Bioresponsive supramolecular hydrogels for haemostasis, infection control and accelerated dermal wound healing

Namitha K. Preman,[a] Sindhu Priya E.S,[b] Ashwini Prabhu,[c] Sadiya Bi Shaikh,[c] Vipin C,[d,e] Rashmi R. Barki,[c] Yashodhar P. Bhandary,[c] P. D. Rekha,[d] Renjith P. Johnson[a*]

[a]Polymer Nanobiomaterial Research Laboratory, Yenepoya Research Centre, Yenepoya (Deemed to be University), Mangalore, India.

[b] Yenepoya Pharmacy College and Research Centre, Yenepoya (Deemed to be University), Mangalore, India.

[c]Division of Cell and Molecular biology, Yenepoya Research Centre, Yenepoya (Deemed to be University), Mangalore, India.

[d]Division of Biotechnology, Microbiology and Infectious diseases, Yenepoya Research Centre, Yenepoya (Deemed to be University), Mangalore, India.

***Corresponding author:** Polymer Nanobiomaterial Research Laboratory, Yenepoya Research Centre, Yenepoya (Deemed to be University), Mangalore, India; **E-mail:** renjithjohnson@yenepoya.edu.in; **Tel/Fax:** +91 8242204669.

Present address

[e] Relicus Bio Pvt. Ltd, Technology Business Incubator, Anna University, Chennai, 600025-Tamilnadu, India.

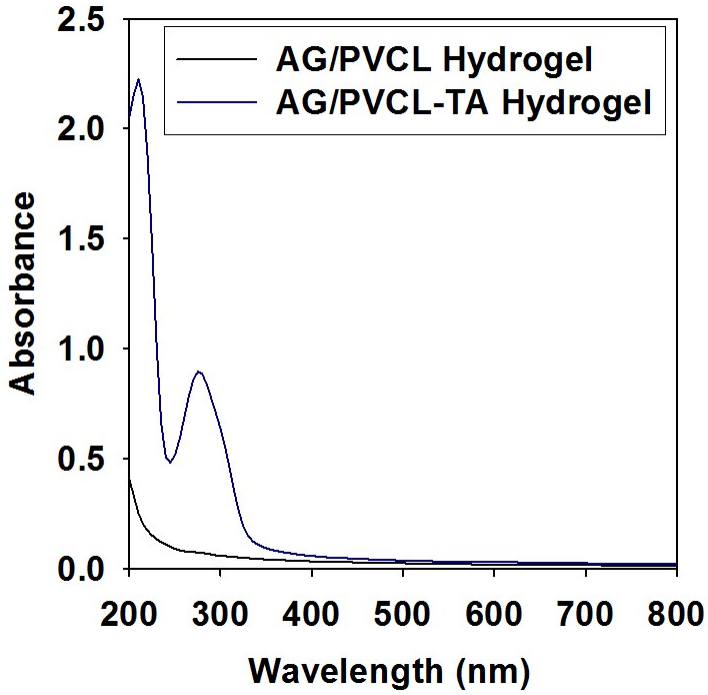

Fig. S1. The UV-vis spectra of AG/PVCL and AG/PVCL-TA SHGs

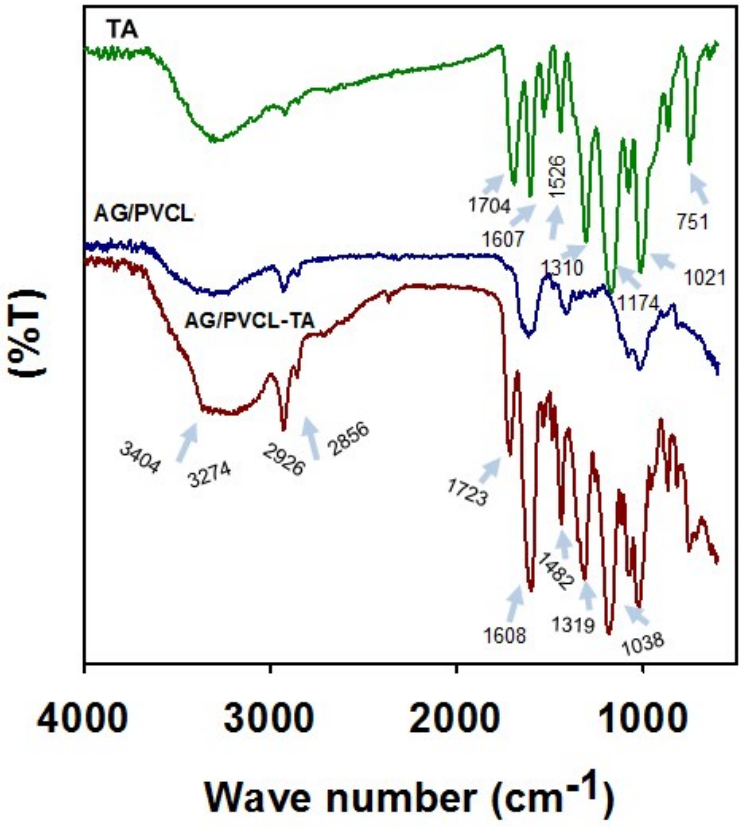

Fig. S2. The FT-IR spectra of TA, AG/PVCL and AG/PVCL-TA SHGs

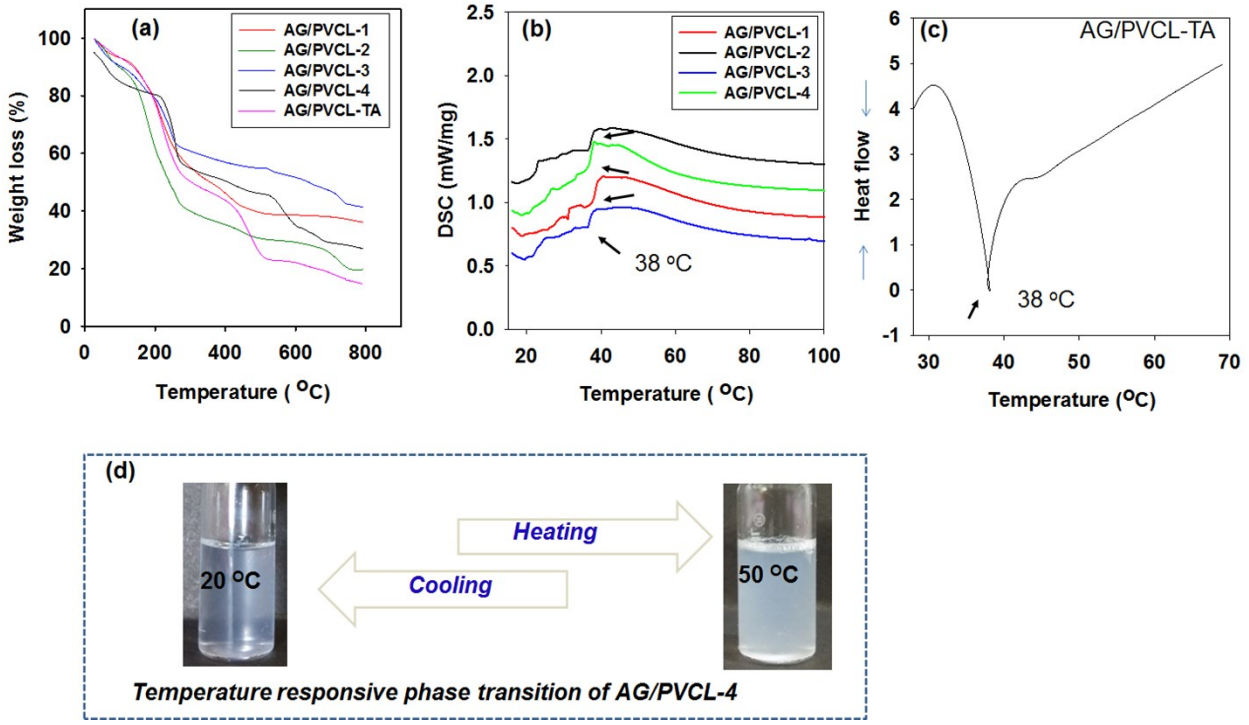

Fig. S3. The TGA **(a)** and DSC **(b)** and **(c)** analysis of AG/PVCL and AG/PVCL-TA SHGs. **(d)**. The visible temperature responsive phase transition of an aqueous dispersion of AG/PVCL SHG during a heating-cooling cycle.

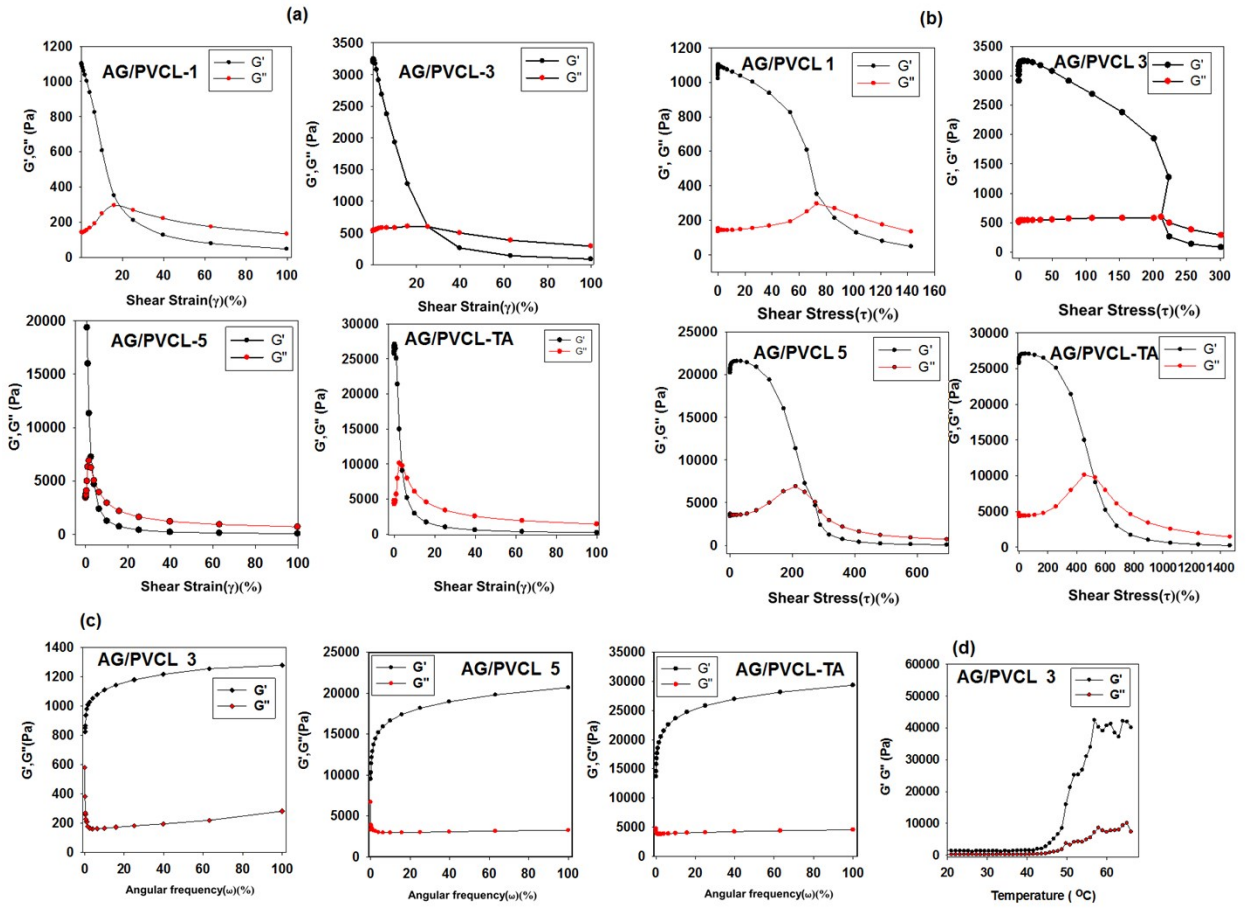

Fig. S4 (a, b). Storage G′ and loss G″ moduli of various SHGs in oscillatory tests (amplitude strain and amplitude stress sweep). **(c)**. The angular frequency sweep, and **(d)** the temperature sweep measurements.

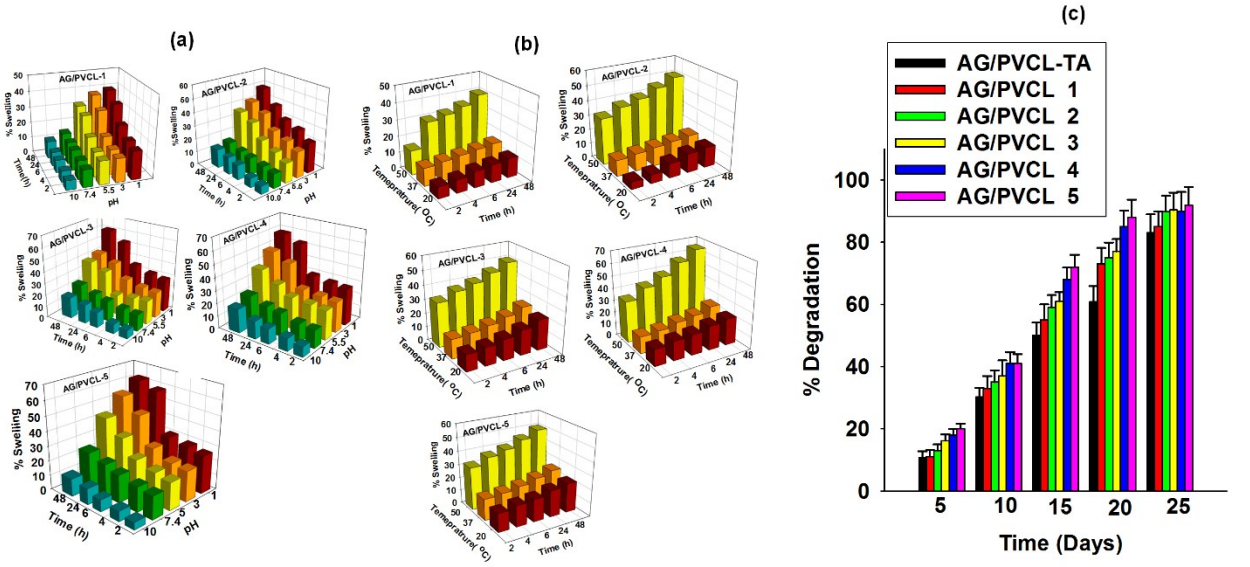

Fig. S5. The pH **(a)** and temperature **(b)** responsive swelling of various AG/PVCL SHGs. **(c)**. The *in vitro* hydrolytic degradation kinetics of AG/PVCL and AG/PVCL-TA SHGs at pH 7.4 and 37 ^{o}C.

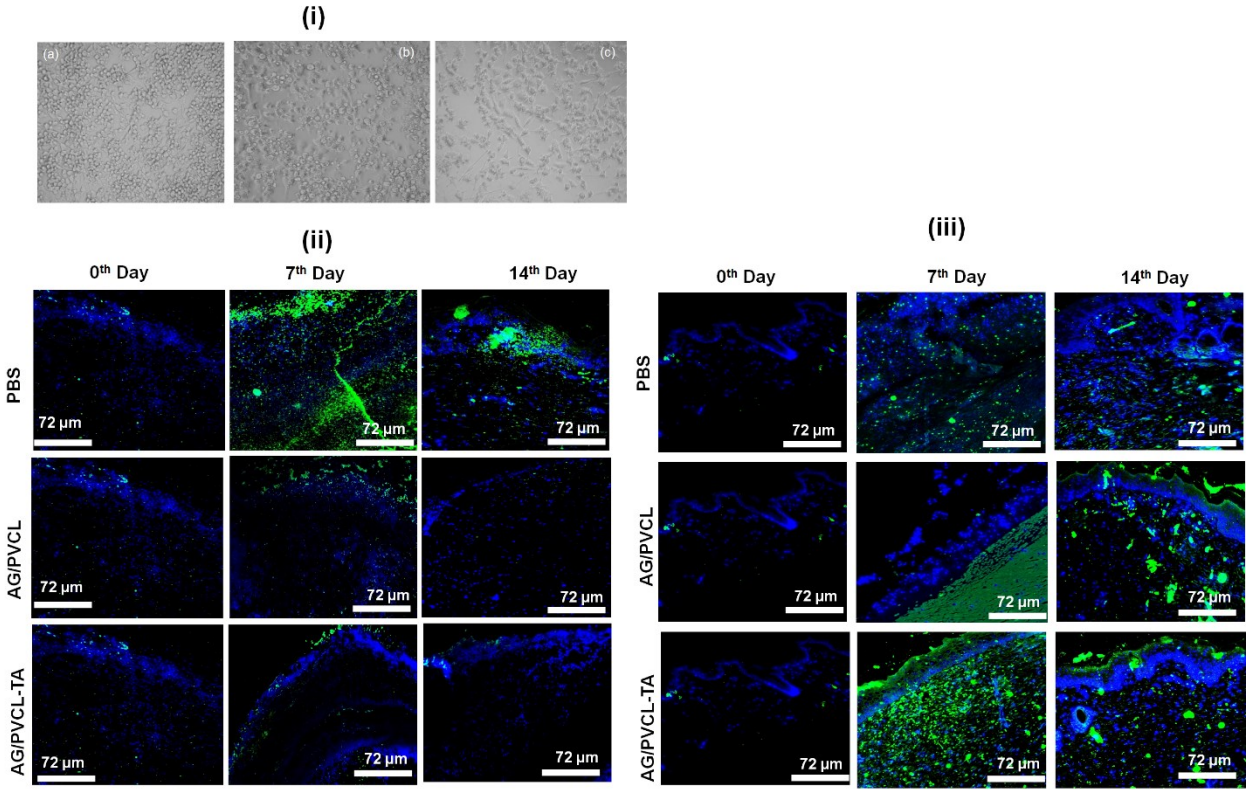

Fig. S6(i). (a). Phase contrast microscopy images of macrophages cells not exposed to LPS (negative control), **(b)**. Exposed to LPS for 24 h and then treated with AG/PVCL-TA conditioned medium and incubated for 24 h, and **(c).** Exposed to LPS (positive control) and treated with normal medium and incubated for 24 h **(ii)**. The IL-23 expression in wound tissues treated with PBS, AG/PVCL and AG/PVCL-TA SHGs at 0^{th}, 7^{th}, 14^{th} day (Scale bar 72 µm) (iii). The TGF-beta expression in wound tissues treated with PBS, AG/PVCL and AG/PVCL-TA SHGs at 0^{th}, 7^{th}, 14^{th} day (Scale bar 72 µm).

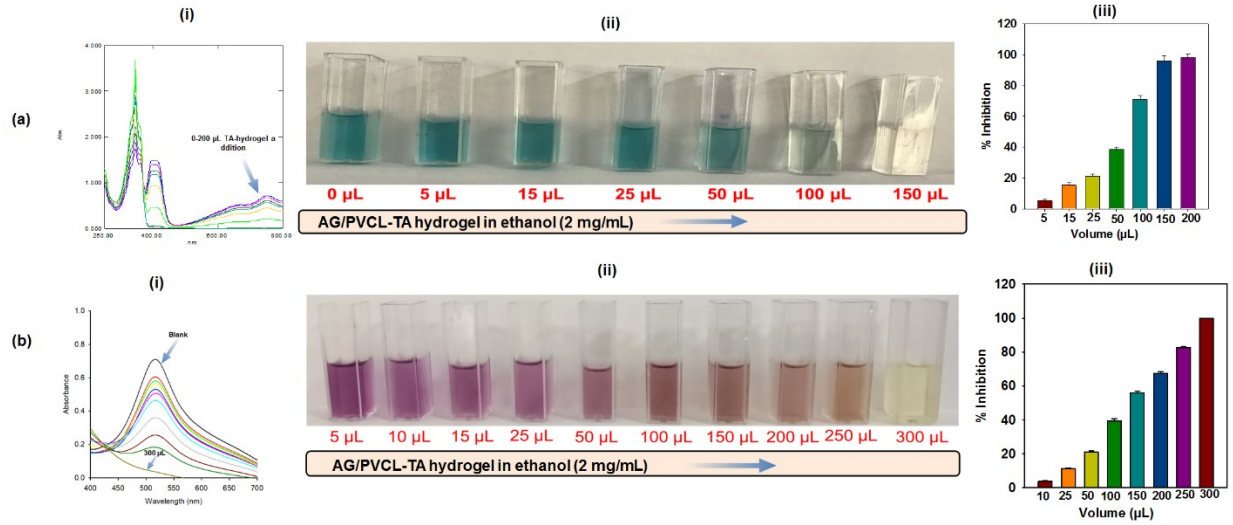

Fig. S7. (a). The $ABTS^{\bullet+}$ radical inhibition performance of AG/PVCL-TA SHG (i). The reduction in UV-vis spectra absorption at λ_{max} = 734 nm. **(ii)**. Radical decolorization and **(iii)**. The percentage of radical inhibition upon addition of ethanolic solution of AG/PVCL-TA. **(b)**. The $DPPH^{\bullet}$ radical inhibition performance of AG/PVCL-TA SHG **(i)**. The reduction in UV-vis spectra absorption at λ_{max} = 518 nm. **(ii)**. Radical decolorization and **(iii)**. The percentage of radical inhibition upon addition of ethanolic solution of AG/PVCL-TA.

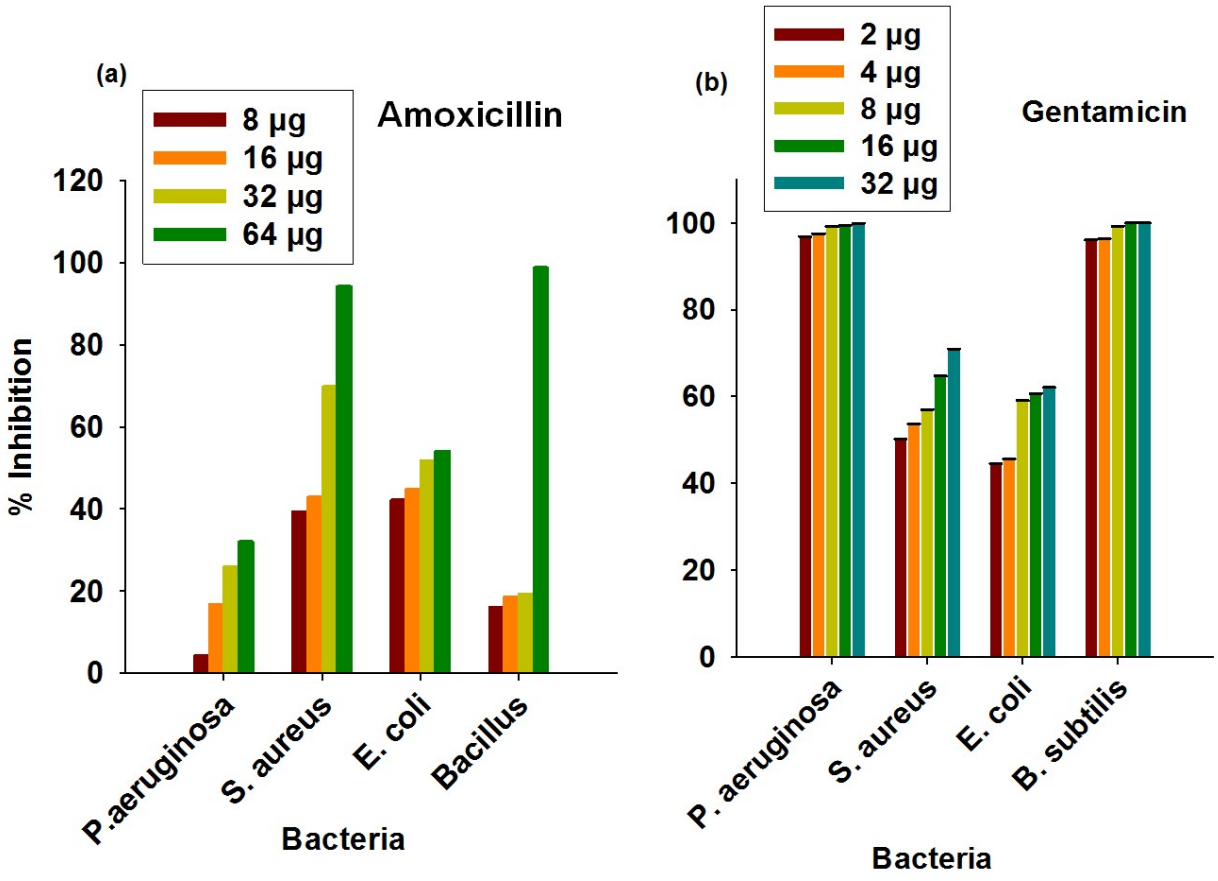

Fig. S8. (a) and (b). The anti-bacterial activity of *E. coli*, *P. aeruginosa, S. aureus*, *B. subtilis* bacteria against amoxicillin and gentamycin (% inhibition plotted with respect to control).

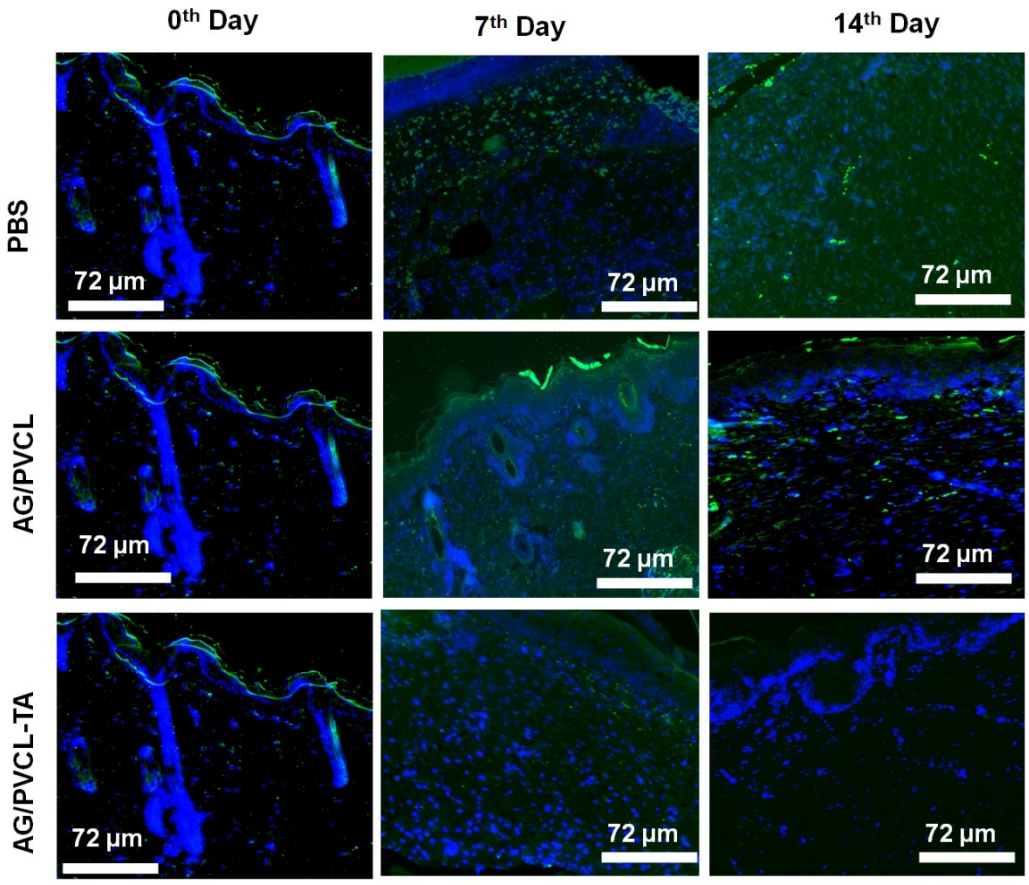

Fig. S9. The angiogenin expression in wound tissues treated with PBS, AG/PVCL and AG/PVCL-TA SHGs at 0^{th}, 7^{th}, 14^{th} day (Scale bar 72 µm).

Experimental procedures

Swelling kinetics: The stimuli-responsive swelling studies of all SHGs were performed under different pH and temperature conditions.[1S] Inorder to assess the pH responsive swelling kinetics, the preweighed SHGs were submerged in phosphate buffer saline in a vial at required pH values (1, 3, 5.5, 7.4, and 10) under room temperature for 48 h. Similarly, to assess the temperature responsive swelling, the SHGs were immersed in DI water at different temperature conditions (27, 37 and 50 ^{o}C) for 48 h at pH 7. 4. The swollen SHGs were taken out in predetermined intervells and the buffer adhered on the surface were removed using a tissue paper, and the percentage water uptake was calculated as a function of pH or temperature at different time intervells by measuring the hydrogel weight. The percentage of swelling was calculated from the following equation and was expressed as mean ± SD ($n = 3$): % swelling = $[(W_2\text{-}W_1)/W_1] \times 100$ %, where, W_1 and W_2 are the initial and final weights, respectively.

In vitro degradation study: The degradation characteristics of the SHGs was performed using standard protocols[1S] in phosphate buffered saline (PBS) of pH 7.4. The lyophilized and pre-weighed (50 mg) hydrogels were immersed in PBS, and placed at constant temperature of 37 ^{o}C in an incubator shaker with a shaking rate of 100 rpm. At specific time intervals, the hydrogel gel samples were removed and lyophilized to determine the dry weight. The degradation kinetics of the hydrogels were calculated by the following equation and was expressed as mean ± SD (n = 3): Mass loss (%) = $(M_0\text{-}M_D)/M_0 \times 100$, where M_0 is the dry mass of the sample, M_D is the residual dry mass of the sample after degradation at a certain time.

Anti-inflammatory assay: The anti-inflammatory activity of AG/PVCL-TA was assessed by measuring nitric oxide (NO) released as nitrite through Griess assay.[2S] The AG/PVCL-TA precursor mixture was incubated with DMEM with 10 % FBS (1 mL) for 24 h. The murine

macrophages cells (RAW 264.7) were seeded at a density of 5000 cells/well and allowed to reach the confluence over 24 h, and 100 ng/mL of lipopolysaccharide (LPS) was added to each well to stimulate NO production. After 5 h, hydrogel conditioned medium was added to each well and incubated for 24 h. The cells treated with LPS alone acted as positive controls and cells without any treatment acted as negative controls. After incubation, the cells were centrifuged and 100 µL supernatants were transferred to a 96 well plate along with 100 µL of Griess reagent, and subsequently, the amount of NO production was quantified by measuring optical density at 540 nm.

Anti-oxidant assays: DPPH assay-A freshly prepared DPPH/ethanol (0.2 mM) solution was used for the measurements. 0.2 mM DPPH radical solution was prepared by dissolving 1.97 mg of DPPH radical in ethanol and the mixture is stirred for 30 minutes in the dark at 37 °C. The blank was prepared by mixing 700 µL of DPPH solution in 1.7 mL ethanol for UV analysis.

DPPH decolorization assay using AG/PVCL-TA: Initially, 2 mg of *AG/PVCL-TA* weighed to 1 mL of ethanol in vial and vortexed for 5 mins. Different volumes of this sample are mixed with the DPPH radical solution and vortexed for a minute. The absorbance at 518 nm was calculated by using UV spectrometer, and the radical inhibition percentage was calculated. The DPPH radical scavenging activity was calculated as $I = [1 - (Ai - A_j)/A_c] \times 100\%$, where A_c is the absorbance of DPPH solution without test sample, A_i is the absorbance of test sample mixed with DPPH solution, and A_j is the absorbance of the test sample without DPPH solution. Each sample was tested three times.

ABTS assay- For conducting ABTS decolorization assay, the reagents prepared as follows. Initially, 7 mM ABTS radical cation stock solution was prepared by dissolving 8 mg of ABTS in 1 mL of water (Solution A), and 13.2 mg of potassium persulphate dissolved in 10 mL of water

(Solution B). In the next step, 0.5 mL of solution A and 0.5 mL solution B were mixed from the above stock and allowed to react in room dark at room temperature for 16 hours before further experiments. The concentration of ABTS and potassium persulphate in the mixture are 7 mM and 2.45 mM, respectively.

Preparation of Trolox stock solution: 1. 5 mM Trolox stock solution were prepared by dissolving 7.5 mg of Trolox in 20 mL of ethanol by gentle sonication. Different concentrations of Trolox samples were prepared by adding 0 to 20 µL Trolox in 1 mL ethanol, and the standard curve is generated.

ABTS decolorization assay using Trolox (TEAC assay): Initially, $ABTS^{\circ+}$ radical solution is prepared by pipetting 100 µL $ABTS^{\circ+}$ radical solution in 2 mL of ethanol. 2 mL of the radical solution is then mixed with different concentration of Trolox in ethanol in such a way that to get the final volume to 3 mL. The blank sample is prepared by adding 2 mL of $ABTS^{\circ+}$ in 1000 µL of ethanol. Required amount of Trolox mixed in $ABTS^{\circ+}$ radical solution and vortexed for 6 minutes. The absorbance at 734 nm was calculated by using UV spectrometer for different Trolox concentrations and the radical inhibition percentage is calculated.

ABTS decolorization assay using AG/PVCL-TA: Initially, 2 mg of AG/PVCL-TA is weighed to 2 mL of ethanol in vial and vortexed for 5 mins. Different volumes of this sample are mixed with the $ABTS^{\circ+}$ radical was solution vortexed for a minute and the absorbance at 734 nm was calculated by using UV spectrometer, and the percentage radical inhibition was calculated.

Antibacterial activity assay: The antibacterial activity of the AG/PVCL-TA hydrogel was tested against four bacteria namely *Pseudomonas aeruginosa* (MCC 2080), *Escherichia Coli* (ATCC 25175), *Staphylococcus aureus* (ATCC 25932), *Bacillus subtilis* (ATCC 6633). The antibacterial activities and minimal inhibitory concentration, MIC of AG/PVCL-TA hydrogel under defined

test conditions, inhibits the visible growth of the bacterium were determined by broth dilution method. MIC value was determined according to the reference.[3S] Microplate reader technique via absorption of O. D was used to find out the antibacterial activity of TA loaded hydrogel. Initially 2.1g of Muller Hinton and 1.8 g of agar powder were dissolved in 100 mL distilled water in a 500 mL conical flask was covered by cotton and autoclaved at 120 ^{o}C,15lbs pressure for 15 minutes. The suspension of *Pseudomonas aeruginosa*, *Escherichia Coli*, *Staphylococcus aureus*, *Bacillus subtilis* was made in Luria-Bertani broth making O.D of 1×10^{8} CFU. MIC determinations were done in triplicate (n =3), with AG/PVCL-TA Hydrogel and gentamicin, amoxicillin used as positive controls for Gram-positive bacteria and Gram-negative bacteria. The AG/PVCL-TA Hydrogel stocks were prepared to 10 mg/mL solution in water. The hydrogel stock was serially diluted 5 µg/mL, 10 µg/mL, 15 µg/mL 20 µg/mL 25 µg/mL, 30 µg/mL, 40 µg/mL across the 96-well plates. Gram-positive and Gram-negative bacteria were cultured in Mueller-Hinton broth at 37 °C overnight. 5 µL of this bacterial inoculum was added to the wells of the 96-well plate. Total volume was kept 200 µL by considering the above-mentioned concentration. All the plates were covered and incubated at 37 °C for 24 h. Finally, after incubation absorption readings were taken by using a microplate reader (Flustar Omega BMG Labtech, Germany) at 600nm O.D and MIC were determined.

Cytocompatibility assays

***MTT assay*:** The *in vitro* cytotoxicity of the SHGs was assessed through MTT assay towards NIH 3T3 fibroblast cell lines. The SHGs and SHG-TA precursor mixtures with different concentrations were incubated with 1 mL of DMEM (supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin for 24 h. The conditioned medium was then removed and the cytotoxicity of the leachable was tested. The 3T3 fibroblasts were grown in DMEM with 10 % fetal bovine

serum, antibiotic-antimycotic solution containing penicillin, streptomycin and amphotericin B were kept in a humidified CO_2 incubator. For MTT assay, 5000 cells in 100 µL complete media were seeded in each well of 96 well plates and incubated for 24 h. Subsequently, the medium was replaced with calculated concentrations of the SHG conditioned medium and the cells were incubated for 24 h in a humidified 5 % CO_2 incubator. Finally, 100 µL of MTT solution (1 mg/mL) was added to each well in dark and the cells were incubated for 4 h. The formazan crystals were solubilized using 100 µL DMSO and the absorbance was measured at 570 nm using a multimode microplate reader. Untreated cells served as a negative control, all assays were conducted in triplicate, and the data were plotted as mean ± SD. The and cell viability was determined using the following equation: Cell viability=Absorbance of the test x 100/Average absorbance of the negative control).

***Trypan blue exclusion assay*:** The AG/PVCL-4 and AG/PVCL-TA SHGs precursor mixture was with different concentrations were incubated with 1 mL of DMEM for 24 h. The 3T3 cells were seeded onto 24 well plates at a seeding density of 10,000 cells per well and then exposed to 100 µL of hydrogel conditioned medium. After 24 h incubation, the cells were trypsinised and then resuspended in 1X PBS. Subsequently, 10 µL of Trypan blue (0.4 %, w/v) solution was mixed with 10 µL of cell suspension, loaded into a haemocytometer, and examined immediately under an inverted microscope. The number of viable and dead cells was counted and percentage cell proliferation with respect to untreated control was calculated. The experiments were conducted in triplicate, and the data were plotted as mean ± SD and cell viability was determined using the following equation: Cell viability (%) = number of viable cells/total number of cells x 100.

***Live/dead cell staining assay*:** Live and dead NIH 3T3 cells after treatment with the SHG (AG/PVCL and AG/PVCL-TA) conditioned medium were qualitatively distinguished by

sequential staining with acridine orange and ethidium iodide. The cells were seeded onto each well of 24 well plates, followed by one-day incubation in 1 mL of hydrogel conditioned medium at 37 °C in a 5% CO_2 incubator. After incubation, the medium was removed and cells were washed with 1X PBS, and incubated in staining solution containing 2 μg/mL each of acridine orange and ethidium bromide for 15 min at 37 °C in dark and subsequently washed with 1X PBS. The cells were visualized and imaged under green and red channels using fluorescence imager (ZOE, BioRad, USA).

References

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[2S]. N.S. Bryan, M.B. Grisham, Methods to detect nitric oxide and its metabolites in biological samples. Free Radic. Biol. Med. 43 (2007), 645-57.

[3S] A. Kavanagh, S. Ramu, Y. Gong, M.A. Cooper, M.A. Blaskovich, Effects of microplate type and broth additives on microdilution MIC susceptibility assays. Antimicrob. Agents Chemother. 63 (2019) 01760-18.

Table S1. The primers used in the PCR experiments

Sl.No	Primers	Forward	Anti-sense
1	Mouse COL1	5′ACGGCTGCACGAGTCACAC-3	5′ GGCAGGCGGGAGGTCTT -3′
2	Mouse COL3	5′GTTCTAGAGGATGGCTGTACTAAACACA-3′	5′ TTGCCTTGCGTGTTTGATATTC-3′
3	β-actin	5′ACTATTGGCAACGAGCGGTT-3′	5′CAGGATTCCATACCCAAGA AGGA-3′