Synthesis and fabrication of surface-active microparticles using membrane emulsion technique and rapid conjugation of model protein via strain-promoted azide-alkyne click chemistry in physiological conditions

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1. Experimental

2.1. Materials

2-[2-(2-Chloroethoxy)ethoxy]ethanol (96% Sigma), sodium azide (NaN_3 ≥99.99% Sigma), sodium iodide (≥99.5% Sigma), tin(II) 2-ethylhexanoate (92.5-100.0% Sigma), Dibenzocyclooctyne-acid (DBCO-acid 95% Sigma), Poly(vinyl alcohol) (PVA, Mw 13,000-23,000, 87-89% hydrolyzed, Sigma), Triethanolamine (≥99% Sigma), Trifluoroacetic acid (TFA, reagent grade, 99% Sigma), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (HEPES, ≥99.5% Sigma), (Ethylenedinitrilo)tetraacetic acid (EDTA, sigma), Bradford reagent (coomasie blue, sigma), Human Serum Albumin (HSA, ≥99% Sigma) and all other chemicals were used as received without further purifications. DBCO-PEG4-Maleimide (DBCO-mal >95% Click Chemistry tools) was dissolved in DMSO to a final concentration of 0.015M. ε-caprolactone (97% Sigma) was degassed with nitrogen before use.

2.2. Producing an Azide functionalised initiator for ring opening polymerisation.

2-[2-(2-Chloroethoxy)ethoxy]ethanol (9 g, 0.053 moles), sodium azide (6.9 g, 0.106 moles, 0.5eq) and sodium iodide (2 g, 0.1eq) were dissolved in distilled water (25 mL). The reaction was heated at reflux for 72 hours. The product was extracted from the aqueous layer using ethyl acetate (3x 20 mL.) Organic layers were combined and the solvents were removed under reduced pressure to yield compound **1**; 2-[2-(2-azidoethoxy)ethoxy]ethanol as a light pink liquid (6.406 g, 71%). 1H NMR (400 MHz, DMSO) δ 4.60 (t, J = 5.3 Hz, 1H), 3.62 – 3.57 (m, 2H), 3.58 – 3.51 (m, 4H), 3.48 (dd, J = 9.4, 4.3 Hz, 2H), 3.44 – 3.40 (m, 2H), 3.39 – 3.35 (m, 2H). 13C NMR (101 MHz, CDCl3) δ 72.52 (s), 70.60 (s), 70.32 (s), 69.98 (s), 61.65 (s), 50.61 (s).

1.3. Ring opening polymerisation of ε-caprolactone

ε-caprolactone (9.742 g, 0.08mol) was heated to 85°C. 2-[2-(2-azidoethoxy)ethoxy]ethanol initiator **1** (100 mg, 0.0005mol, 1eq) and tin(II) 2-ethylhexanoate (0.023 g, 0.1eq) were added to the reaction mixture under an atmosphere of nitrogen, and stirred for 13 hours. The solution was allowed to cool to room temperature, and the solid mixture recrystallized from DCM using cold methanol. The precipitate was collected using a Buchner funnel to yield the desired compound **2**; Polycaprolactone azide (PCL-N_3) as a white solid (7.868 g, 81%).

1.4. Strain promoted Azide-Alkyne Cycloaddition.

Dibenzocyclooctyne-acid (10 mg, $3*10^{-5}$mol, 1eq) was added to PCL-N_3 **2** (44.2mg, $1.5*10^{-6}$mol, 0.1eq) dissolved in $CDCL_3$. Commercial PCL (15.2mg $1.5*10^{-6}$mol, 0.1eq) was used as a control. The reaction was monitored using Diffusion-Ordered Spectroscopy (DOSY) NMR. A spectrum was recorded every 30 minutes for 6 hours. The resulting product was analysed using FT-IR to confirm completion of the reaction.

1.5. Microparticle Production

PCL-N_3 Microparticles were formulated using membrane emulsification, producing oil in water emulsion of particle droplets, which were solidified by solvent evaporation. RPM values were calculated using manufacturer guidelines. PCL-N_3 **2** was dissolved in DCM (10 mL) at varying concentrations and injected into 1% PVA solution (150 mL) at a rate of 0.70ml/min, through a 15μm pore sized ringed membrane. Slow evaporation of solvent produced solidified particles, the aqueous layer was

removed by centrifugation and the particles were washed with water (500 mL) and freeze dried to yield desired compound **3** as a fine white powder (0.464g 46%). Microparticles size was evaluated using Image J software.

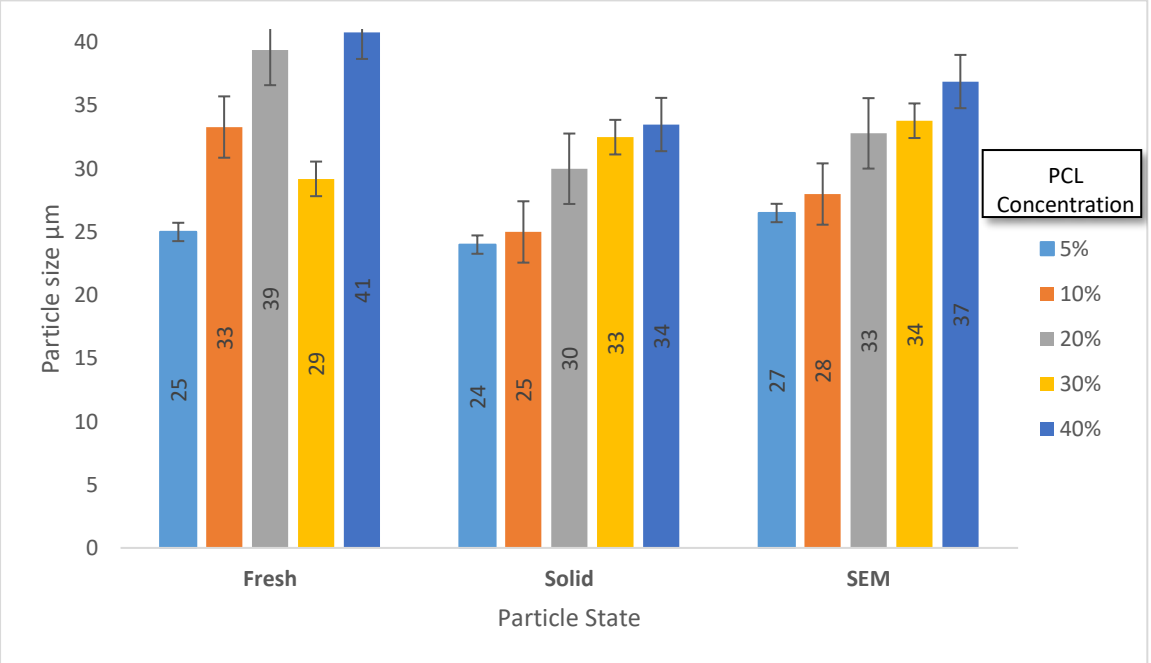
1.6. Synthesis of DBCO-peg$_4$-maleimide-HSA conjugates

DBCO-peg$_4$-maleimide and Human Serum Albumin were conjugated utilising SPAAC chemistry. Human serum albumin (100mg, 1.5*10^{-6}mol, 1eq) was dissolved in 0.1M HEPES, 5mM EDTA, pH7. 125µl DBCO-maleimide dissolved in DMSO (0.015M, 2eq) and Triethanolamine (4mM) added and reacted at room temperature for 24 h with gentle agitation. RP-HPLC (Agilent technology) with Aeris widepore column (XB-C18 Phenomenex ltd) was used to assess the conjugation reaction. 5µl of reaction material was injected into the HPLC device with the following chromatography conditions: mobile phase A ddH2O + 0.1% TFA, mobile phase B HPLC grade ACN + 0.1% TFA, gradient 20%-50% Solvent B over 9 minutes, 95% Solvent B 1 minute and re-equilibrated to 20% Solvent B over 3 minutes. The flow rate was kept constant at 1ml/min. The absorption was measured at 214 nm.

1.7. Synthesis of HSA immobilized PCL-N$_3$ microparticles

PCL-N$_3$ Microparticles **3** (15.3mg, 6.4*10^{-7} mol, 1eq) were mixed with 1ml of HSA-DBCO-peg$_4$-maleimide conjugate solution (6.25*10^{-4} mol, 1eq) and allowed to react for 12 hrs with gentle agitation. Particles were washed 10 times with distilled H_20 (5mL) and purified using centrifugation via viva spin columns (Sartorius, 100,000MWCO, 500µl). After purification particles were freeze dried to yield a fluffy white powder (0.0177g). Protein concentration was determined by Bradford assay for all washes and on the freeze dried particles. PCL-N$_3$ Microparticles conjugated to DBCO-maleimide with no protein and PCL-N$_3$ Microparticles conjugated to a protein with no DBCO-maleimide linker were used as controls. For fluorescently labelled microparticles Human serum albumin (150mg, 2.25*10-6mol, 1eq) was dissolved in Sodium Carbonate buffer (0.1M pH9) 300µl DBCO-maleimide dissolved in DMSO (0.015M, 2eq) and 2µlTriethanolamine (4mM) added. Fluorescein isothiocyanate isomer I (FITC) dissolved in DMSO at 1mg/ml was added slowly with gentle agitation in 5µl aliquots at a ratio of 50µl per 1ml of protein solution, the reaction was carried out for 24hrs at 4^{0}C. Unbound FITC was removed via PD10 desalting column (GE healthcare) with sodium carbonate buffer as eluent, fractions collected were assessed for protien content via bradford assay and fluorescence using a microplate reader (Ex 485 Em 520) and combined to produce FITC labelled HSA sample. PCLN$_3$ or PCL microparticles were mixed with HSA/FITC solution at a ratio of 1:1 and left to react for 10min, 1hr, 2hr, and 12hrs. Particles were washed 10x with Distilled water and fluorescently imaged.

2. Results and discussion



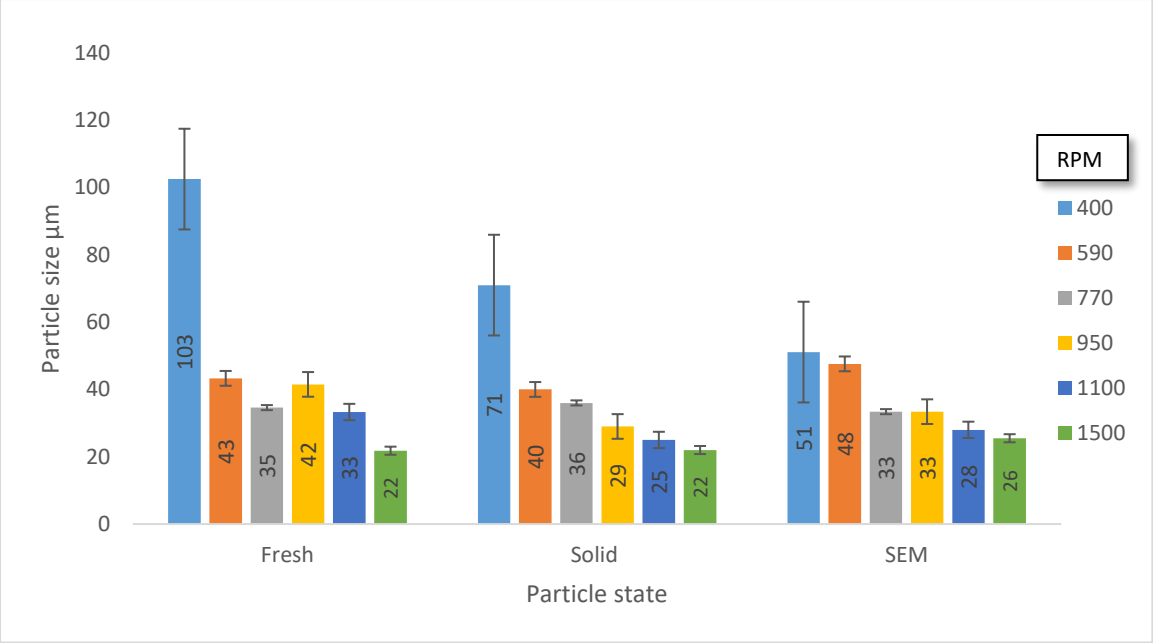


Figure S1: Effect of increasing polymer concentration and rotation speed on the resulting average particle size of microparticles produced from membrane emulsification.

		Fresh			Solid			SEM		
PCL N_3	**RPM**	**Span**	**CV**	**SD**	**Span**	**CV**	**SD**	**Span**	**CV**	**SD**
10%	400	0.54	0.21	21.60	0.73	0.35	23.08	1.29	0.51	26.10
10%	590	0.67	0.28	12.48	0.61	0.24	9.84	0.45	0.17	8.32
10%	770	0.36	0.15	5.22	0.27	0.13	4.84	0.69	0.19	6.43
10%	950	0.46	0.17	7.36	0.38	0.14	4.20	0.35	0.14	4.85
10%	1140	0.39	0.14	4.96	0.33	0.14	3.64	0.22	0.11	3.22
10%	1500	0.45	0.19	4.26	0.40	0.16	3.67	0.52	0.18	4.80
5%	1140	0.40	0.16	4.02	0.41	0.14	3.46	0.34	0.14	3.95
10%	1140	0.39	0.14	4.96	0.33	0.14	3.63	0.22	0.11	3.22
20%	1140	0.33	0.13	5.26	0.33	0.14	4.23	0.33	0.14	4.65
30%	1140	0.34	0.12	3.78	0.43	0.17	5.72	0.39	0.16	5.48
40%	1140	0.37	0.13	5.39	0.34	0.14	4.80	0.32	0.12	4.75

Figure S2: Increasing PCL-N_3 polymer concentrations and increasing rotation speed for the paddle blade were tested to assess the effect on polymer morphology and size distribution. A microparticle population is completely monodisperse with a span of 0, but are considered monodisperse with a span below 1. Above rotations speeds of 400rpm all parameters produced monodisperse particle populations and a controlled and defined tunable size range was easy to achieve.

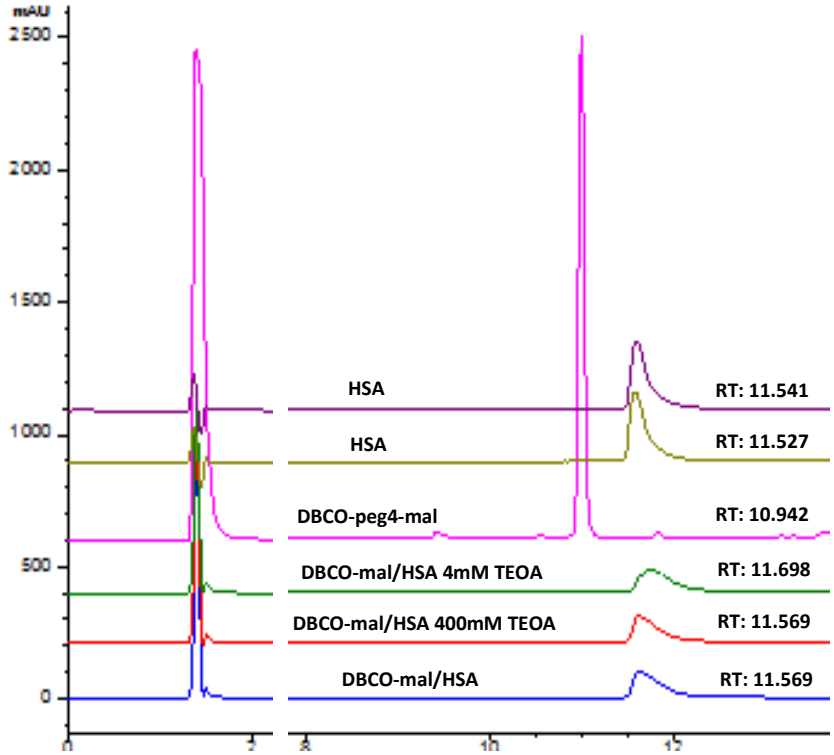


Figure S3: HPLC analysis of Human Serum Albumin (HSA) and DBCO-peg$_4$-maleimide in comparison to conjugates reacted with or without Triethanolamine (TEOA).

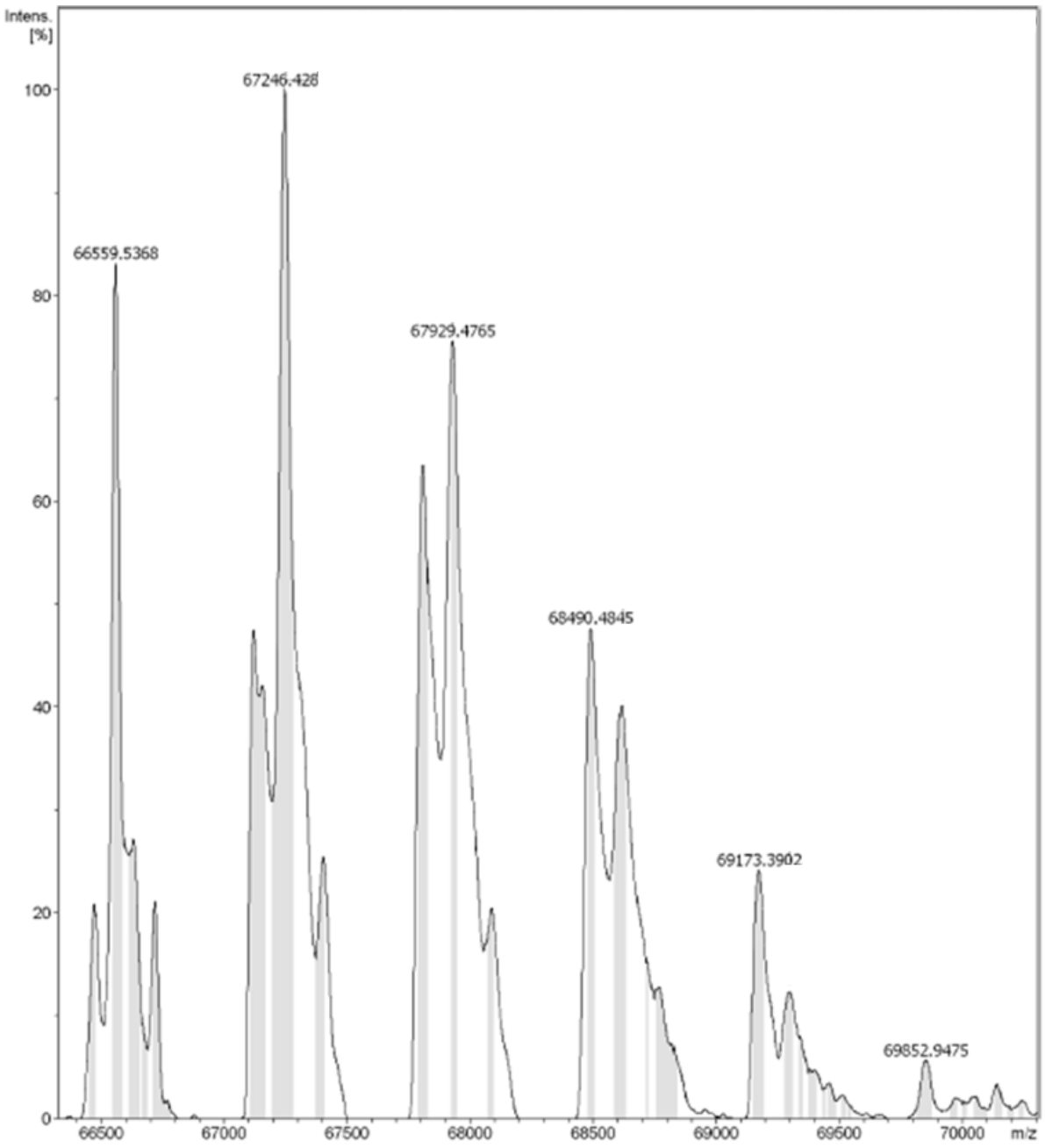


Figure S4: Liquid Chromatography Mass Spec analysis showing the addition of app 680 MW to the native Human Serum Albumin protein peak at 665559 (MW 66.5kDa) and each additional glycosylation peak. The Molecular Weight of DBCO-peg$_4$-maleimide is 674.74g/mol suggesting the successful thiol/maleimide reaction between the DBCO-peg4-maleimide linker and the free thiol present at cysteine 34 of Human Serum Albumin.

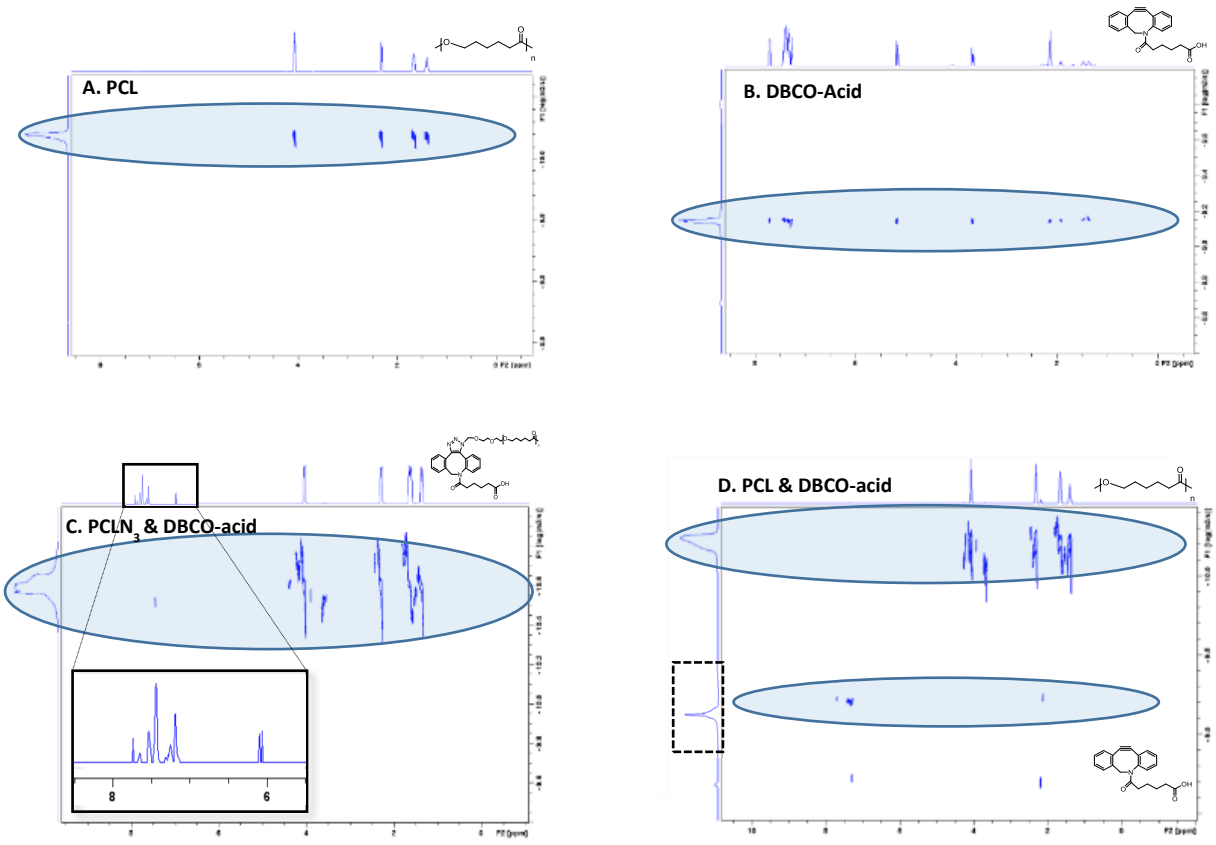


Figure S5: DOSY NMR spectra of commercial (A) PCL, (B) DBCO-acid and the corresponding reactions of DBCO-acid with either(C) PCL-N_3 showing parallel species for the *PCL-N_3 and DBCO-acid, which confirm successful click reaction, whereas D) PCL, showing an attempted reaction as control by reacting PCL (with no azide functional group) with the DBCO-acid, confirming the presence of more than one species and therefore unsuccessful click reaction as expected*