The Reverse of Polymer Degradation: *In situ* crosslinked gel formation through disulphide cleavage[†]

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Supporting Information

Synthesis of Disulphide-Based Dimethacrylate Branching Agent (DSDMA)

As adopted from Li *et al.*, *Macromolecules*, 2005, **38**, 8155-8162, bis(2-hydroxyethyl) disulphide (BHEDS (Sigma), 10g, 64mmol, 1.0 equiv) and triethylamine (Sigma) (54ml, 38mmol, 6.0 equiv) were dissolved in 150mL of anhydrous dichloromethane (DCM, HPLC grade). The flask containing the solution was then immersed in an ice bath, and methacryloyl chloride (Sigma)(20.0g, 193mmol, 3.0 equiv) was added dropwise to the stirred DCM solution. The resulting heterogeneous mixture was stirred within an ice bath for 30 minutes and then room temperature for 24 hours followed by filtration to remove the triethylamine hydrochloride product. The solvent was removed by evaporation, and the crude product was dissolved in chloroform. This solution was three times with an aqueous solution of 0.1M Na₂CO₃, followed by three washings with water. The purified organic solution was dried using anhydrous MgSO₄, and the chloroform was removed under reduced pressure.



SI Figure 1. ¹H NMR characterisation of the DSDMA monomer

Synthesis of PEG-Based Disulphide Hyperbranched Polymer

Poly(ethylene glycol) methyl ethyl methacrylate (PEGMEMA)(Mw 475 Sigma)(6g, 12.63mmol, 60 4.21mmol, equiv). **DSDMA** (1.223g)20 equiv) and ethylene glycol diacrylate (EGDA)(Sigma)(1.086g, 4.21mmol, 20 equiv) were added to a 2 neck round bottomed flask. 0.0312ml of the initiator ethyl α-bromoisobutyrate (EBriB)(Sigma) was added (41.06mg, 210µmol, 1 equiv) to the monomers, along with 0.0110ml of the ligand 1,1,4,7,7-Pentamethyl-diethylenetriamine (PMDETA)(Sigma)(18.2mg, 105µmol, **0.5 equiv**) and CuCl₂ (Sigma)(14.2mg, 105µmol, **0.5 equiv**). 20ml of Butanone (Sigma, HPLC grade) was added to the flask which was subsequently degassed by bubbling argon through the solution for 20 minutes. To start the reaction the reducing agent Lascorbic acid (AA)(Sigma)(2.31mg, 13.2µmol, 0.0625 equiv) was then added to the solution via prior dissolution in water and addition by pipette. The reaction was carried out over an oil bath at 50°C and stirred at 800rpm, for the desired reaction time. Air was allowed into the flask to stop the reaction, before purification by precipitation in hexane to remove un-reacted DSDMA. After removal of the hexane, the polymer was re-dissolved in acetone and dialized against distilled water (MWCO - 6000, Spectrapor) for several days. The purified polymer was then freeze dried to yield a viscous polymer ready for subsequent analysis. At all stages of the purification the polymer was protected from light.

Gel Permeation Chromatography (GPC) analysis

At intervals throughout the reaction process, small samples were removed and prepared for analysis by gel permeation chromatography. The sample was removed from the reaction mixture under positive pressure of argon, using a glass syringe and needle with leur fitting. The sample was diluted in dimethylformamide (DMF)(HPLC grade, Fisher Scientific) and passed through an alumina column (alumina for chromatography, Sigma) followed by filtration with a 0.2µm filter. The molecular weight and molecular weight distribution of each sample was determined using a Varian 920-LC instrument with a refractive index detector (RI). Chromatograms were run at 50 °C using DMF as eluent with a flow rate of 1 ml/min. Calibration was performed with linear polystyrene standards.



SI Figure 2 – Gel Permeation Chromatography traces taken after 4hours 30 minutes of reaction time and at the end point (6hours), where a final Mw of 31.4KDa was obtained after 32% monomer conversion.

¹H Nuclear Magnetic Resonance (NMR) spectroscopy analysis.

The purified DSDMA monomer and final PEG-based disulphide polymer were dissolved in deuterated chloroform and analysed at 300 MHz using a Bruker spectrometer. All chemical shifts are reported in ppm relative to TMS.



SI Figure 3. ¹H NMR characterisation of the PEG-based polymer, with subsequent peak identification



SI Figure 4. Integration of the ¹H NMR peaks **d**, **e**, **c** and **h** for determining the polymer composition (see formula below).

The degree of branching of the polymer was calculated using the peak integral values and the following formula:

 \mathbf{z} - Amount of PEG = $\mathbf{h}/34$ $\mathbf{x} + \mathbf{y}$ - Total free vinyl groups = $\mathbf{d} + \mathbf{e}$ $\mathbf{m} + \mathbf{n}$ - Total branching = $\mathbf{c} - (2\mathbf{z}) - (6[\mathbf{x}+\mathbf{y}])$ 6

Where **h**= 145.24, **c**= 25.54 and the vinyl peaks of **d** and **e** are 0.47, 0.68, 0.54, 0.53

PEG = 59% Total Vinyl = 30% Total Branching = 11%The degree of branching of the polymer was calculated using the peak integral values (see SI Figure 3 for peak identification) and the following formula:

 \mathbf{z} – Amount of PEG = $\mathbf{h}/34$

 $\mathbf{x} + \mathbf{y} - \text{Total free vinyl groups} = \mathbf{d} + \mathbf{e}$

 $\mathbf{m} + \mathbf{n}$ – Total branching = $\underline{\mathbf{c} - (2\mathbf{z}) - (6[\mathbf{x}+\mathbf{y}])}$ 6

PEG = 59% Total Vinyl = 30% Total Branching = 11%

The ability to control in situ DE-ATRP

The synthesis method described above allows the use of a 40:1 multi-vinyl monomer to initiator ratio, which leads to a hyperbranched structure, but with low PDI (1.68). However, a further reaction to demonstrate the controlled nature of *in situ* DE-ATRP was performed where the amount of catalyst and reducing agent were reduced as follows: PMDETA (9.12mg, 52.6µmol, **0.25 equiv**),CuCl₂ (7.08mg, 52.6µmol, **0.25 equiv**) and AA (2.31mg, 13.2µmol, **0.0625 equiv**). This produced a slower reaction, whereby samples for GPC analysis were withdrawn throughout the process (plotted in SI Figure 4).



SI Figure 5 – (a) Narrow GPC peaks, typical of *in situ* DE-ATRP, allow the formation of branched structures while suppressing gelation as noted by the steady growth in Mw (b) and PDI (c) during the reaction process.

Crosslinked Gel Formation

Solutions of reduced L-Glutathione (GSH)(Sigma) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP)(Sigma) were prepared at concentrations of 5mM and 20mM in distilled water(dH₂O), 30 minutes prior to use and degassed by bubbling argon through for 10 minutes. During this time the polymer was weighed directly into vials. The appropriate volume of reducing agent, or dH₂O control, was added into the vials to give a final concentration of 10mg/ml. For TEM analysis, these solutions were left at room temperature for 20 minutes then a 10 μ l volume was dropped onto copper TEM grids and allowed to dry. For GPC analysis equal volumes of each solution were diluted in the GPC eluent DMF, ran through a 0.2 μ m filter and sampled as explained above.

Effect of Incubation Time on Crosslinked Gel Formation

Gel permeation chromatography was also used to analyse the time taken for crosslinking to occur. As explained above, two samples were prepared for GPC analysis however; one sample was run after ten minutes of incubation with 20mM TCEP and the other was run after 20 minutes. SI Figure 5 shows how even after 10 minutes a large shift in molecular weight is already observed, however the low molecular weight peaks of the polymer still partially remain. However after 20 minutes almost complete nanogel formation has taken place.



SI Figure 6. GPC traces showing the effect of incubation time on gel formation. After 10 minutes of 20mM TCEP incubation a large shift in Mw is observed, which appears completed after 20minutes.

Supplementary TEM images

Control (polymer in water)



SI Figure 7. A 10mg/ml concentration of the PEG based polymer in dH_2O alone, at three different magnifications (a) 10,000 times, (b) 25,000 times, and (c) 70,000 times zoom.

5mM Glutathione



SI Figure 8. TEM images of various samples of nano sized gels formed via 5mM GSH at magnifications of (a) 15,000x, (b) 4,000x, (c) 7,000x and (d) 5,000x with measurements of various areas taken.



SI Figure 9. TEM images of the same area of the TEM grid, through progressive zooming through (**a**) 10,000x, (**b**) 15,000x, (**c**) 25,000x, (**d**) 40,000x, (**e**) 50,000x and (**f**) 60,000x.

20mM Glutathione



SI Figure 10. TEM images of the gels of nanometer dimensions prepared with 20mM GSH showing a more pronounced effect of crosslinking. Images were taken at a magnification of (a) 2,500x, (b) 4,000x, (c) 5,000x, (d) 15,000x, (e) 30,000x and (f) 50,000x.

5mM TCEP



SI Figure 11. TEM images of nanogels prepared in using 5mM TCEP, showing more fine extensions connecting globular structures. Images were taken at magnifications of (a) 5,000x, (b) 8,000x, (c) 15,000x, and (d) 40,000x. Measurement of the fibres shows values at approximately 40nm for fibre width.

20mM TCEP



SI Figure 12. TEM images of highly crosslinked gels via preparation in 20mM TCEP. Images taken at magnifications of (a) 5,000x, (b) 15,000x, (c) 15,000x, and (d) 40,000x.

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SI Figure 13. Example of ¹H NMR (top) and ¹³C NMR (bottom) (D₂O, 25 °C, 400 MHz) spectra of PEG based polymer. The spectra (top) shows disappearance of vinyl group peaks (5.8-6.2ppm) 20 min after the addition of reducing agent (TCEP), indication of successful Michael addition reaction.

This is further supported by ¹³CNMR spectra (bottom) showing no peak for (C=C) peaks and appearance of (C-C-S) peak at 27ppm.

Effect of the Polymer and the Gel on Cell Metabolic Activity

NIH3T3 fibroblasts were grown in Dulbecco modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (P/S) at 37°C with 5% CO₂ using standard cell culturing techniques. Cells were seeded at 20,000 cells/well of a 48 well plate and allowed to adhere for 12hrs before experimentation. Polymer solutions at the relevant concentrations were made up fresh prior to analysis followed by the addition of TCEP at 20mM to form the gels. Both non reduced and reduced (gel) polymer were added in triplicate to the test wells, along with the media vehicle solution alone to control wells and incubated for 24 hours. To analyze the cell metabolic activity of the fibroblasts the PrestoBlue TM (Invitrogen) was used according to manufacturers protocol. After incubation the fluorescence of the supernatant was read (Infinite M200 plate reader, Tecan – equipped with I-control 1.6 software) at 540/650 excitation and emission wavelengths respectively. All values are plotted as a percentage of the control cells.



SI Figure 14. NIH3T3 cells viability when cultured with PEG based polymer and corresponding crosslinked gel at different concentration for 24 hrs, Values are mean \pm stdev, N=3). Neither polymer nor crosslinked gel shows toxicity toward NIH 3T3 cells at the concentrations tested.