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Electronic Supplementary Information

Dually crosslinked injectable hydrogels of poly(ethylene glycol) and poly[(2-dimethylamino)ethyl methacrylate]-b-poly(N-isopropyl acrylamide) as a wound healing promoter†

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Synthesis of Cl-PEG-Cl

PEG (4000 g/mol, Aldrich) was transformed into activated halide terminated PEG (Cl-PEG-Cl) by the esterification reaction with 4-(chloromethyl) benzoyl chloride. PEG (15 g, 0.00375 mol) was dissolved in dry toluene (250 mL) and 4-(chloromethyl) benzoyl chloride (Cl-Bz-Cl, 0.00975 mol, 1.875 g) was added in the solution under nitrogen atmosphere. The round bottom flask was capped with rubber septum and the temperature of the flask was kept at 5-10 ^{o}C by an ice bath. Subsequently, triethyl amine (0.00975 mol, 1.35 mL) was added into the round bottom flask drop-wise via the syringe. The admixture was stirred at room temperature for 6 h. The insoluble mass was then filtered off. The solid mass was recovered by removing the toluene with rotary evaporator at 60 ^{o}C. The solid mass was then dissolved in deionised water and centrifuged for 10 min at 5000 rpm. The transparent supernatant solution was collected and lyophilized to obtain the product. The ^{1}H NMR spectrum of the product confirmed the formation of Cl-PEG-Cl (Figure S1).

Synthesis of PDMA-*b*-NIPAM

Synthesis of PDMA-Based Macroinitiator

The synthesis of PDMA-*b*-NIPAM was reported earlier by us.[1] Briefly, DMA (20 gm) was dissolved in DMF (20 mL). RAFT agent S-1-Dodecyl-S´-(α,α´-dimethyl-α´´-aceticacid) trithiocarbonate (0.6 g, 16.4 x10^{-4} mol) was added at room temperature. The mixture was degassed by purging nitrogen for about 20 min and then AIBN (0.016 g, 9.6 x10^{-5} mol) was added under nitrogen atmosphere. The reaction vessel was sealed by rubber septum. Polymerization was conducted at 70 ^{o}C under constant stirring for 10 h. The monomer conversion was ca. 83%. The polymerized mixture was cooled to room temperature and then precipitated in excess hexane. The DMF was removed by rotary evaporator and again washed with hexane. The polymer was then dried in vacuum oven at 60 ^{o}C for 24 h. The GPC derived

molecular was 12000 g/mol and PDI was 1.3. This macro initiator is termed as PDMA-RAFT.

Synthesis and Characterization of PDMA-*b*-PNIPAM

PDMA-*b*-PNIPAM block copolymer was synthesized by the polymerization of NIPAM using PDMA-RAFT as macro initiator as reported earlier. A typical example of synthesis of PDMA-*b*-PNIPAM is as follows. PDMA-RAFT (6 g, 0.0005 mol) was dissolved in DMF (20 mL). Next, NIPAM (14 g) was added in the solution with stirring for 1 h. Then the reaction mixture was purged with nitrogen for about 20 min. AIBN (0.012 g, 7.2x10-5 mol) was then added under nitrogen atmosphere. The reaction vessel was sealed with septum and secured by copper wire. The reaction mixture was stirred at 70 ^{o}C for 8 h. The viscous mixture was precipitated in mixture of hexane and diethyl ether. The polymer was again re-dissolved in minimum amount of DMF and precipitated in diethyl ether. The polymer was again then solubilized in water and dialyzed by 1.2 kDa cut-off dialysis bag followed by lyophilisation to obtain pure product. The conversion was 70 %. The copolymer was characterized by GPC and ^{1}H NMR spectroscopy. The GPC (Figure S2) derived molecular weight was 32000 g/mol and 1H NMR derived molecular weight was 44000 g/mol.

The following equations were used for the determination of molecular weight and composition of the copolymer by ^{1}H NMR Figure S1):

$$\text{DMA (mol)} = \frac{I_a}{I_a + I_b} \times 100 \qquad (1)$$

where I_a is the area of integral of methyl hydrogen (6H, δ=2.33 ppm) attached to nitrogen [-$N(CH_3)_2$] of PDMA chains and I_b is the integral of methyl hydrogen (6H, δ=1.2 ppm) attached to nitrogen [-$N(CH_3)_2$] of PNIPAM chains. Molecular weight of the block

copolymers were determined by considering the GPC derived molecular weight of PDMA which is 12000 g/mol (repeat unit 77) by the following equation:

$$M_{n\,NMR} = \frac{77 \times I_b \times 113}{I_a} + 12000 \qquad (2)$$

where 113 is the molecular weight of NIPAM and 12000 is the molecular weight (in g/mol) of PDMA macro RAFT agent obtained by GPC (Figure S1).

Characterization of the Copolymer and Cl-PEG-Cl

PDMA macroinitiator and the PDMA-*b*-PNIPAM copolymer were characterized by GPC using a Waters model 2695 separation module coupled with Waters 2414 refractive index detector and Waters Styragel columns (HR 0.5 DMF, HR 4E DMF and HR 5 DMF). HPLC grade DMF (Spectrochem, India) was used as eluent at a flow rate of 0.8 mL/min at room temperature. Polystyrene standards were used for calibration.

Bruker 200 MHz spectrometer was used to record the ^{1}H NMR spectra of the copolymer and Cl-PEG-Cl ^{1}H NMR spectra. ^{1}H NMR spectra were recorded at 25 ^{0}C in $CDCl_3$ and TMS was used as the internal reference.

Characterization of the hydrogels by ^{13}C NMR spectroscopy and by FT-IR spectroscopy

The hydrogels were washed with water several times and then lyophilized. ^{13}C NMR (200 MHz, Bruker) spectra of the prepared hydrogel were recorded at 25 ^{0}C. FT-IR spectra were recorded using Agilent Cary 600 series FTIR at room temperature The IR spectra of the dried hydrogels were obtained by grinding with KBr.[1,2]

Determination of Sol fraction

Sol fraction was determined by immersing preweighed dry hydrogels in DMF for 48 h at 30 ^{o}C. After 48 h hydrogels were removed gently and extracted with methanol to remove DMF.

The hydrogels were dried at 45 ^{o}C and weighed. The same procedure was repeated three times. The sol fraction was determined by following equation:

$$\text{Solfraction (\%)} = \frac{m_d - m_{ex}}{m_d} \times 100 \qquad (3)$$

where m_d and m_{ex} are the masses of the dried and extracted samples, respectively.

Determination of Swelling

DMF extracted hydrogels were dried by lyophilisation and weighed. Hydrogels were submerged into PBS of pH 7.4 and aqueous acidic solution of pH 5 separately at fixed temperature in an incubator shaker (INFORS AGCH-4103) at shaking speed 100 rpm. The hydrogels were removed from the solution after certain time; excess water was wiped off and the weight of the swollen hydrogels was determined. Equilibrium swelling was measured when the weight of the swollen hydrogels remained constant after 5 h.

The temperature responsive swelling of the hydrogels was also determined. The fully water swollen (equilibrium swelling) hydrogels were again submerged in aqueous solution of pH 5 and 7.4 separately. The hydrogels (in triplicate) were then placed at constant temperature 17 ^{o}C in an incubator shaker (100 rpm). The samples were equilibrated for 1.5 h at the temperature and then weighed after removing the excess surface water. After that the temperature was raised up to 37 ^{o}C and the same procedure was followed to determine the temperature dependent swelling measurement. The cyclic variation of swelling property with temperature was determined similarly.[1] The swelling was determined by the following equation:

$$\text{Swelling (\%)} = \frac{m_s - m_d}{m_d} \times 100 \qquad (4)$$

where m_s and m_d are the masses of the swollen and the dry hydrogel respectively.

Degradation Study

The degradation behaviour of the hydrogels was studied in PBS solution of pH 7.4 and in mildly acidic (pH 5) aqueous solution. All the preformed hydrogel were lyophilized and accurately weighed. The pre-weighed hydrogels were immersed in PBS pH 7.4 and aqueous acidic solution of pH 5 separately. The degradation study was performed at shaking rate 100 rpm at 37 ^{o}C. After specific time interval, gel samples were removed and lyophilized to determine the dry weight. The degradation of the hydrogels was obtained by the following equation:

$$\text{Mass loss } (\%) = \frac{m_0 - m_D}{m_0} \times 100 \qquad (5)$$

where m_0 is the dry mass of the sample, m_D is the residual dry mass of the sample after a certain time of degradation.

Model gelation reaction

A model reaction was performed with PNIPAM homomopolymer and Cl-PEG-Cl. The PNIPM homopolymer and Cl-PEG-Cl were separately solubilized in PBS (pH 7.4). The temperature of the two solutions was ca. 25 ^{o}C. Two solutions were mixed in different proportions and kept at 25 ^{o}C. There is no gelation. On the other hand, two solution of PDMA homopolymer and Cl-PEG-Cl formed gels instantaneously at 25 ^{o}C.

Morphology by SEM

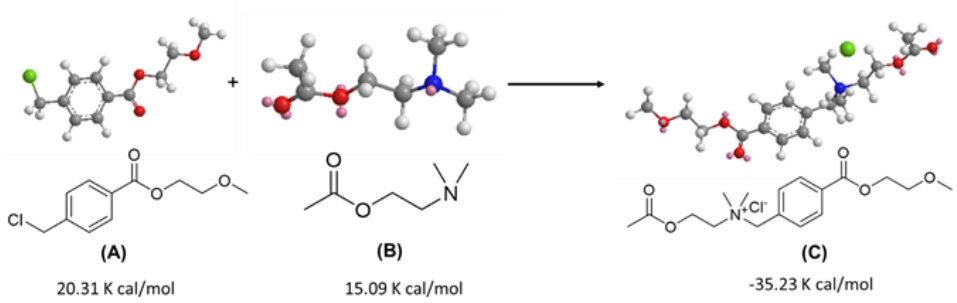
The as prepared hydrogels (in their swollen condition) were frozen liquid nitrogen. The frozen hydrogels were then subjected to freeze-drying. The samples were then stored in $CaCl_2$ filled desiccator for SEM analysis. The dried samples were again frozen with liquid N_2 and fractured for cross-sectional SEM analysis. This process was performed to avoid pore

shrinkage. The freeze-dried samples were then subjected to gold coating in a sputter coater (LEICA EM ACE 200 gold coater) and then visualized through SEM (FESEM, JSM-7100F).

Determination of Temperature Change During Gelation of Cl-PEG-Cl and the Copolymer

Published procedure was followed with some modification for the determination of temperature change during gelation.[3] Temperature change during gelation was determined where the initial temperature of the injectable solution was kept at 25 ^{o}C. A Cl-PEG-Cl (0.06 g in 0.12 mL PBS) and copolymer (0.06 g, 0.88 mL PBS) solutions (12% w/v total) were taken in teflon coated glass vials. These solutions were kept in an incubator to attain a constant temperature of 25 ^{o}C. The temperature of the solutions was monitored by a temperature probe (Oakton, pH 700), touching the surface. The two solutions were then mixed at once and the change of temperature was monitored. The viscosity of the solution increased while the temperature was also increased to 25.7-25.9 ^{o}C after 3 min of reaction and remained constant for ca. 10 min. We recoded this temperature change of 0.7-0.9 ^{o}C. It is mentioned that the temperature rise might have been underestimated as the heat loss of the system and the heat capacity of the probe was ignored.

We also calculated the energy change of the molecules by MM2 method using Chen3D pro (Perkin Elmer) software (scheme 1). The change of total energy before and after the reaction was calculated. The change of temperature for total 1 g of solution, containing Cl-PEG-Cl (0.06 g, $3x10^{-5}$ mol) and copolymer (0.06 g, $1.15x10^{-4}$ mol reactive DMA groups) calculated. Hence the total $3x10^{-5}$ mol reactants will be involved in the reaction. The total mass of the mixture was 1g.



Scheme 1. Representation of approximate three dimensional structures of reactants (A) chloride terminated molecule and (B) amine molecule and the product (C) quaternized amine molecule with calculated energy in their minimal energy conformation obtained by MM2 simulation.

The temperature change of the above mentioned reaction mixture during complete gelation could be obtained by the following equation:

$$\Delta H = mass\ of\ solution \times specific\ heat\ capacity\ water \times temperature\ change \quad (6)$$

where ΔH =-292 KJ/mol as calculated from the MM2 simulation. Hence, the rise of temperature was calculated to be ca. 2.1 ^{o}C for 1 g of solution from equation 6.

Hemocompatibility of Hydrogel

For assessment of hemocompatibility of the hydrogels we have perform in vitro hemolysis assay with whole human blood, 3 ml of defibrinated human blood was collected and centrifuged at 1500 rpm for 10 min. RBC pellet was washed thrice with 0.01 M PBS and diluted to 6 ml to prepare RBC stock solution. Hydrogels (400 µL each) (HG-1, HG-2, HG-4) were saturated overnight at 37°C with 0.01M PBS. incubated with RBC stock solution (300 µL each) at 37 °C for 3 h. Collected supernatant and centrifuged at 2500 rpm for 5 min. 100 µl of released haemoglobin in the supernatant was analysed at 540 nm using UV−visible spectrophotometer (Bio-RAD 680, USA). All the experiments were performed in triplicates.

Percent haemolysis was calculated with respect to haemolysis caused by negative control (PBS) and positive control (De-ionized water), as shown in the following equation:

$$\text{Hemolysis (\%)} = \frac{(\text{Sample}_{540\text{ nm}} - \text{negative control}_{540\text{ nm}})}{(\text{Positive control}_{540\text{ nm}} - \text{negative control}_{540\text{ nm}})} \text{ X } 100 \qquad (6)$$

In vitro Cytocompatibility of the Hydrogels

To estimate the cell viability using MTT assay, HepG2 cells were grown in 25 cm2 tissue culture flasks in 5%, CO_2 atmosphere at 37 °C with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin-streptomycin solution. Briefly, hydrogels (100 µl each) (HG-1, HG-2, HG-3, HG-4) were prepared and saturated overnight at 37°C with 0.01M PBS. Seeded with 10^4 cells per well in a 96-well plate and incubated in a CO2 incubator at 37 °C for 24 h. Cells seeded on polystyrene tissue culture plates were used as a control. Media was replaced with 10 µL of 5% MTT solution and incubated for 4 h followed by addition of 100 µL DMSO. Absorbance was measured at 595 nm on microplate spectrophotometer (Bio-RAD 680, USA). All the experiments were performed in triplicates. Cell viability was calculated with respect to controls, where only media represents negative control, cells seeded on polystyrene tissue culture plates represent positive control (eqn. 7).

$$\text{Cell survival} = \frac{\text{Sample}_{595\text{ nm}} - \text{negative control}_{595\text{ nm}}}{\text{Positive control}_{595\text{nm}} - \text{negative control}_{595\text{ nm}}} \times 100 \qquad (7)$$

Adhesion of Platelet on Hydrogel by SEM

Scanning electron microscopy (SEM) was used to visualize platelet adhesion on hydrogel HG-2 as reported with slight modification.[4,5] Briefly, HG-2 was saturated overnight with 0.1M PB. Platelets were isolated from human citrated blood. PRP (50 µL; 1 × 107 platelets) was co-incubated with HG-2 for 1h at 37 °C, 5% CO_2. Platelets incubated on coverslips were

used as control. After rinsing with 0.1M PB (pH 7.4), samples were fixated in 2.5% glutaraldehyde in 0.1 M PB at 4 °C for 2-3 h. Samples were rinsed and post fixated in 1% osmium tetroxide for 1 h. Next samples were washed & dehydrated by increasing acetone concentration (50-100%) 20 minutes each. After drying, the specimens were treated with 100% HMDS at room temperature for 5 minutes and mounted on aluminum stubs with adhesive carbon tape. Samples were coated with thin gold layer, by means of a sputter-coater (SC7640, Polaron Equipment, England, UK). The samples were observed under an environmental, variable pressure Scanning Electron Microscope (Carl Zeiss EV0-40, Cambridge, UK) at a voltage of 20 kV and a working distance of 10 mm. The morphologies of adhered platelets were characterized according to platelet shape.[6]

Table S1. Compositions of Hydrogels

Hydrogel gel	Cl-PEG-Cl (50% w/v in PBS, pH 7.4) (mL)	PDMA-*b*-PNIPAM (10% w/v in PBS, pH 7.4) (mL)	PBS (pH 7.4) (mL)
HG-1	0.106	1.61	-
HG-2	0.214	1.07	0.432
HG-3	0.256	0.86	0.6
HG-4	0.298	0.64	0.778

Table S2. Rheological Properties of the Hydrogels. The Hydrogels were Obtained by the Injection of 12% (w/w) Mixture of the Prepolymers into the Parallel Disk of Rheology Instrument (Figure 1) and the Loss Modulus and Storage Modulus were Noted After 30 Min.

Hydrogel gel	Gelation time (min) 25 ^{o}C/37 ^{o}C	Loss modulus (G", Pa) 37 ^{o}C/25 ^{o}C	Storage modulus (G', Pa) 37 ^{o}C/25 ^{o}C
HG-1	7.5/3	46/28	6845/4176
HG-2	4.0/2.1	318/3	16811/1610
HG-3	3.6/1.5	2380/17	26500/2960
HG-4	2.8/1	1691/50	40750/6752

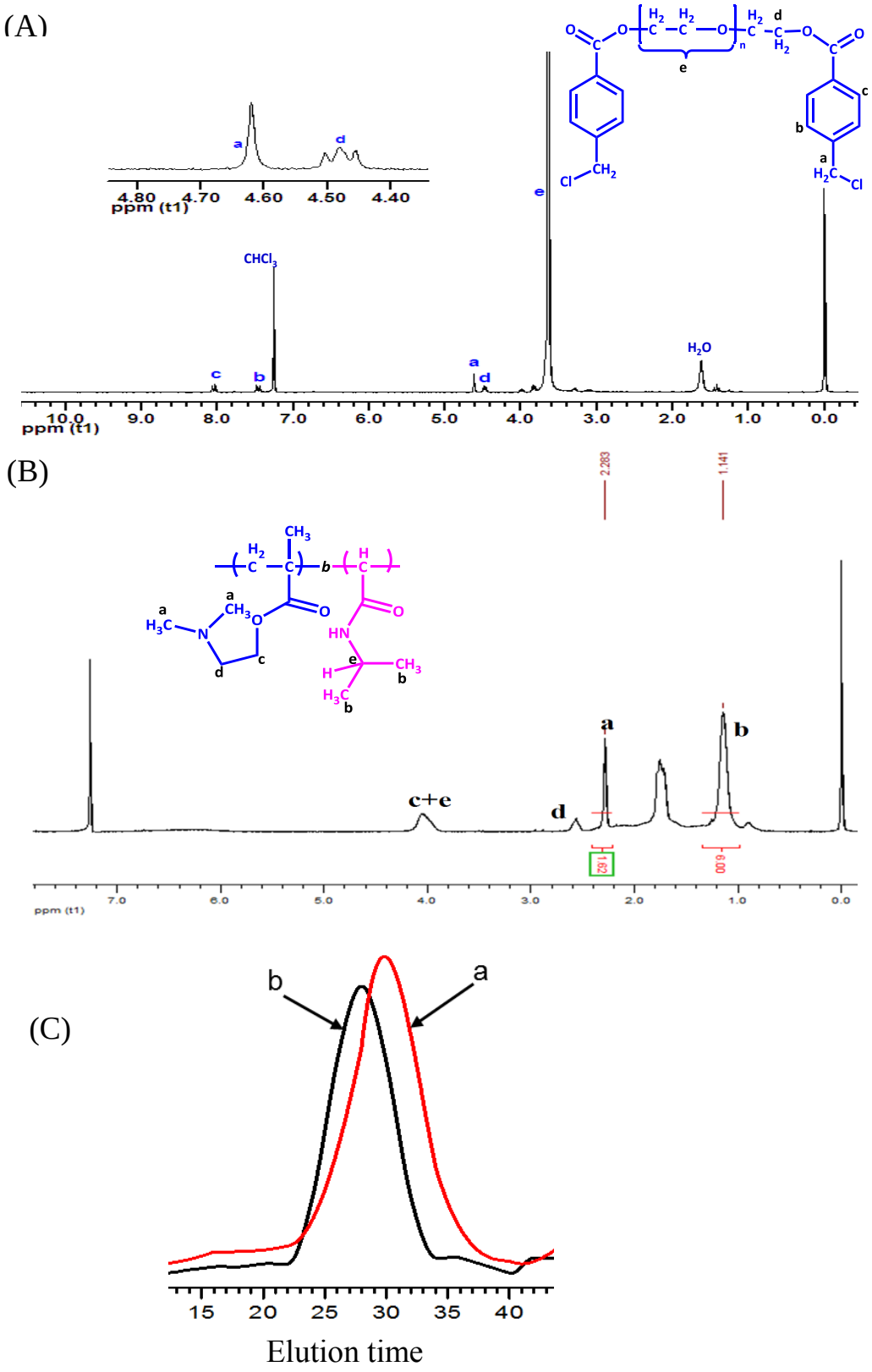


Figure S1. ^{1}H NMR (200 MHz) of (A) Cl-PEG-Cl and (B) PDMA-*b*-PNIPAM. Spectra were taken in $CDCl_3$ using TMS as standard. Figure (C) GPC traces of (a) PDMA macroinitiator and (b) PDMA-*b*-PNIPAM copolymer. GPC was performed using DMF as an eluent at flow arte 0.8 mL/min.

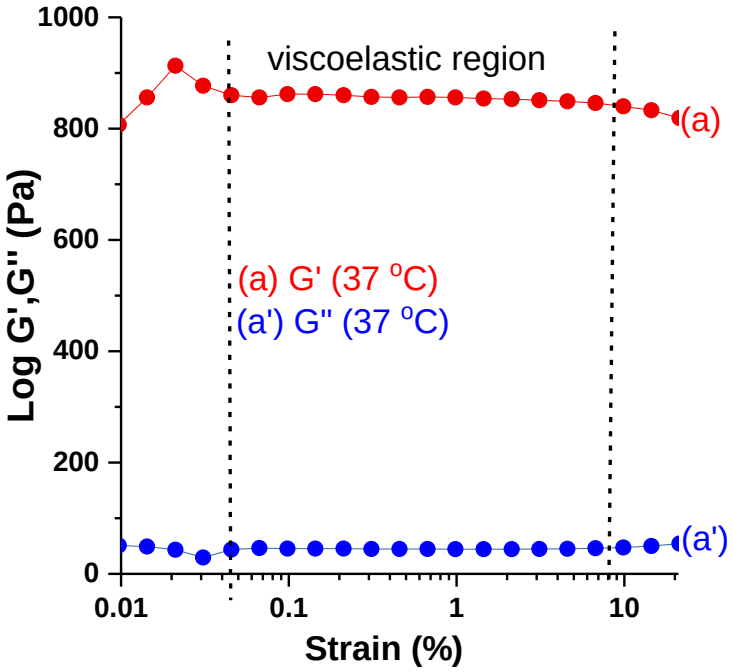


Figure S2. Strain sweep experiment (1 Hz) with fully hydrated HG-2 hydrogel showing viscoelastic region.

Figure S3A shows the IR spectra of the extracted gels. A band at 1725 cm^{-1} is appeared due to ester carbonyl (C=O) stretching vibration of PDMA groups. The bands at 1640 cm^{-1} and 1550 cm^{-1} are due to amide I (C=O of amide) and amide II (N-H of amide) bands respectively. The bands at ca. 1100 cm^{-1} and 943 cm^{-1} appeared due to the C-O and C-C stretch, and CH_2 rock and C-C stretch of PEG chains in the gels.

Majorly, the ^{13}C NMR of the hydrogels exhibited broad signal at δ value 174-180 ppm which indicates presence of ester carbonyl (from PDMA) and amide carbonyl carbons. The signals at 135 ppm, 71 ppm, 63 ppm, 57 ppm, 54 ppm and 45 ppm are assigned to the aromatic ring carbon, $^{*}CH_2$-O of PEG, adjacent -$^{*}CH_2$ of quaternized N of PDMA, -N$^{*}CH_2$ of PDMA, (polymer backbone carbons), and N-$^{*}CH_2$ of PNIPAM respectively (Figure S3B).

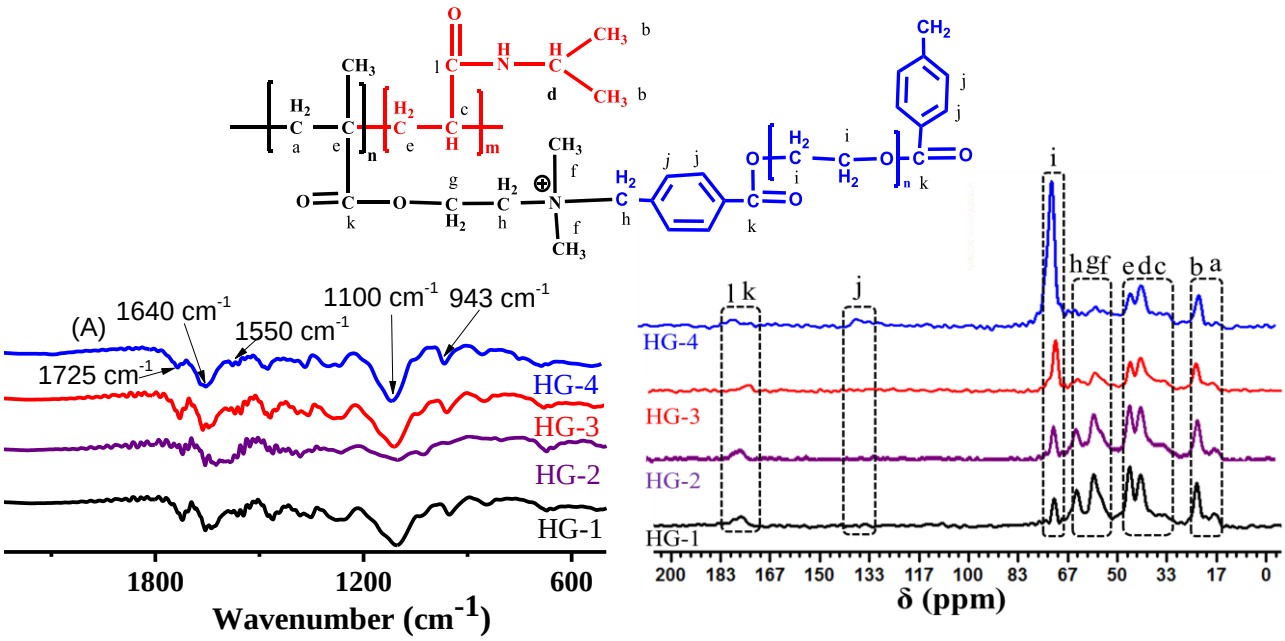


Figure S3. (A) IR spectra and (B) solid state ^{13}C NMR spectra of the hydrogels.

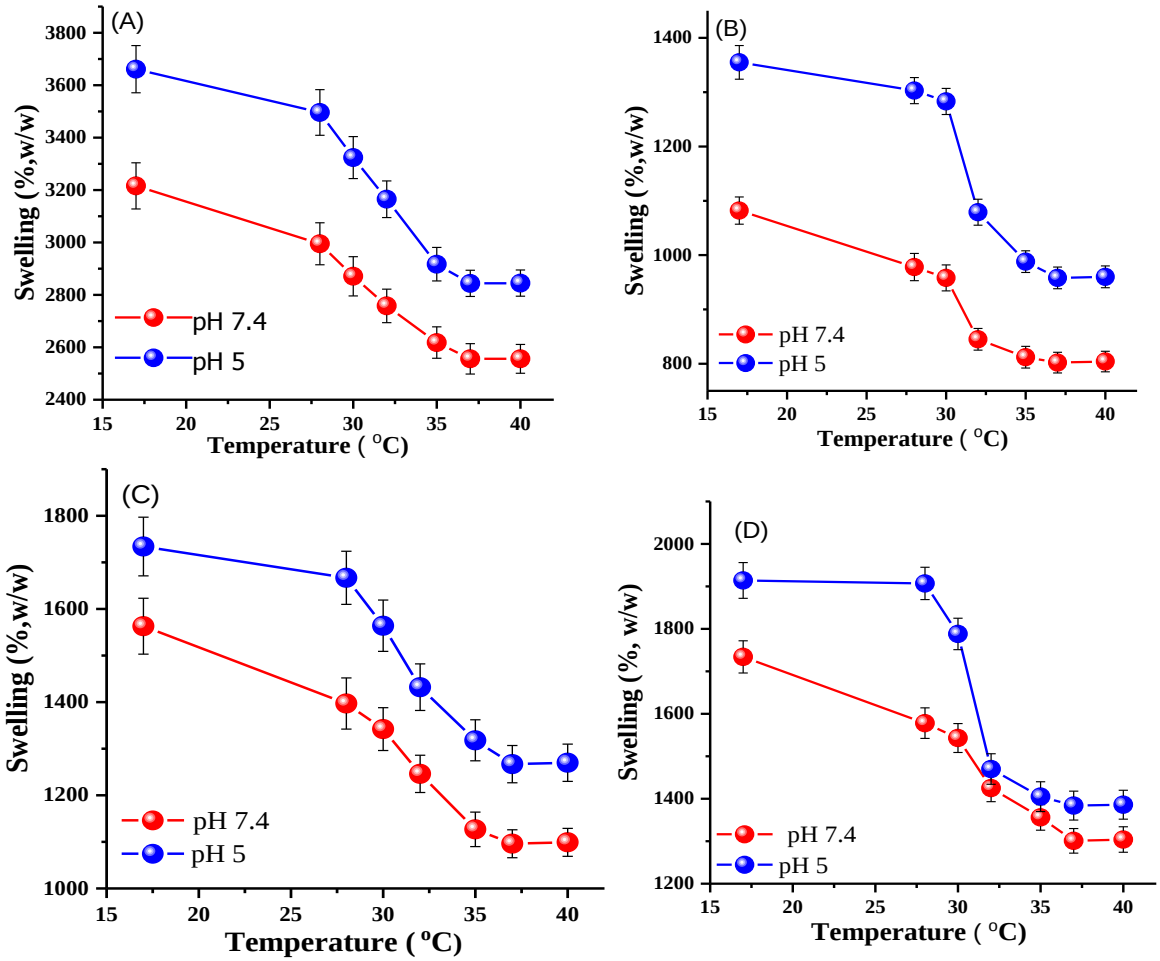


Figure S4. Effect of temperature and pH on the equilibrium water swelling of the (A) HG-1, (B) HG-2, (C) HG-3 and (4) HG-4 hydrogels.

Figure S4 shows the pH and temperature responsive S_w of the hydrogels. The PNIPAM chains in the hydrogels start to shrink at around 22-25 °C which leads to de-swelling. The de-swelling ended around 40 °C. The marked effect is seen around 28-34 °C. The S_w values of the hydrogels were higher at lower pH at wide range of temperature. The HG-4 gave low pH response in the temperature range 30-40 °C whereas others gave almost similar pH response owing to lower amount of copolymer in the HG-4 hydrogel.

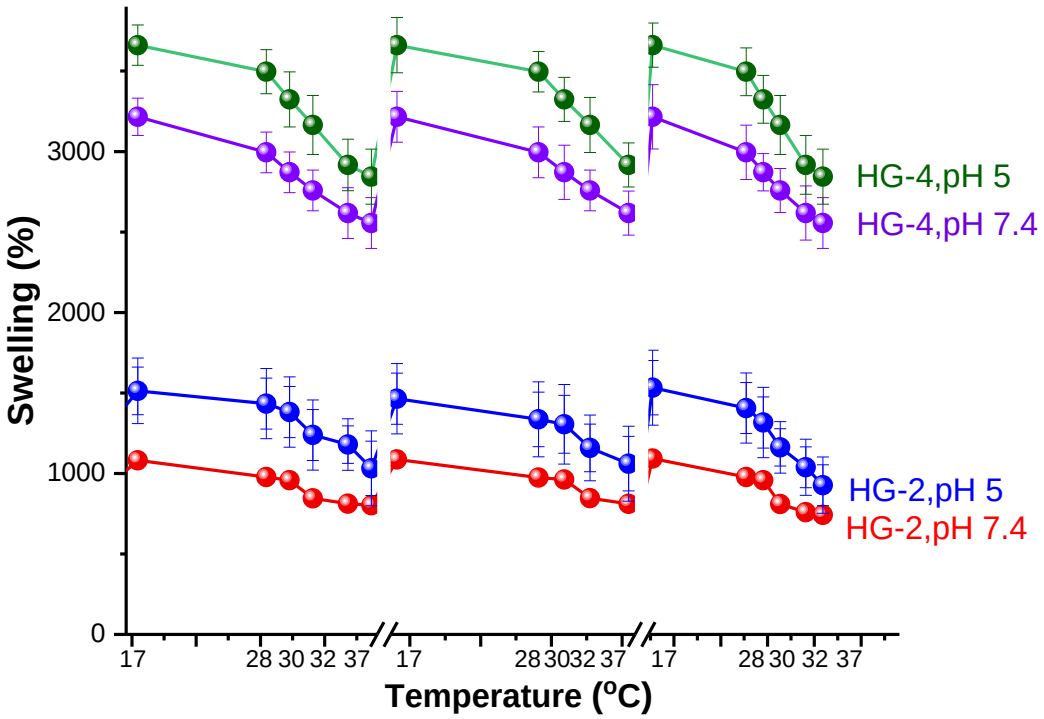


Figure S5. Change of equilibrium water swelling of representative HG-2 and HG-4 in heating-cooling cycle at two different pH.

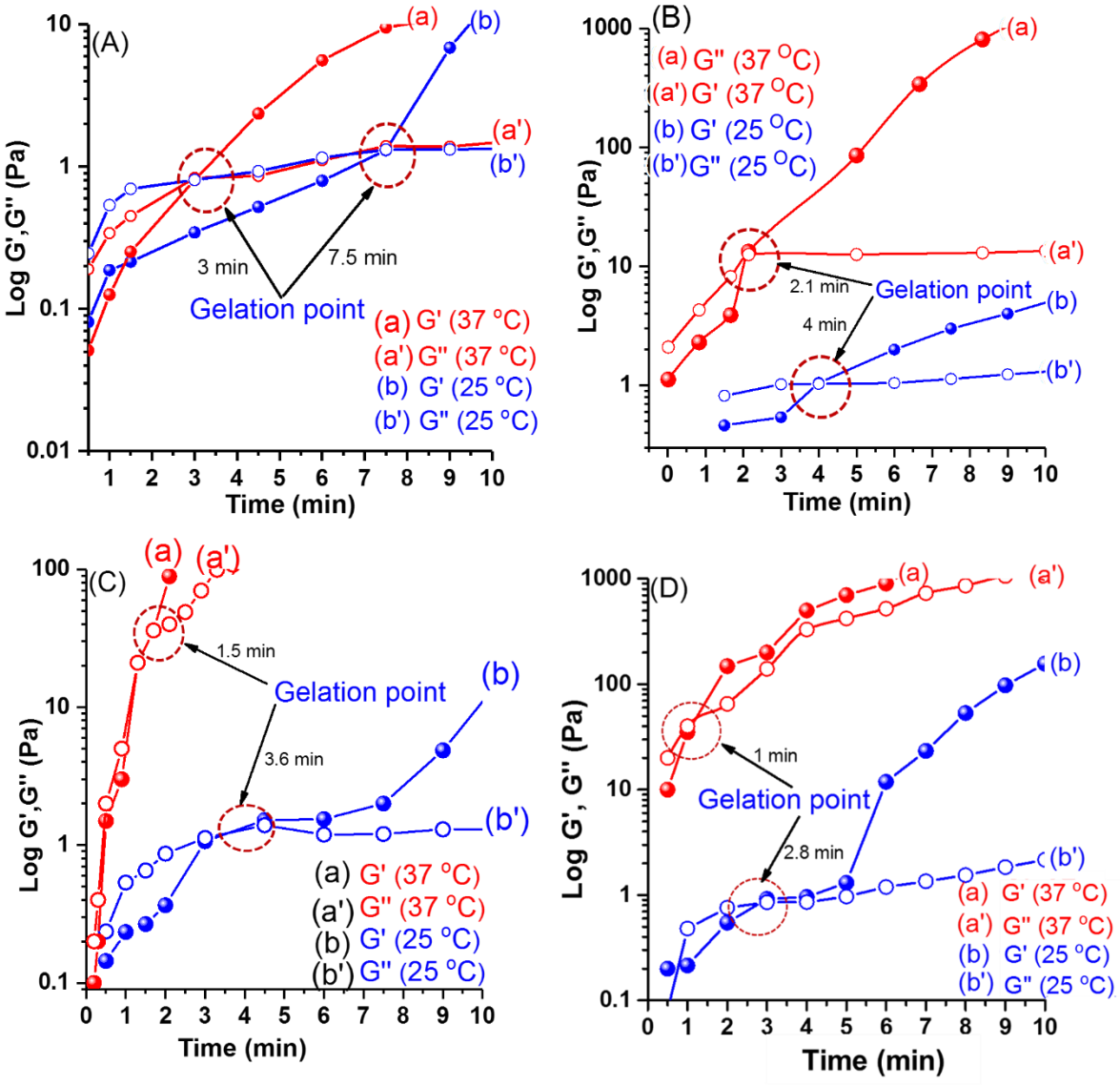


Figure S6. Variation in *G′* and *G′′* with time in an extended scale (Figure 1 in main text) showing intersection region (gelation time).

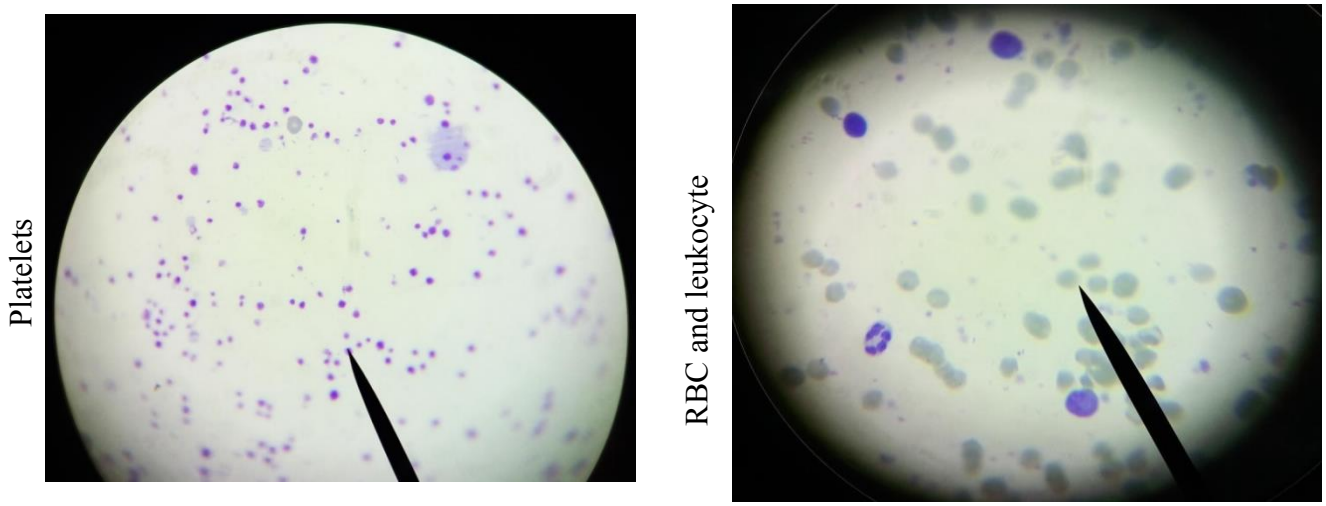


Figure S7. Optical microscope images of freshly isolated platelets (Left) and mixture of RBC and WBC (Right).

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