Supplementary information

Bioactive and Biodegradable Vitamin C stearate-based Injectable hydrogel alleviate experimental inflammatory arthritis

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1. Materials

6-O-Stearoyl-L-ascorbic Acid (SAA) and 9-aminoacridine (9AA) were procured from Tokyo Chemical Industry (TCI), Esterase (from porcine liver, 100 and 400 U/ml) (Sigma Aldrich), Matrix Metalloproteinase-9 (MMP-9), XTT (sodium 3′-[1- (phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate) purchased from Sigma Aldrich India. Rabbit polyclonal Tet-2 antibody (A1526), rabbit polyclonal NUR77 (NR4A1) antibody (A6676), rabbit polyclonal antibody to Nf-κβ (A3108) was obtained from Abclonal. Rabbit polyclonal COL4a1 antibody (PAS149Hu01) obtained from cloudclone. Freund's complete adjuvant was obtained from Sigma-Aldrich. Collagen Type II was obtained from Elastin Products Company Inc.

2. Methods

2.1. Synthesis of SAA hydrogel

2.1.1. Synthesis of SAA hydrogel

Synthesis process was earlier reported in our laboratory [1], Briefly, 40 mg of 6-O-Stearoyl-L-ascorbic Acid (SAA) (4% w/v) was heated with 1 ml of ethanol-distilled water (DW) mixture with the volume ratio of 1: 4 respectively. This mixture was mildly kept on heating up to 110-120 °C with stirring until SAA was completely dissolved. Heating and stirring were stopped as soon as SAA was dissolved, and the mixture was left undisturbed overnight to enable the SAA self-assembled into a SAA hydrogel. The glass vial was inverted to verify the gelation; the SAA hydrogel remained attached to the bottom part of the glass vial bottom without any gravitational flow was observed which indicates the formation of hydrogel. Similarly, 9-Aminoacridine-loaded SAA hydrogel (9AA-loaded SAA-hydrogel were synthesized using the same method. To formulate 9AA-loaded SAA-hydrogel was mixed with SAA individually.

2.2. Characterization of SAA-hydrogel

2.2.1. Zeta potential

The zeta potential of the blank SAA-hydrogel, 9AA-loaded SAA-hydrogel were measured using Zetasizer Nano ZSP; Model-ZEN5600; Malvern Instruments Ltd, Malvern, UK, using phase analysis light scattering (PALS) technique.

2.2.2. Field emission scanning electron microscopy

Field emission scanning electron microscopy (FE-SEM) was performed by using model JEOL JSM-7600F field emission scanning electron microscope Tokyo, Japan for assessing the surface morphology of the SAA-hydrogel, 9AA-loaded SAA-hydrogel and hydrogel incubation with esterase. A 20 kV acceleration voltage was maintained. A very small amount of freshly synthesized blank SAA-hydrogel, 9AA-loaded SAA-hydrogel that had been loaded with 9AA was lyophilized, positioned on carbon tape that was linked to aluminum grids, and subsequently coated with a thin layer of platinum conductive material using an auto fine coater (JEOL JEC-3000FC; Tokyo, Japan) for 90 seconds at 30 Pascals. Similarly, SAA hydrogel incubated with esterase and its morphology checked by FE-SEM.

2.2.3. Rheology of hydrogel

MCR302 (advanced Rheometer) parallel plate (PP-25) geometry at a measuring distance of 0.2 mm at 37°C has been used for detection of linear viscoelastic region, loss modulus, storage modulus, and thixotropic characteristics.

2.2.4. Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR)

To determine the loading of 9AA into the SAA-hydrogel, the ATR-FTIR was performed. To evaluate the physical and chemical interactions between 9AA, SAA and 9AA-loaded SAA-hydrogel using the ATR-FTIR system, a small quantity of SAA, 9AA, and 9AA-loaded SAA-hydrogel were placed on the sample stage. Cary Agilent 660 IR spectrophotometer and scanning was performed in IR range from 400–4000 cm-1.

2.2.5. High-Resolution Mass Spectrometry of SAA hydrogel

High-Resolution Mass Spectrometry (HR-MS) was employed to assess the breakdown of the blank SAA hydrogel using Agilent 6546 LC/Q-TOF spectrometer in positive (ESI^+) ion mode.

2.2.6. Determination of drug loading capacity and encapsulation efficiency

A calibration curve for 9AA was generated for encapsulation effectiveness (EE) and drug loading (DL). From 9AA stock solution series of dilutions made, and absorbance was calculated using a Shimadzu UV-2600 UV-Vis spectrophotometer between the wavelength range of 200-400 nm. Drug loading capacity was determined, freshly prepared l ml SAA-hydrogel (40 mg SAA, 4 mg 9AA with 200µl Ethanol and 800µl distilled water) was centrifuged at 16,000 RPM for 15 min, The absorbance was measured to determine the amount of free 9AA in the collected supernatant

before it had been adequately diluted. According to the UV spectroscopy evaluation, drug loading was 16% and drug encapsulation efficiency was 95.53% which is excellent and ideal for a drug delivery system. Drug loading and entrapment efficiency was calculated using following formula:

$$\text{Amount of 9 - Aminoacridine - loaded in SAA - Hydrogel} = \text{Amount of 9 - Aminoacridine taken} - \text{9 - Aminoacridine present in supernatant}$$

$$Encapsulation\ Efficiency\ (\%) = \frac{\text{Amount of 9 - Aminoacridine in SAA hydrogel}}{\text{Total amount of 9 - Aminoacridine taken}} X100$$

$$Drug\ Loading\ (\%) = \frac{\text{Amount of 9 - Aminoacridine in SAA hydrogel}}{\text{Total amount of SAA hydrogel used}} X100$$

2.2.7. Ultraviolet-visible (UV-Vis) spectroscopy

Different concentrations of 9AA were read from 200 to 400nm using Shimadzu UV-2600 for plotting 9AA standard curve to determine the drug loading and encapsulation efficiency.

2.2.8. *In-vitro* drug release

Drug release kinetics for 9AA-SAA hydrogel was studied at 37 °C in PBS pH 7.4 through the dialysis bag method. Briefly, a known amount of 9AA drug loaded in SAA hydrogel was transferred into a previously activated dialysis bag with the desired length after washing with PBS (dialysis membrane, Himedia) and kept in 200 mL of PBS, pH 7.4, and stirred at 100 rpm at 37 °C. At predetermined time intervals of 1d, 2d, 3d……& 20d, 1 mL of solution from the receptor compartment was taken and the same volume of PBS was added back to retain sink conditions. The concentration was calculated by a UV–Vis spectrophotometer and plotted against different time points [1].

2.2.9. Enzyme-responsive *in-vitro* drug release

The enzyme responsive drug release was performed by the previously reported method with slight modifications. In brief, esterase (from porcine liver, 100 and 400 U/ml) (Sigma Aldrich) and MMP-9 (1µg/ml and 5µg/ml) was added at pre-defined time points into the dialysis tubing of 9AA-SAA hydrogel. The samples were withdrawn at predefined time points and analyzed by using UV-Vis spectrophotometer at the same wavelength of standard curve of 9AA.

2.2.10. *In-vitro* cytocompatibility assay

(a) Trans-well method

The cytocompatibility of SAA hydrogel was measured by XTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate) assay. It was measured against hTERT-BJ cells. The cells were seeded at the concentration of 1 × 104 in 12-transwell insert plates. Cells were treated with various concentrations of SAA hydrogel for 24 h. Cytocompatibility was determined by following the modified protocol of assay kit purchased from Invitrogen. XTT was incubated for 4 h, and the % cell viability was calculated by measuring the absorbance at 570–595nm [1].

(b) Co-incubation method

By following the protocol of Ou et. al., 2021 with slight modification we have performed XTT assay [2]. For extracting the liquids of SAA hydrogels, 5 mg/mL SAA hydrogel were added to complete media under stirring condition at 37 ℃ for 24 h. The resulting solutions were passed through a 0.22μm filter before being co-incubated with cells in a 96-well plate for 24hrs. Absorbance of XTT assay was taken at 570-595nm was determined using an automatic microplate reader.

3. *In-vivo* efficacy of 9AA-loaded SAA hydrogel in Collagen induced arthritis in Wistar rats

Study groups and treatment regimen

3.1. Ethical Statement

Female Wistar rats (140−160 g), 6−8 weeks old, were purchased from Central Animal facility, National Institute of Pharmaceutical Education and Research, S.A.S. Nagar, Punjab. Rats were housed in Central University of Punjab and Bathinda, Bathinda animal care facility under an ambient temperature of 25 ± 1 °C with 12 h light/dark cycles after initial acclimatization for about 1 week. They had free access to standard rodent pellet diet and water ad libitum. All procedures for using experimental animals were checked and permitted by the “Institutional Animal Ethics Committee (IAEC)” of Central University of Punjab and Bathinda that is fully accredited by the Committee for Purpose of Control and Supervision on Experimental Animals (CPCSEA) Chennai, India. Project approval number for this study is CUPB/IAEC/2022/68.

3.2. Development of Collagen induced arthritis (CIA) in Wistar rats

Specifically, collagen type II from bovine nasal septum was solubilized in 0.05M acetic acid at concentration of 2mg/mL and emulsified with same volume of complete Freund’s adjuvant

(CFA) composed of 1mg/mL *Mycobacterium tuberculosis* H37Ra and placed on ice before use. Rats were immunized by injecting intradermally 100µl of previously prepared emulsion at 1.5cm away from base of the tail. It was reported that rats immunized with collagen type II emulsified in complete Freund's adjuvant showed significant development of foot pad and ankle joint swelling, which is indicative of arthritis development. Whereas rats immunized with collagen type II in incomplete Freund's adjuvant showed no significant increase in foot pad and ankle swelling, on this basis we have selected to use complete Freund's adjuvant over incomplete adjuvant [3,4].

3.3. *In-Vivo* Therapeutic Study

To evaluate the therapeutic potential of 9AA-SAA hydrogel in the CIA model in female Wistar rats, the rats were distributed in 6 groups containing 6 rats each by keeping the average weight of every group as close as possible. The treatment was started after the development of arthritis symptoms (after 14 days). The study was divided into 2 parts; the basic objective was to evaluate the short-term and long-term protection of our 9AA-SAA hydrogel. For study, the animals were given 4 doses of 9AA-SAA hydrogel by keeping a gap of 2 days between the doses, and the animals were sacrificed on the 21st day. Then we observed the animals for the next 21days to study the recurrence as well as the long-term protection from RA.

4. Treatment Regimen

To study the effect of treatment with 9AA-SAA hydrogel against CIA, 30 female Wistar rats were randomly allocated to 6 groups of 6 rats each.

Group I (Control group). Rats received basal diet.

Group II (CIA group). This group served as a collagen-induced arthritis (CIA) group.

Group III (9AA-SAA hydrogel). Group III served as the treatment group rats CIA also received 9AA-SAA hydrogel at the dose of 10mg/kg body weight intraarticular *(i.a.)* from onset of disease once daily (equivalent to 10mg/kg body weight of 9AA) intra-articular *(i.a.)* from 14th day after immunization.

Group IV (9AA group). This group served as the CIA group and rats also received 10mg/kg 9AA intraarticular *(i.a.)* from 14th day after immunization.

Group V (SAA hydrogel group). Group V served as the CIA group and rats also received 10mg/kg blank SAA hydrogel (equivalent to 10mg/kg body weight of 9AA) from 14th day after immunization.

Group VI (Safety group). Rats received an equivalent dose of blank SAA hydrogel. All the rats were anaesthetized with mild anesthesia and sacrificed by cervical dislocation on 21st day.

4.1. Radiological Analysis

Radiological assessment of paw joints was conducted using an X-ray machine. Anesthetized rats were positioned on a radiographic plate, and a controlled X-ray beam was directed to create images of the paw joints on a radiological plate. This procedure was carried out using an X-ray machine (Siemens Kinescope-H) model with an exposure set at 38kVp for a duration of 6.5mAs.

4.2. Disease activity index

4.3. Weight variation

Percentage variation in the weight of the experimental animals has been determined using following formula:

$$Weight\ variation\ (\%) = \frac{Final\ weight - Initial\ weight}{Final\ weight} X100$$

4.4. Disease Activity Index (Mean arthritis severity score)

Evaluation of arthritis severity: Mean arthritis severity scores of different groups on 21st day (day of sacrifice). The severity of the arthritis was determined after every third day by a clinical score measurement from 0 to 4 as follows: 0, no signs of arthritis; 1, swelling of one group of joints (namely, wrist, or ankle joints); 2, two groups of swollen joints; 3, three groups of swollen joints; 4; swelling of the entire paw. To examine the severity of the disease, measurements of rat joint's(paw)diameter were carried out with digital Vernier's callipers (Yamayo, Japan) before and after onset of disease every third day. Evaluation of joint inflammation was performed by an observer having no knowledge of treatment protocol[5].

Groups	Mean arthritis severity scores
Control	0

CIA	15.23
CIA+ 9AA-SAA hydrogel	3.23
CIA+ 9AA	68.34
CIA + SAA hydrogel	14.98
Control + SAA hydrogel	0

5. Histological Staining

5.1. Hematoxylin and eosin staining

Rats were anesthetized and sacrificed on 21st day of the study by using isoflurane anesthesia. Rat's ankle joints were removed and fixed in 4% formalin. After decalcification in 5% formic acid the joints were embedded in paraffin. Section of 5µm thickness cut and stained for hematoxylin and eosin and observed under Olympus microscope[6].

5.2. Safranin-O staining

Proteoglycan content of cartilage was stained with safranin O staining was performed on 5µm thick joint sections. The sections were deparaffinised in xylene followed by rehydration in graded series of alcohol to water. These were then stained with hematoxylin and destained with acid ethanol. Sections were then stained with 0.001% fast green (FCF) and rinsed with 1% acetic acid solution. Further, these were then stained with 0.1% safranin O stain for 20min. Sections then dehydrated with increasing concentration of ethanol. Then dipped in xylene and mounted with DPX and observed under Olympus microscope under 10X magnification[3].

5.3. Toluidine blue staining

Cartilage was specifically stained with toluidine blue, 5µm thick joint sections were deparaffinised and rehydrated in decreasing concentration of ethanol followed by water. The sections were stained with 0.4% toluidine blue stain and counterstained with 0.001% fast green (FCF). The sections were dehydrated with increasing concentration of ethanol and xylene. Sections were mounted with DPX and observed with Olympus microscope under 40X magnification[3].

5.4. Mast cells staining

Deparaffinised joint sections were rehydrated with decreasing concentration of ethanol followed by water, and staining with 0.1% toluidine blue at pH 2.3 for 20min. Then sections

were dehydrated with increasing concentration of ethanol followed by xylene, mounted with DPX and observed with Olympus microscope under 40X magnification[3].

5.5. Immunohistochemistry

Immunohistochemistry of NUR77 (NR4A1), Tet-2, IL-1B and Nf-κβ was carried out using paraffin embedded 5μm thick sections. The sections were deparaffinised three times in xylene, followed by rehydration in graded ethanol and water. These were then subjected for antigen retrieval in 10mM citrate buffer (pH 6.0) for 10-15min and then incubated with hydrogen peroxide to avoid nonspecific staining by neutralizing the endogenous peroxidase activity. These were washed with PBS for three times each for five minutes, incubated with 1% BSA for 45 min. After washing with PBS these were incubated with rabbit polyclonal Tet-2 antibody (A1526,1:200, Cloudclone), rabbit polyclonal NUR77 (NR4A1) antibody (1:200, A6676, Abclonal), rabbit polyclonal antibody to NF-κB (A3108, 1:200, Abclonal), rabbit polyclonal COL4a1 antibody (1:200, PAS149Hu01, Cloudclone), in humidified chamber for overnight at 4°C. Following washing with PBST (PBS pH 7.4, 0.005% Tween 20) sections was incubated with HRP conjugated secondary antibody for 2–3 h. At last, these were treated with 3,3, - diaminobenzidine (DAB) followed by counterstaining with hematoxylin and mounted with DPX and covered with coverslips. We used negative control in which we incubated the tissue section in antibody diluent only (i.e., phosphate buffer saline) further we used secondary antibody and DAB. Hence, it concludes that staining is only because of the primary antibody bound to the antigen. The observation was carried out under Olympus bright-field microscope using 40X magnification [1,3].

5.6. Protein Content

Protein content was determined by the Bradford method using bovine serum albumin (BSA) as a standard as described earlier with slight modification[5].

5.7. Statistical Analysis

Results were expressed as mean ± S.D. All data were analyzed using two-way anova analysis of variance followed by Tukey's test. Values of $^{***}p \leq 0.001$, $^{\#\#\#}p \leq 0.001$ were considered as significant. All statistical analyses were performed using graph pad prism 9 software (Graph Pad Software, Inc., San Diego, CA, USA).

6. Results

6.1. Zeta potential of hydrogel

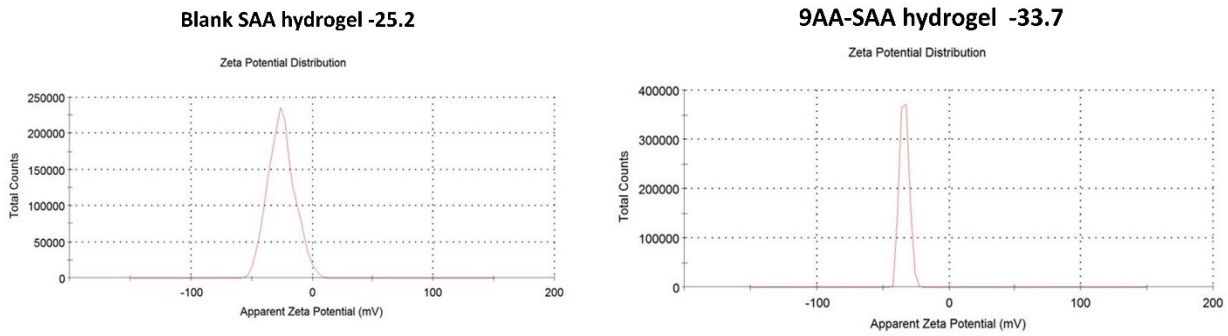

Figure S1. Zeta potential value of blank SAA hydrogel and 9AA-SAA hydrogel

6.2. HR-MS of SAA hydrogel

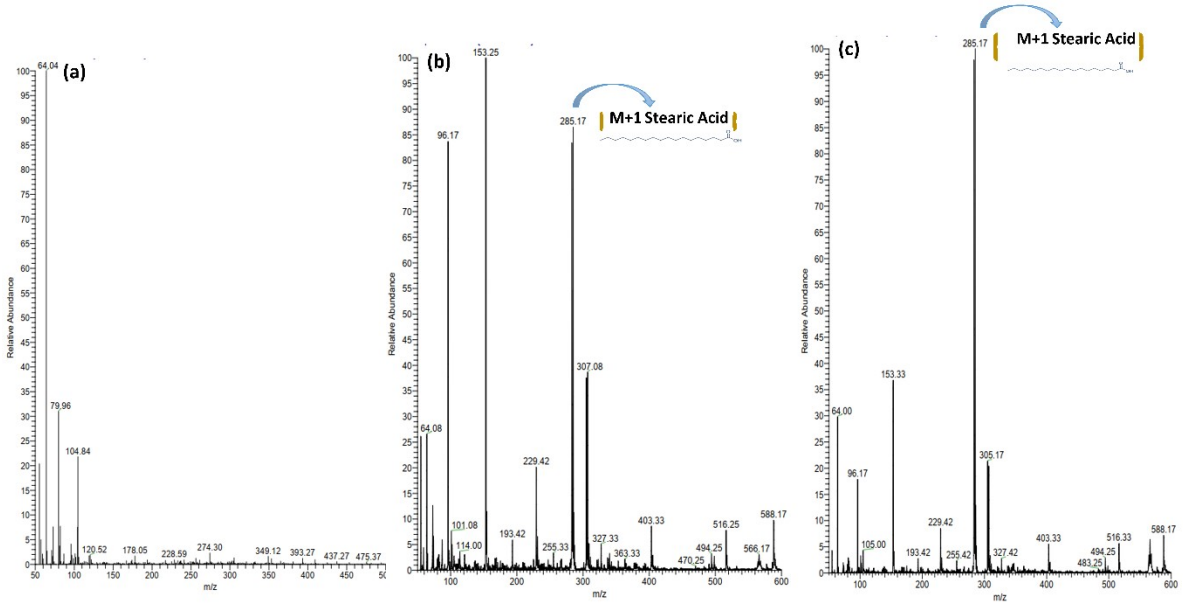

Figure S2. HR-MS of SAA hydrogel (a) without esterase (b) with esterase (100 unit) (c) with esterase (400 unit)

6.3. Cytocompatibility of hydrogel

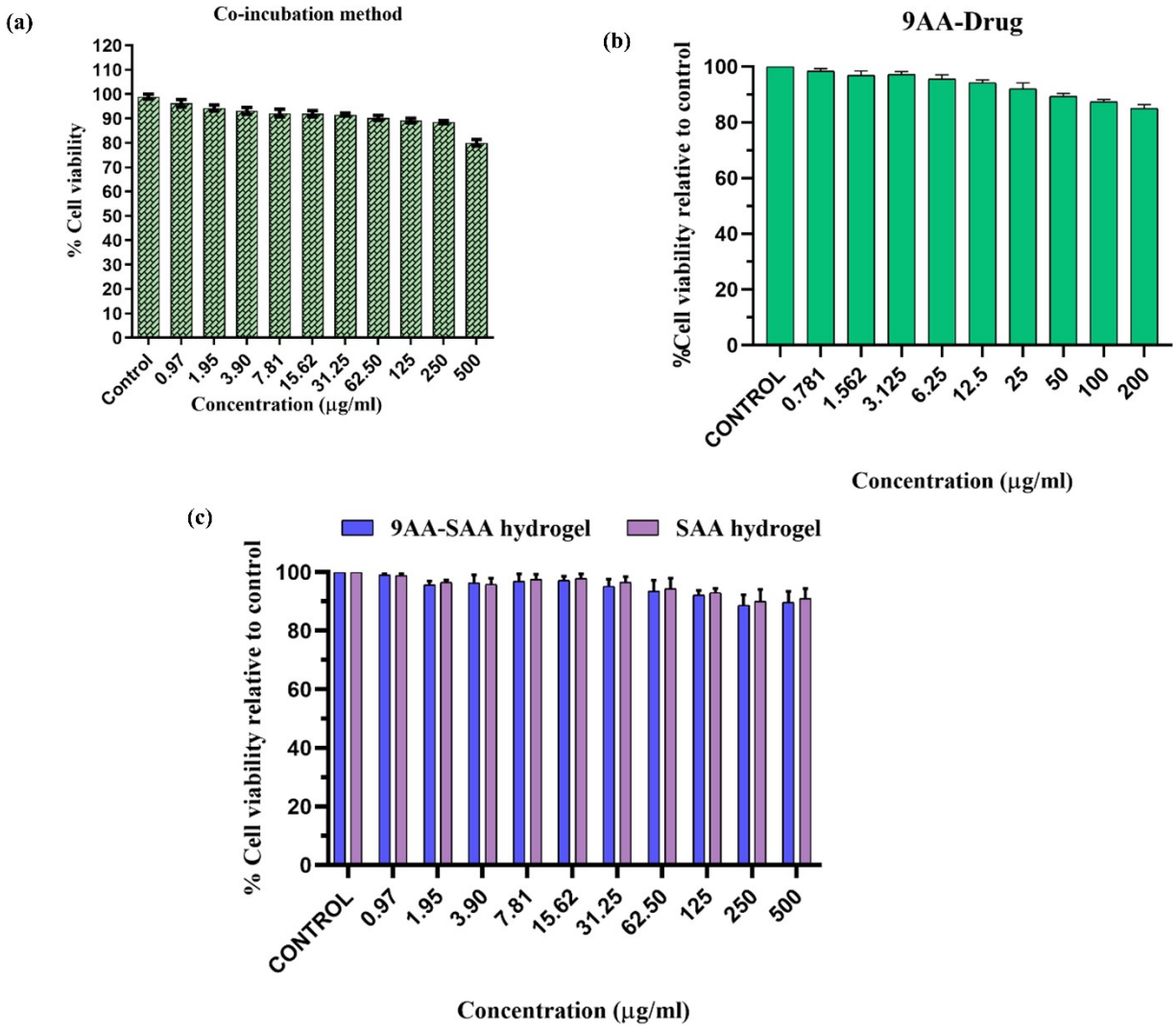

Figure S3. Cytocompatibility of hydrogel **(a)** SAA hydrogel **(b)** 9AA and **(c)** 9AA-SAA hydrogel

6.4. Protective effects of 9AA-SAA hydrogel in LPS induced RAW264.7 cell line

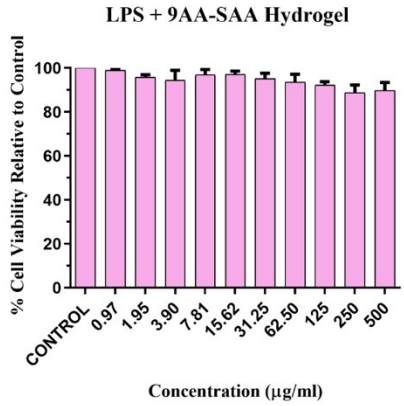

Figure S4. Cytocompatibility assay of 9AA-SAA hydrogel on LPS induced RAW 264.7 cell line for 24 hrs. Experiments were performed in triplicates n=3. Data were represented as mean ± SD of three independent sets of observations. Significant differences were indicated by ***p≤0.001, **p≤0.01, *p≤0.05.

6.5. Mast cells imaging

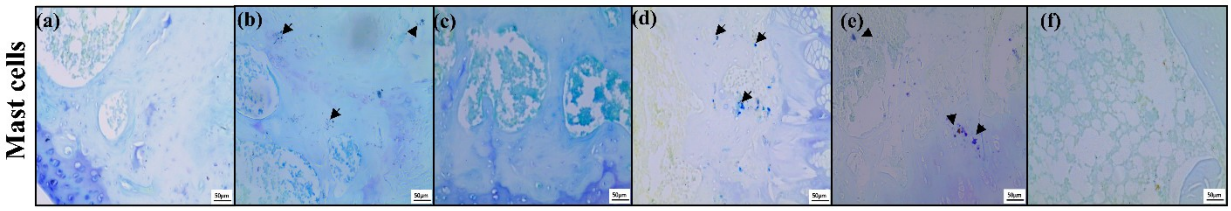

Figure S5. Representative images of mast cells staining. ((a) Control, (b) CIA, (c) 9AA-SAA hydrogel, (d) CIA + 9AA, (e) CIA + SAA hydrogel, (f) Control + SAA hydrogel).

6.6. Quantitative analysis of various staining of cartilage

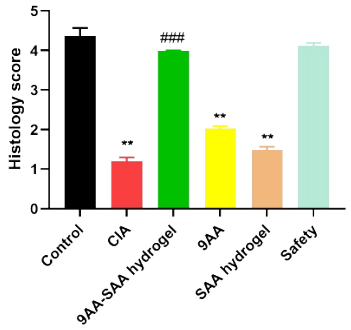

Figure S6. Histology scoring of various staining

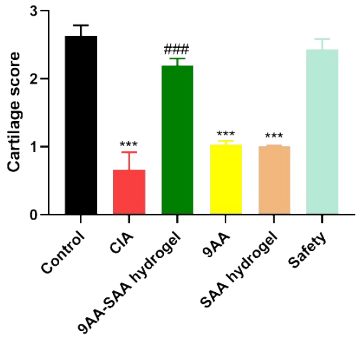

Figure S7. Cartilage thickness scoring.

6.7. Immunohistochemistry scoring

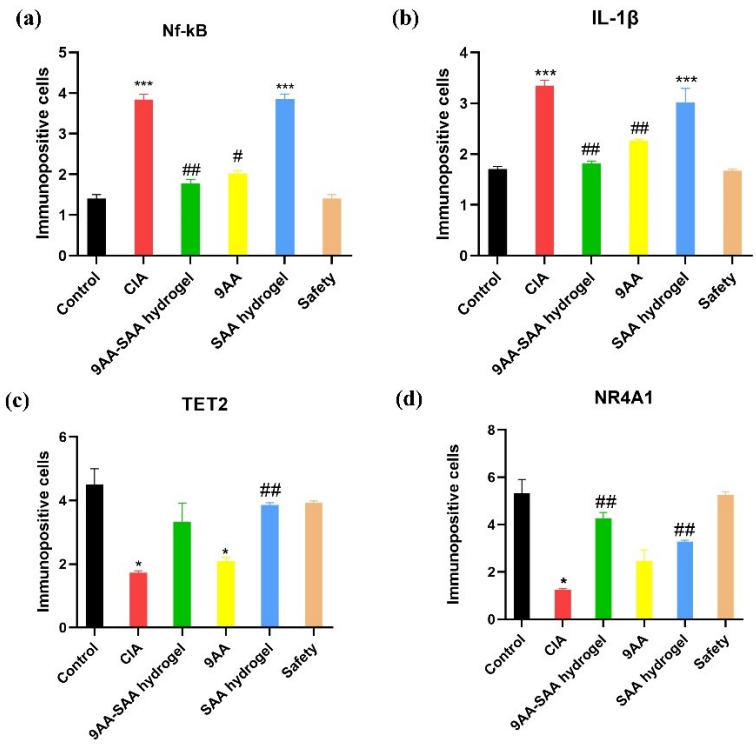

Figure S8. Immunohistochemistry scoring of various protein. (Control, CIA, 9AA-SAA hydrogel, CIA + 9AA, CIA + SAA hydrogel, Control + SAA hydrogel).

7. References

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