ELECTRONIC SUPPLEMENTARY INFORMATION FOR

Enzymatically crosslinked dendritic polyglycerol nanogels for encapsulation of catalytically active proteins

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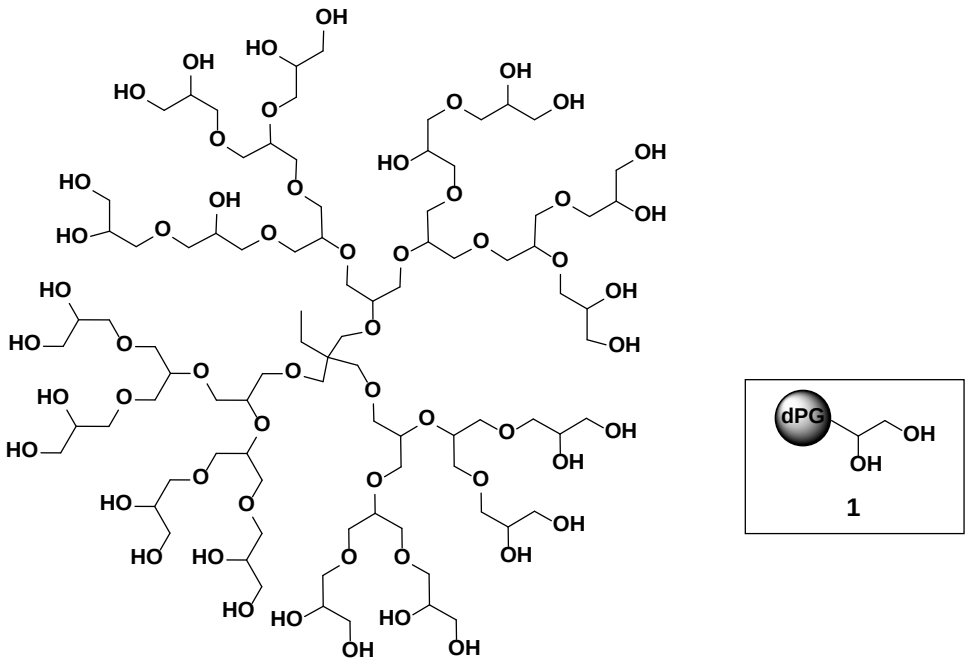
Contents

1. Materials and Methods

1.1. *Materials*

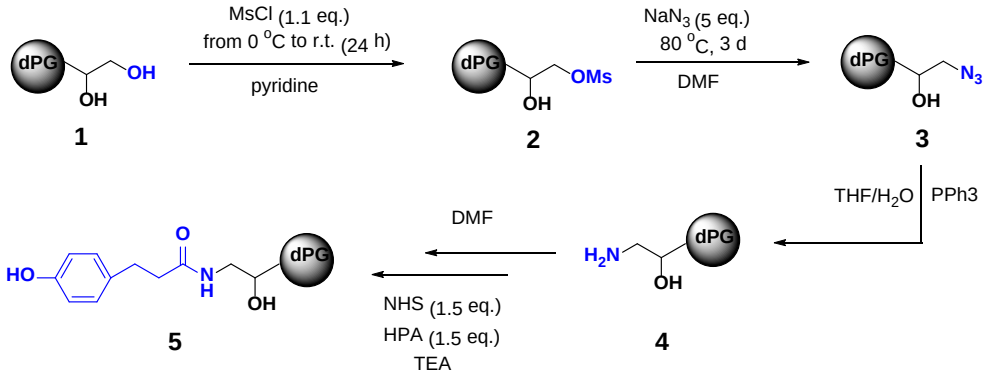
Dendritic polyglycerol (dPG, Mw 6 kDa) was synthesized by anionic, ring-opening multi-branching polymerization of glycidol according to literature (**Scheme S1**).[1, 2] Polyethylene glycol (PEG, Mw 6 kDa) was obtained from Acros Organics. All other general chemicals were purchased from Sigma-Aldrich without further purification. Some of them were given with abbreviated names as follows: dimethylformamide (DMF), dichloromethane (DCM), dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), triethylamine (TEA), 4-dimethylaminopyridine (DMAP), methanesulfonyl chloride (MsCl), triphenylphosphine (PPh_3), N-Hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), tyramine (TA), 4-nitrophenyl chloroformate (PNC), and 3-(4-hydroxyphenyl) propionic acid (HPA).



Scheme S1. Representative structure of dPG – the shown structure represents only one possible isomer or one part of dPG scaffold (Mw 6 kDa).

1.2. *Synthesis of dPG-HPA*

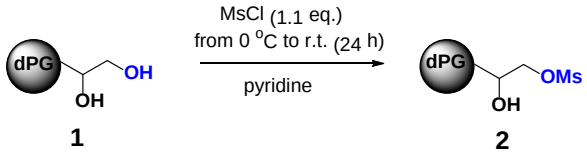
In general, dPG was first converted to dPG-NH_2 in three steps (mesylation, nucleophilic substitution, and reduction); dPG-HPA was then obtained by amide coupling between HPA and dPG-NH_2, as shown in **Scheme S2**.[3-5]



Scheme S2. Synthetic route of dPG-HPA.

1.2.1. PG-NH_2 synthesis

1.2.1.1.The preparation of mesylpolyglycerol (dPG-OMs)



Scheme S3. Synthesis of dPG-OMs.

The reaction was carried out under the protection of argon atmosphere and extra drying conditions. dPG was converted to mesylpolyglycerol (dPG-OMs) with 2%, 4%, 6%, and 8% mesyl group loading, respectively. In general, 5 g dPG (67.56 mmol –OH on dPG scaffold) was

dried at 65 ^{o}C under vacuum for 12 hours to exclude residual water. Then, 100 mL abs. pyridine was added to dissolve the honey-like dPG, stirred with a magnetic plate. The mesylation reaction was initiated when MsCl solution (1.15 eq.) was slowly and dropwise added into dPG solution at 0 ^{o}C. Subsequently, the mixture was stirred in the thawing cooling bath for 20 hours. A yellowish-brown mixture was finally obtained. The solvents were removed by vacuum and the crude mixture was further purified by dialysis in MeOH to receive more than 90% yield. The final product dPG-OMs was confirmed by **^{1}H NMR** (500 MHz, MeOD or D_2O (data not shown here), δ): 4.55-3.40 (m, $\underline{CH_2}$ and $\underline{CH}$, PG scaffold); 3.30-3.17 (m, $\underline{CH_3}$, mesyl groups), 1.38 (m, $\underline{CH_2}$CH3 of starter); 0.87 (m, CH2$\underline{CH_3}$ of starter).

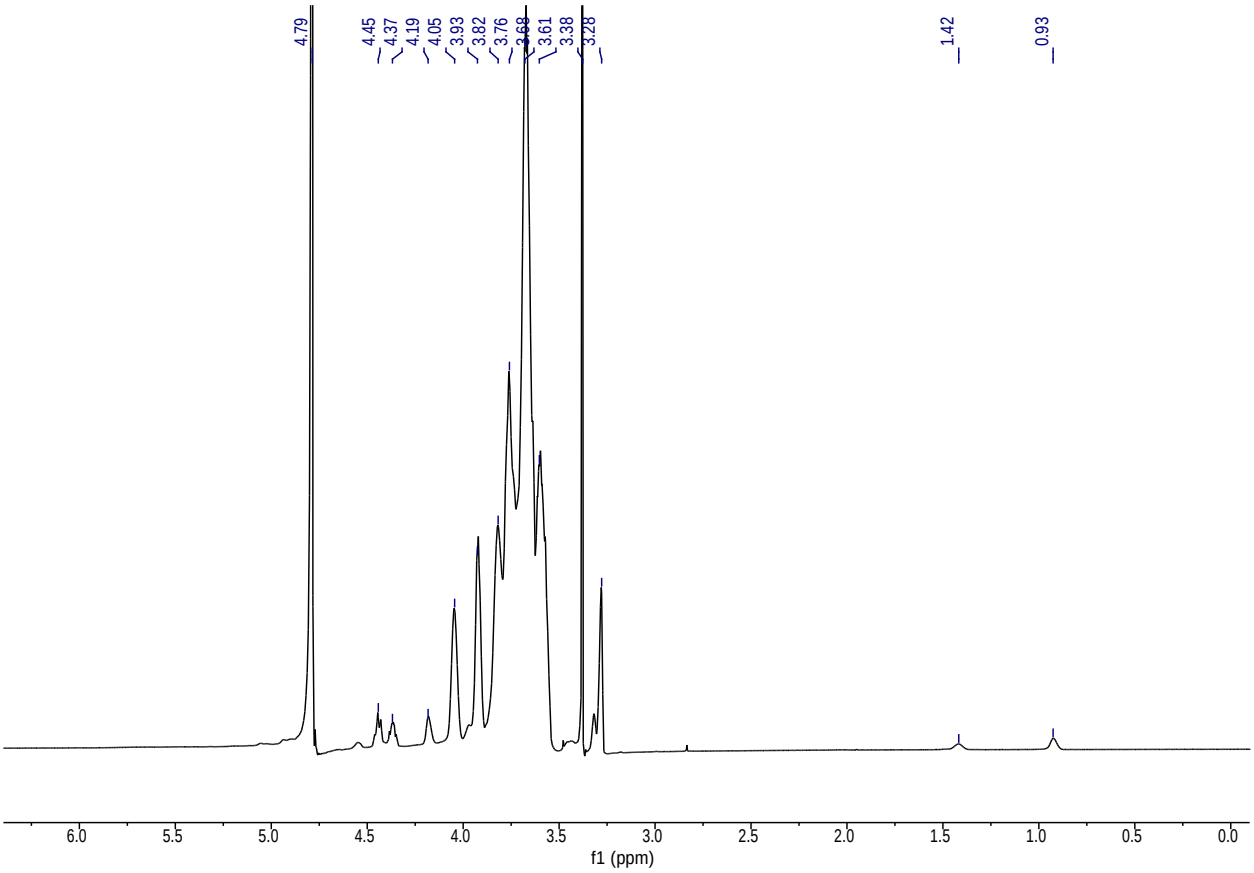


Figure S1. ^{1}H NMR spectra of dPG-2%OMs (500 MHz, D_2O).

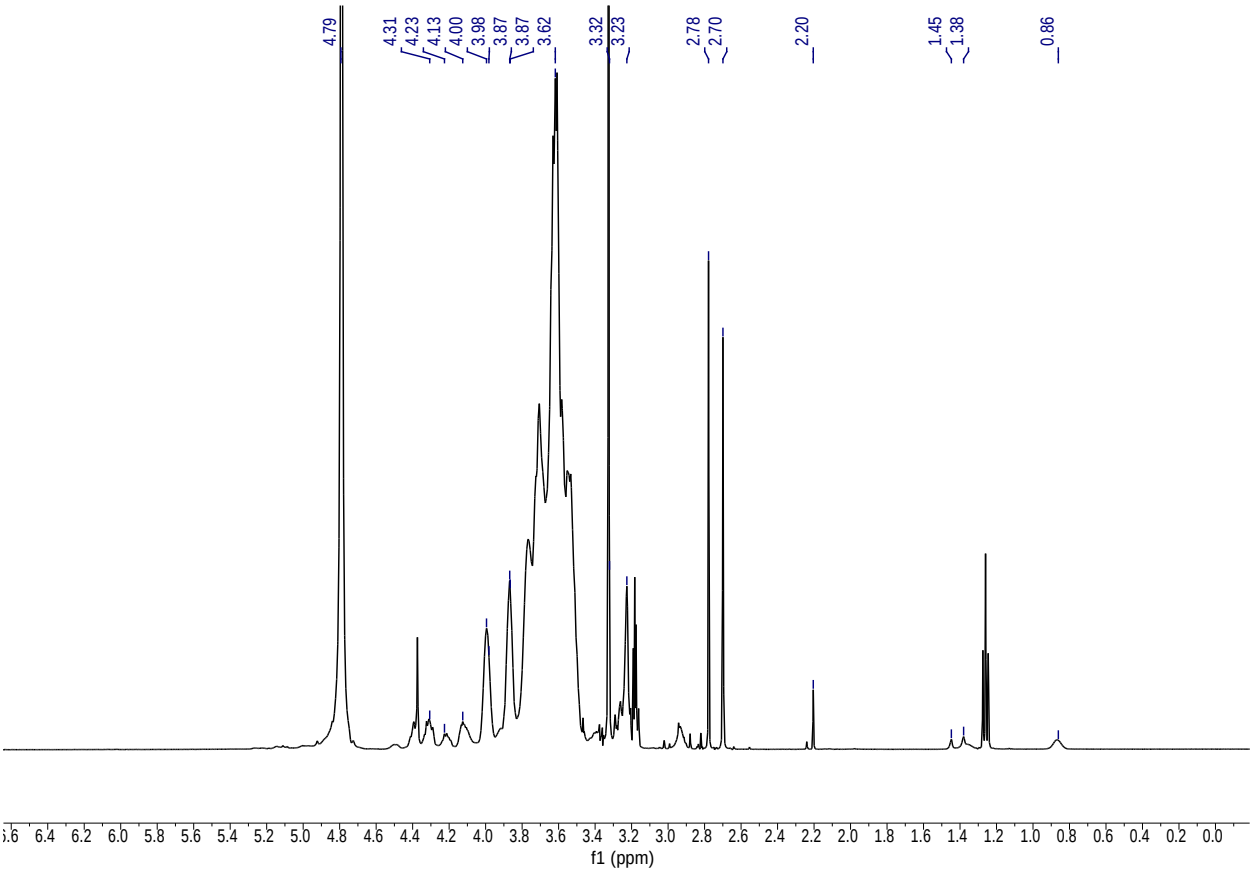


Figure S2. ^{1}H NMR spectra of dPG-4%OMs (500 MHz, CD_3OD).

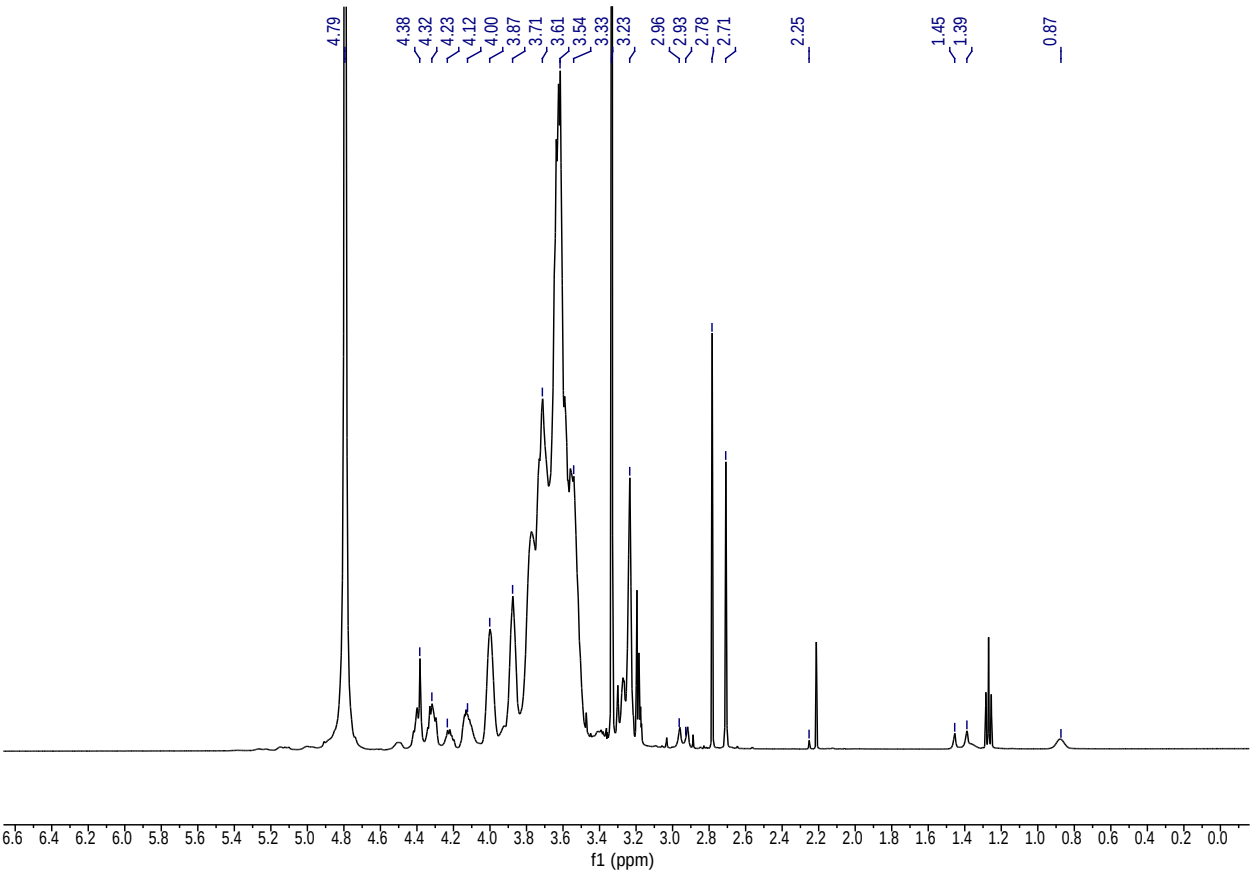


Figure S3. ^{1}H NMR spectra of dPG-6%OMs (500 MHz, CD_3OD).

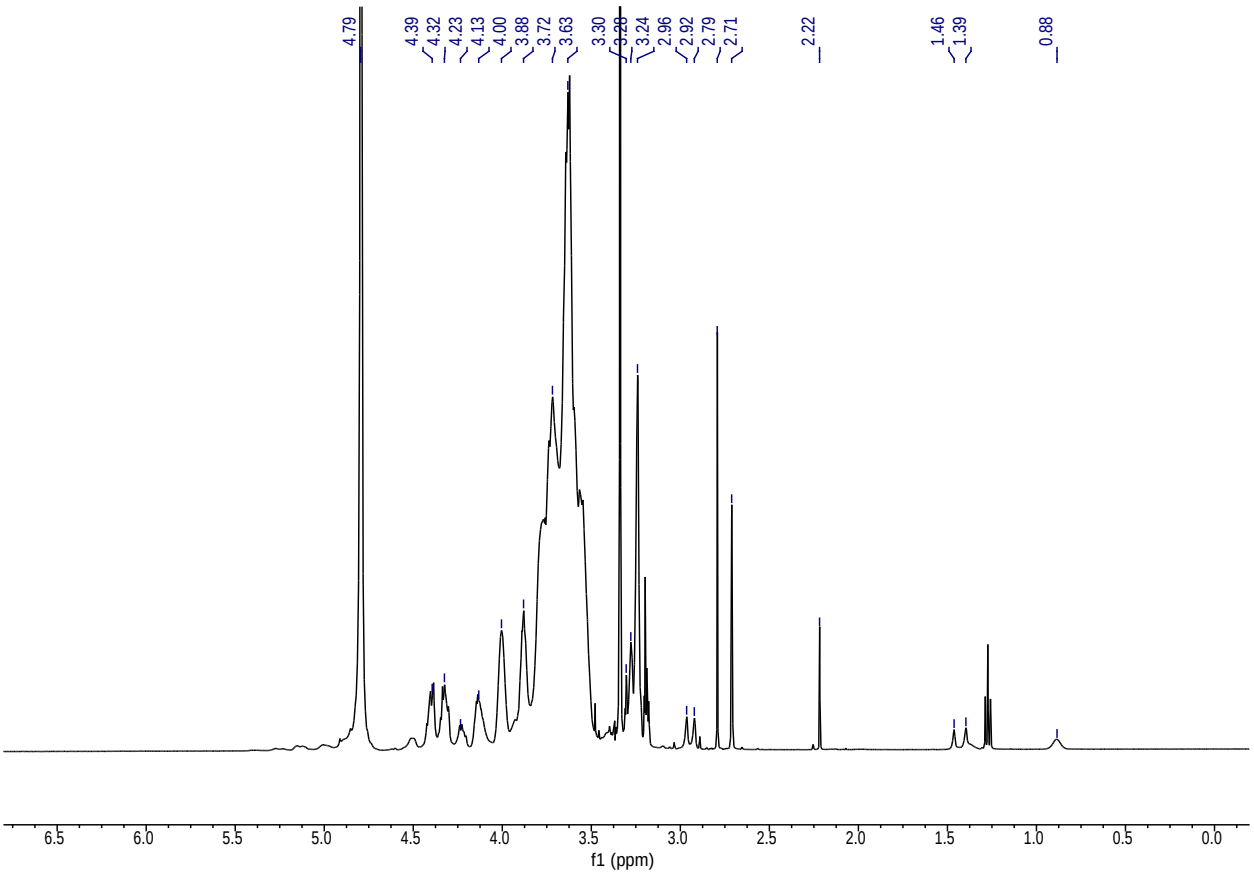
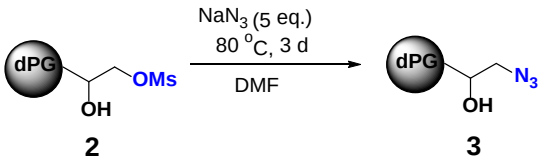


Figure S4. ^{1}H NMR spectra of dPG-8%OMs (500 MHz, CD_3OD).

1.2.1.2.The preparation of polyglycerolazide (dPG-N_3)



Scheme S4. Synthesis of dPG-N_3.

The preparation of dPG-N_3 was achieved through nucleophilic substitution of dPG-OMs with sodium azide. Typically, 5 g dPG-OMs (2, 4, 6, and 8% –OMs group laoding, respectively) were dissolved in p.a. DMF (100 mL) in a one-necked flask. Subsequently, 5 eq. NaN_3 were added and the mixture was heated at 80 ^{o}C for 3 days behind a transparent security wall. The reaction was stopped by cooling down the mixture and filtrating the white residue of excess NaN_3 solid.

The obtained reddish-brown residue was further concentrated in vacuum, with the protection of plastic spatula, to remove solvents at temperature below 40 ^{o}C. The residue was then purified by dialysis in MeOH to achieve pure product with over 90% yield. dPG-2, 4, 6, and 8%N_3 was characterized by both ^{1}H NMR and FT-IR. **^{1}H NMR** (500 MHz, MeOD, δ): 4.55-3.40 (m, $\underline{CH_2}$ and $\underline{CH}$, PG scaffold); 1.37 (m, $\underline{CH_2}CH_3$ of starter); 0.87 (m, CH2$\underline{CH_3}$ of starter). **IR** ν_{max}/cm^{-1}: 3355, 2871, 2100, 1646, 1455, 1268, 1064, 930, 867.

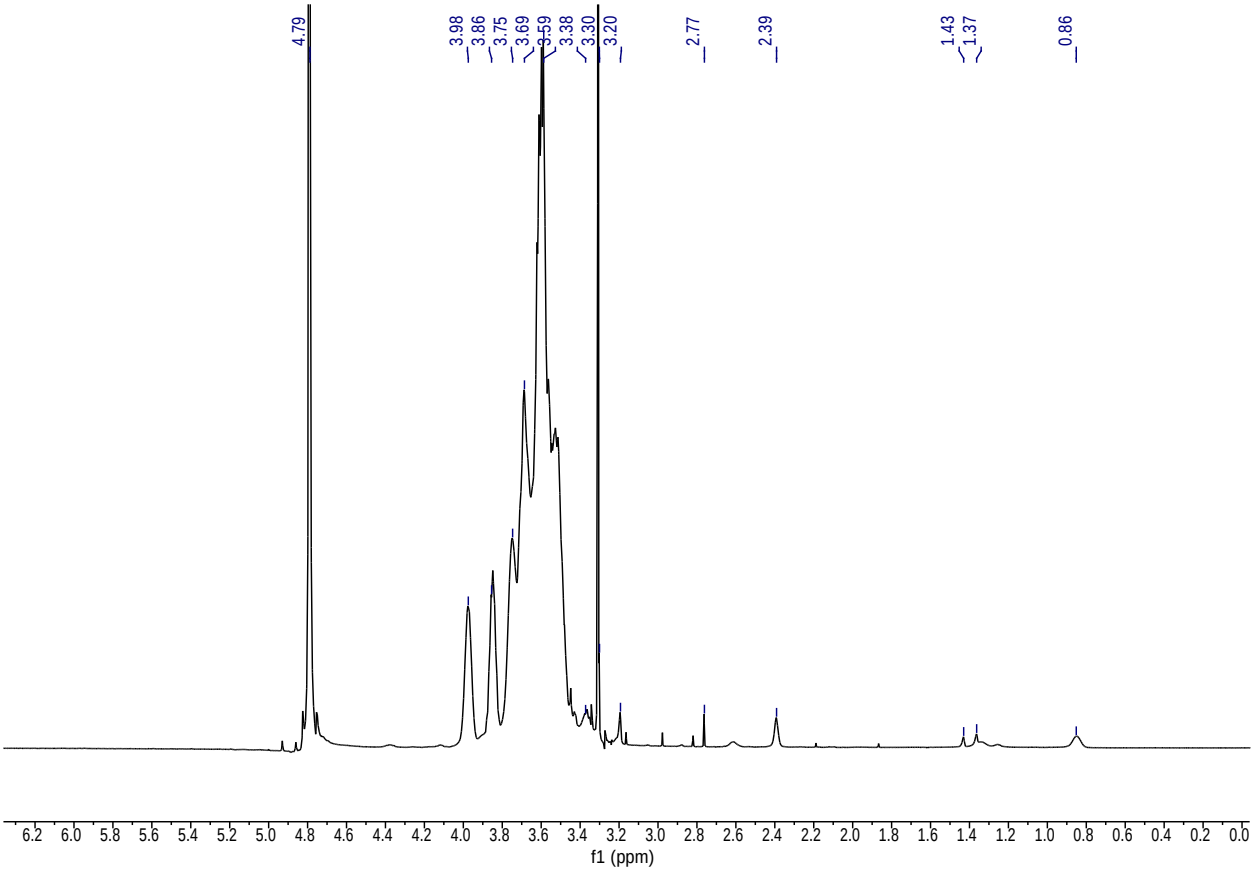


Figure S5. ^{1}H NMR spectra of dPG-4%N_3 (500 MHz, CD_3OD).

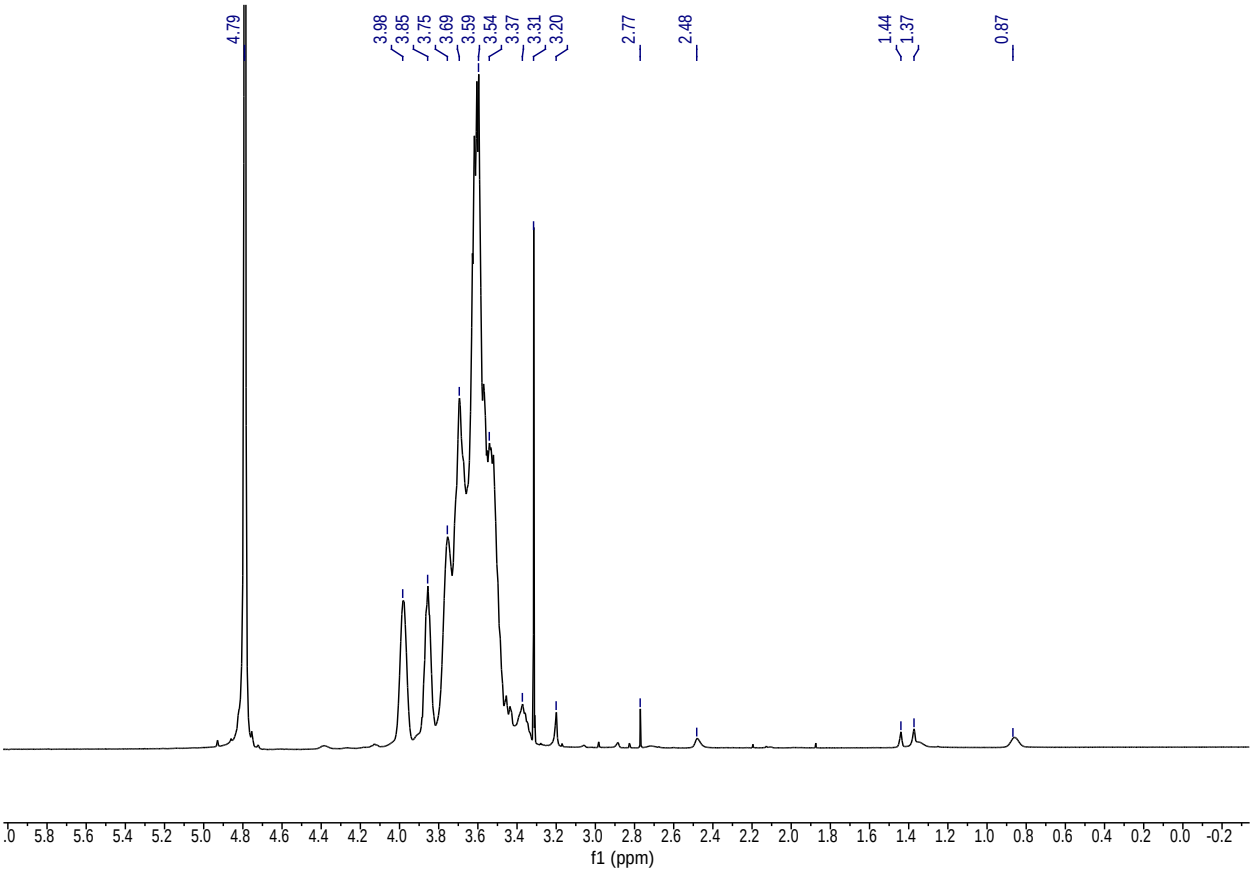


Figure S6. ^{1}H NMR spectra of dPG-6%N_3 (500 MHz, CD_3OD).

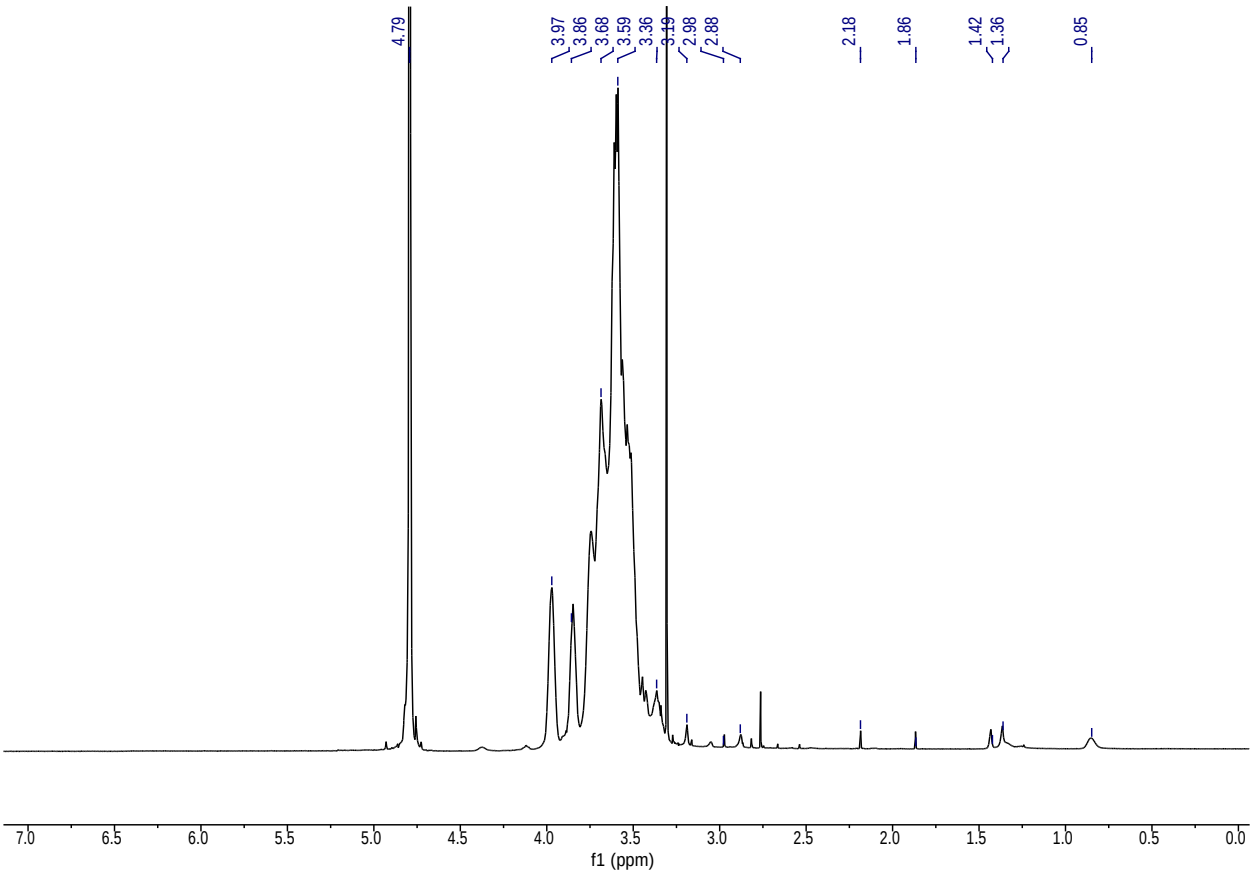


Figure S7. ^{1}H NMR spectra of dPG-8%N_3 (500 MHz, CD_3OD).

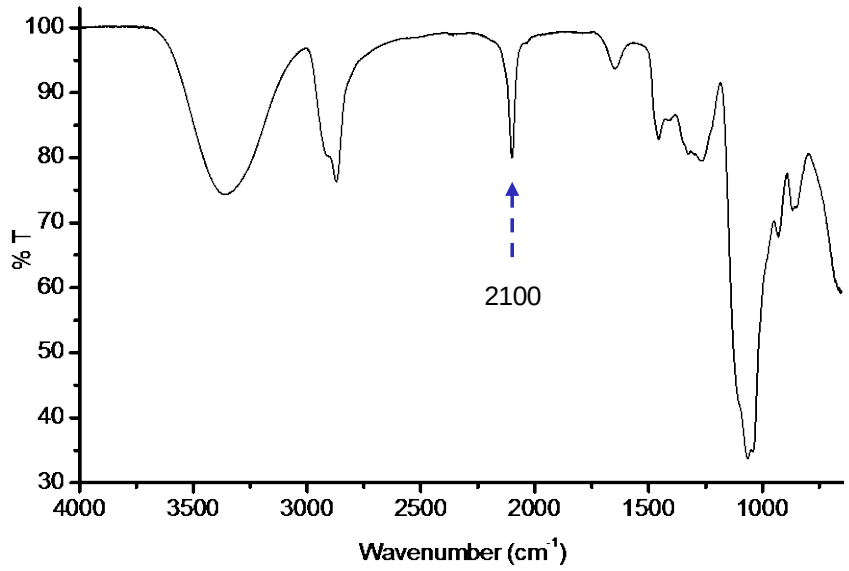
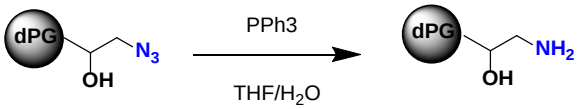


Figure S8. IR spectra of a typical dPG-N_3 (here corresponds to dPG-4%N_3): The appearance of band at 2100 represents the conjugation of azide groups on dPG scaffolds.

1.2.1.3. The preparation of polyglycerylamine (dPG-NH_2)



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Scheme S5. Synthesis of dPG-NH_2.

In a 2 liter one-necked flask equipped with magnetic stirring, 4 g dPG-NH_2 was dissolved in THF/H_2O mixture with volume ration at 7/3. More than 3 eq. PPh_3 was then added to the mixture, followed up by addition of sufficient THF/H_2O solution to obtain a transparent reaction media. Since N_2 was produced from the reaction, and the flask was not completely closed to avoid high pressure. After 12 hours, some amount of H_2O was dropwise added into reaction media until clear solution was observed, which could avoid precipitation of the partially reduced product. The azide groups of dPG were repeatedly monitored during the reaction by FTIR until they were

fully reduced. The final product of dPG-NH_2 was obtained by dialysis of the crude mixture in MeOH, and they were confirmed by ^{1}H NMR and FTIR, respectively. **^{1}H NMR** (500 MHz, D_2O or CD_3OD (data not shown here), δ): 4.4-3.30 (m, $\underline{CH_2}$ and $\underline{CH}$, PG scaffold); 3.16-2.96 (m, O$\underline{CH}$-NH_2); 2.83-2.54 (m, $C\underline{H_2}$-NH_2), 1.32 (m, $\underline{CH_2}CH_3$ of starter); 0.87 (m, CH2$\underline{CH_3}$ of starter). **IR** ν_{max}/cm^{-1}: 3333, 2870, 1644, 1549, 1516, 1454, 1326, 1244, 1087, 932, 834.

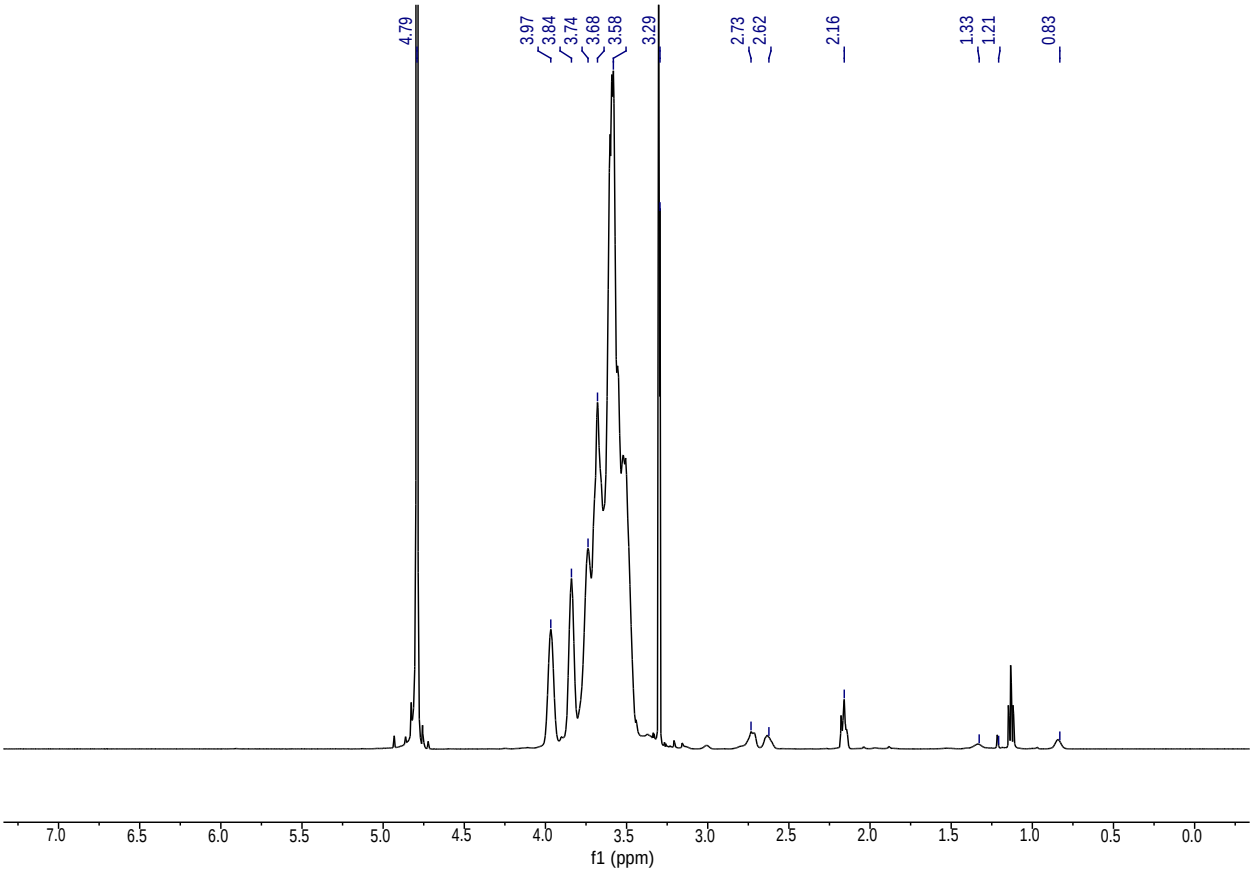


Figure S9. ^{1}H NMR spectra of dPG-2%NH_2 (500 MHz, CD_3OD).

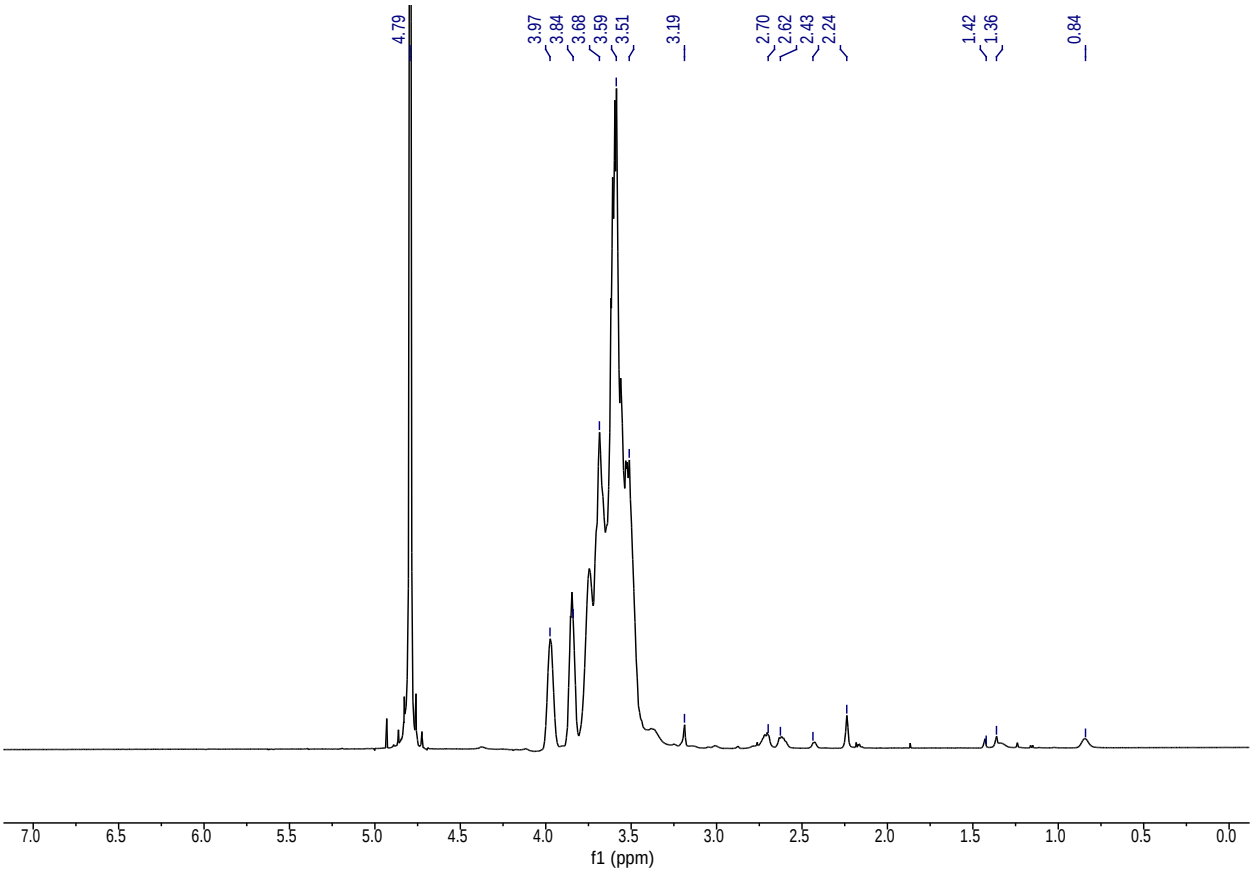


Figure S10. ^{1}H NMR spectra of dPG-4%NH_2 (500 MHz, D_2O).

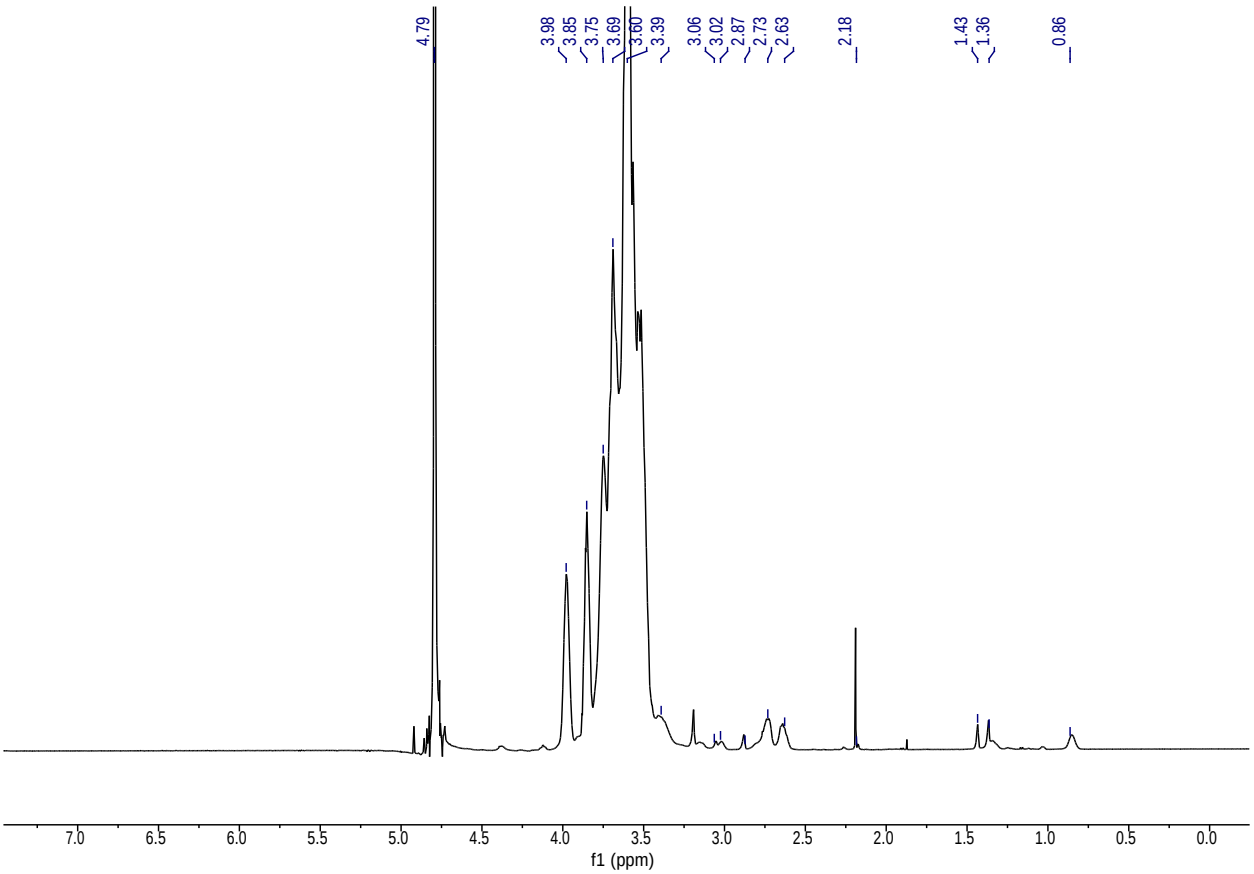


Figure S11. ^{1}H NMR spectra of dPG-6%NH_2 (500 MHz, D_2O).

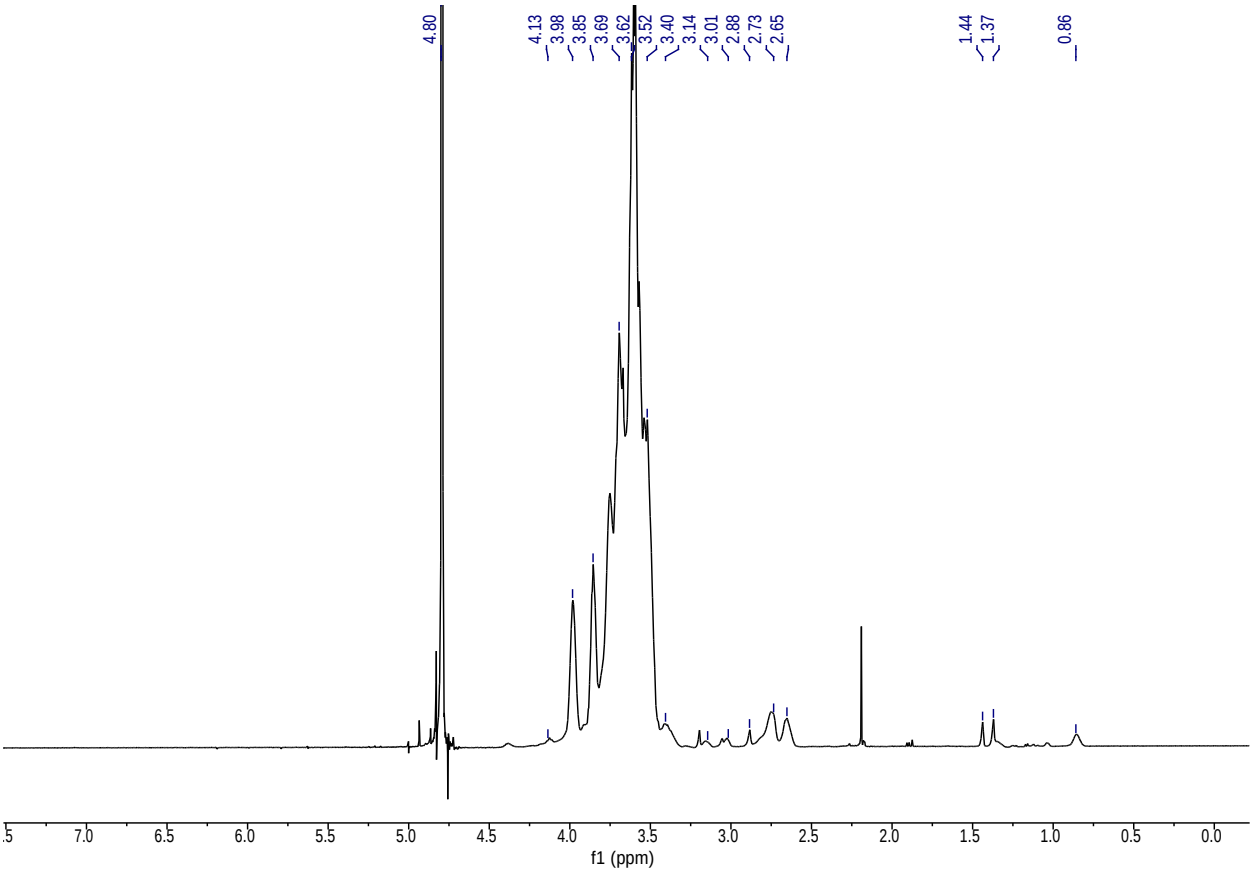


Figure S12. ^{1}H NMR spectra of dPG-8%NH_2 (500 MHz, D_2O).

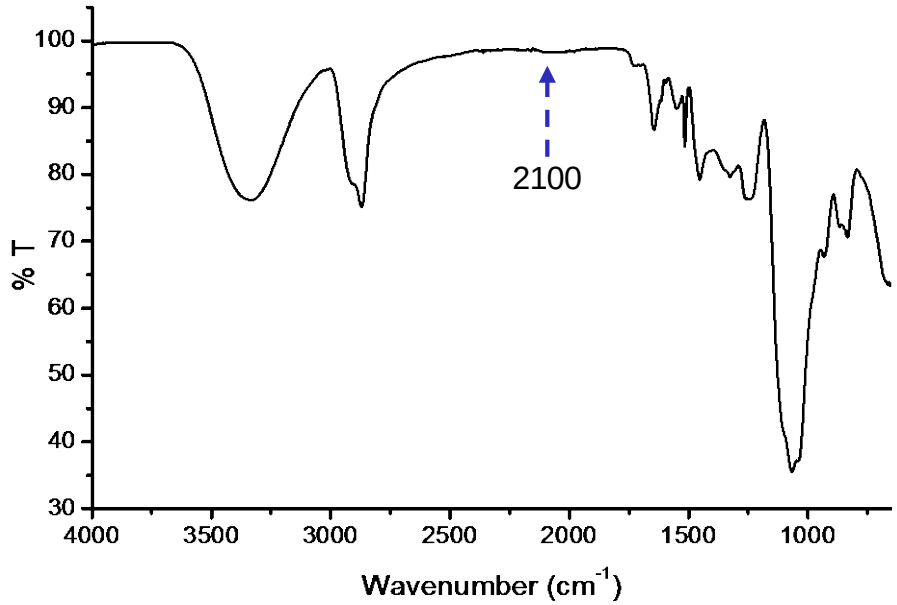
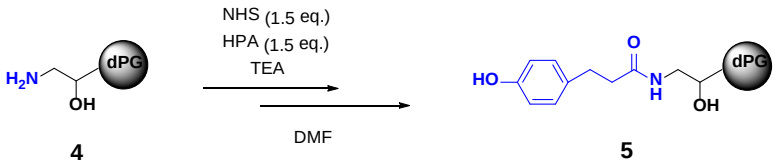


Figure S13. IR spectra of a typical dPG-NH_2 (here corresponds to dPG-2%NH_2). The disappear of band at 2100 cm^{-1} indicate that all azide groups were converted to amine groups.

1.2.2. dPG-HPA synthesis



Scheme S6. Synthesis of dPG-HPA.

The synthesis of dPG-2, 4, 6, and 8%HPA was achieved through amide coupling between dPG-2, 4, 6, and 8%NH_2 and HPA (1.5 eq.) according to the previously described method.[3] Here we described only the synthesis of dPG-2%HPA as an example (the others are in the same procedure): Prior to the amide coupling, an HPA-NHS ester was synthesized by mixing 3-(4-hydroxyphenyl) propionic acid (HPA, 3 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 3 mmol), triethylamine (TEA, 3 mmol) and N-hydroxysuccinimide (NHS, 2.5 mmol) in 70 mL abs. DMF. The reaction was performed at 0 ^{o}C with the protection of argon, and stirred for 12 hours. HPA-NHS ester was purified by column (87% yield). Immediately after the purification, 1.74 mmol HPA-NHS was further re-dissolved into a 50 mL abs. DMF. Then 4 g dPG-2%NH_2 (1.08 mmol –NH_2 and 52.97 mmol –OH on dPG scaffold) solutions (in 50 mL abs. DMF) were slowly added into HPA-NHS media. After 12 hours, the mixture was concentrated in vacuum and further purified by dialysis in MeOH, and dPG-2%HPA was finally obtained with over 90% yield and confirmed by ^{1}H NMR and FTIR. ^{1}H NMR spectra was described in manuscript. **IR** ν_{max}/cm^{-1}: 3316, 2873, 1732, 1645, 1549, 1512, 1451, 1357, 1246, 1072, 1024, 832.

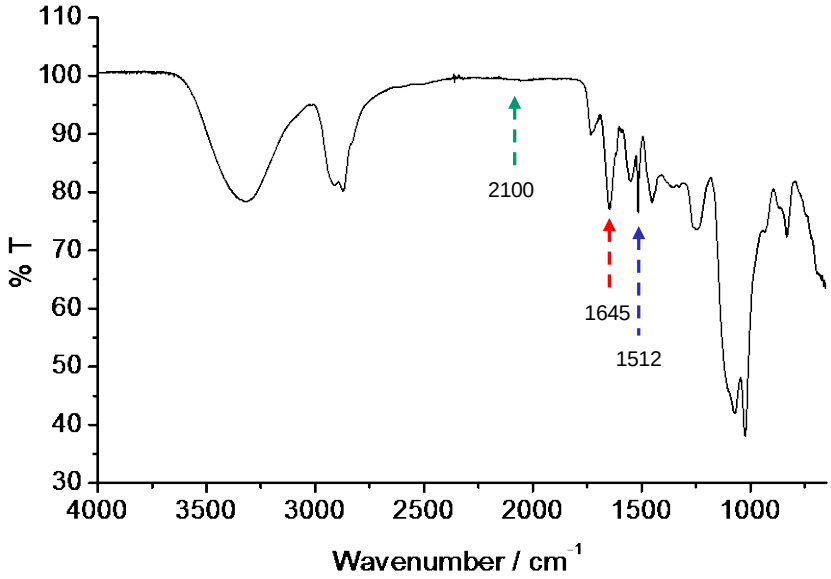
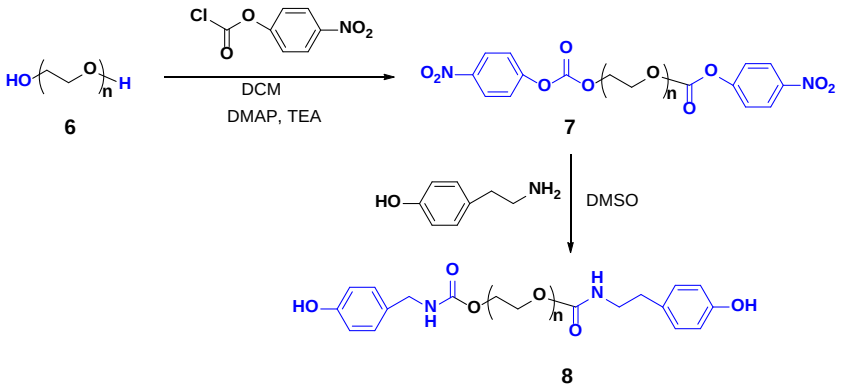


Figure S14. IR spectra of a typical dPG-HPA (here corresponds to dPG-8%NH_2): the appearance of bands at 1512 (blue dash line) and 1645 cm^{-1} (red dash line) representing the formation of amide bonds from the conjugation of HPA molecules.

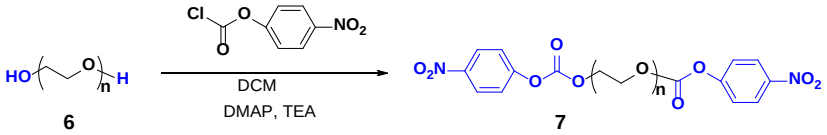
1.3. Synthesis of PEG-TG

PEG-TG was synthesized according to the modified procedure,[6, 7] by activating the terminal hydroxyl groups of PEG, and subsequently conjugating TA to the amine reactive PEG-PNC, as shown in **Scheme S7**.



Scheme S7. Synthetic route of PEG-TA.

1.3.1. The preparation of poly(ethyleneglycol)-*p*-nitrophenyl carbonate ester (PEG-PNC)



Scheme S8. Synthesis of PEG-PNC.

Briefly, 10 g PEG (1.67 mmol) was dried under vacuum for 12 hours at 60 ^{o}C in a 250 mL three-necked flask. After drying, the flask was cooled down by an ice-bath, then 100 mL abs. dichloromethane (DCM) was added to dissolve PEG. The PEG solution was then pre-mixed with catalytic amount of DMAP and 1 mL TEA for 20 minutes. PNC (5 mmol) solution in abs DCM was slowly and dropwise added into the mixture under the protection of argon. The reaction was carried out for 12 hours, and then the mixture was filtrated to remove salts. The residue was precipitated in cold diethyl ether three times, and the resulting PEG-PNC product was obtained with 82% yield, further dried under vacuum before use. **^{1}H NMR** (500 MHz, CD_3Cl_3, δ): 8.25 – 7.30 (d, CH=CH, Ar); 3.56 (s, CH_2CH_2, PEG backbone).

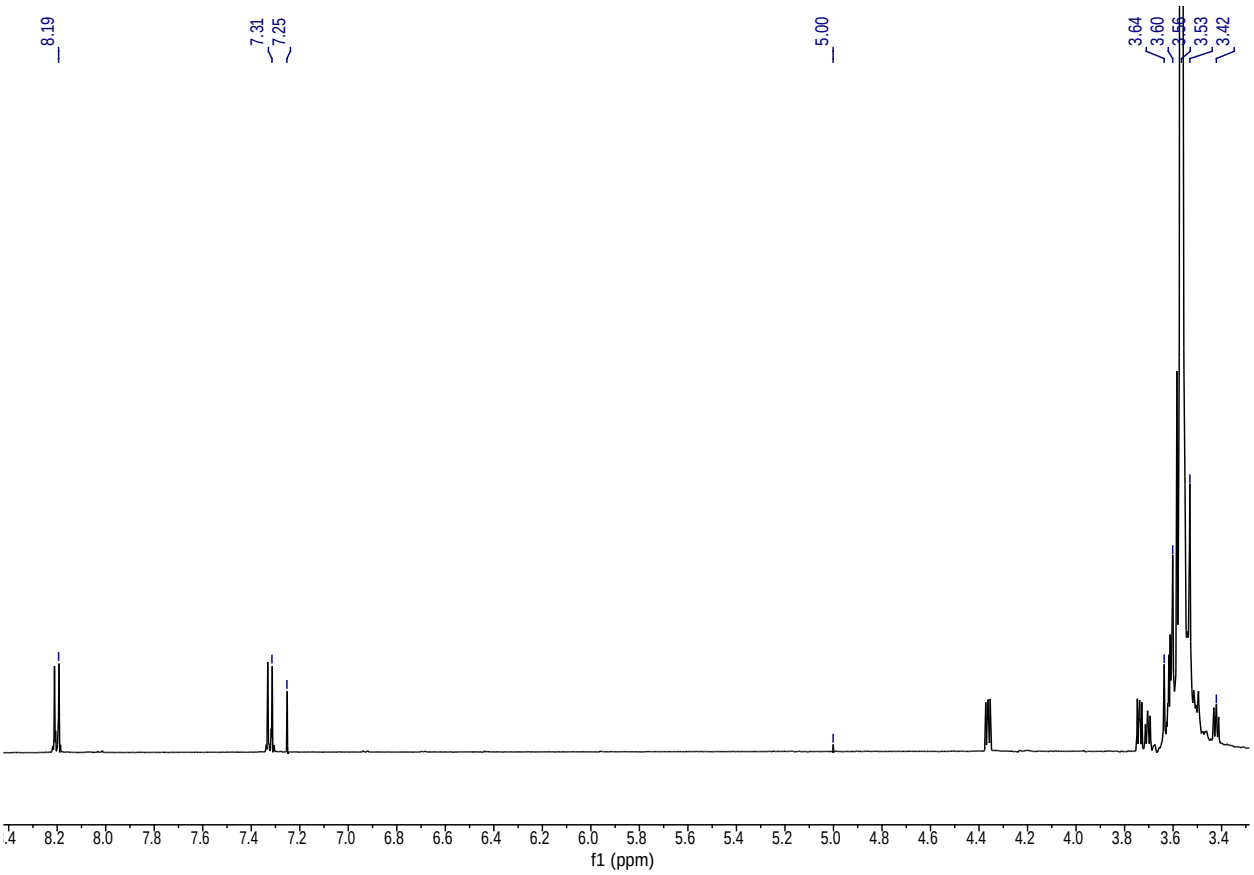
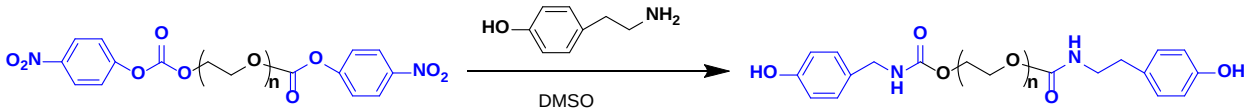


Figure S15. ^{1}H NMR spectra of PEG-PNC (500 MHz, $CDCl_3$).

1.3.2. The preparation of poly(ethyleneglycol)-tyramine (PEG-TA)



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Scheme S9. Synthesis of PEG-TA.

Typically, to a 100 mL one-necked flask, 8 g PEG-PNC (1.33 mmol) was dissolved in 50 mL DMSO. Subsequently, 273 mg tyramine (TA, 1.5 eq.) was added into the mixture and the reaction was stirred for 12 hours at room temperature. The resultant mixture was precipitated in cold diethyl ether three times, followed up with dialysis in MeOH. The product was dried in

vacuum before use and confirmed by ^{1}H NMR. **^{1}H NMR** (500 MHz, DMSO-d_6): 7.00 - 6.62 (d, CH=CH, Ar); 3.51 (s, CH_2CH_2, PEG backbone).

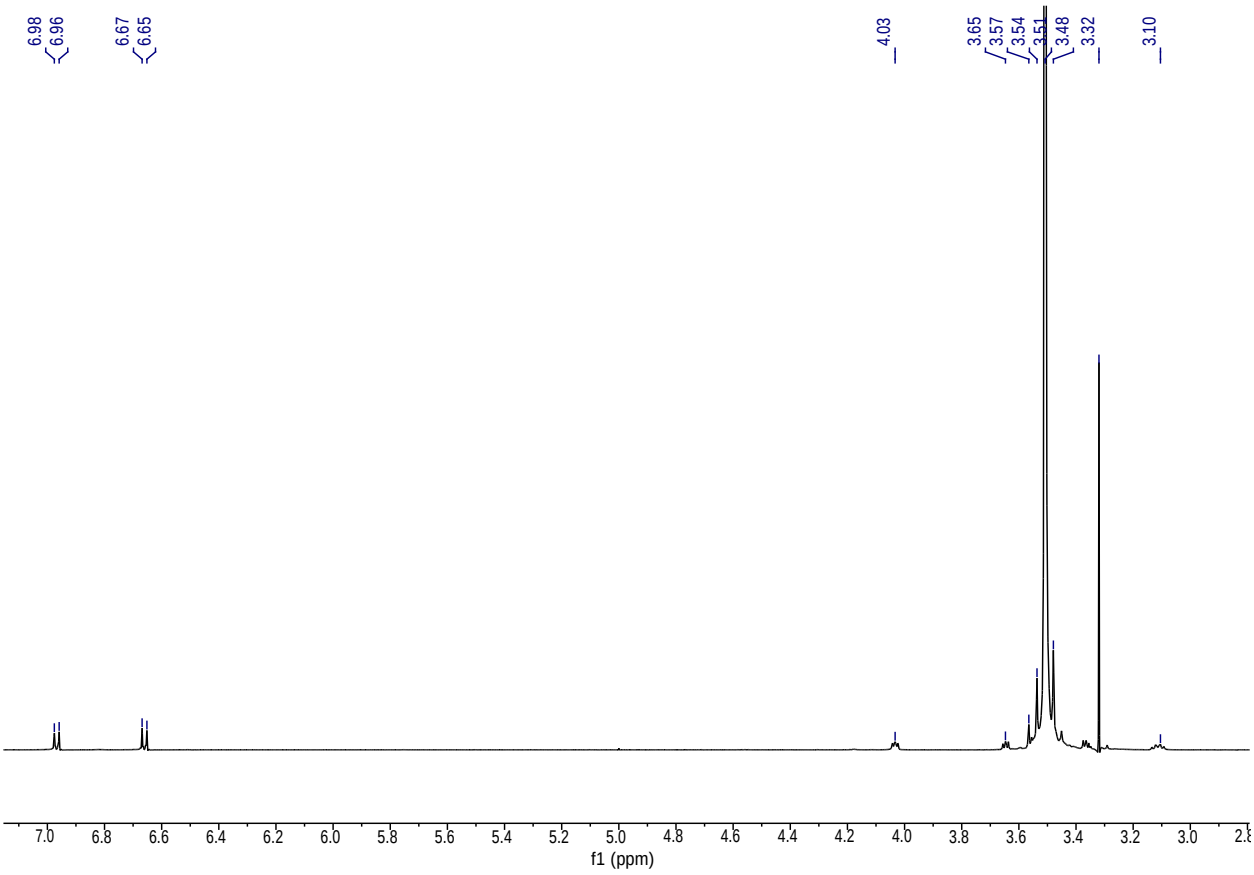

Figure S16. ^{1}H NMR spectra of PEG-TA (500 MHz, DMSO-d_6).

2. Nanogel characterization

dPG nanogels were characterized by cyro-TEM and DLS, respectively.

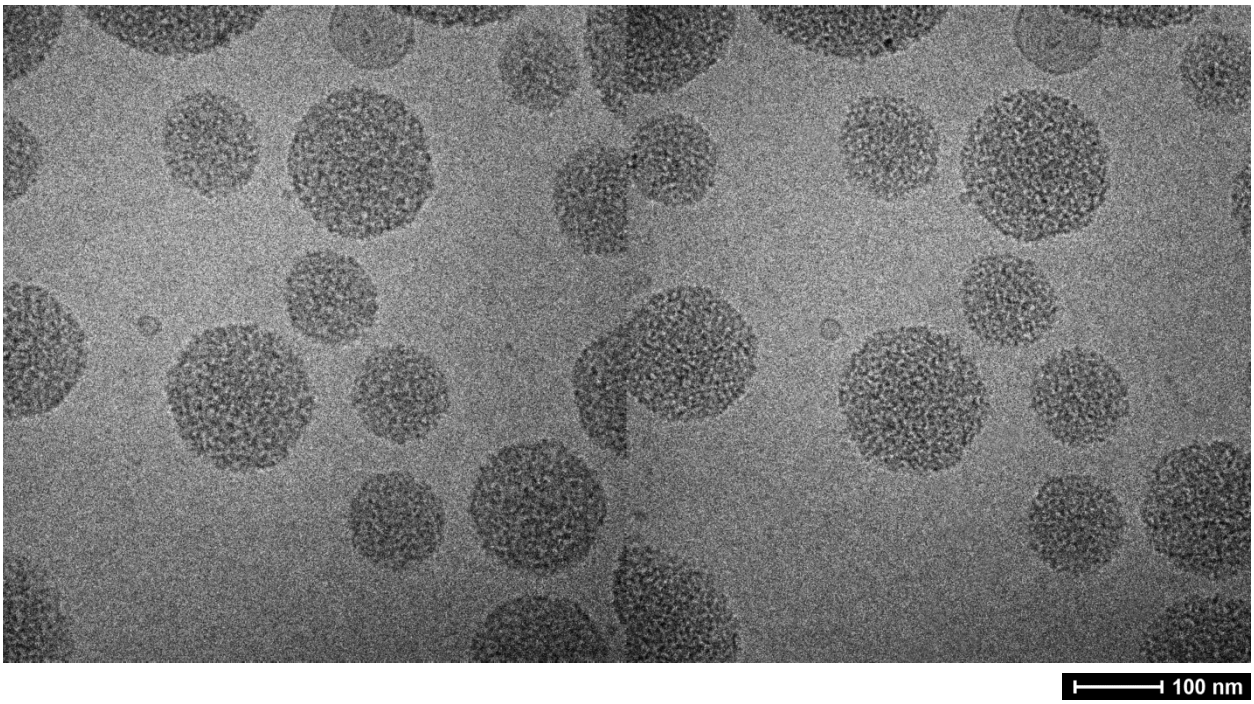

Figure S17. Stereo cryo-TEM image pairs (8° tilt increment) of a dPG nanogel. The spherical shape, the porous network character of the particles and the native volume distribution in the frozen (i.e. vitrified) solvent matrix are clearly visible. The crosslinking conditions are 100 mg/mL dPG-2%HPA, 0.25 mg/mL HRP, and 14 mM H_2O_2.

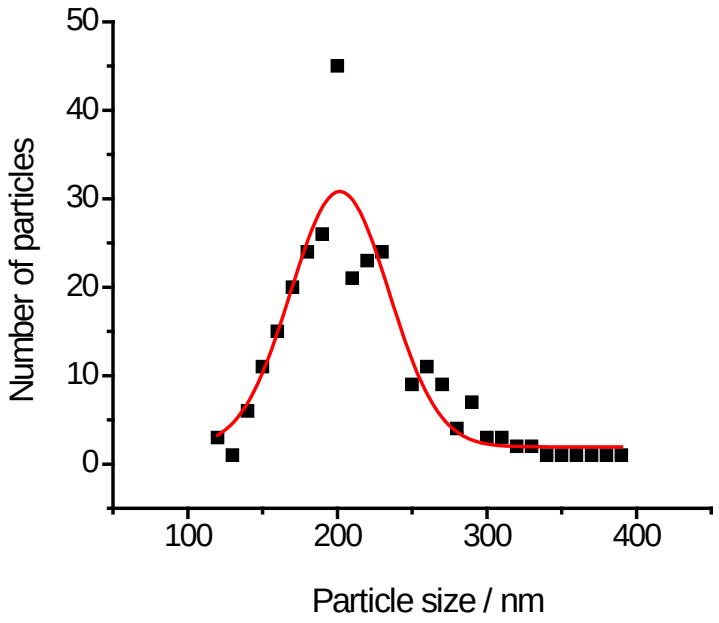

Figure S18. Particle size distribution of dPG nanogels which was obtained by measuring 275 particles from twelve different cryo-TEM images using of ImageJ software (National Institute of Health, USA). The data were fitted by NLFit (Gauss) in OriginPro8 software.

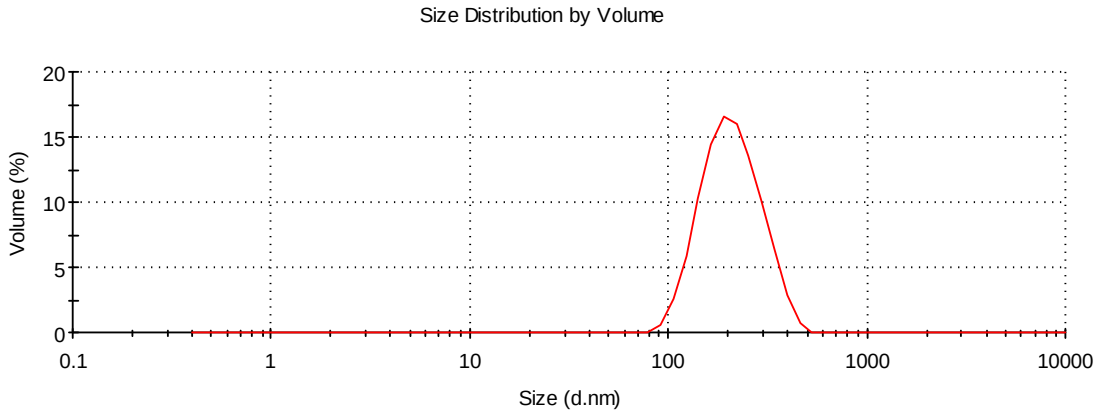

Figure S19. DLS data of dPG nanogels used to encapsulate both HRP and CalB. The crosslinking conditions are 100 mg/mL polymer, 14 mM H_2O_2, 1 mg/mL HRP, and 0.6 mg/mL CalB, respectively.

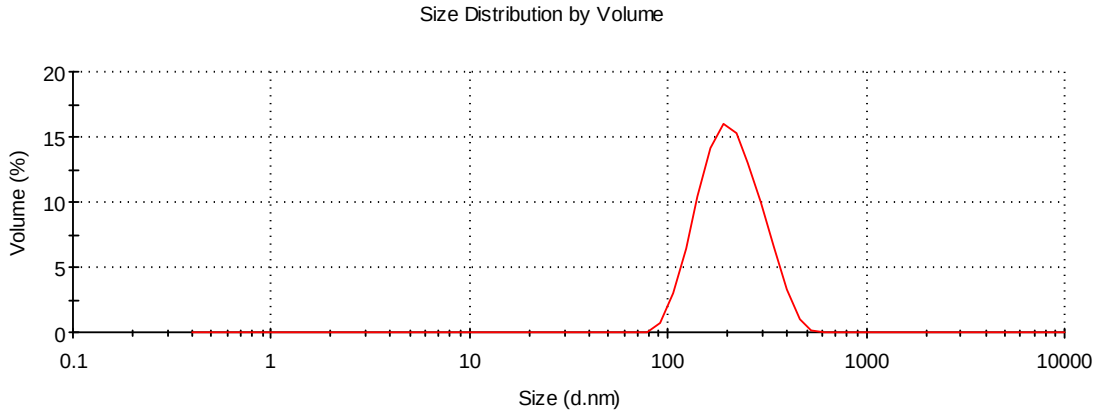

Figure S20. DLS data of dPG nanogels used to encapsulate both HRP and CalB at 72 mM H_2O_2. The other crosslinking conditions are 100 mg/mL polymer, 1 mg/mL HRP, and 0.6 mg/mL CalB, respectively.

3. H_2O_2 detection

The H_2O_2 detection was performed by Pierce™ quantitative peroxide assay kits according to the previous used procedure.[3] Typically, nanogels were formed in an inverse miniemulsion with 100 mg/mL dPG-2%HPA, 0.25 mg/mL HRP, and 14 mM H_2O_2. After 2 hours, the residual solvents and surfactants in emulsion were first washed several times with cyclohexane by centrifugation. Nanogels were then further washed by MeOH and water to transfer nanogels into water. All MeOH and water that were used for the washing were collected, and subsequently a fraction of mixture was subjected to the H_2O_2 assay. On the other hand, in order to double-check H_2O_2 consumption in the same crosslinking conditions as dPG nanogels but without emulsion, a volume of 200 uL dPG hydrogels were enzymatically formulated accordingly. Then 1 mL water

was added into the hydrogels to extract residual H_2O_2. After 2 hours, the residual H_2O_2 content from hydrogels were determined by H_2O_2 assay.

The H_2O_2 assay was operated at room temperature. In general, 200 µL fraction of solution that was withdrawn from the incubation solution of nano- and hydrogels was thoroughly mixed 1 mL working reagent (WR) solution. After 20 min, the mixture was subjected to Uv-vis, recorded at 560 nm. The amount of residual H_2O_2 content was calculated from the two ranges of calibration curves with 0.1 – 1 µM and 0.5 – 10 µM, respectively (**Figure S21**).

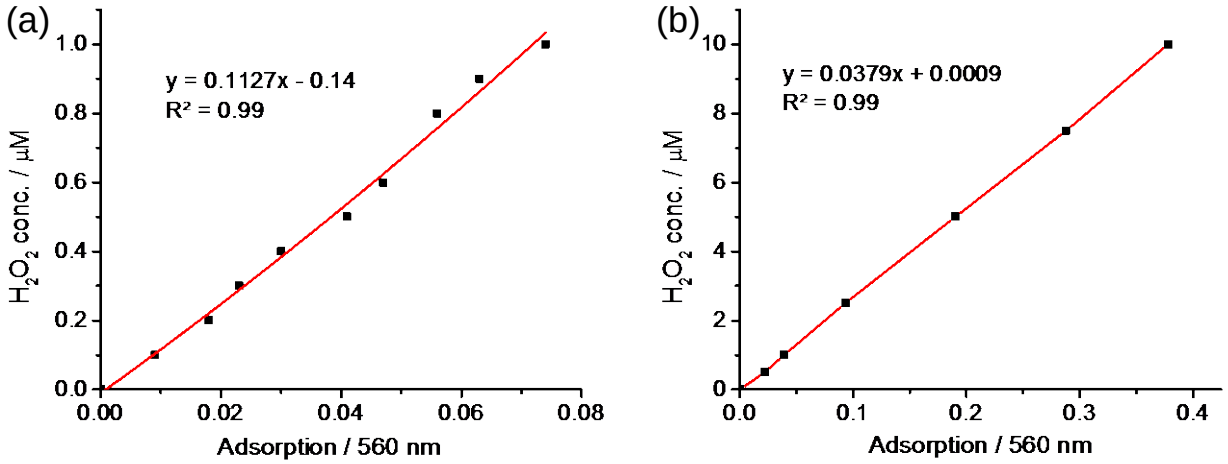

Figure S21. Calibration curves for H_2O_2 determination in the range of a) 0.1 – 1 µM and b) 0.5 – 10 µM, respectively.

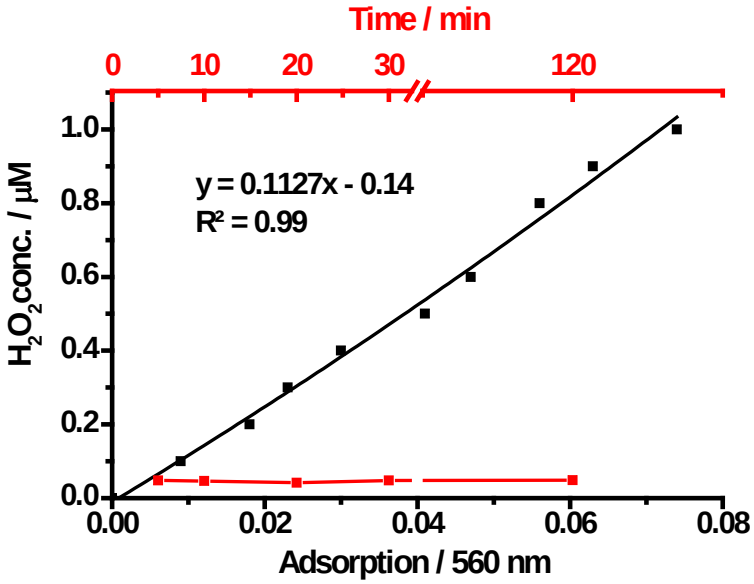

Figure S22. Two-hour real time monitoring of residual H_2O_2 level (red dots) from the hydrogels that were crosslinked with the same condition as nanogels (100 mg/mL dPG-2%HPA, 0.25 mg/mL HRP, and 14 mM H_2O_2).

4. Calculation of the half life time of HRP

The HRP thermal stability is reflected by its half-life ($t_{1/2}$) time, which is defined as, in the half-life time, 50% of initial HRP activity retains under defined condition. The half-life time can be calculated form **Equation S1**:[8, 9]

$$\frac{E(t)}{E(0)} = exp^{-kt} \qquad \textbf{(Equation S1)}$$

Where E(t) is the HRP activity at any time t, E(0) is the HRP initial activity, thus E(t)/E(0) is as a function of chronological time during the continuous operation, for example, incubation at 50 °C; k is the rate constant of deactivation (h^{-1}).

When t = $t_{1/2}$, the Equation S1 can be simply deduced to **Equation S2**:[8-10]

$$t_{1/2} = \frac{\ln 2}{k} \qquad \textbf{(Equation S2)}$$

According to Equation S1, data from the Figure 2b can be re-plotted and linearly fitted as **Figure S23.**

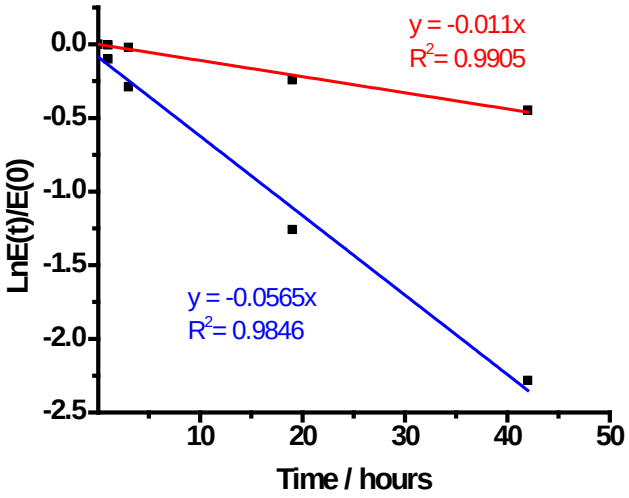

Figure S23. Linearly fitted graphs of data in Figure 2b by Equation S1, where blue line represents the fitting of native HRP, and the red line shows the fitting of immobilized HRP.

Based on the fitted data in Figure S23, k for native HRP and immobilized HRP at 50 °C are 0.0565 and 0.0110, respectively. Therefore, the half-life of native HR is calculated by Equation S2 as approx. 12 hours, while that of HRP encapsulated in nanogels is approx. 67 hours at 50 °C.

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