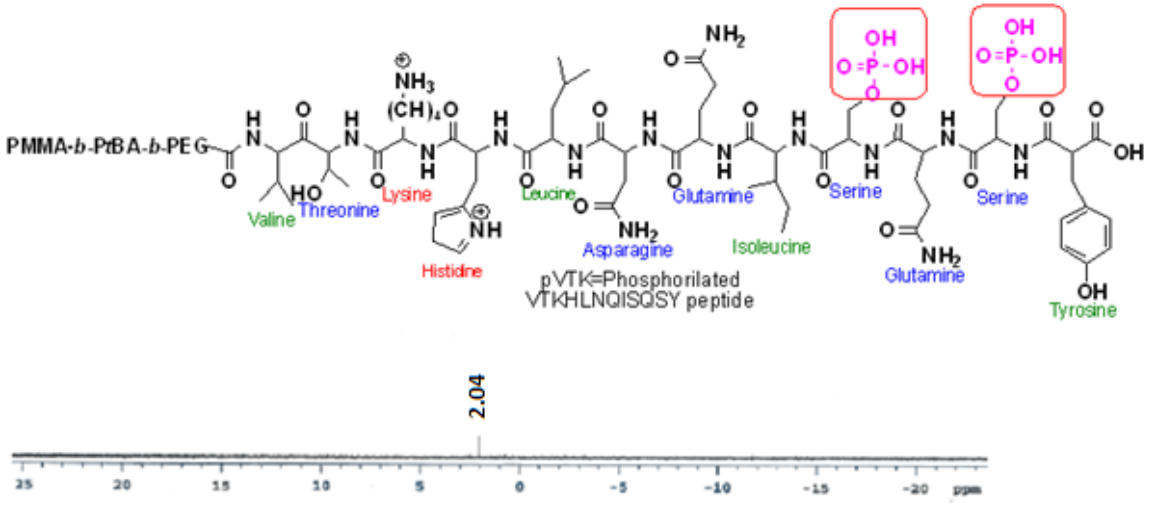

Figure S16: ^{31}P NMR spectrum of compound 7 in DMSO-d6

^{31}P NMR (283 MHz, CD_3SOCD_3): δ 2.04.

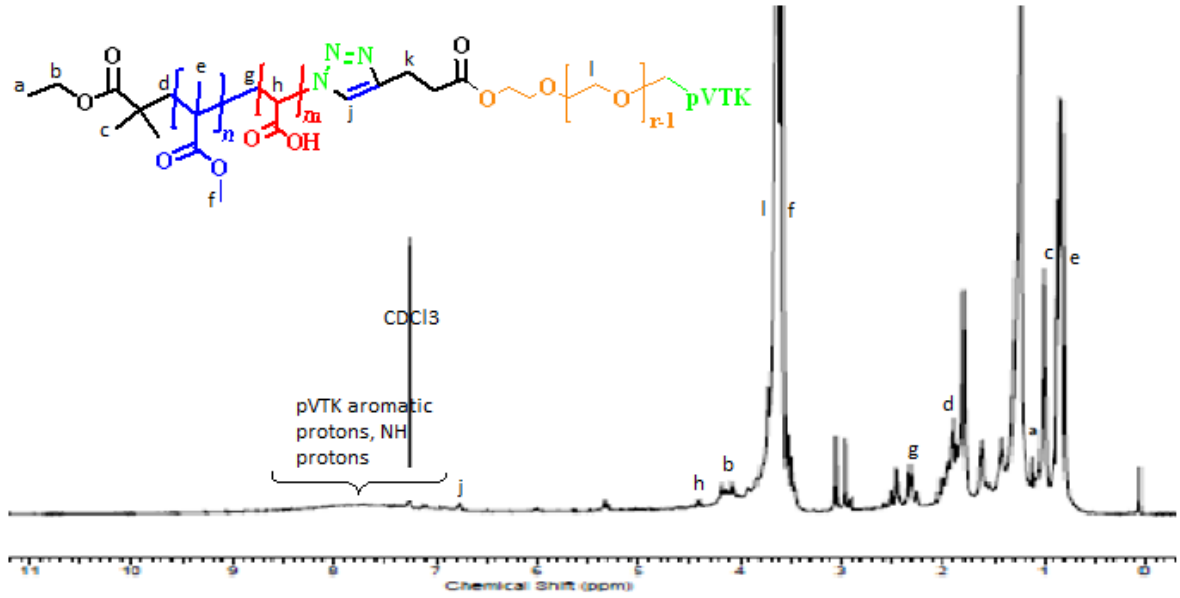

Figure S17: ^1H NMR spectrum of compound 8 in $CDCl_3$

^1H NMR after acid-hydrolysis confirms the disappearance of Boc methyl protons at 1.44 ppm and all other peaks intact.

^1H NMR (700 MHz, $CDCl_3$): δ 0.75-0.90 (bs, 69H, H_c, Valine CH_3), 1.00 (bs, 26H, H_e), 1.11 (s, 4H), 1.24 (s, 110H, including H_a), 1.36-145 (m, 16H), 1.61 (bs, 15H), 1.74-2.06 (m, 50H, including H_d), 2.22-2.60 (m, 16H, H_g), 2.96 (s, 3H), 3.04 (s, 4H), 3.42-3.54 (m, 100H, H_f), 3.54-3.75 (s, 450H, PEG protons, H_l), 3.86-3.94 (m, 8H, H_k), 3.98-4.24 (m, 16H, including H_b), 4.32-4.64 (m, 7H, including H_h), 4.88 (bs, 1H), 5.02 (s, 1H), 5.28 (s, 1H), 5.33 (s, 2H), 6.03 (s, 2H), 6.54-7.12 (m, 6H), 7.30-8.50 (m, 20H, including H_j, COOH peaks).

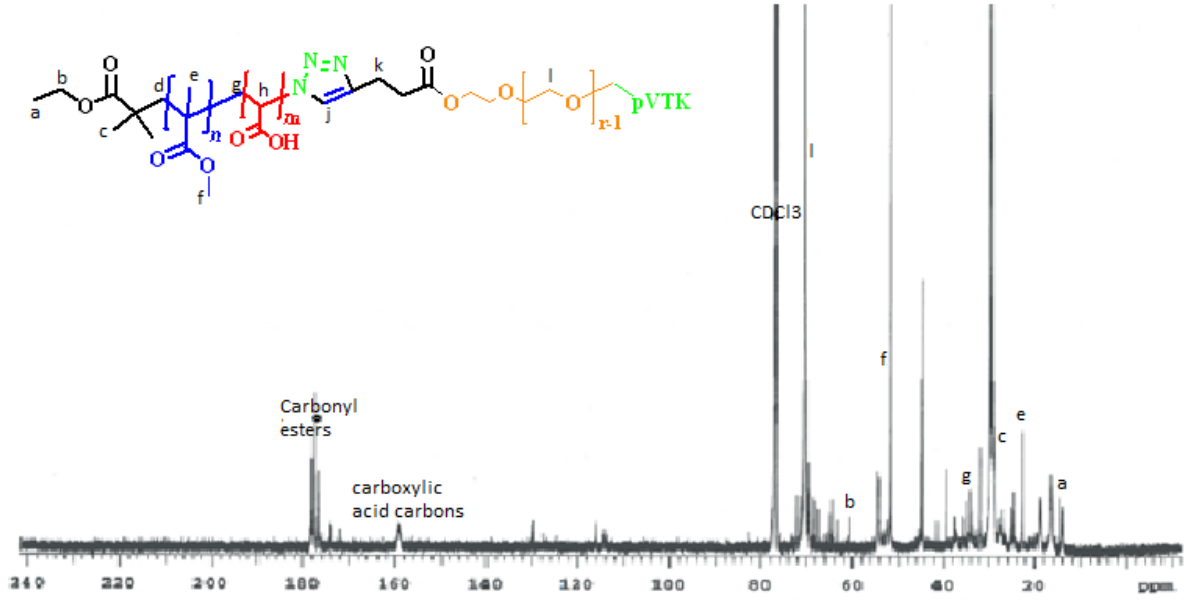

Figure S18: ^{13}C NMR spectrum of compound 8 in $CDCl_3$

^{13}C NMR (175 MHz, $CDCl_3$): δ 13.99, 14.88 (C_a), 16.45, 18.73, 22.63, 24.85 (C_c), 25.15, 27.16, 27.86, 29.07, 29.19, 29.30, 29.40, 29.69 (C_e), 31.86, 32.68, 33.53, 33.96, 34.09, 34.99, 35.71, 35.84 (C_g), 37.32, 37.60. 39.50, 41.37, 44.51, 44.85, 45.48, 51.77 (C_f), 52.50, 53.38 (C_d), 54.13, 54.37, 60.48 (C_b), 63.29, 64.50, 64.73, 65.08, 65.56, 66.00, 67.41, 67.99, 68.33, 68.53, 69.57 (C_k), 69.93, 70.15, 70.42 (PEG carbons, C_l), 71.36, 72.30, 73.24, 75.99, 82.64, 114.38, 114.71, 116.03, 127.73, 129.61, 129.81, 129.87, 130.00, 159.21, 172.05, 173.88, 174.19, 174.26, 176.14, 176.96, 177.12, 177.77, 178.07, 178.36.

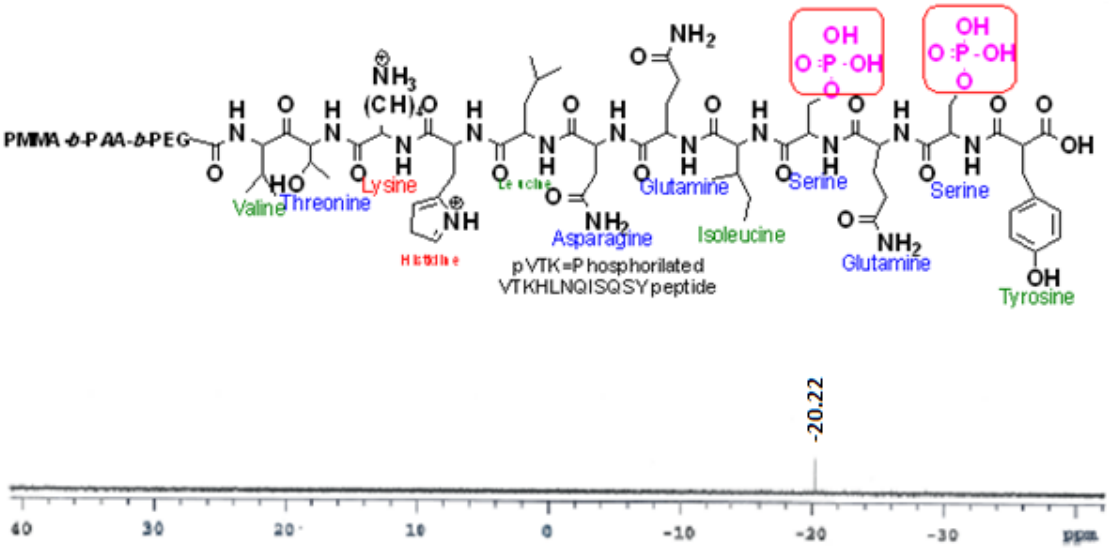

Figure S19: ^{31}P NMR spectrum of compound 8 in $CDCl_3$

^{31}P NMR (283 MHz, $CDCl_3$): δ -20.22.

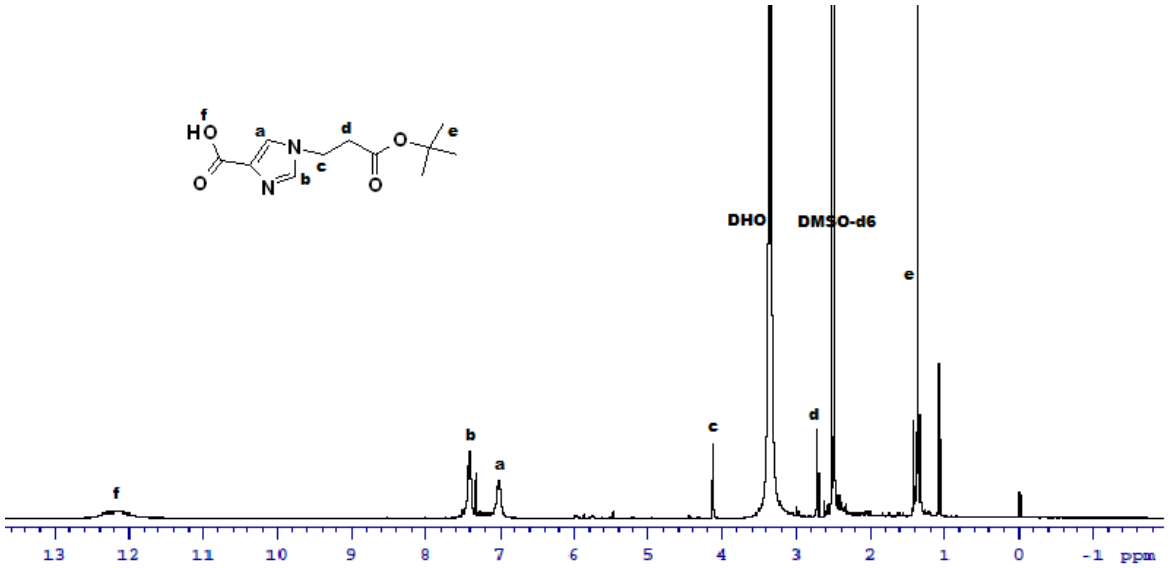

Figure S20 ^{1}H NMR of compound 9 in DMSO-d6 (before purification)

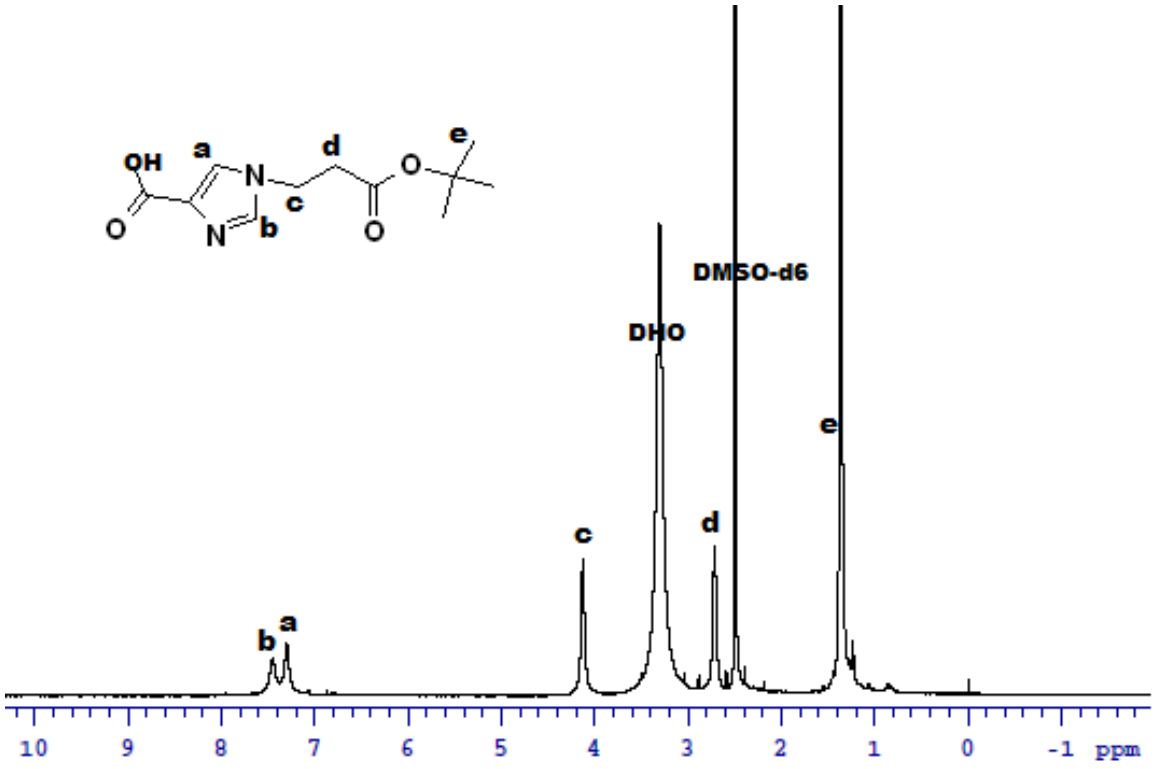

Figure S21 ^{1}H NMR of compound 9 in DMSO-d6 (after purification)

In ^{1}H NMR, the aromatic protons were observed at 7.30 and 7.45 ppm, whereas tertiary butyl group was observed at 1.38 ppm.

^{1}H NMR (700 MHz, DMSO-d6): δ 1.36 (s, 9H, C-$(CH_3)_3$, H_e), 2.71 (bs, 2H, CH_2, H_d), 4.12 (bs, 2H, CH_2, H_c), 7.30 (bs, 1H, Ar-CH, H_a), 7.45 (bs, 1H, Ar-CH, H_b).

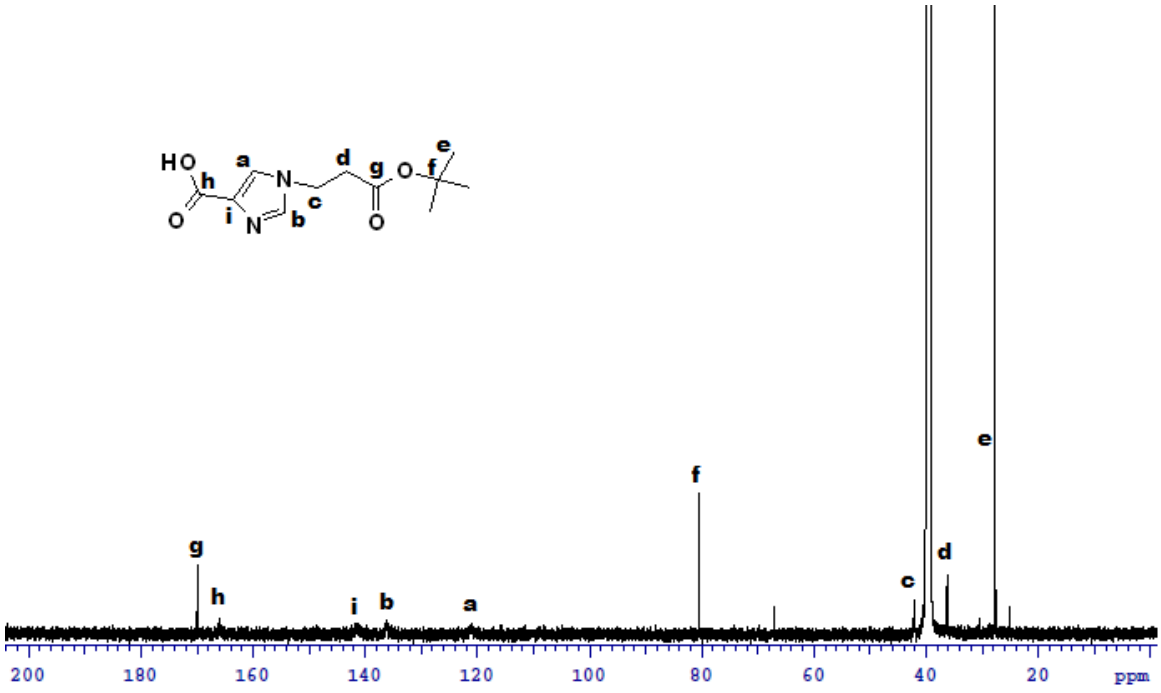

Figure S22 ^{13}C NMR of compound 9 in DMSO-d6

^{13}C NMR (175 MHz, DMSO-d6): δ 27.67 (C_e), 36.26 (C_d), 42.16 (C_c), 80.43 (C_f), 121.08 (C_a), 136.24 (C_b), 141.49 (C_i), 166.07 (C_h), 169.93 (C_g).

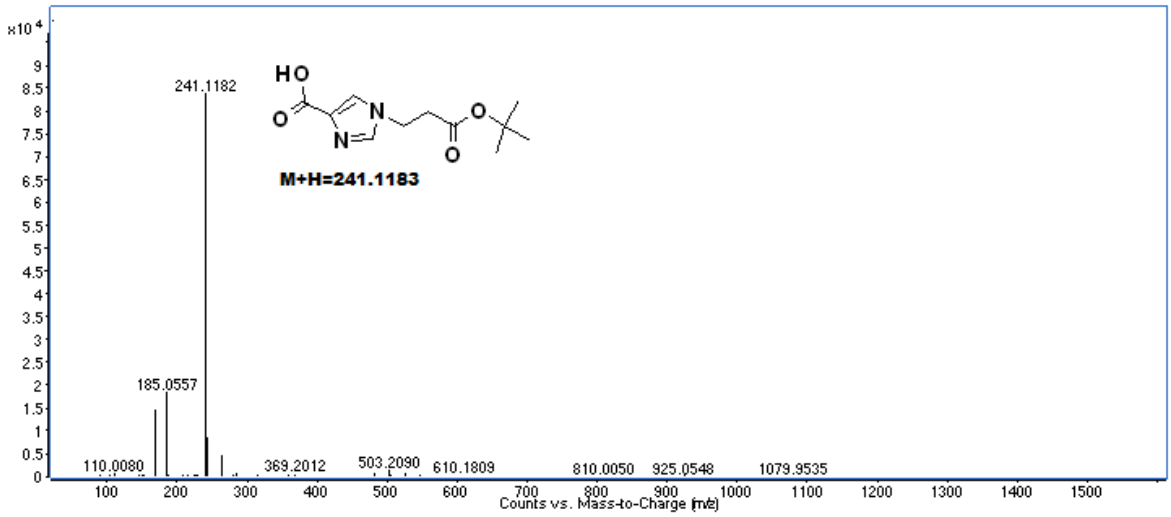

Figure S23 HR-MS (ESI-MS) of compound 9

HR-MS $[M+H]^+$ $C_{11}H_{17}N_2O_4$ calcd 241.1188, obsd 241.1186.

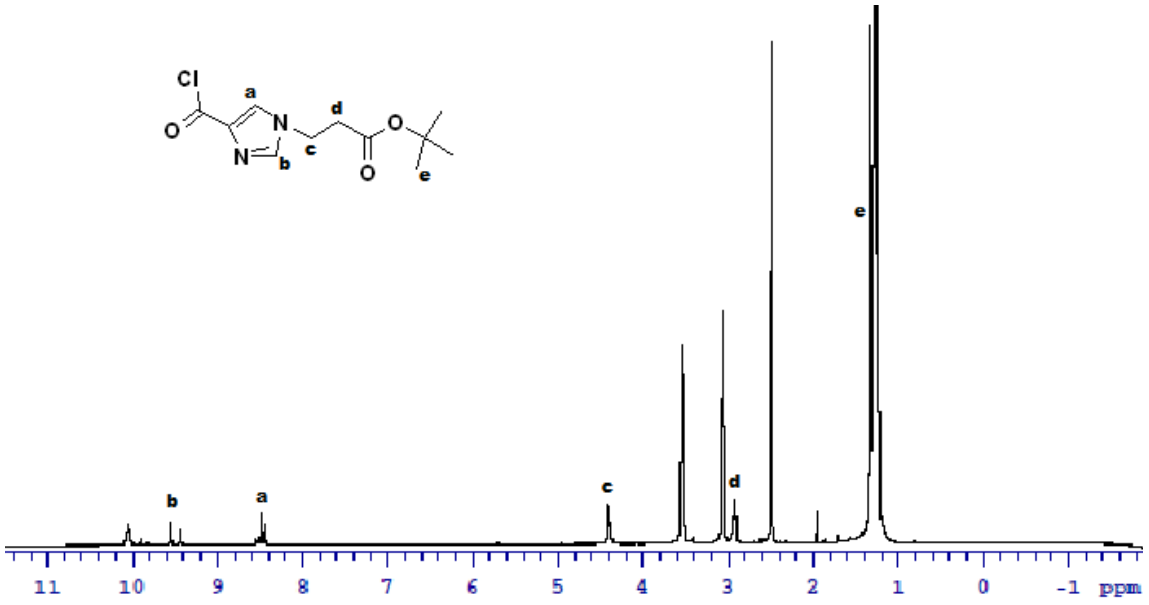

Figure S24 [1]H NMR of compound 10 in DMSO-d6 (non-purified material, DIPEA peaks observed)

^{1}H NMR shows that the aromatic protons were shifted to 8.45 and 9.45 ppm. The aromatic protons shifting to down field is attributed to acid chloride formation.

^{1}H NMR (700 MHz, DMSO-d6): δ 1.28 (s, 9H, C-(CH_3)$_3$, H_e), 2.92 (bs, 2H, CH_2, H_d), 4.22 (bs, 2H, CH_2, H_c), 8.45 (bs, 1H, Ar-CH, H_a), 9.45 (m, 1H, Ar-CH, H_b).

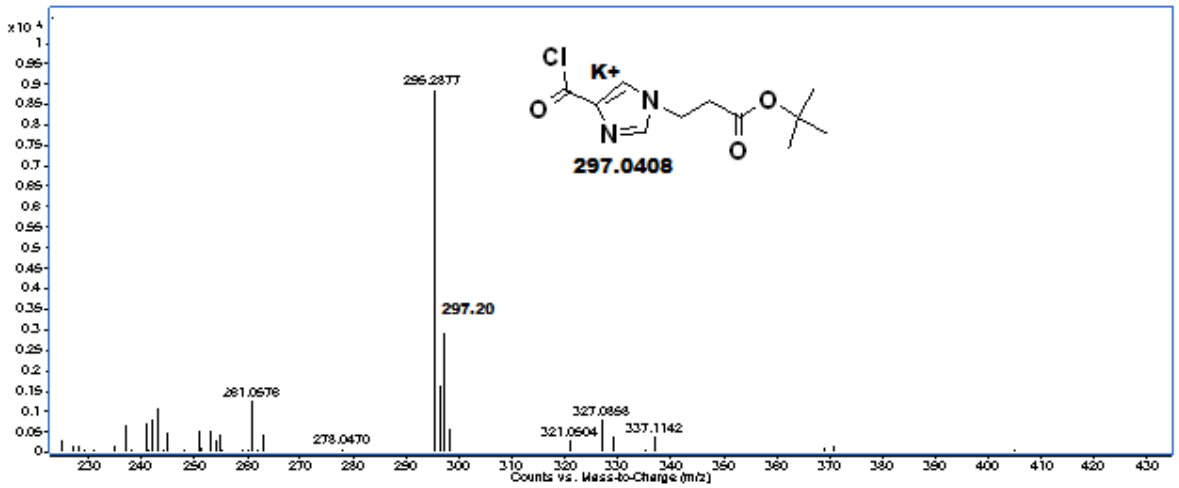

Figure S25 ESI-MS of compound 10 (non-purified material)

ESI-MS $[M+K]^+$ $C_{11}H_{15}ClKN_2O_3$ calcd 297.0408, obsd 295.2877.

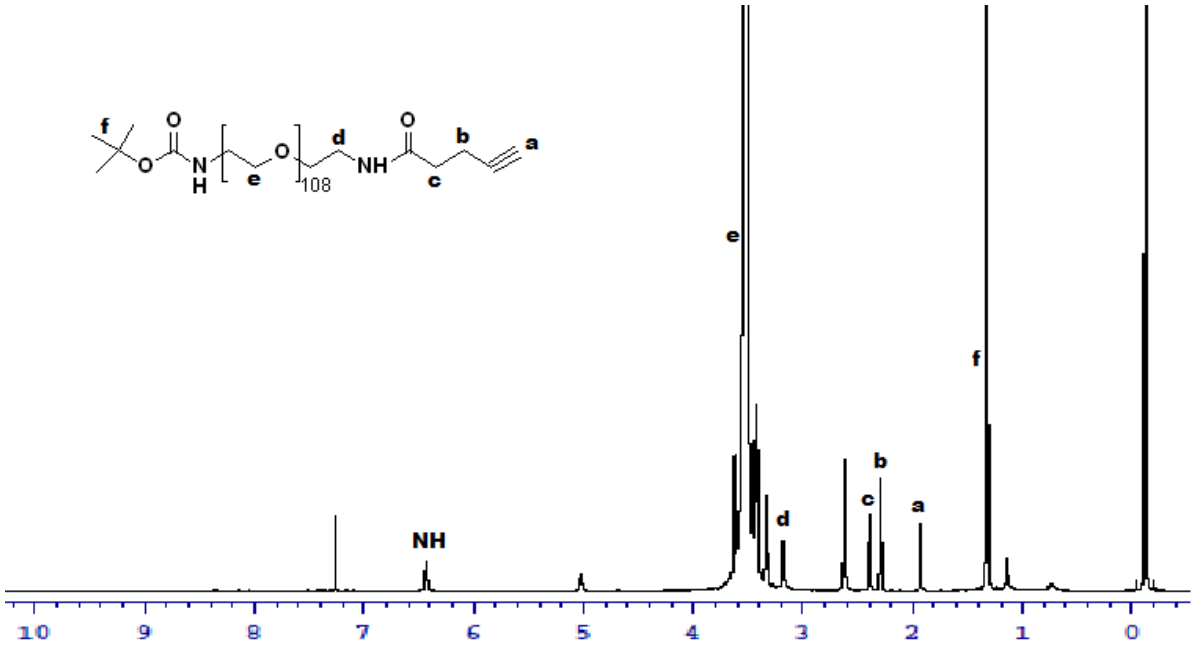

Figure S26 ^{1}H NMR of compound 11 in $CDCl_3$

^{1}H NMR confirms the coupling; its characteristic peak for alkyne proton was observed at 1.92 ppm and Boc protons were observed at 1.36 ppm.

^{1}H NMR (700 MHz, $CDCl_3$): δ 1.32 (a, 9H, C-$(CH_3)_3$, H_f), 1.92 (t, 1H, alkyne proton, j = 2.1 Hz, H_a), 2.29 (t, 2H, CH_2, j = 7.0 Hz, H_b), 2.39 (t, 2H, CH_2, j = 7.0 Hz, H_c), 2.62 (bs, 2H), 3.19 (d, 2H, HN-CH_2, j = 4.2 Hz, H_d), 3.32-3.36 (m, 2H, CH_2CH_2-CO-O), 3.40-3.65 (m, 450H, OCH_2CH_2, H_e), 5.02 (bs, 1H), 6.41 (bs, 1H, NH).

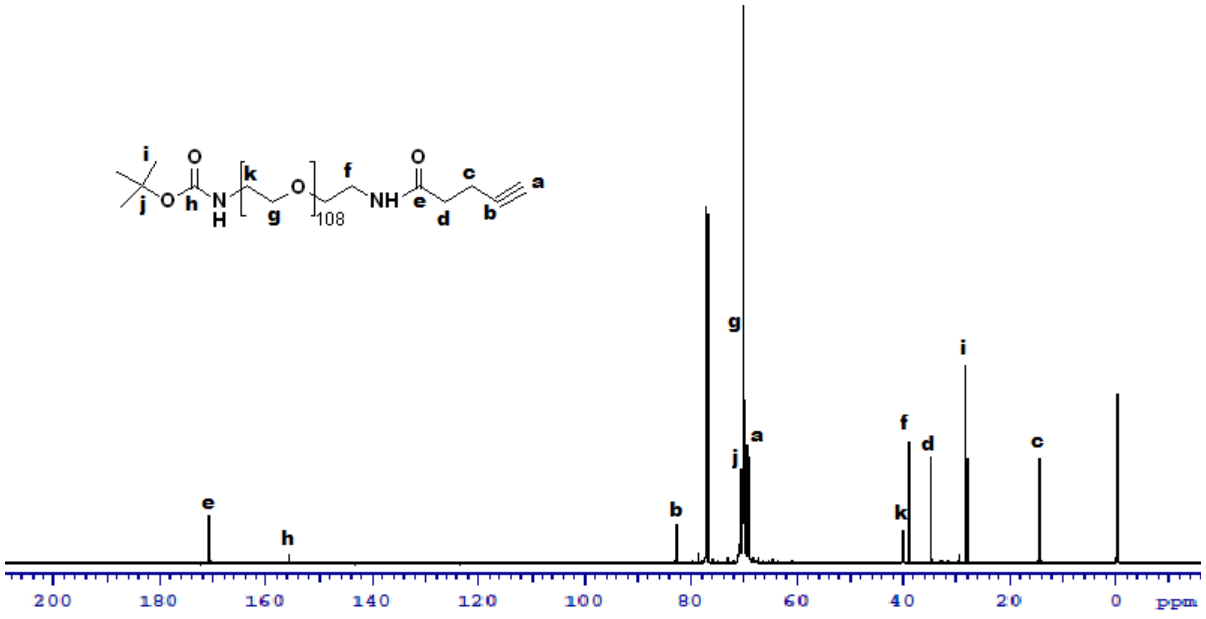

Figure S27 ^{13}C NMR of compound 11 in $CDCl_3$

^{13}C NMR (175 MHz, $CDCl_3$): δ 14.45 (C_c), 28.08 (C_i), 34.74 (C_d), 38.90 (C_f), 39.97 (C_k), 68.97 (C_j), 69.43 (C_a), 69.811, 69.86, 70.19 (C_g), 70.91, 82.76 (C_b), 155.8 (C_h), 170.62 (C_e).

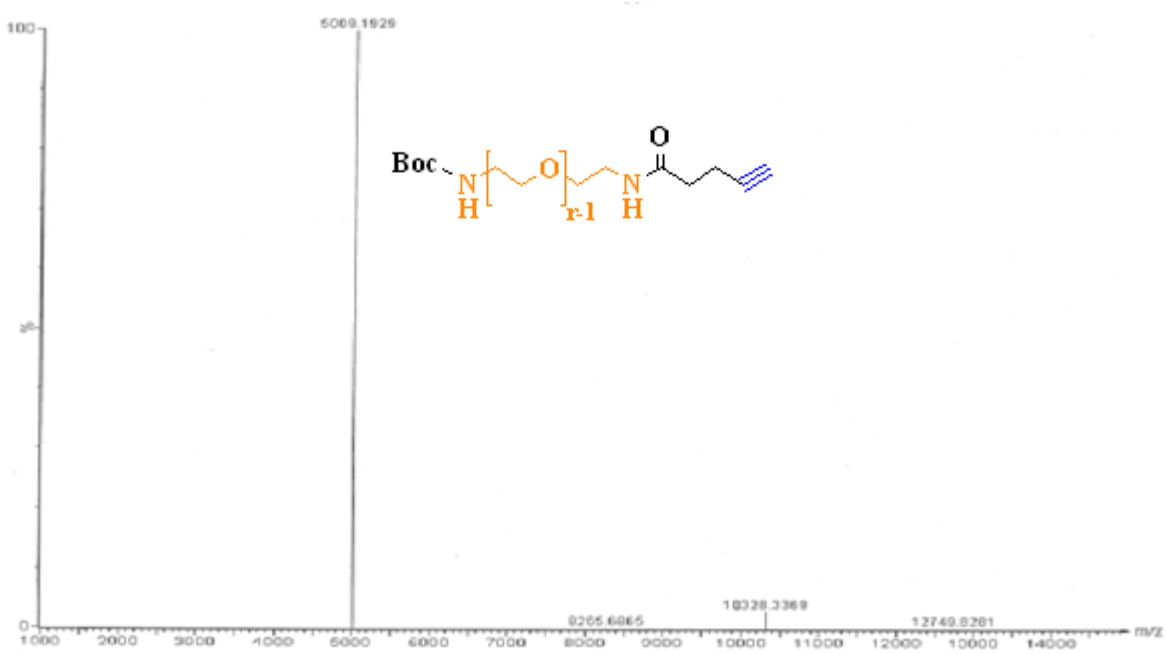

Figure S28 MALDI-spectrum of compound 11

MALDI $[M]^+$ Alk-PEG-NHBoc, obsd 5009.19. Note: Boc group might not survive during the analysis

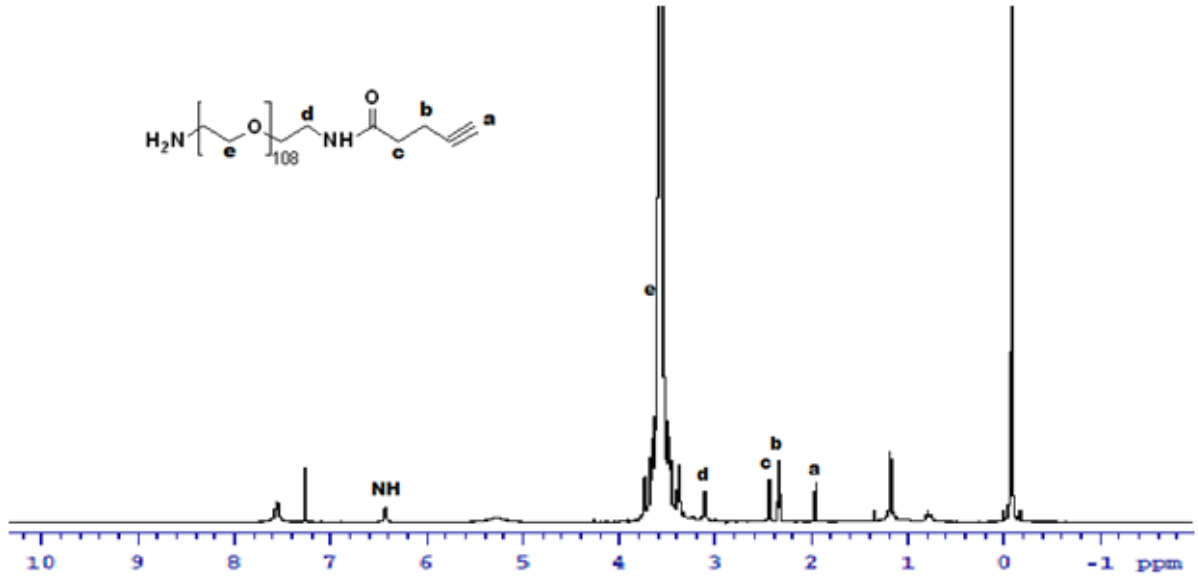

Figure S29 ^1H NMR of compound 12 in $CDCl_3$

^{1}H NMR confirms the Boc deprotection as its characteristic peak at 1.36 ppm was disappeared.

^{1}H NMR (700 MHz, $CDCl_3$): δ 1.96 (t, 1H, alkyne proton, j = 2.1 Hz, H_a), 2.34 (t, 2H, CH_2, j = 7.0 Hz, H_b), 2.44 (dt, 2H, CH_2, j = 7.0, 2.1 Hz, H_c), 3.11 (bs, 2H, HN-CH_2, H_d), 3.36-3.40 (m, 2H, CH_2CH_2-CO-O), 3.42-3.72 (m, 450H, OCH_2CH_2, H_e), 5.26 (bs, 1H), 6.43 (bs, 1H, NH), 7.55 (bs, 2H, NH_2).

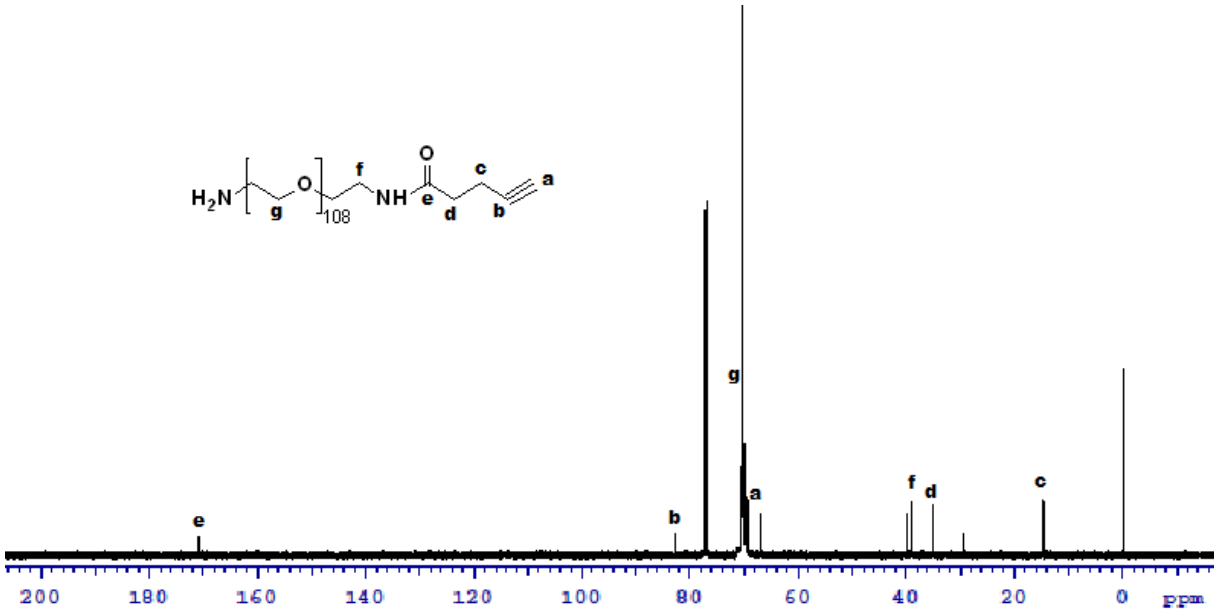

Figure S30 ^{13}C NMR of compound 12 in $CDCl_3$

^{13}C NMR (175 MHz, $CDCl_3$): δ 14.59 (C_c), 29.42, 34.90 (C_d), 39.05 (C_f), 39.82, 66.78, 69.08 (C_a), 69.56, 69.63, 69.66, 69.74, 69.80, 69.86, 70.19 (C_g), 70.91, 82.86 (C_b), 170.83 (C_e).

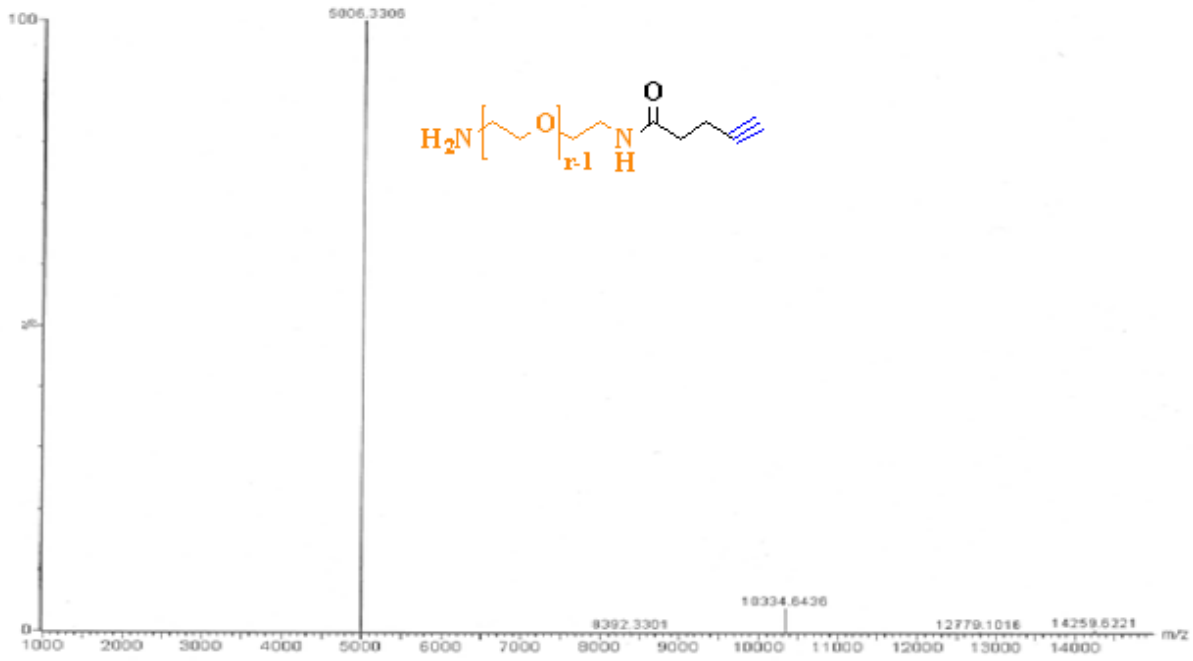

Figure S31 MALDI-spectrum of compound 12

MALDI $[M]^+$ Alk-PEG-NH_2 obsd 5006.33.

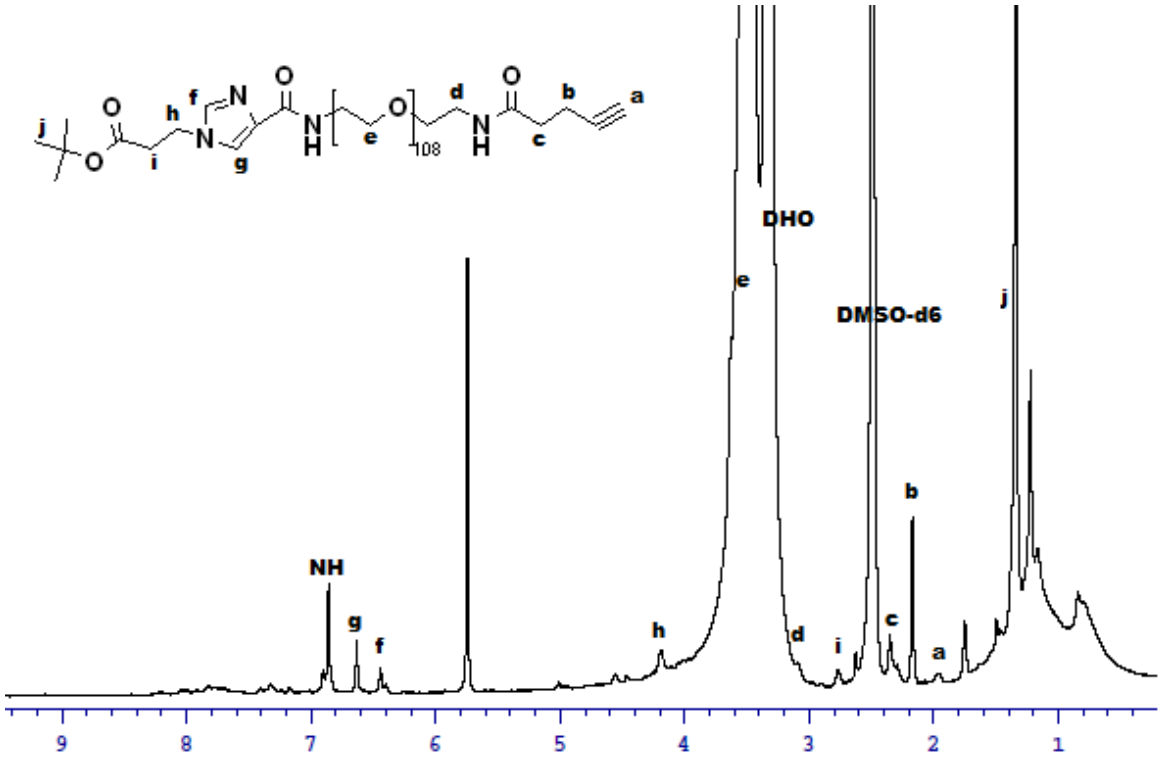

Figure S32 ^{1}H NMR of compound 13 in $CDCl_3$

^{1}H NMR confirms the coupling; its characteristic peak for alkyne proton was observed at 1.96 ppm, imidazole aromatic protons at 6.43 & 6.62 ppm and the Boc methyl protons were observed at 1.36 ppm.

^{1}H NMR (700 MHz, $CDCl_3$): δ 1.36 (s, 9H, $C(CH_3)_3$, H_j), 1.96 (bs, 1H, alkyne proton, H_a), 2.18 (bs, 2H, CH_2, H_b), 2.38 (t, 2H, CH_2, j = 7.0 Hz, H_c), 2.78 (bs, 2H, CH_2, H_i), 3.09 (bs, 2H, HN-CH_2, H_d), 3.36-3.40 (m, 2H, CH_2CH_2-CO-O), 3.40-3.80 (m, 450H, OCH_2CH_2, H_e), 4.18 (bs, 2H, CH_2, H_h), 4.45-4.55 (m, 2H), 5.76 (bs, 2H), 6.43 (bs, 1H, NH), 6.64 (bs, 1H, H_f), 6.84 (bs, 1H, H_g), 7.36 (bs, 1H, NH).

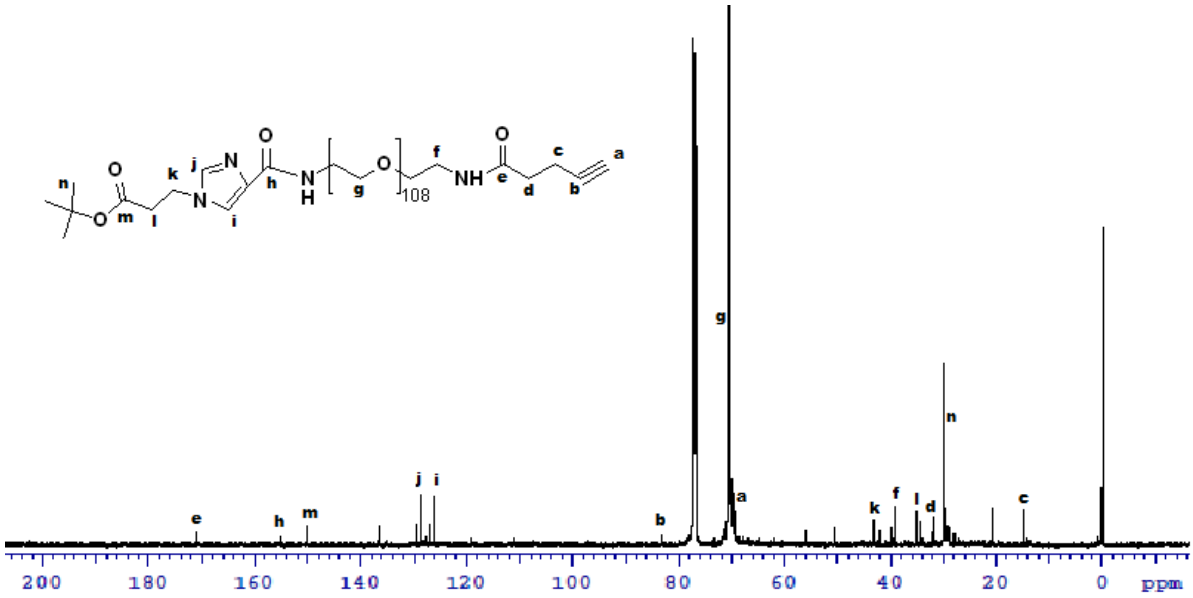

Figure S33 ^{13}C NMR of compound 13 in $CDCl_3$

^{13}C NMR (175 MHz, $CDCl_3$): δ 14.75 (C_c), 20.77, 29.01, 29.59, 29.84 (C_n), 31.88 (C_d), 34.29, 35.07 (C_l), 39.18 (C_f), 39.74, 42.09, 43.19 (C_k), 50.44, 55.95, 61.98, 69.14 (C_a), 69.78, 70.13, 70.46 (C_g), 71.40, 73.28, 76.10, 77.95, 83.04 (C_b), 119.04, 126.13 (C_i), 126.90, 127.63, 128.65 (C_j), 129.52, 136.45, 149.93 (C_m), 155.15 (C_h), 170.90 (C_e).

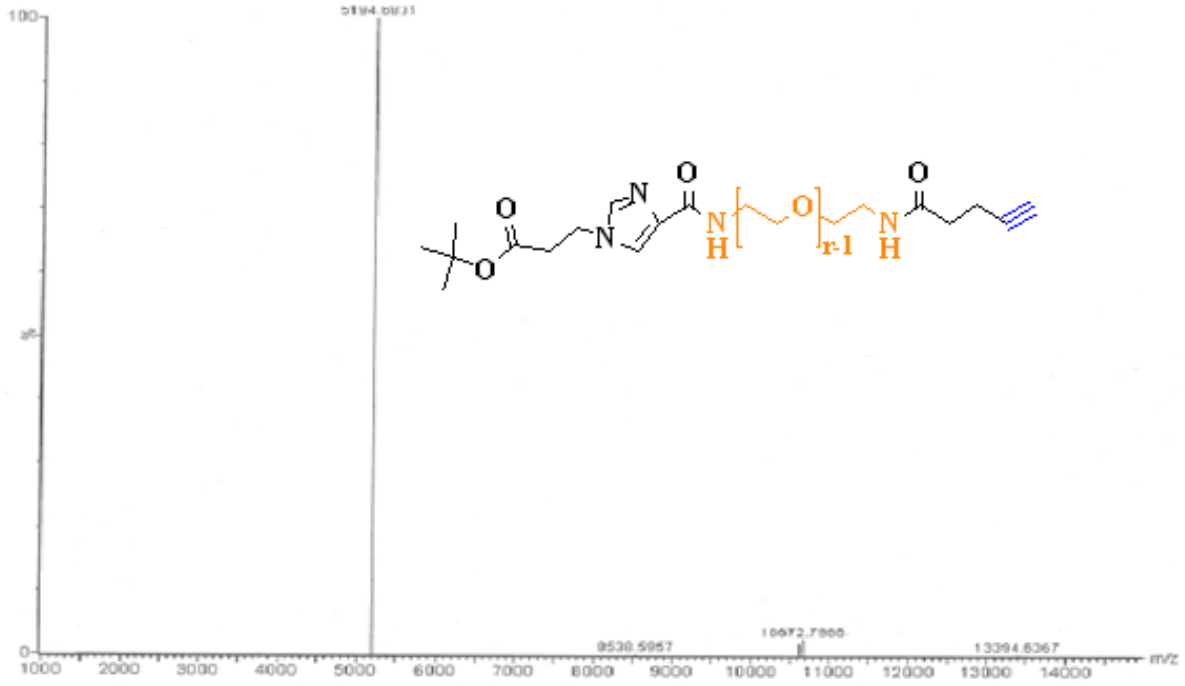

Figure S34 MALDI-spectrum of compound 13

MALDI $[M]^+$ Alk-PEG-Imidazole-ester obsd 5194.68.

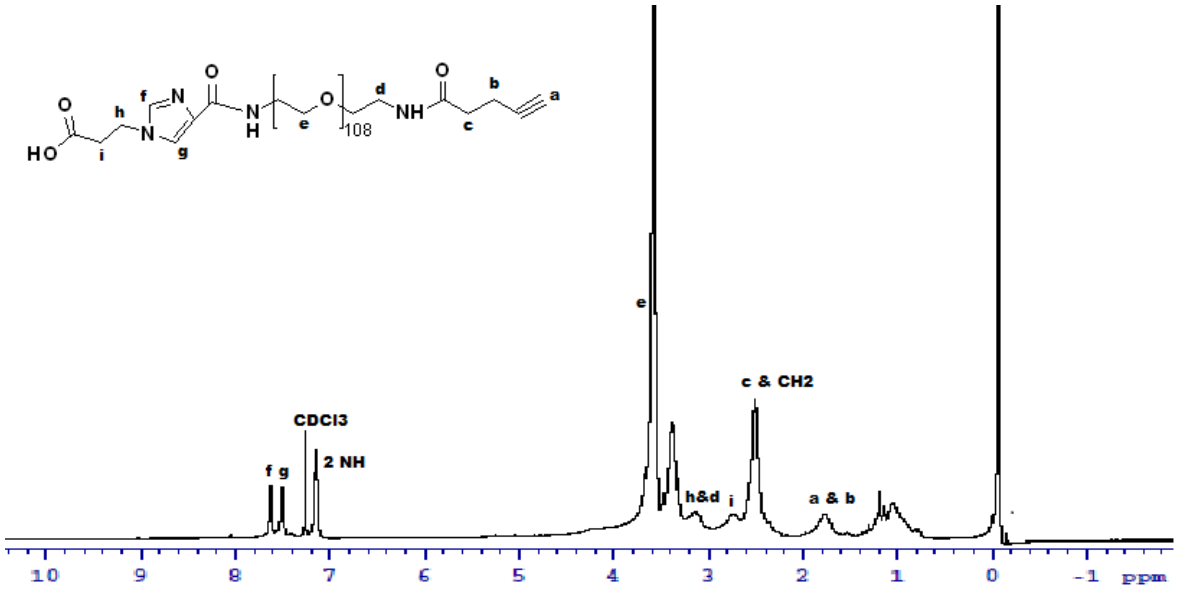

Figure S35 ^1H NMR of compound 14 in $CDCl_3$

^{1}H NMR confirms the Boc deprotection as its characteristic peak at 1.36 ppm disappeared and all other peaks remained intact.

^{1}H NMR (700 MHz, $CDCl_3$): δ 1.96 (bs, 1H, alkyne proton, H_a), 2.20 (bs, 2H, CH_2, H_b), 2.38 (bs, 2H, CH_2, H_c), 2.68 (bs, 2H, CH_2, H_i), 3.40-3.80 (m, 450H, OCH_2CH_2, H_e), 4.45-4.55 (m, 2H), 5.21 (bs, 2H), 5.56 (bs, 1H), 6.82 (bs, 1H, H_f), 7.14 (bs, 1H, H_g), 7.36 (bs, 1H, NH).

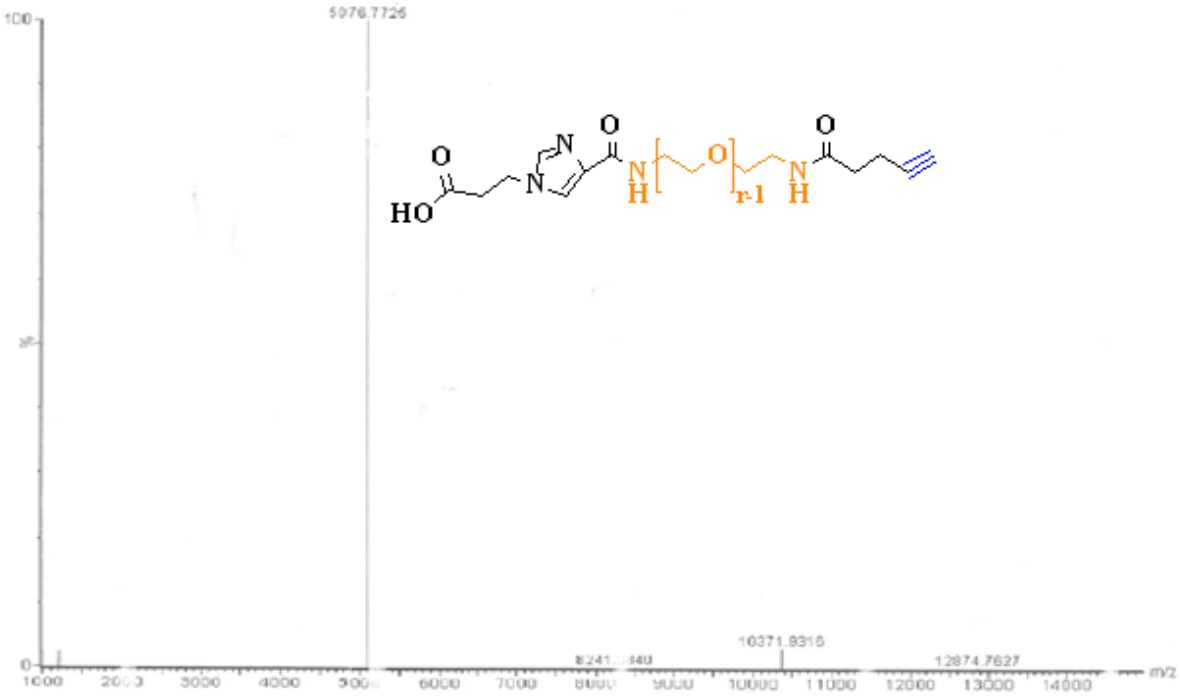

Figure S36 MALDI-spectrum of compound 14

MALDI $[M]^+$ Alk-PEG-Imidazole-acid obsd 5076.77.

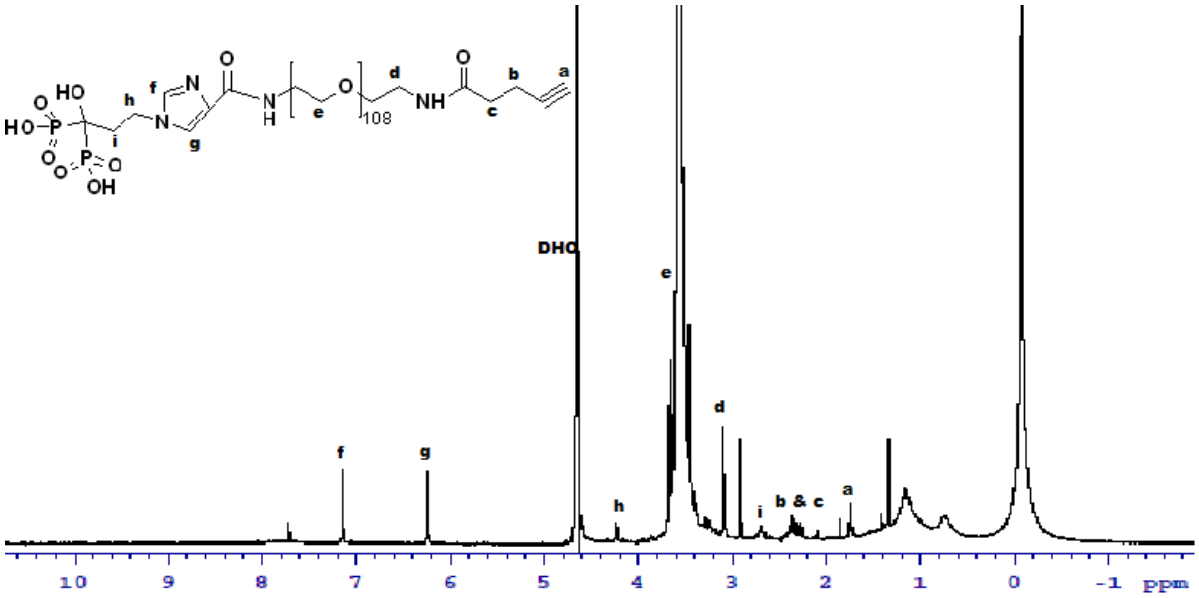

Figure S37 ^{1}H NMR of compound 15 in D_2O

In ^{1}H NMR, the aromatic peaks were observed at 6.24 and 7.18 ppm, and the alkyne peak was observed at 1.84 ppm.

^{1}H NMR (700 MHz, $CDCl_3$): δ 2.00 (t, 1H, alkyne proton, j = 7.0 Hz, H_a), 2.39 (t, 2H, CH_2, j = 7.5 Hz H_b), 2.50 (dt, 2H, CH_2, j = 6.5, 2.0 Hz, H_c), 2.76 (t, 2H, CH_2, j = 5.5 Hz, H_i), 3.20 (bs, 2H, HN-CH_2, H_d), 3.40-3.50 (m, 4H, CH_2CH_2-CO-O), 3.52-3.84 (m, 430H, OCH_2CH_2, H_e), 4.16-4.26 (m, 2H, CH_2, H_h), 6.52 (bs, 1H, NH), 7.58 (bs, 1H, H_f), 7.68 (bs, 1H, NH), 7.80 (bs, 1H, H_g).

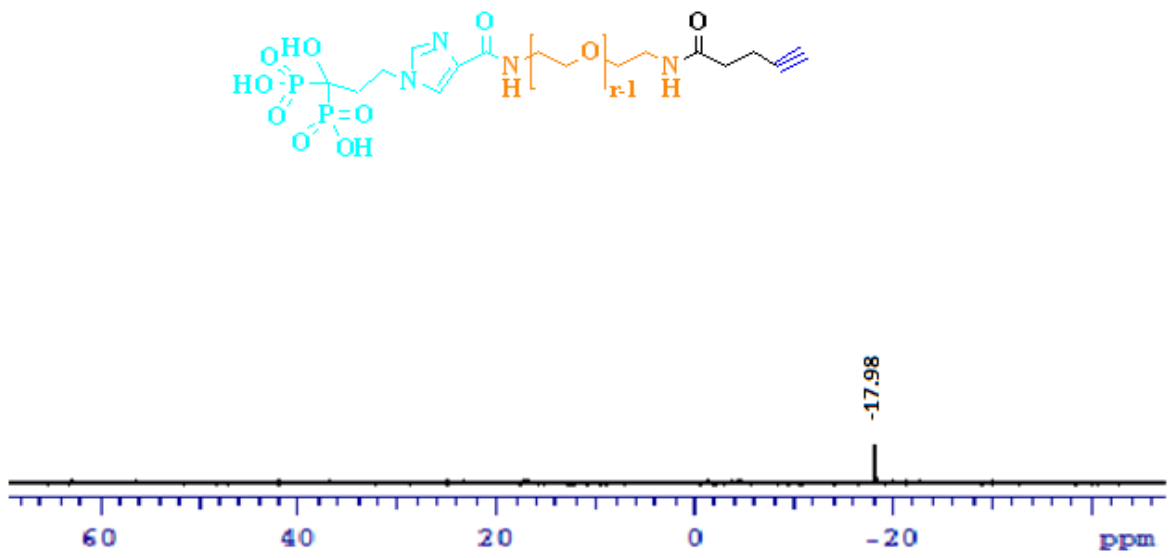

Figure S38 ^{31}P-NMR of compound 15 in D_2O

The ^{31}P NMR confirms the phosphorylation and its phosphonate peaks were observed at -17.98 ppm. ^{31}P NMR (283 MHz, $CDCl_3$): δ -19.98.

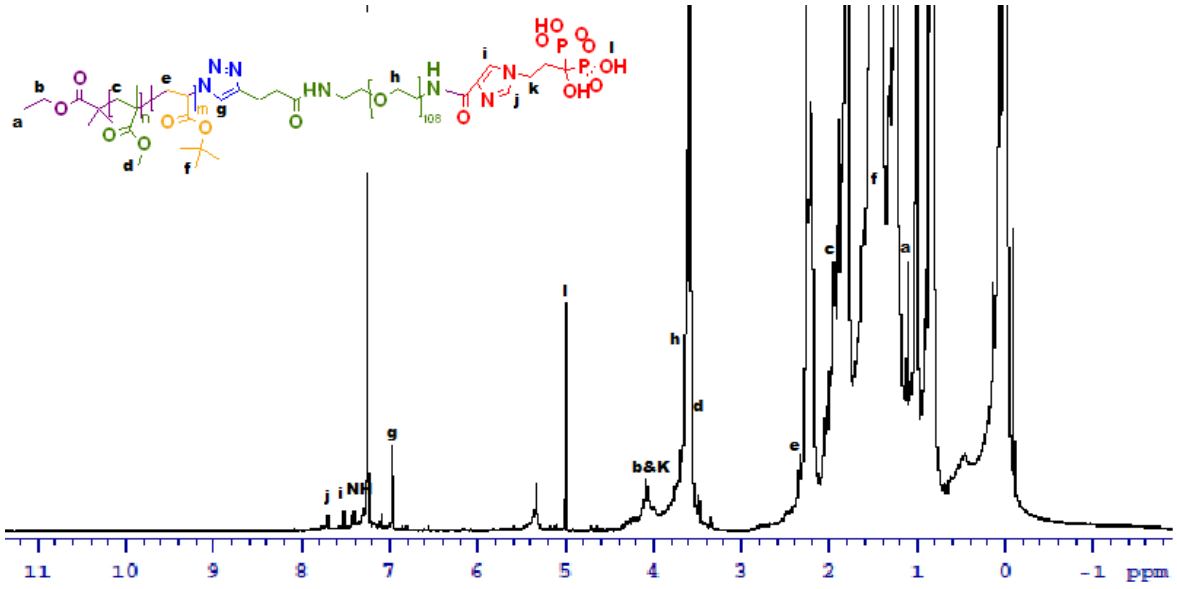

Figure S39 ^{1}H NMR of compound 16 in $CDCl_3$

^{1}H NMR confirms the coupling, the characteristic peaks from both the addendums were observed. The imidazole aromatic peaks were observed at 7.52 and 7.76 ppm, whereas the PMMA and PEG protons were observed at 3.50-3.70 ppm.

^{1}H NMR (700 MHz, $CDCl_3$): δ 7.72 (bs, 1H, H_j), 7.52 (bs, 1H, H_i), 7.42 (bs, 2H, NH), 6.98 (bs, 1H, triazole ring proton, H_g), 5.34 (bs, 2H), 5.00 (s, 2H, O=P-OH), 4.15 ($CH_2C\mathit{H}$-triazole, H_k and CO-O-$C\mathit{H}_2$, H_b), 4.05 (2H, CH_3-$C\mathit{H}_2$-O-CO), 3.64 (bs, O-$C\mathit{H}_2$-$C\mathit{H}_2$, H_h), 3.59 (s, CO-O-CH_3, H_d), 2.42 (2H, $C\mathit{H}_2$, H_e), 2.12 (2H, $C\mathit{H}_2$, H_c), 1.36 (9H, CO-O-C($C\mathit{H}_3$)$_3$, H_f), 1.22 (s, 3H, -CH_2-$C\mathit{H}_3$, H_a).

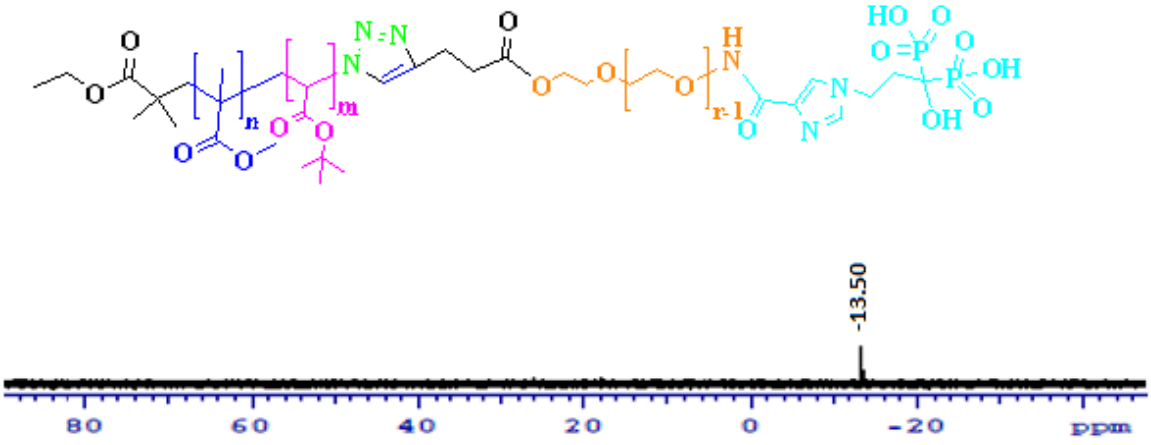

Figure S40 ^{31}P NMR of compound 16 in D_2O

The ^{31}P NMR shows that the phosphonate group was shifted to -13.50 ppm after the coupling to aliphatic PMMA-*b*-P*t*BA conjugate.

^{31}P NMR (283 MHz, $CDCl_3$): δ -13.50

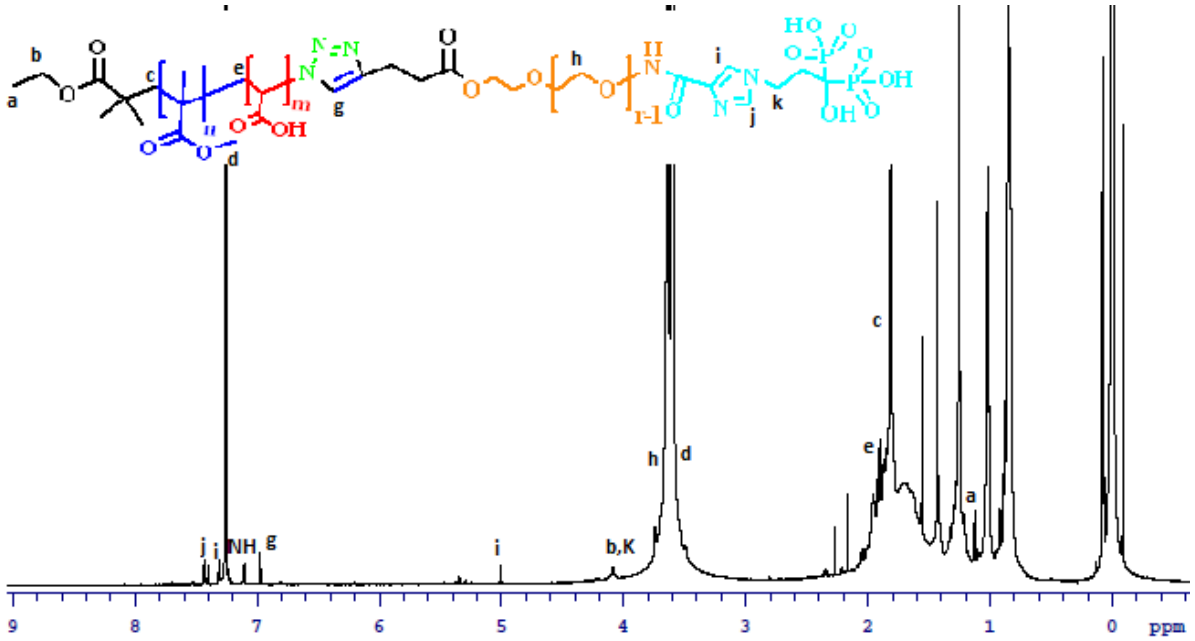

Figure S41 ^{1}H NMR of compound 17 in $CDCl_3$

^{1}H NMR after acid-hydrolysis, we observed the disappearance of the methyl protons of the *tert*. butyl groups at 1.38 ppm and observed unaffected PMMA protons around that region.

^{1}H NMR (700 MHz, $CDCl_3$): δ 7.43 (bs, 1H, H_j), 7.40 (bs, 1H, H_i), 6.97 (bs, 1H, NH), 6.97 (bs, 1H, triazole ring proton, H_g), 5.36 (bs, 2H), 5.00 (s, 2H, O=P-OH), 4.15 (CH_2CH-triazole, H_k and CO-O-CH_2, H_b), 4.05 (2H, CH_3-CH_2-O-CO), 3.63 (bs, O-CH_2-CH_2, H_h), 3.57 (s, CO-O-CH_3, H_d), 2.42 (2H, CH_2, H_e), 2.12 (2H, CH_2, H_c), 1.22 (s, 3H, -CH_2-CH_3, H_a).

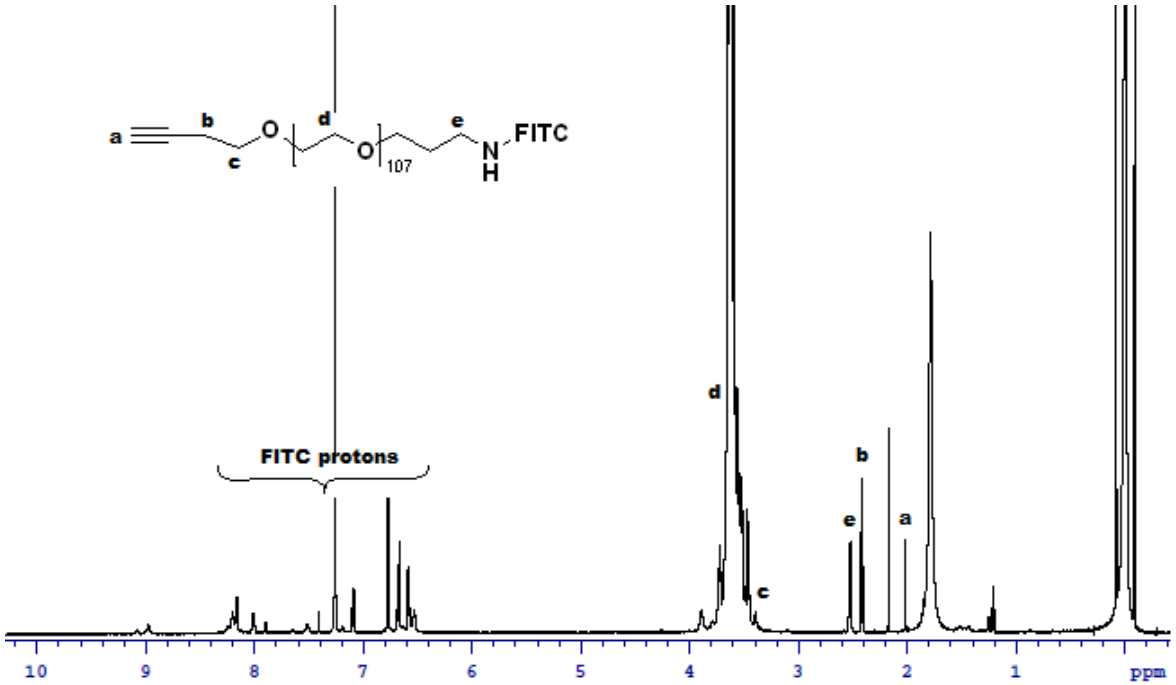

Figure S42 ^1H NMR of compound 18 in $CDCl_3$

^{1}H NMR shows the FITC aromatic protons at 6.50-9.10 ppm, and the alkyne peak was observed at 2.02 ppm.

^{1}H NMR (700 MHz, $CDCl_3$): δ 1.55 (p, 2H, J = 4.5 & 11.00 Hz, CH_2), 1.74 (p, 2H, J = 4.5 & 11.00 Hz, CH_2), 1.95 (t, 1H, J = 1.5 Hz, alkyne proton), 2.19 (t, 4H, J = 5.0Hz, CH_2), 3.44 (q, 4H, J = 7.0 Hz, CH_2), 3.48-3.55 (m, 8H, CH_2CH_2-CO-O), 3.55-3.72 (bs, 430H, OCH_2CH_2), 4.74 (t, 4H CO-O-CH_2-CH_2-O), 6.12-8.40 (m, 10H, FITC aromatic protons).

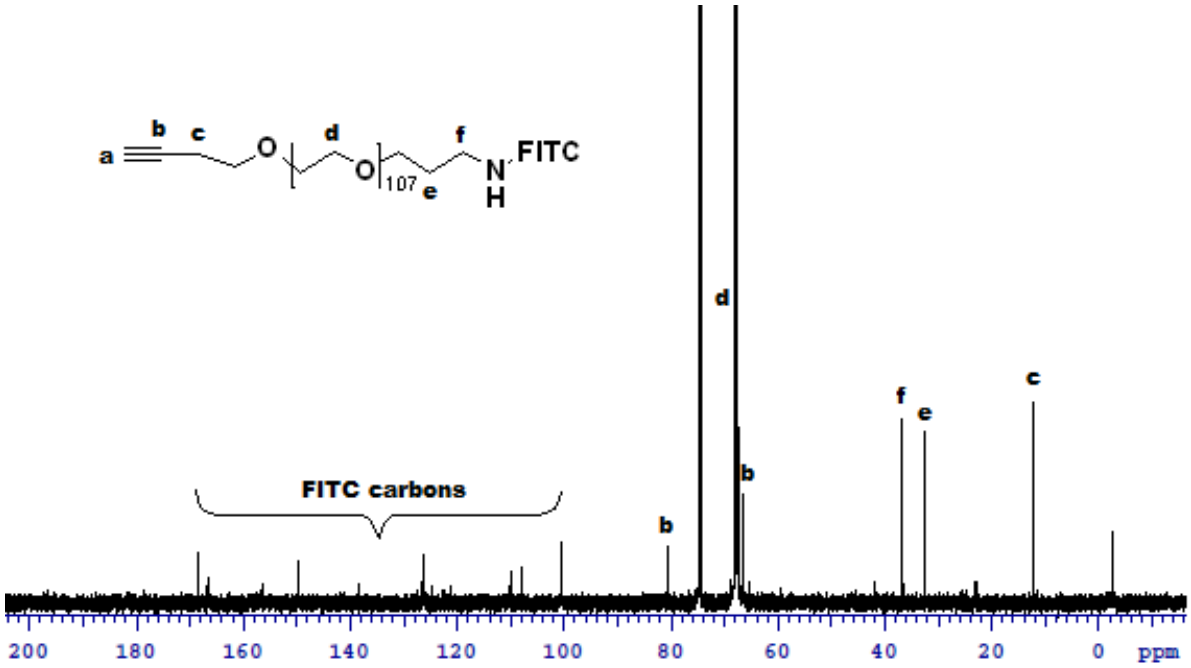

Figure S43 ^{13}C NMR of compound 18 in $CDCl_3$

^{13}C NMR (175 MHz, $CDCl_3$): δ 18.15, 24.72, 27.93, 29.29, 29.62, 64.97, 68.63, 69.61, 69.85, 70.17, 70.51, 71.45, 72.39, 84.09, 95.22, 97.07, 103.02, 108.87, 172.64, 231.37. MALDI $[M]^+$ *Alkyne-PEG-FITC* obsd 5376.

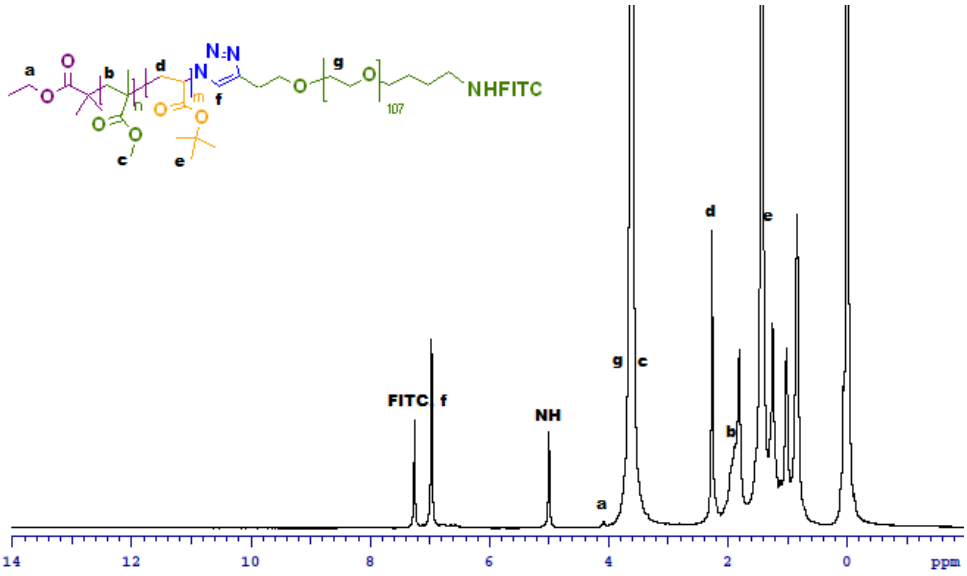

Figure S44: ^{1}H NMR of Compound 19 (PMMA-b-PtBA-PEG-NHFITC)

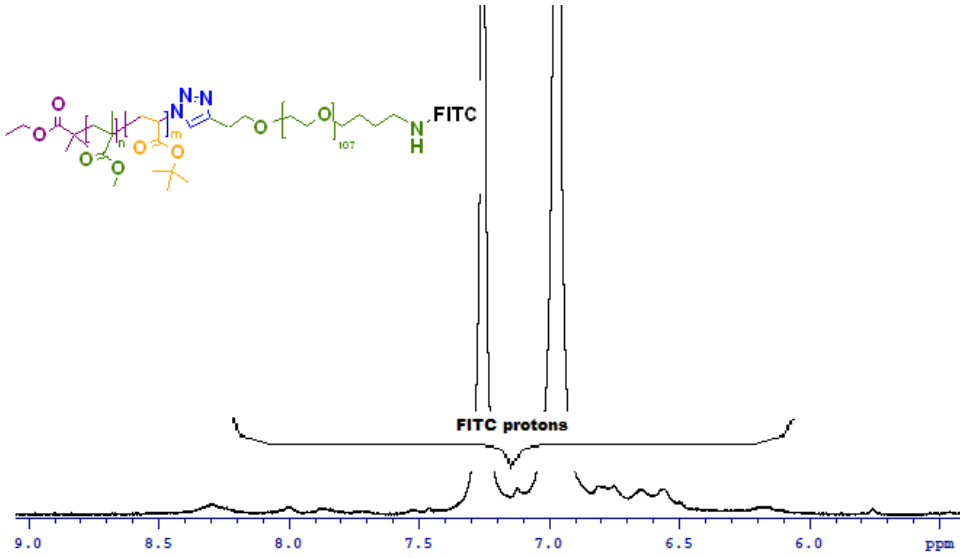

Figure S45 1H NMR of Compound 19 (expanded region, 5.5-9.0 ppm)

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.46 (bs, 1H, triazole ring proton), 4.25 (CH_2CH-triazole and CO-O-CH_2), 4.05 (2H, CH_3-CH_2-O-CO), 3.64 (bs, O-CH_2-CH_2), 3.59 (s, CO-O-CH_3), 3.38 (s, 3H, CH_2-CH_2-O-CH_3), 3.05 (2H, triazole-CH_2-CH_2-CO-O), 2.76 (2H, triazole-CH_2-CH_2-CO-O), 2.26 (CH_2-CH-CO), 1.93-0.83 (6H, O-CO-C(CH_3)$_2$, CO-O-C(CH_3)$_3$, CH_2-CCH_3).

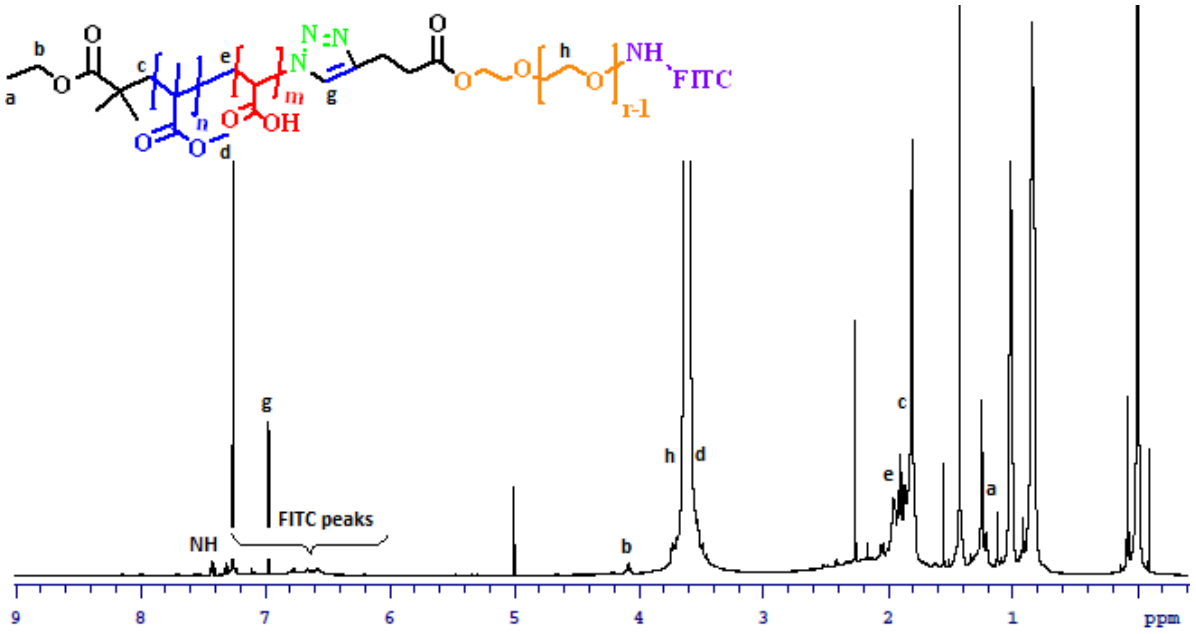

Figure S46 ^{1}H NMR of Compound 20 (PMMA-b-PAA-PEG-NHFITC) in $CDCl_3$

^{1}H NMR showed complete loss of the *tert*-butyl protons at 1.36 ppm.; ^{1}H NMR (700 MHz, $CDCl_3$): δ 7.72 (bs, 1H, H_j), 7.52 (bs, 1H, H_i), 7.42 (bs, 2H, NH), 6.98 (bs, 1H, triazole ring proton, H_g), 5.34 (bs, 2H), 5.00 (s, 2H, O=P-OH), 4.15 (CH_2CH-triazole, H_k and CO-O-CH_2, H_b), 4.05 (2H, CH_3-CH_2-O-CO), 3.64 (bs, O-CH_2-CH_2, H_h), 3.59 (s, CO-O-CH_3, H_d), 2.42 (2H, CH_2, H_e), 2.12 (2H, CH_2, H_c), 1.22 (s, 3H, -CH_2-CH_3, H_a).

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