Supporting Information

Bioinspired adenine-dopamine immobilized polymer hydrogel adhesives for tissue engineering

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Experimental Section

Materials

Adenine and triethylamine was obtained from Tokyo chemical industry (TCI); dopamine hydrochloride, 2-bromoethylamine, trifluoroacetic acid, N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), methyl trifluoroacetate, 2,2-dimethoxypropane, polyacrylic acid (450 KDa) and alginic acid (160 kDa) were purchased from Merck (Sigma) and used without further purification. Dichloromethane (DCM), chloroform, methanol, petroleum ether, dimethylformamide, benzene and acetonitrile (ACN) were purchased from VWR Chemicals. Deuterated DMSO-d_6, D_2O, MeOD-d_4 and $CDCl_3$ were obtained from Cambridge Isotope Laboratories for ^{1}H NMR and ^{13}C NMR spectroscopic studies.

Methods

^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker DRX 400 NMR and Bruker AV 500, respectively, using DMSO-d_6, D_2O and MeOH-d_4 as solvents. Mass spectroscopy of the compounds was performed with Bruker Autoflex III TOF/TOF MALDI analyzer. Optical properties of the compounds were investigated using Shimadzu UV-VIS-near IR Spectrophotometer and Agilent Cary Eclipse Fluorescence Spectrophotometer. Morphologies of the gels were investigated by using JEOL JSM-6701F Field Emission Scanning Electron Microscopy (FE-SEM) after casting the freeze-dried gel on a carbon tape directly and coating with a conducting layer of platinum.

Synthesis

Compounds 1-3,[1] and dopamine acetonide[2] were synthesized and characterized via previously reported procedure (SI Scheme 1).

Synthesis of 8-bromo-9-N-bocethylamineadenine (4)

N-Bocethylamine adenine (1.5 g, 5.39 mmol) was dissolved in DMF (25 mL) under N_2 atmosphere and N-Bromosuccinimide (1.92 g, 10.78 mmol) was subsequently added in the reaction mixture. The reaction mixture was allowed to stir at room temperature for 48 hr while the color of the reaction turned red with time. After completion of the reaction, DMF was removed under reduced pressure and the crude product was purified on a silica gel column with EtOAc: hexane (80:20) as an eluent.Yield = 0.780 g (40%). ^{1}H NMR (500 MHz, MeOD-d_4, δ) : 8.17 (s, 1H), 4.31 (t, 2H), 3.48 (t, 2H), 1.30 (s, 9H). ^{13}C NMR (126 MHz, MeOD-d_4, δ): 158.14, 156.10, 153.76, 152.41, 129.10, 120.72, 80.14, 45.78, 40.26, 28.62. HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{12}H_{17}BrN_6O_2$, 357.0667; found, 357.0669 (^{79}Br), 359.0649 (^{81}Br).

Synthesis of tert-butyl (2-(6-amino-8-((2-(2,2-dimethylbenzo[d][1,3]dioxol-5-yl)ethyl)amino)-9H-purin-9-yl)ethyl)carbamate (5)

Compound 4 (0.4 g, 1.12 mmol) was dissolved in butanol and excess dopamine acetonide (1.95 g, 10.08 mmol) was added in the mixture under N_2. Triethylamine (0.56 g, 5.60 mmol) was added in the reaction vessel and mixture was heated at 100 °C for 72 hr. The product formation was monitored using thin layer chromatography (TLC). After 72 hr, the reaction was stopped, cooled to room temperature and excess solvent was removed under reduced pressure. The crude mixture was dissolved in EtOAc (50 mL), washed with 10% aqueous citric acid (2 x 30 mL), aqueous $NaHCO_3$ (2 x30 mL), brine (2 X 30 mL) solutions, followed by water (2 x 30 mL), the organic fraction was dried over anhydrous sodium sulfate, filtered and excess solvent was removed under reduced pressure. Pure compound was obtained after column chromatography using EtOAc/hexane [95:5] as an eluent, yield = 0.13 g (25%). ^{1}H NMR (500 MHz, MeOD-d_4, δ): 7.99 (s, 1H), 6.71 – 6.67 (m, 2H), 6.63 (d, J = 7.7 Hz, 1H), 4.03 (t, J = 6.0 Hz, 2H), 3.62 (t, J = 7.4 Hz, 2H), 3.33 (t, J = 5.7 Hz, 2H), 2.92 (t, J = 7.4 Hz, 2H), 1.61 (s, 6H), 1.32 (s, 9H). ^{13}C NMR (126 MHz, MeOD-d_4, δ): 158.35, 153.72, 153.05, 151.52, 150.03, 148.97, 147.40, 133.71, 122.32, 118.70, 117.85, 110.00, 108.94, 80.28, 45.68, 41.87, 39.73, 36.31, 28.63, 25.90. HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{23}H_{31}N_7O_4$, 470.2510; found, 470.2513.

Synthesis of 4-(2-((6-amino-9-(2-aminoethyl)-9H-purin-8-yl)amino)ethyl)benzene-1,2-diol (6)
Compound 5 dissolved in trifluoroacetic acid (TFA)/ dichloromethane (DCM) (2 mL, 1:1) mixture and H_2O (0.1 mL) was added and the mixture was stirred for 5-6 hr at room temperature. Product formation was monitored by TLC. After completion of the reaction, volatile solvents were removed under reduced pressure, to obtain a white precipitate on addition of diethyl ether, Yield = 0.070 g (90%). ^{1}H NMR (500 MHz, MeOD-d_4, δ):8.22 (s, 1H), 6.71 – 6.67 (m, 2H), 6.58 (dd, J = 8.0, 2.1 Hz, 1H), 4.39 (t, J = 5.6 Hz, 2H), 3.67 (t, J = 7.3 Hz, 2H), 3.36 – 3.32 (m, 2H), 2.88 (t, J = 7.3 Hz, 2H). ^{13}C NMR (126 MHz, MeOD-d_4, δ):154.41, 151.36, 148.30, 146.33, 144.91, 144.26, 131.77, 121.12, 117.02, 116.42, 49.51, 49.34, 49.17, 49.00, 48.83, 48.66, 48.49, 45.76, 40.32, 39.49, 35.42. HRMS-ESI (m/z): $[M+H]^+$ cacld for $C_{15}H_{19}N_7O_2$, 330.1673; found, 330.1671.

Polymer functionalization

Polymer (PAA – 450 KDa or Alg – 160 Kda, 1 g) was dissolved in distilled water (100 mL) to give a concentration of 1% w/v and purged with N_2 for 30 min. Subsequently, 10 eq. (with respect to the total weight of polymer) of N-hydroxysuccinimide (NHS) followed by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 10 eq.) were added to the polymer solution and stirred for 1-2 hr at room temperature. Adenine derivative (**3 or 6**, 10eq. with respect to the total weight of polymer) was added to the mixture, stirred for 15 hr at room temperature and a pH 5.5 was maintained to prevent the oxidation of catechol moiety. The reaction mixture was dialyzed (MW cut off – 3000) for 4 days against distilled water. The dialyzed solution was collected and freeze-dried to give a white powder. The functionalization was confirmed by ^{1}H NMR and UV-Vis spectroscopy. Full characterization data for all polymers (**P1** – **P4**) are given in the supporting information.

Hydrogel synthesis

The modified polymer (0.1 g) was dissolved in PBS to give the final concentration of 5 % (w/v). After the complete dissolution, methylenebisacrylamide (BAAm, 0.0062 g) was added to the solution at 0.31 % w/v followed by ammonium persulfate (APS, 0.0125 g) at 0.62 % w/v. The mixture was stirred under N_2 atmosphere for 1 hr, transferred to a mold and kept at 70 °C for 6-7 hr for gelation.

Rheological studies

The rheological studies performed with the hydrogel disk samples (13 mm x 4 mm) using MCR 102 rheometer (Anton Paar) with 25 mm parallel plate geometry disc and 1 mm gap at room temperature. To determine the elastic modulus, strain sweep (γ) from 0.01-100 % employed to determine the linear viscoelastic region. Oscillatory frequency sweep was then performed (0.1 – 100 rad/s) with a constant shear strain of 1 % within the linear strain regime. Time sweep was performed at a fixed frequency of 1 Hz and 1 % strain to record the values as a function of time for 5 min.

Swelling behavior

The swelling behavior of the hydrogel was determined using an established method in the literature.[3] The gels were soaked into the PBS buffer and the weight of the swollen gel was measured after certain intervals until it reached equilibrium. The swelling ratio (%) is calculated by the following equation.

$$Swelling\ ratio\ [\%] = \frac{W_s - W_i}{W_i} X\ 100$$

Where W_s and W_i are the weights of swollen and initial gel, respectively.

Adhesion studies

For visual adhesion test to various substrates such as pieces of metal, plastic, leaf, wood and pork skin were selected, hydrogel was placed on top of them for 5 s, pulled up with the help of forceps and checked

the adhesion by monitoring the movement of surfaces with the hydrogel. Adhesive strength was measured using a Texture Analyser TA XT Plus (Stable Micro Systems Ltd.) with the slight modification of reported procedure for gels.[4] Briefly, hydrogel disk samples (13 mm x 5 mm) prepared was compressed and redrawn using a P/35 mm probe (Video S1). Distance mode was set for the target at the distance of 3 mm with a speed of 0.5 mm/s. The holding time from 1st cycle to 2nd cycle was 5 s. at room temperature. Trigger force was 5 g with maximum force of 2 Kg. For the adhesion test on a different substrate, probe and base of machine were pasted with substrates, to be tested, by two-way tape and height was calibrated accordingly. Adhesion, hardness and cohesive strength for the hydrogels were calculated by a force-time plot and the average of 5 readings is reported

Cell line and culture conditions

BHK-21 cells were selected for exploring the uptake and cytotoxicity investigations of pure PAA and functionalized PAA hydrogels. Experiments were conducted using RPMI 1640 cell culture medium supplemented with 10% fetal bovine serum (FBS) and 1% of antibiotic-Antimycotic 100X (Biowest) compound. The cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2 gas. After achieving the desired confluence, cells were enzymatically detached with trypsin - ethylenediaminetetraacetic acid (Trypsin-EDTA) and sub-cultured in a new T75 flask.

Cytotoxicity assay

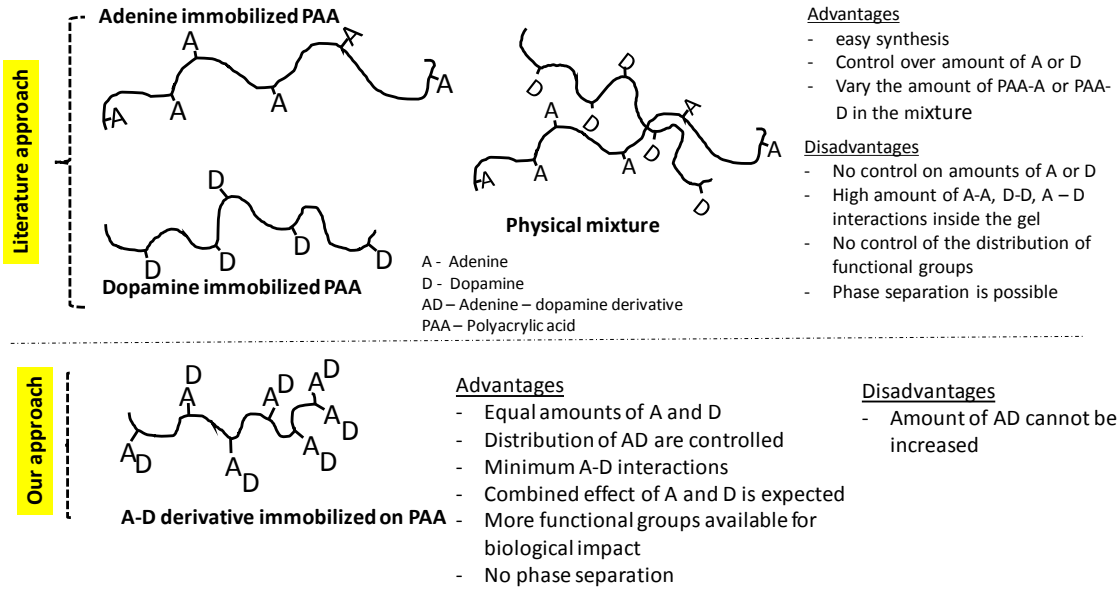
The toxicity of hydrogel or viability of BHK-21 cells was studied by evaluating the metabolic activity using Alamar blue assay (Cell proliferation assay from Promega). Briefly, hydrogel solution (60 µL) was taken in 96 well plate and kept it for gelation at 70 °C for 6 hr under sterilized environment. The obtained hydrogel was soaked with PBS for 10 minutes and excess liquid was drained and the process was repeated 3 times to remove unreacted components. BHK-21 cells (5000 cells/well) were seeded on top of the hydrogel and incubated inside an incubator for 24 hr. The control well contained no hydrogel sample. Alamar blue solution (10 µL) was added to each well, incubated for 2 hr, fluorescence intensity at 600 nm was recorded using a microplate reader using an excitation wavelength of 570 nm. Wells without cells served as the background. Viability was determined with respect to the untreated cells, where hydrogel was not present.

ATP assay

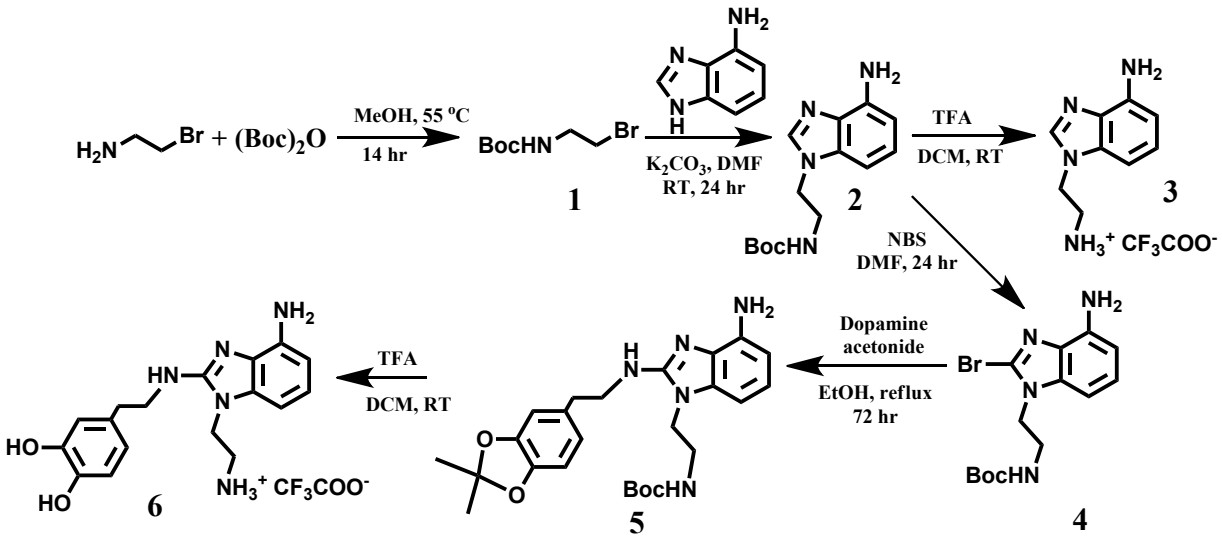
For the ATP assay, 5000 cells per well were plated and treated with 5 % w/v hydrogel in a similar way described for the cytotoxicity assay for a period of 24, 48, 72, and 120 hr. After incubation, the media was removed, and the cells were washed with PBS solution. Fresh PBS (80 µL) solution was added to the wells, followed by ATP assay reagent (20 µL). The plates were incubated for 1 hr and the fluorescence was measured using an excitation wavelength of 485 nm and an emission wavelength of 600 nm. Cells with no hydrogels were considered as controls. In this assay, ATP present in the living cells react with luciferin in the presence of luciferase enzyme and produce oxyluciferin which emits at 560 nm. Thus the observed fluorescent signal is proportional to the concentration of ATP present in the cells.

3D - Cell culture

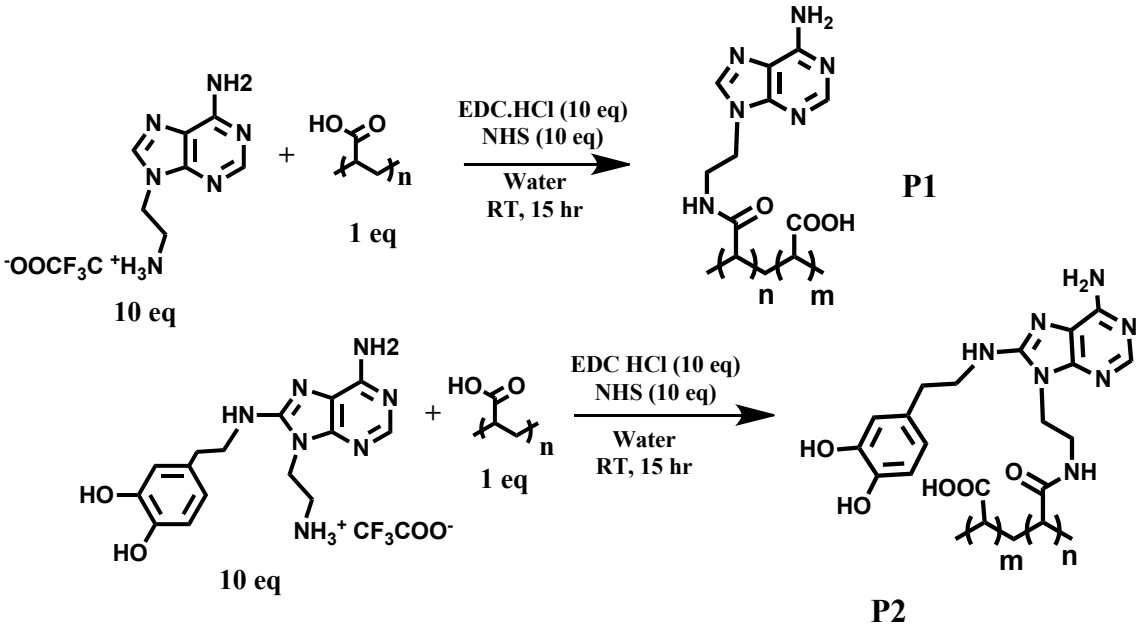
For 3D-cell culture, first hydrogel solution was prepared with all components followed by addition of cells (50,000). Hydrogel solutions with cells were mixed thoroughly to get homogeneity of the cells in the gel solution by vortex mixer. The gel solution (60 µL) with cells immersed in it was taken in each well of a 96 well plate and incubated for gelation at 37 °C for 6-7 hr. Fresh medium (1 mL) was added into the well plates with hydrogels. The cells were incubated for 24 hr at 37 °C in a humidified atmosphere with 5 % CO_2, Alamar blue (10 µL) was added, incubated for 1 hr and the absorbance was measured at 570 nm. Cell viability was calculated with respect to untreated cells.



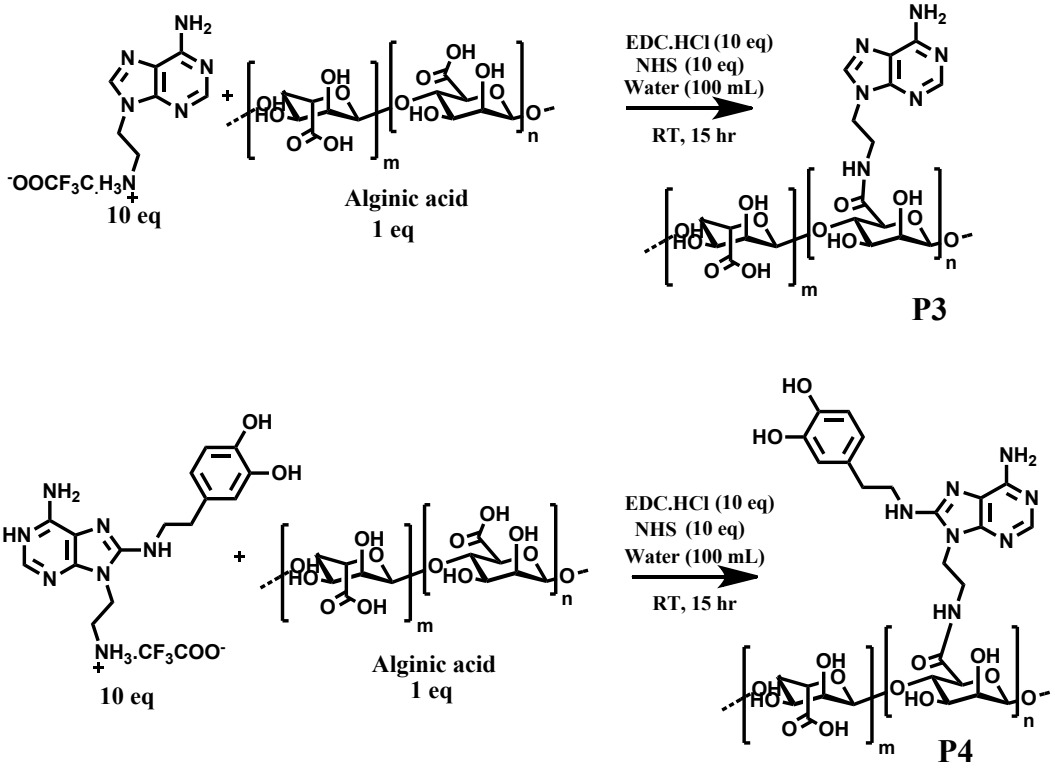
Scheme S1A: Comparison of our design strategy with known polymer based hydrogels.



Scheme S1B. Synthesis of adenine derivatives 3[1] and 6 (dopamine acetonide was synthesized using previously reported protocol).[2]



Scheme S1C. Synthesis of polymer P1 and P2 (adenine derivatives functionalization on PAA matrix using EDC/NHS coupling)[3,5] EDC = 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, NHS = N-Hydroxysuccinimide



Scheme S1D. Synthesis of polymer P3 and P4 (adenine derivatives functionalization on Alg polymer via EDC/NHS coupling)[3, 5]

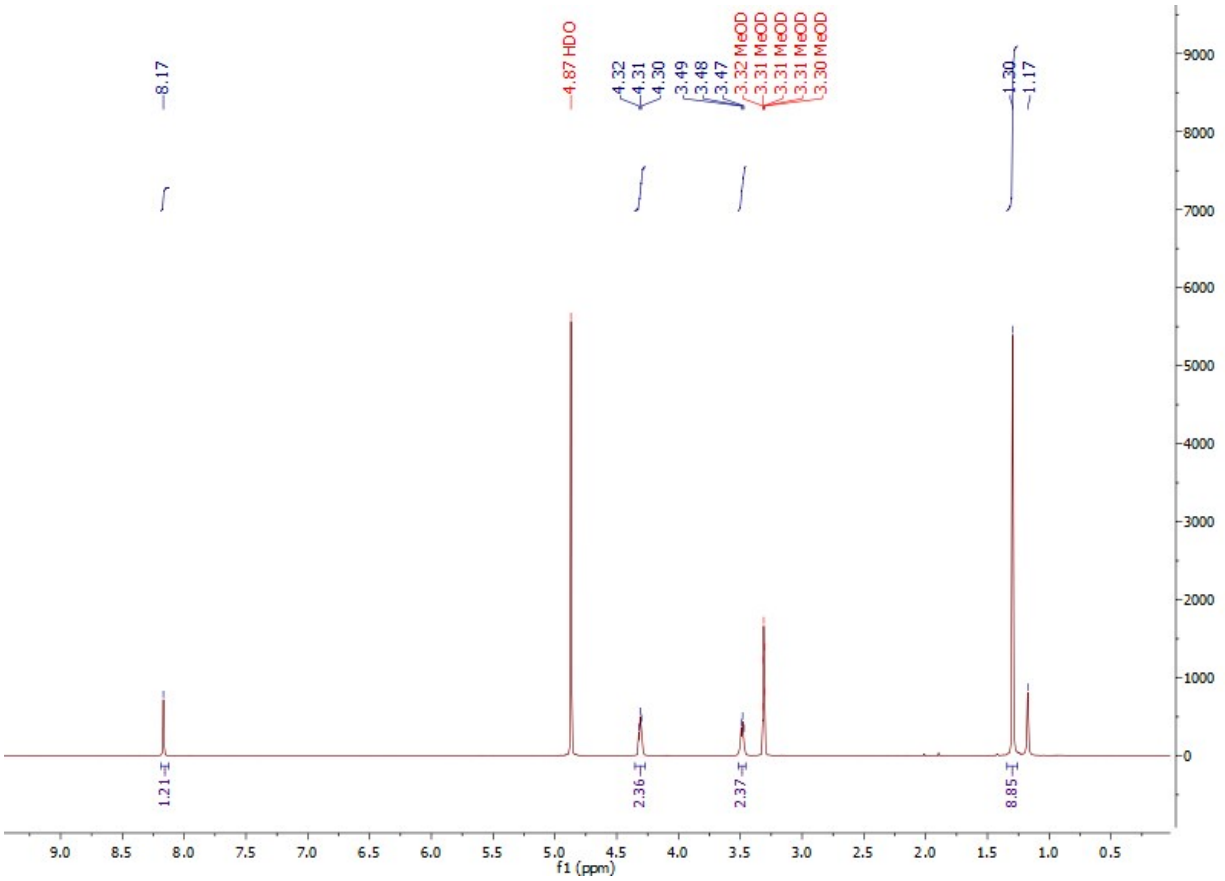


Figure S1. ^{1}H NMR (500 MHz) of 4 in MeOD-d_4.

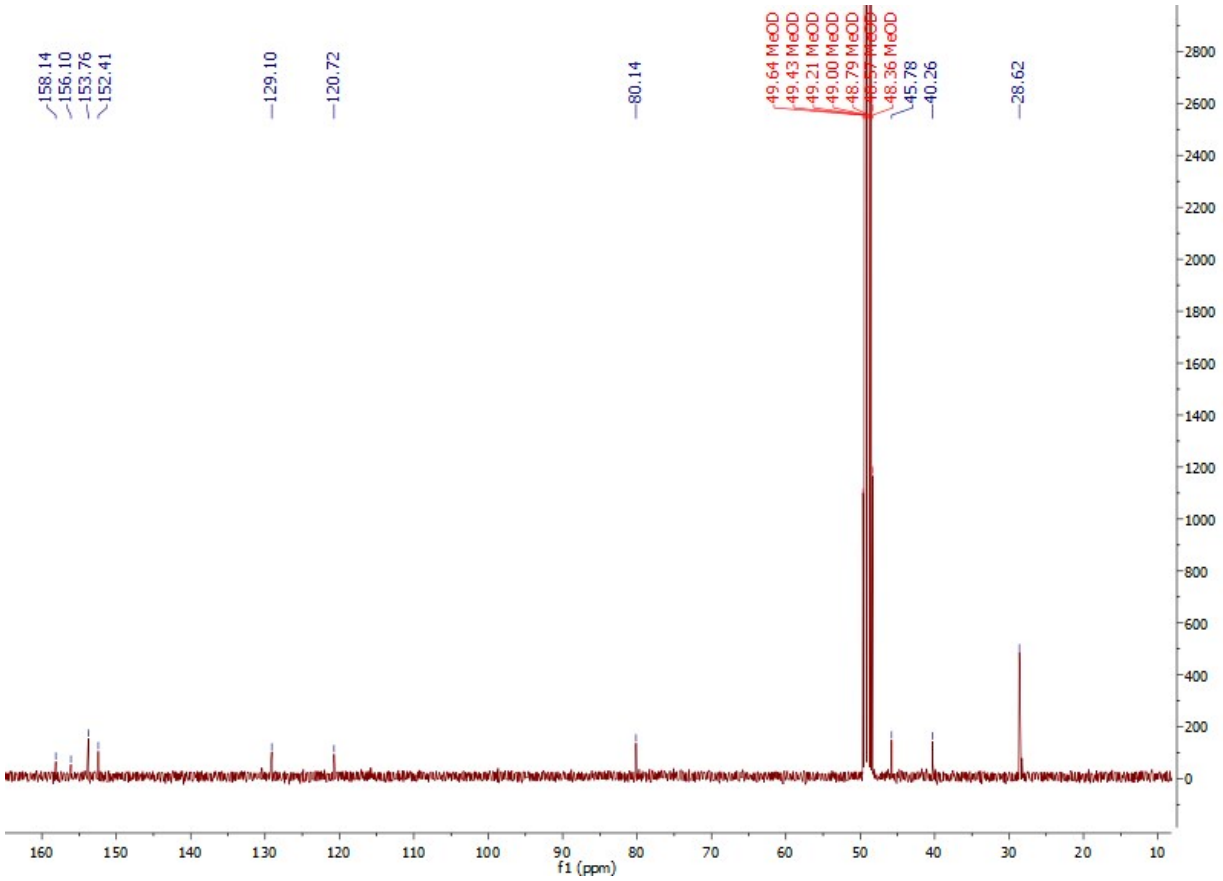


Figure S2. ^{13}C NMR (500 MHz) of 4 in MeOD-d_4.

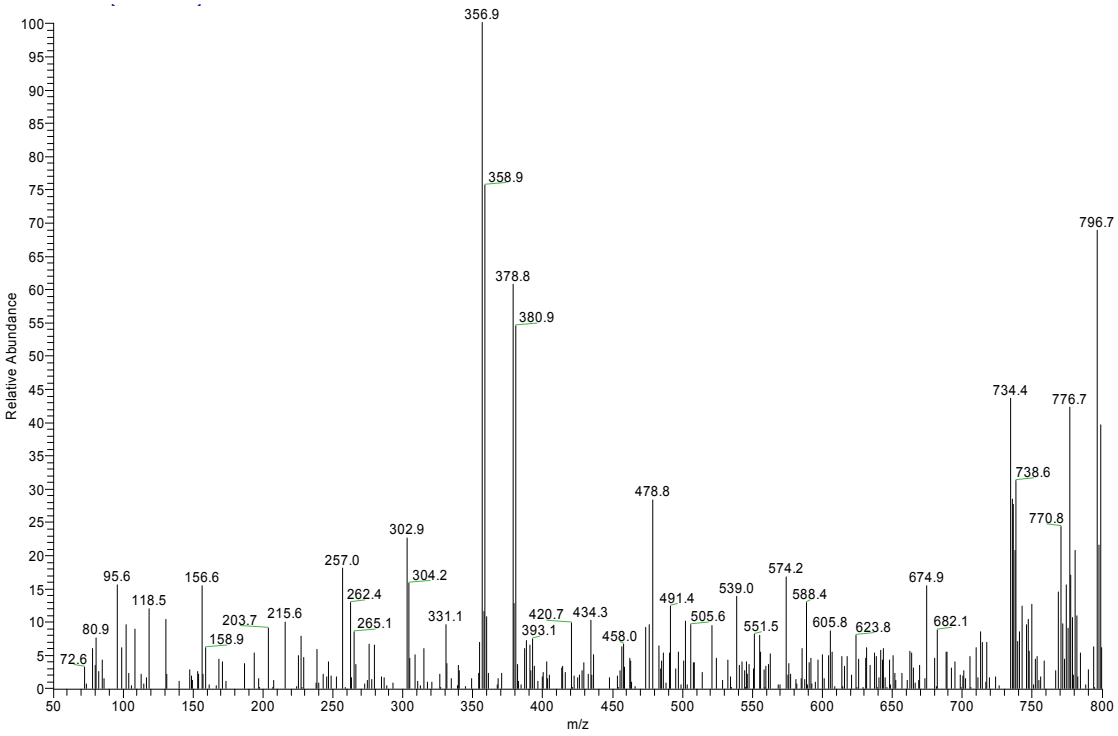


Figure S3A. ESI spectrum of 4.

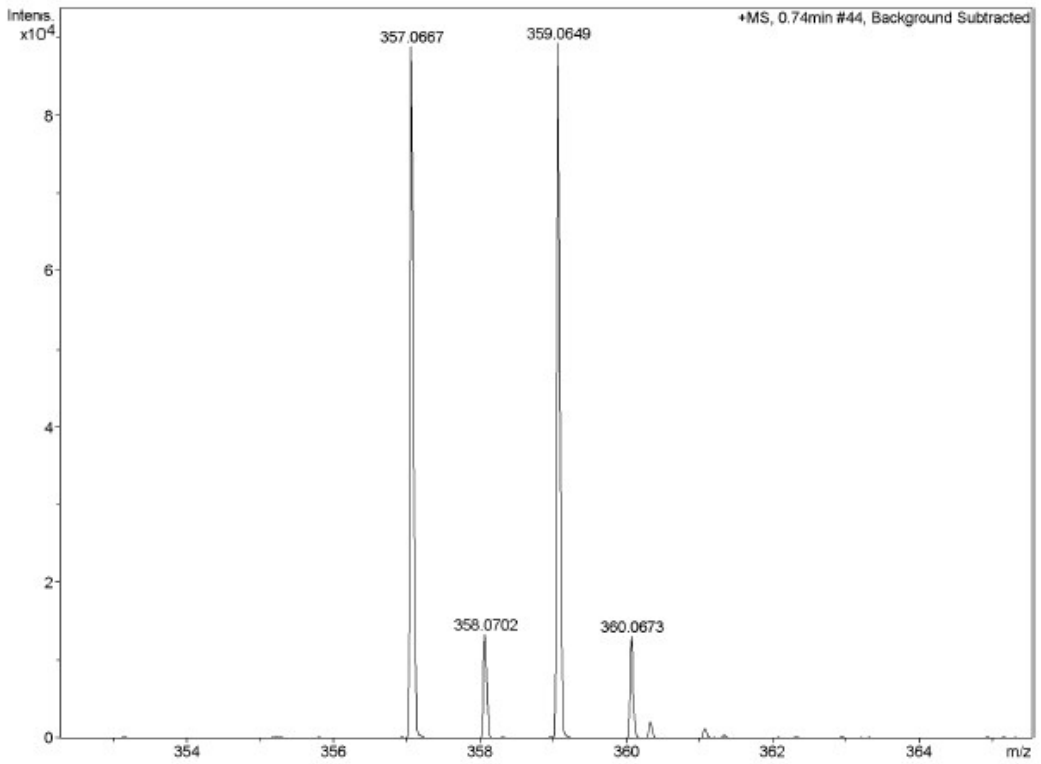


Figure S3B. HRMS-ESI spectrum of 4.

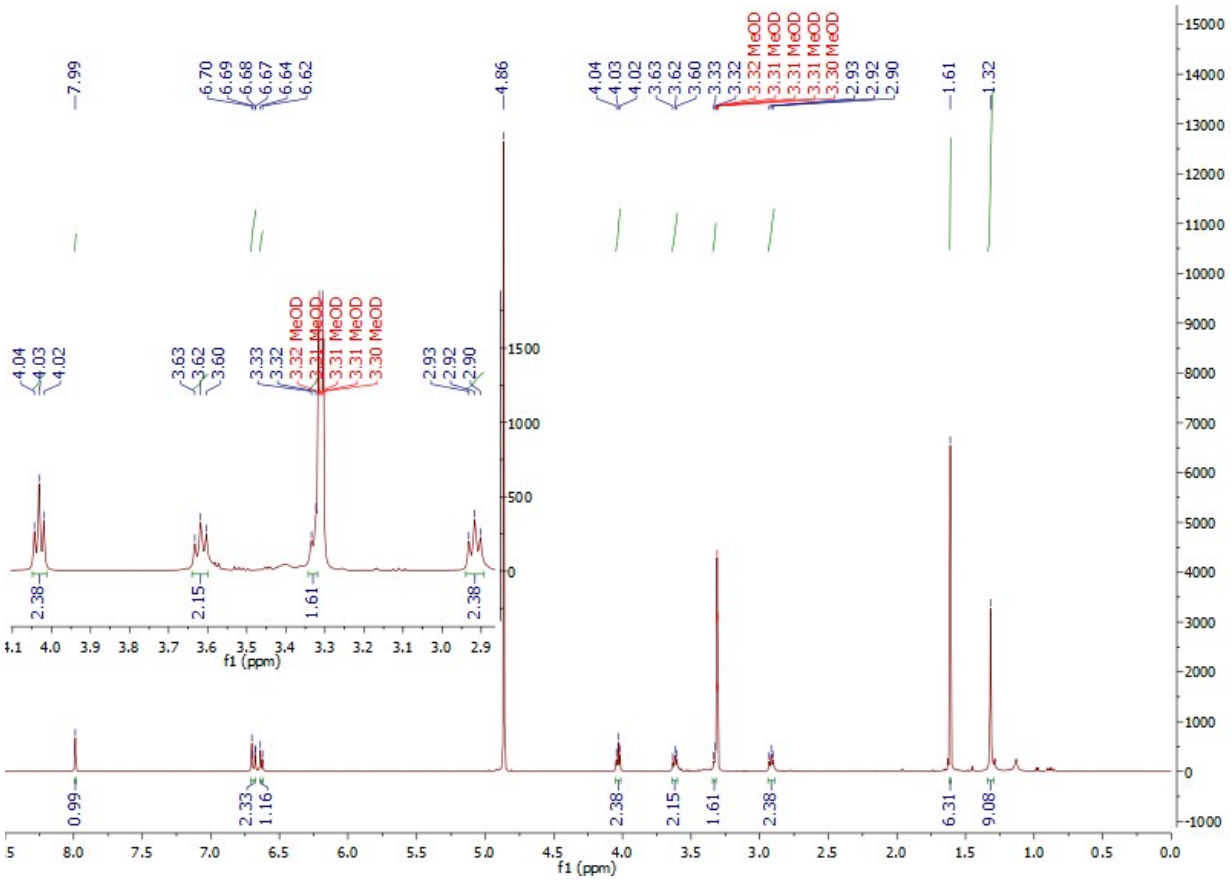


Figure S4. ^{1}H NMR (500 MHz) of 5 in MeOD-d_4.

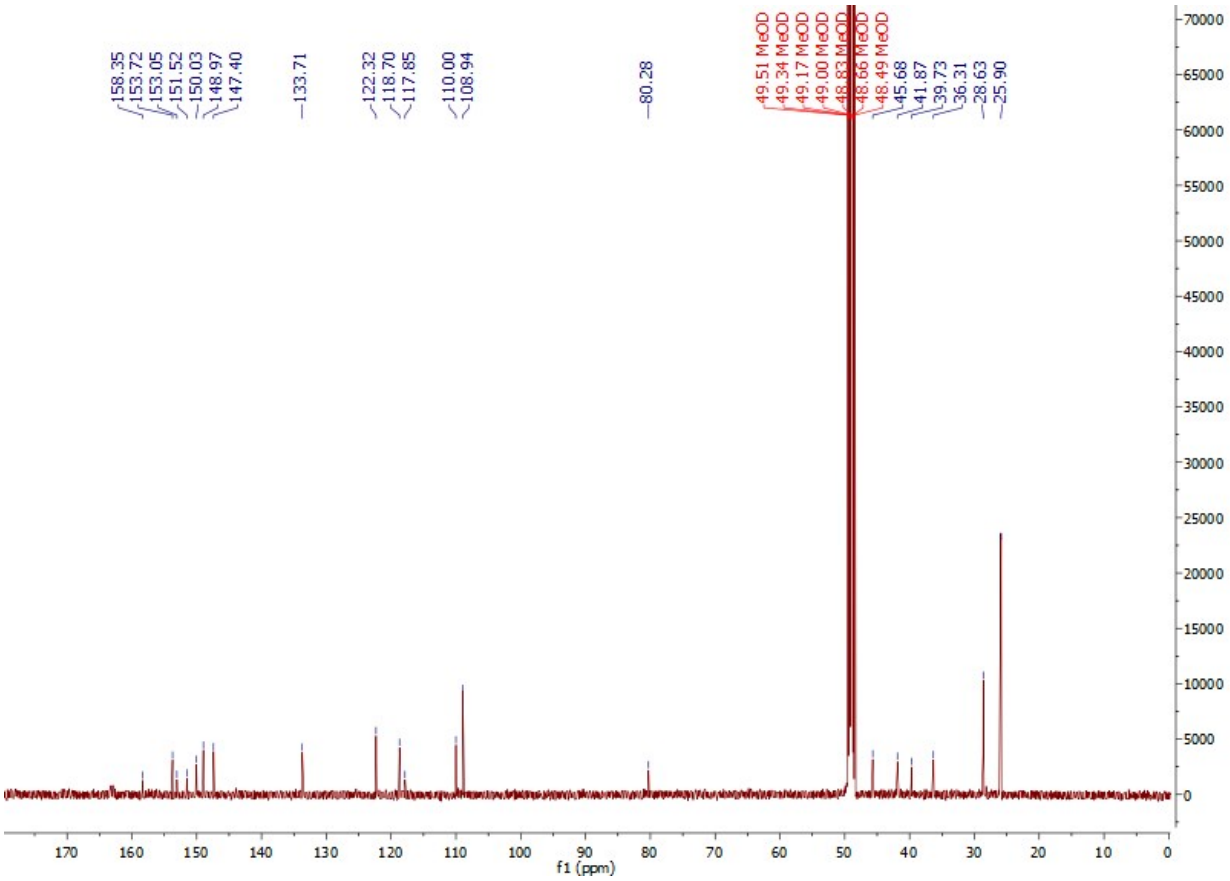


Figure S5. ^{13}C NMR (500 MHz) of 5 in MeOD-d$_4$.

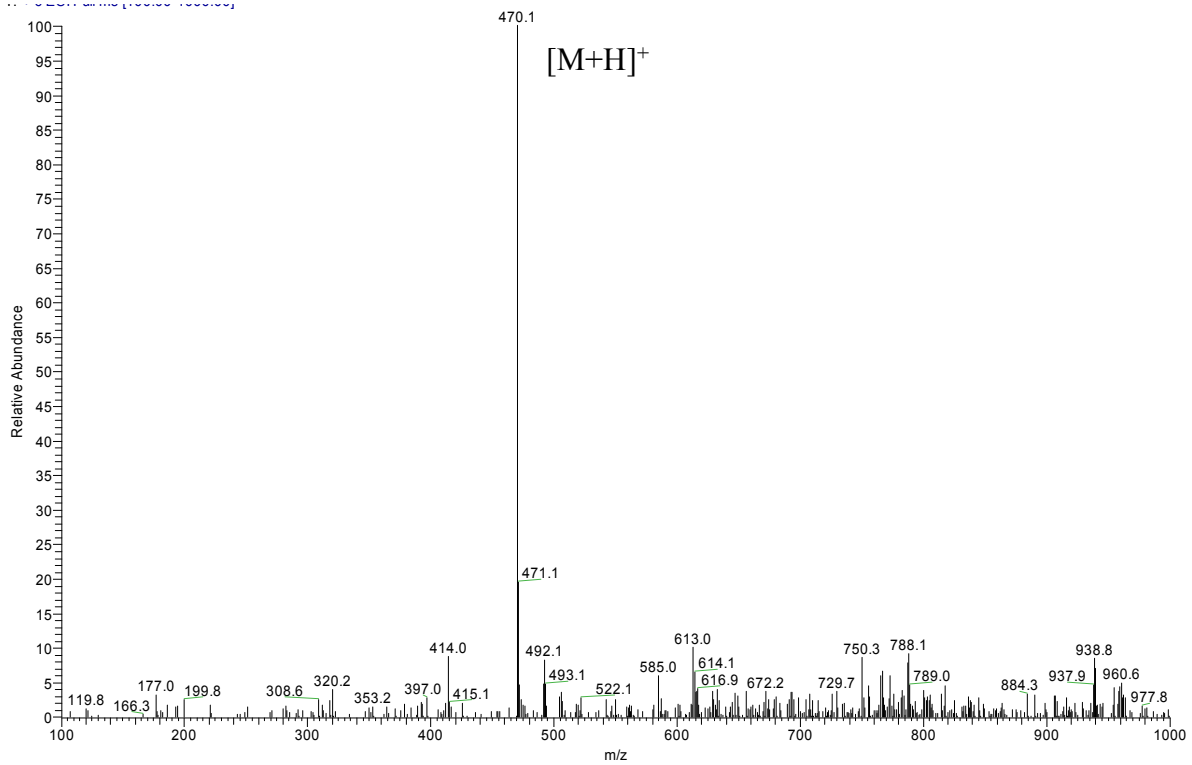


Figure S6A. ESI spectrum of 5.

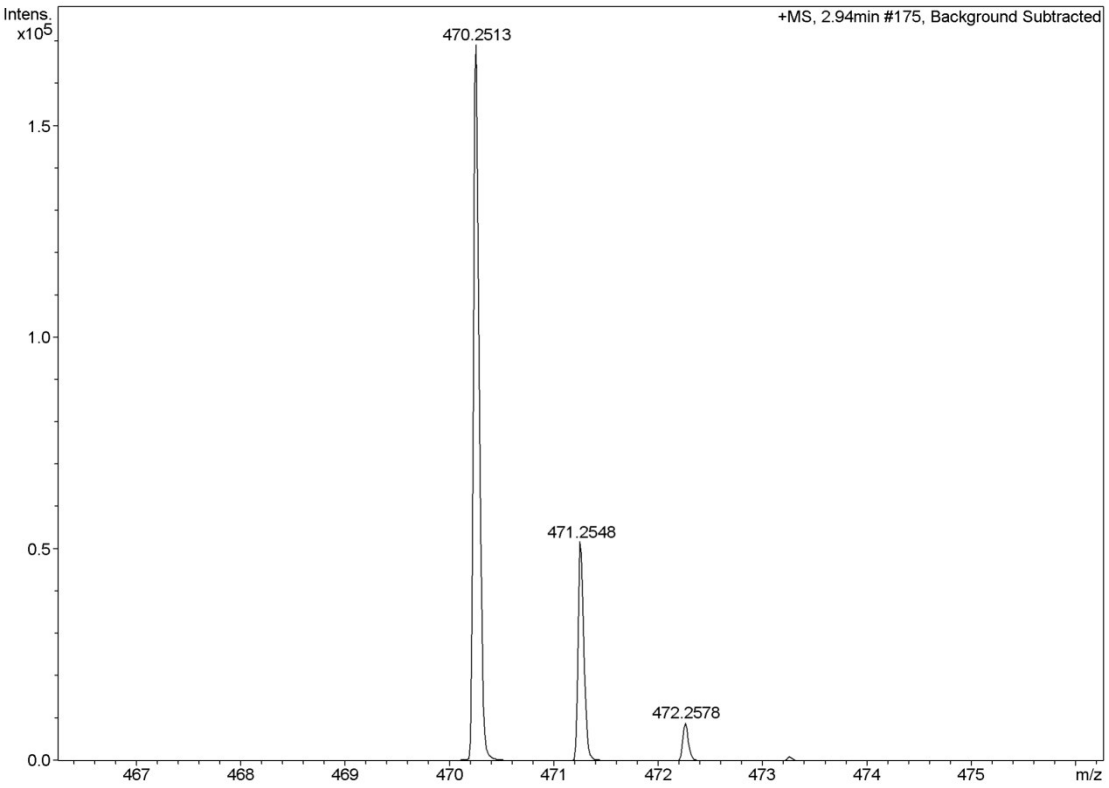


Figure S6B. HRMS-ESI spectrum of 5.

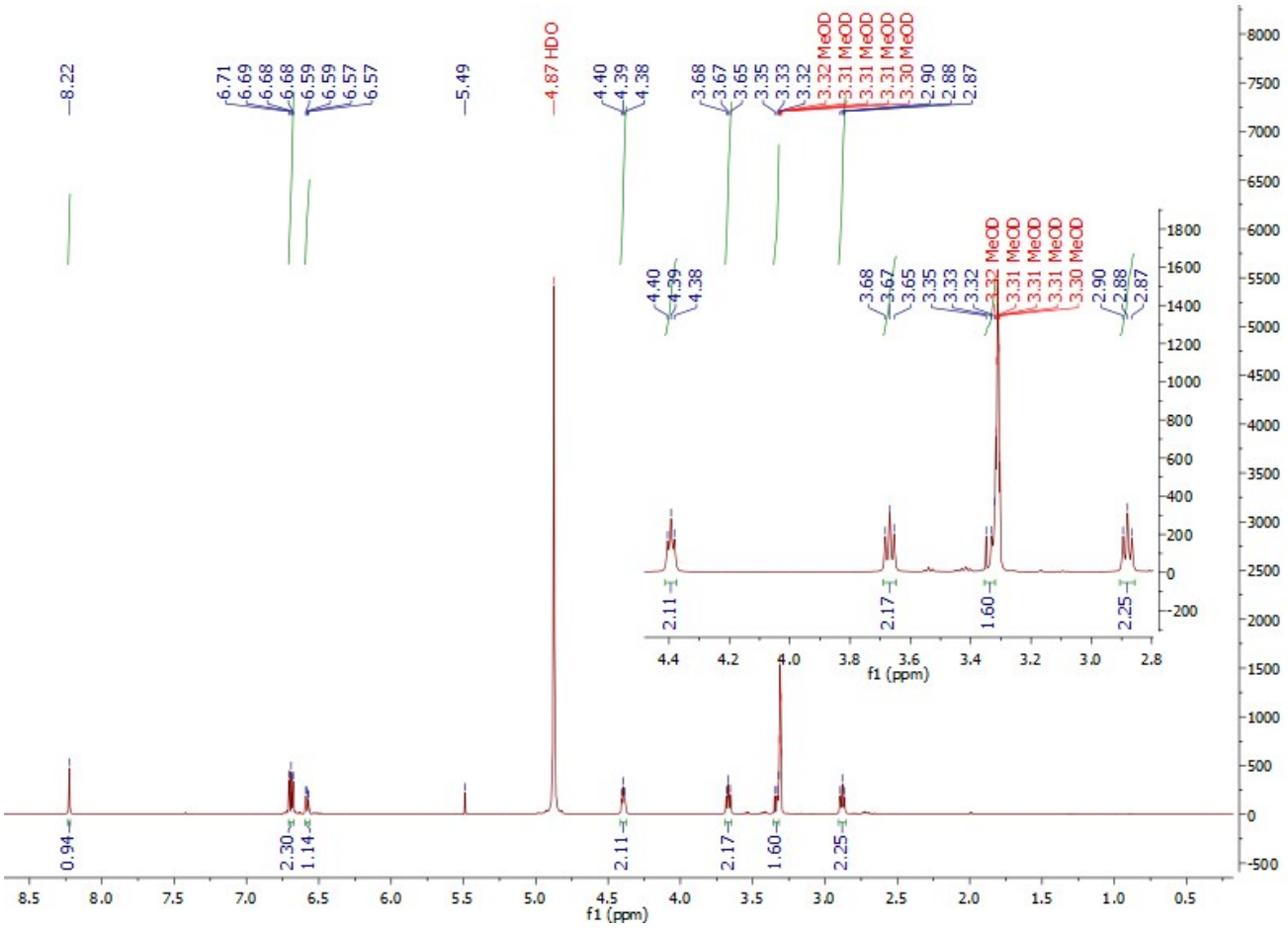


Figure S7. ^{1}H NMR (500 MHz) of 6 in MeOD-d_4.

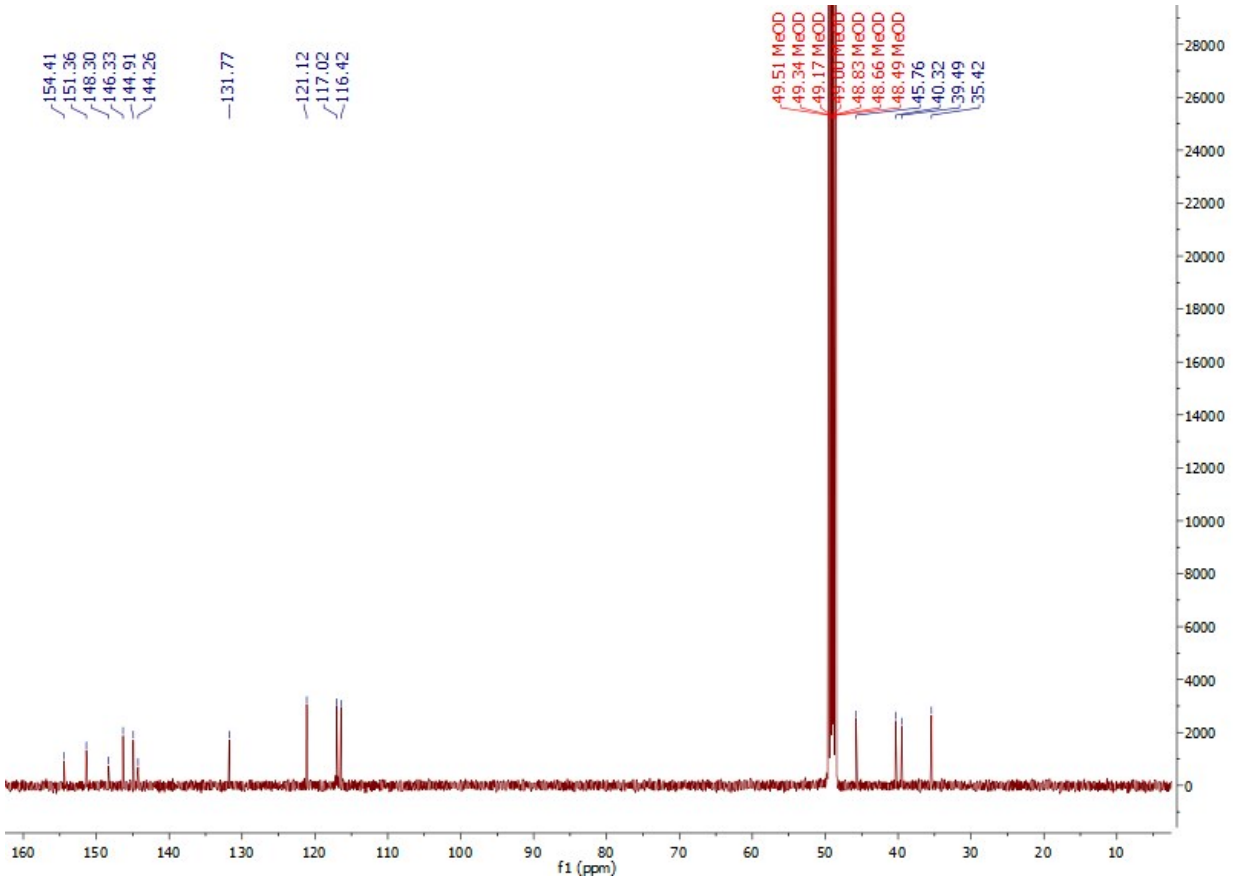


Figure S8. ^{13}C NMR (500 MHz) of 6 in MeOD-d_4.

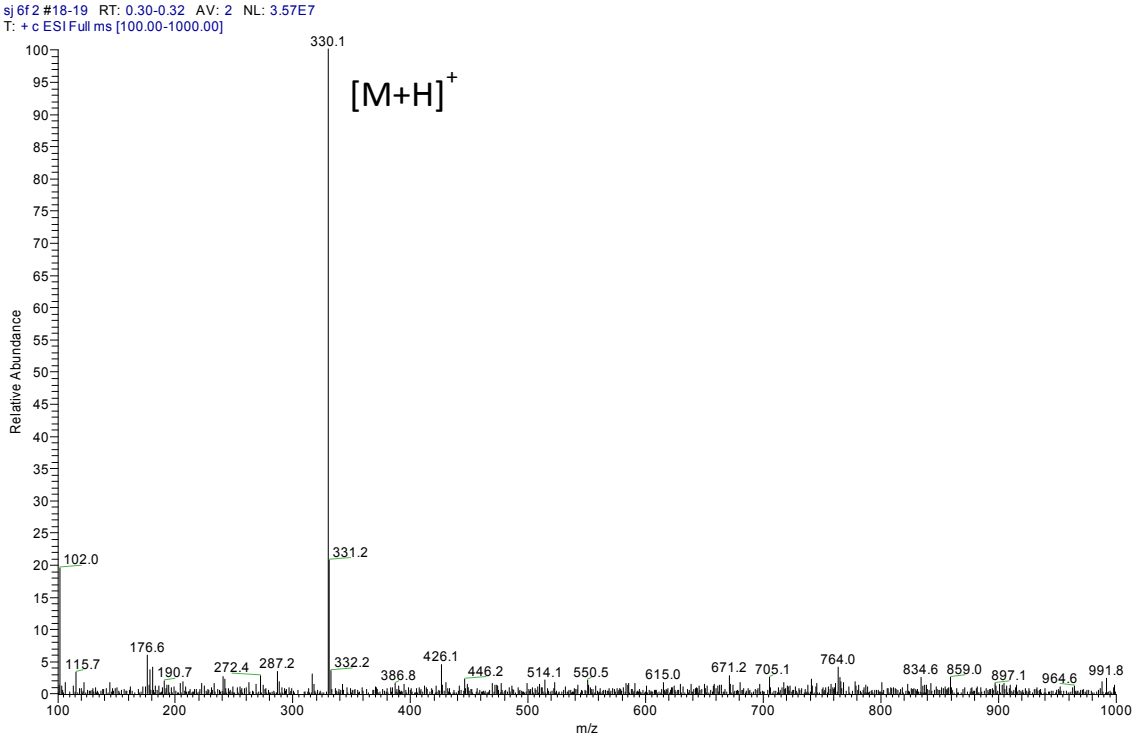


Figure S9A. ESI spectrum of 6.

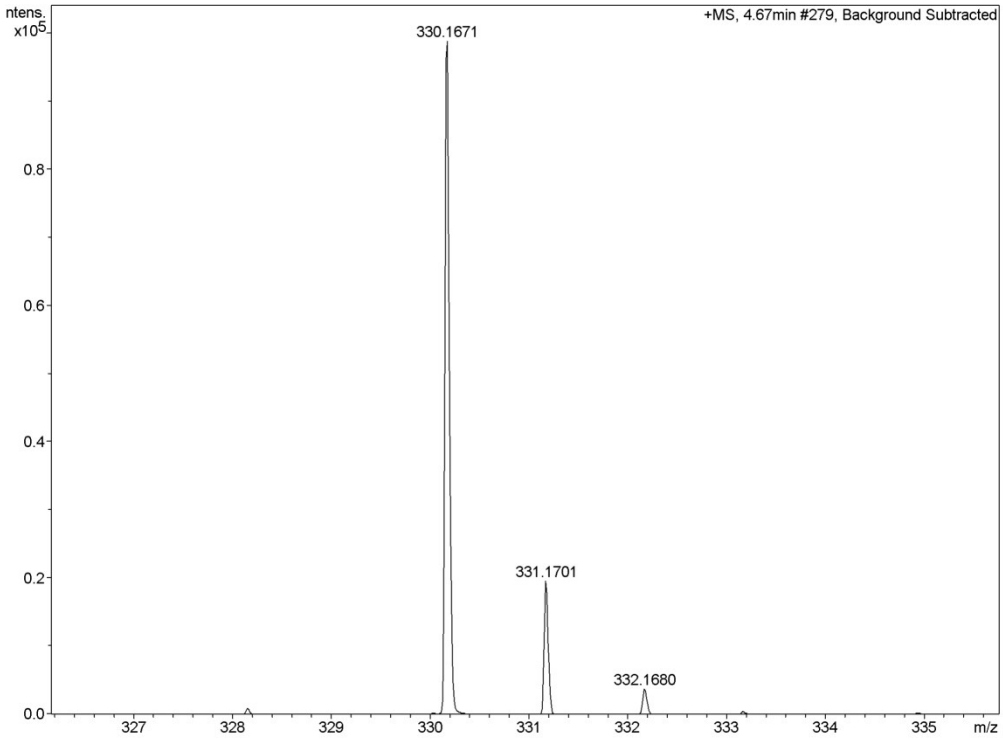


Figure S9B. HRMS-ESI spectrum of 6

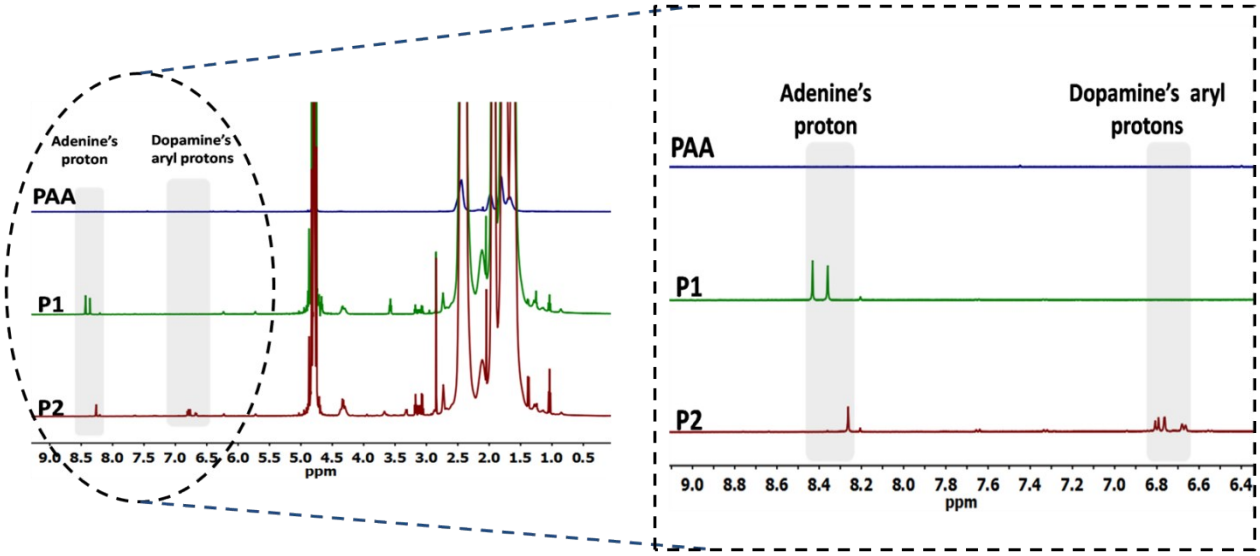


Figure S10. NMR spectra for the PAA, P1 and P2 polymers in D_2O. Aromatic region is expanded for clarity.

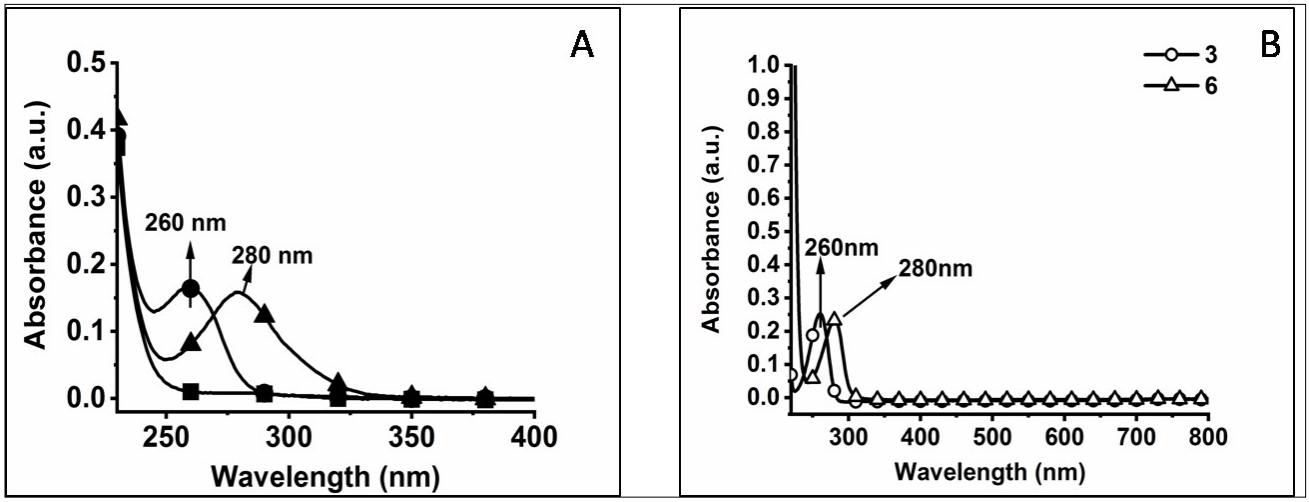


Figure S11.UV-Vis spectra for polymers – P1(—●—), P2(—▲—) and PAA(—■—)(A). UV-spectra of adenine amine (3) and compound 6 (see Scheme S1B) in water (B).

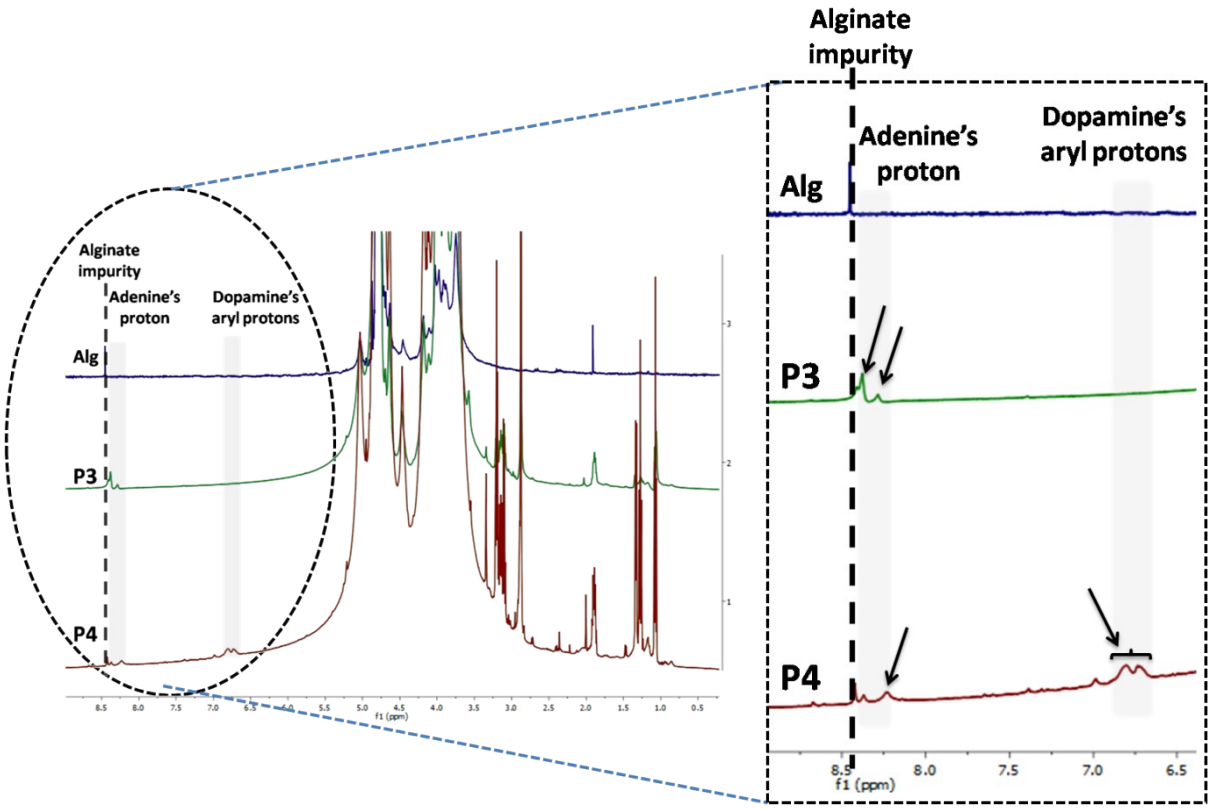


Figure S12. NMR of P3 and P4 polymers in D_2O.

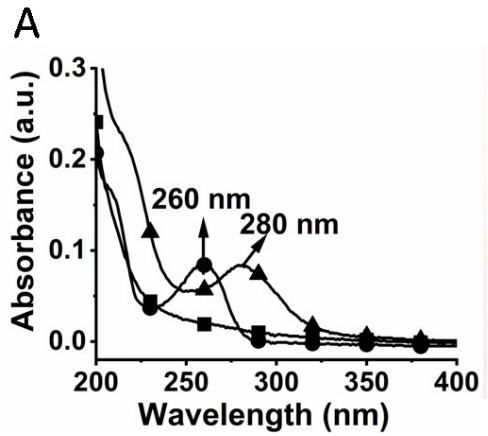


Figure S13. UV-Vis spectra of polymers P3 (—●—), **P4** (—▲—) and Alg (—■—) **polymers (A).**

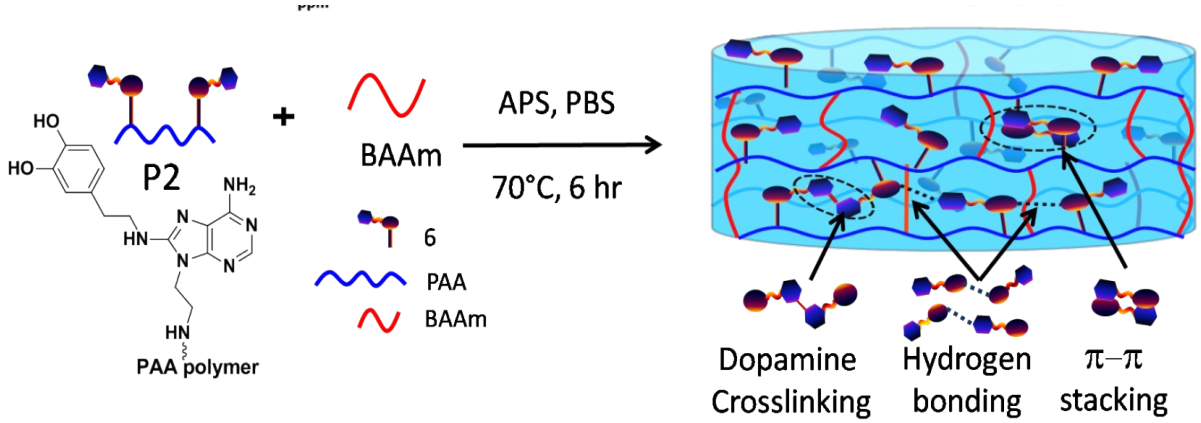


Figure S14. Gel formation with P2 polymer with possible interactions inside the gel.

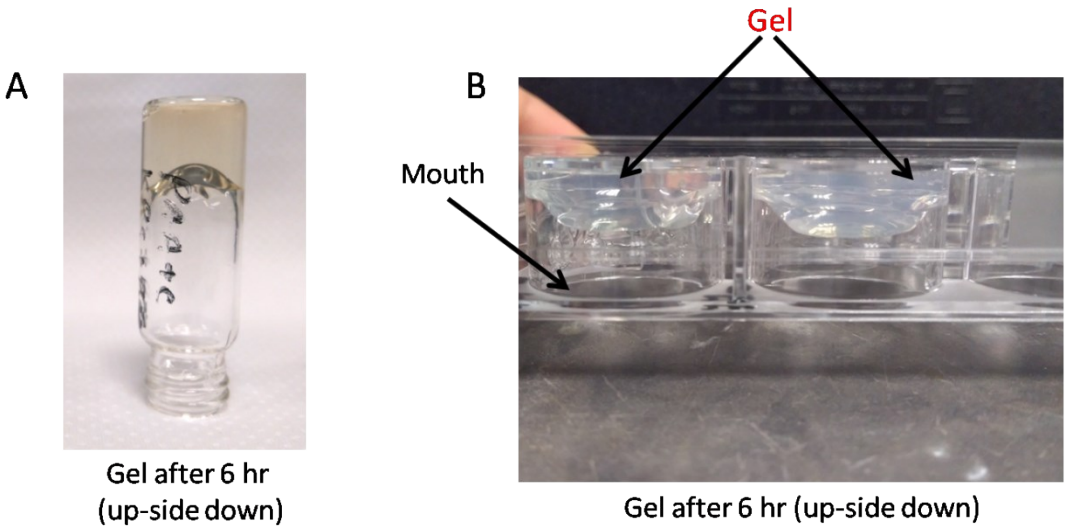


Figure S15. Gelation test after 6hr by changing the position of mold to upside-down format in a bottle (A) and cell culture well (B).

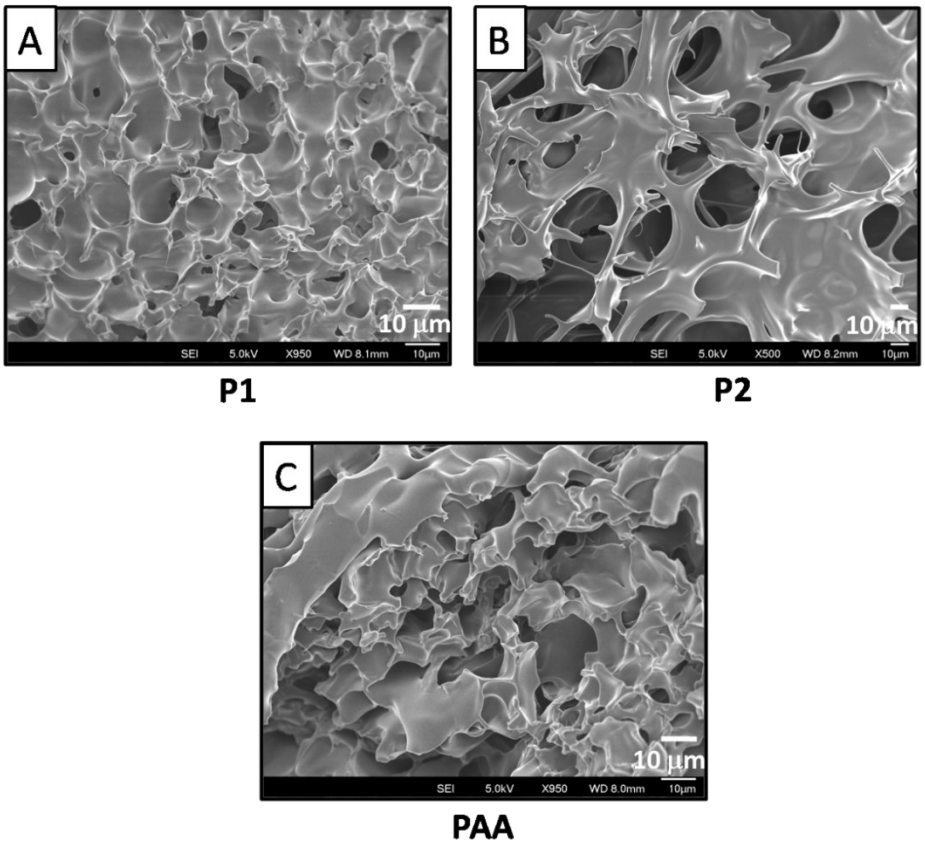


Figure S16. SEM of hydrogels A) P1, B) P2 and C) PAA (before swelling)

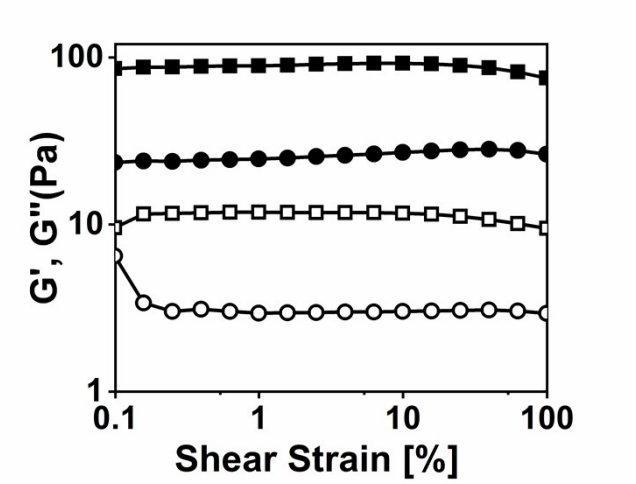


Figure S17. Rheological properties – changes in storage modulus G' for P1 (—□—) and P2 (—o—) and changes in loss modulus G" for P1 (—ᔓ—) and P2 (—ᔕ—) with respect to strain.

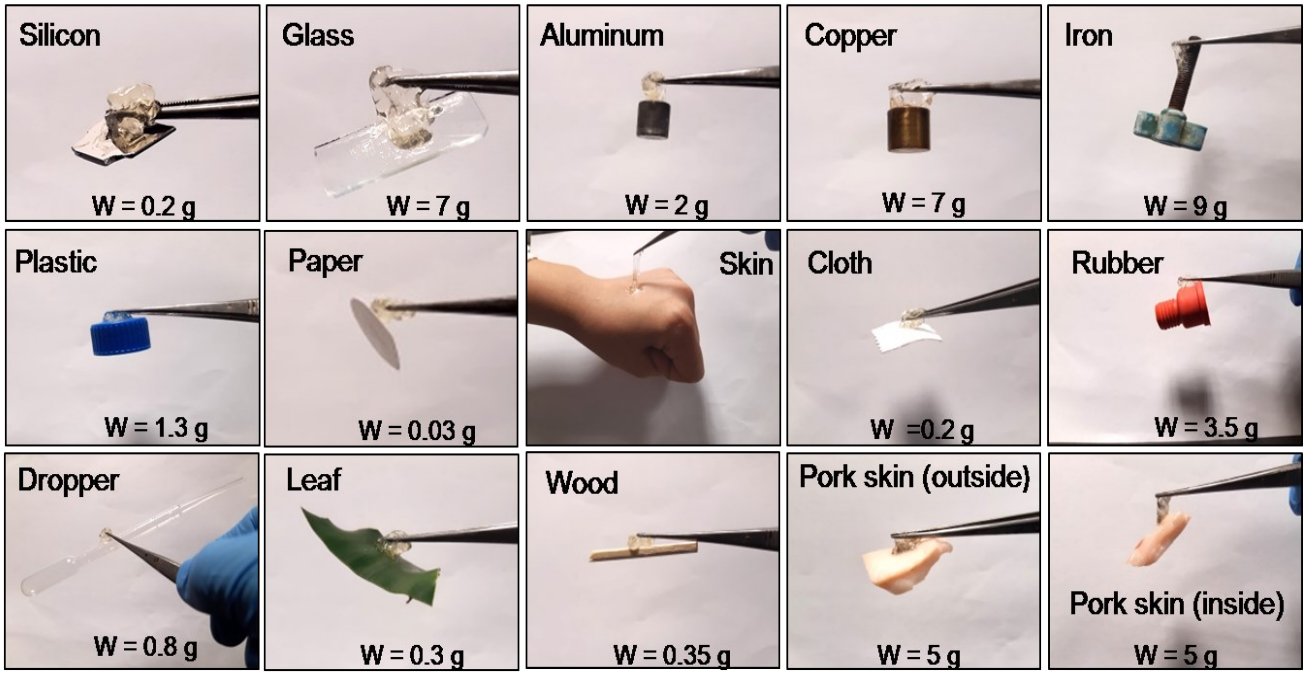


Figure S18. Adhesive behavior of P2 gel on various surfaces like metals, plastic, skin, leaf, paper, rubber, cloth and wood. Hydrogel was placed on top of various substrate surfaces for 5 s and was lifted up to check the adhesion.

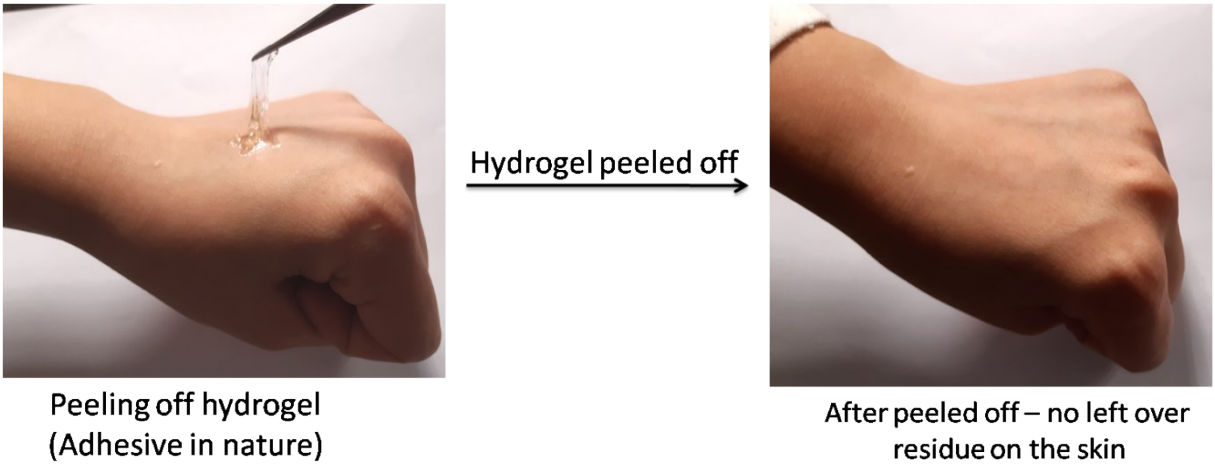


Figure S19. Adhesiveness of P2 gel on human skin and no residue remains behind after peeling off the P2 hydrogel, indicating the strength and adhesiveness.

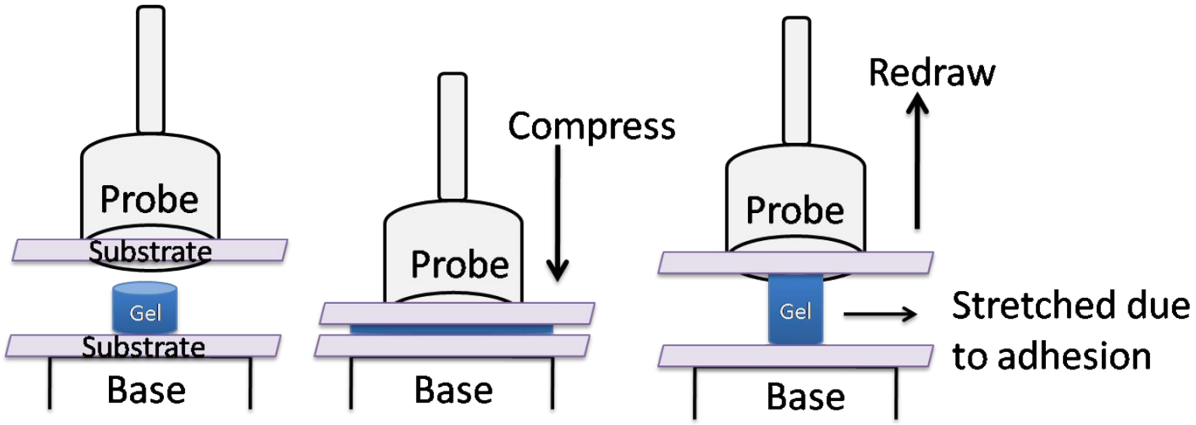


Figure S20. Schematic representation of the adhesion test. (The hydrogels sample dimensions are 13 mm x 5 mm, force used was 2 Kg with a trigger force of 5 g.)

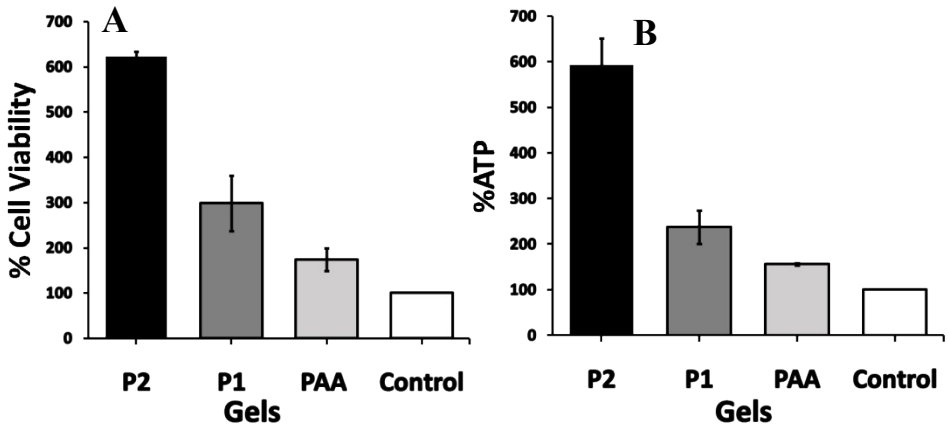


Figure S21.**Cell viability (A) and ATP production (B) assays for BHK-21 cells incubated with hydrogels and control, where no gel was added.**

Figure S22. Optical image of P2P4 (1:1) gel.

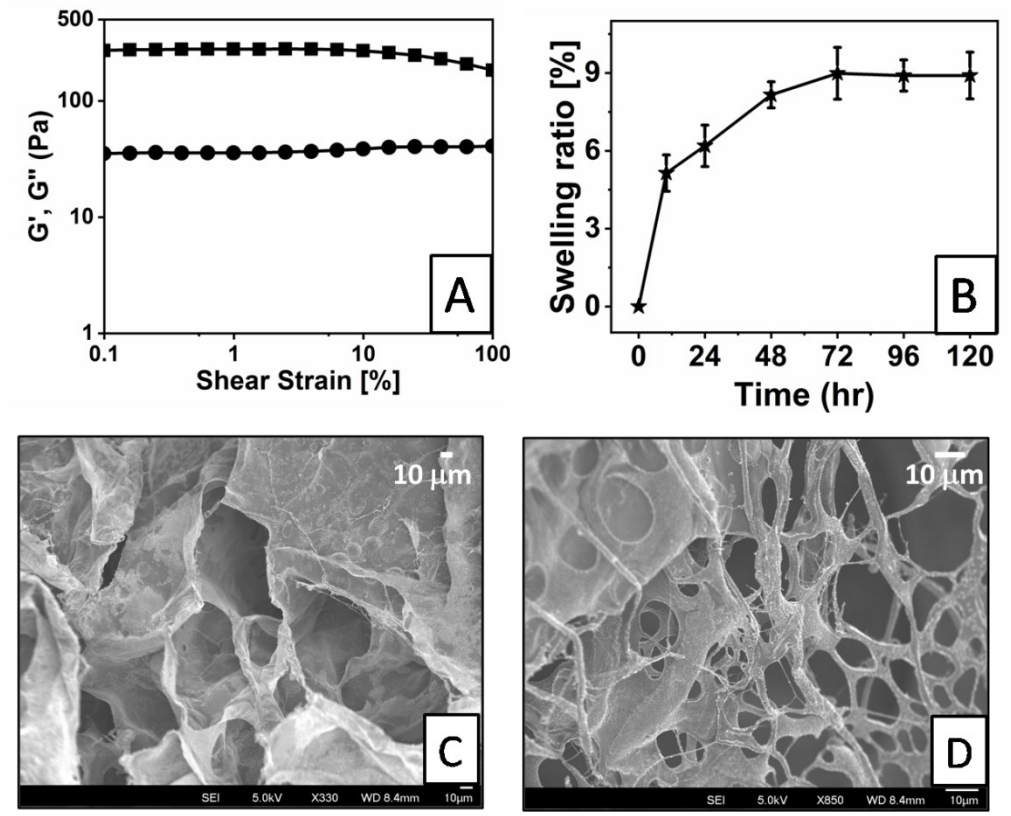


Figure S23. A) Strain sweep analysis of P2P4 hydrogel - storage modulus G' for P2P4 (—o—) and loss modulus G" for P2P4 (—ꝏ—) with respect to strain. B) Swelling ratio of P2P4 gel in PBS for 120 hr and C&D) SEM of swollen freeze dried P2P4 hydrogel.

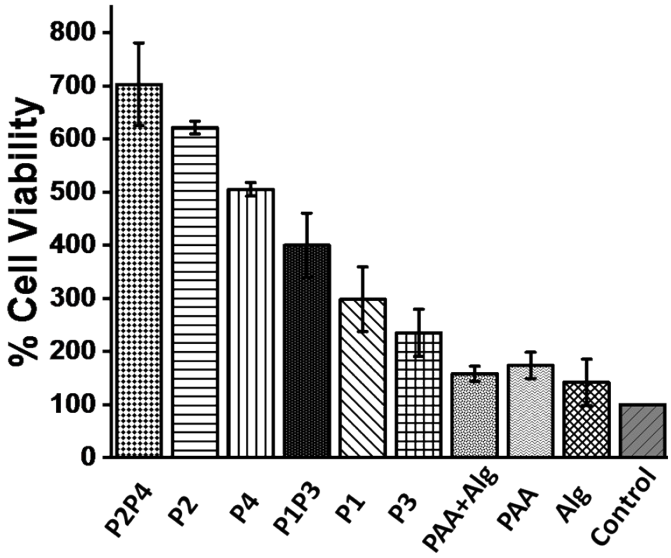


Figure S24. Cell viability assay for different gels/compounds exposed to BHK-21 cells for 24 hr.

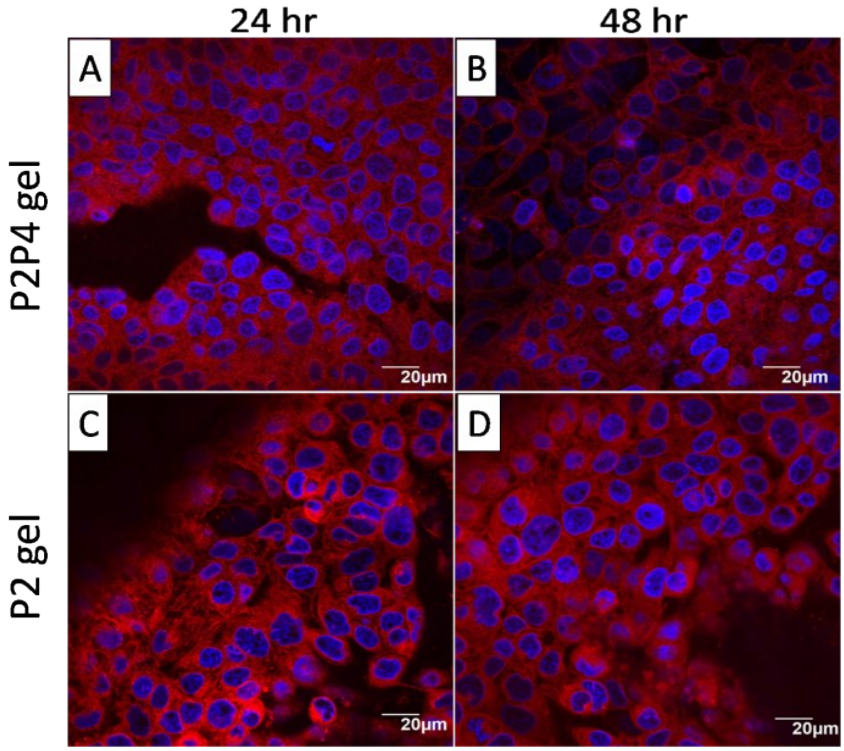


Figure S25. Confocal microscopic images of BHK cells embedded inside the hydrogel P2P4 (A, B) and P2 (C, D) gels in 2D culture after 24 hr (A, C) and 48 hr (B, D).

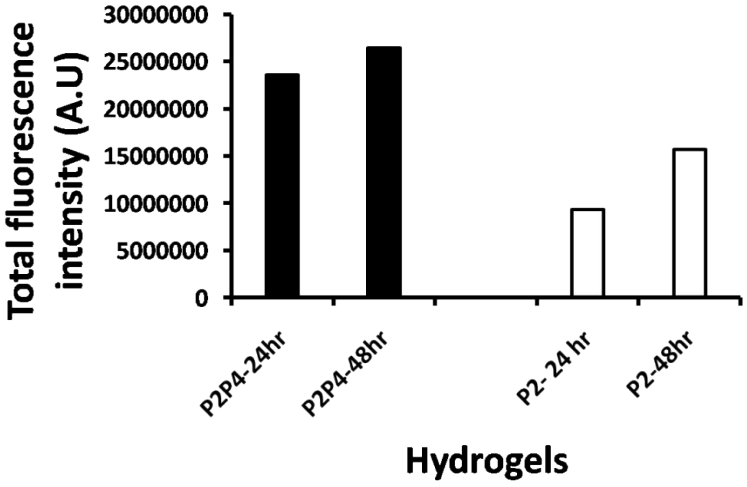


Figure S26. Quantification of fluorescence intensity of nuclear stain at the different time point of cells when seeded into a 2D gel from P2P4 and P2.

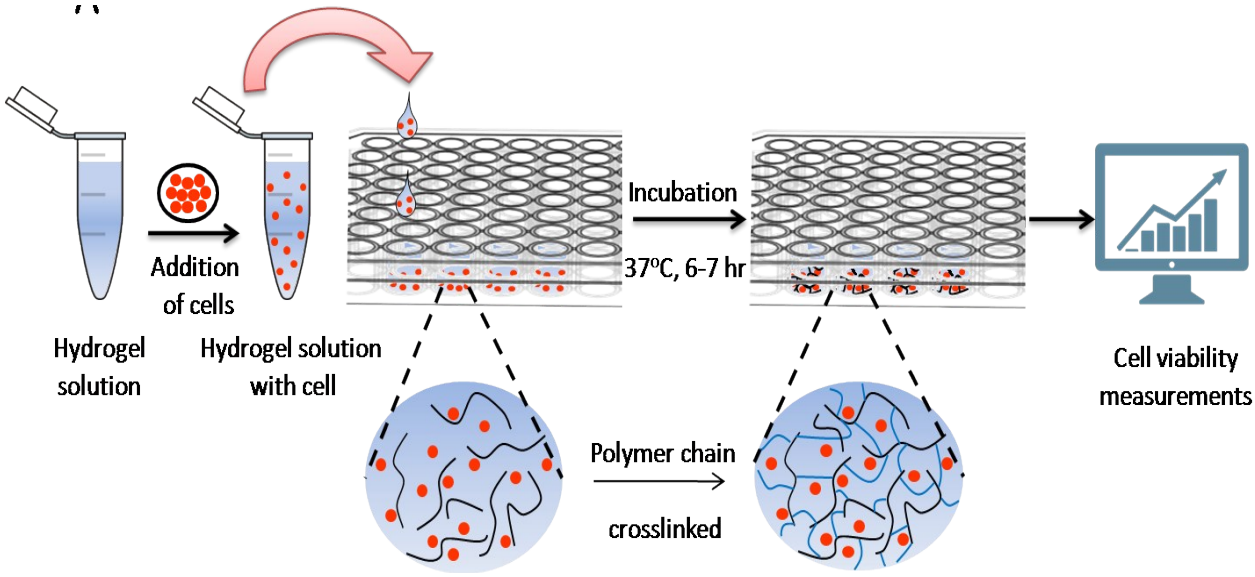


Figure S27. Schematic representation of 3D cell culture used for A431 cell line.

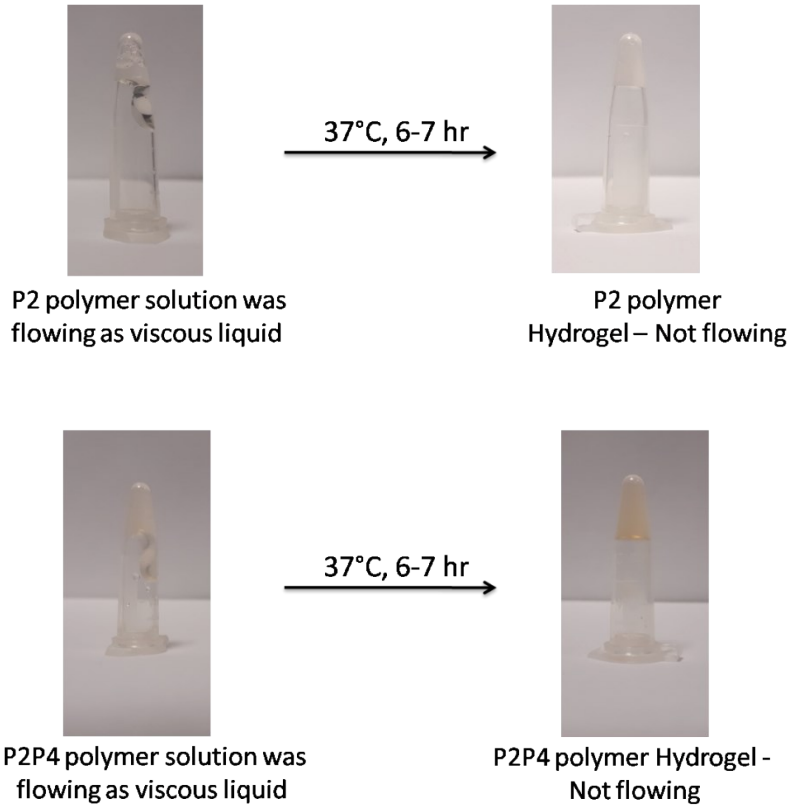


Figure S28. Insitu gelation confirmation test by tube upside down test for P2 and P2P4. **(**Appropriate amount of a gel solution of the desired polymer (60 µL, P2 or P2P4) were taken in a vial and incubated in a water bath at 37 °C for 6 hr).

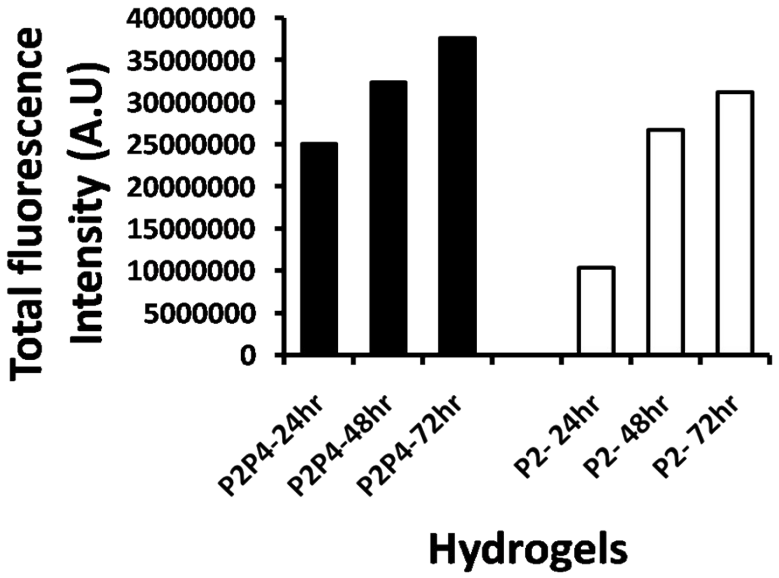


Figure S29. Quantification of fluorescence intensity of nuclear stain at the different time point of cells when seeded in 3D gels prepared from P2P4 and P2.

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