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Development of injectable high molecular weight hyaluronic acid hydrogels for cartilage regeneration

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ABSTRACT

The study focuses on developing hyaluronic acid (1200 kilo Dalton) hydrogels for cartilage regeneration. In spite of being highly biocompatible; a large amount of water absorption and easily degrading nature restricts the use of hyaluronic acid in the field of tissue regeneration. This can be rectified by crosslinking hyaluronic acid with a crosslinking agent such as divinyl sulfone; which results in a biocompatible hydrogel with superior rheological properties. Different amounts of divinyl sulfone have been used for crosslinking hyaluronic acid to get three types of hydrogels with differing properties. Swelling studies, rheology analysis, enzymatic degradation and scanning electron microscopic analysis were conducted on all the different types of hydrogels prepared. Viscoelastic properties of the hydrogel were analyzed so that a hydrogel with better elastic property and stability is obtained. Scanning electron microscopy was used to study the morphology of the HA hydrogels. The cytotoxicity testing was conducted to prove the non-toxic nature of the hydrogels and cell culture studies using adipose mesenchymal stem cells showed better adhesion and proliferation properties in all the three hydrogels. Thus hyaluronic acid hydrogel makes a promising material for cartilage regeneration.

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1. Introduction

Hyaluronic Acid (HA) is a naturally occurring linear polysaccharide with the repeating units of β−1,4-D-glucuronic acid and β−1,3-N-acetyl-D-glucosamine. HA is mainly found in the extracellular matrix of almost all the mammalian connective tissue with reported roles in the rheological, structural, physiological and biological functions of the body. It is highly biocompatible, biodegradable, and elicits very low levels of immune response (1–5). One of the limitations of HA is that it gets degraded rapidly within the tissue, with a residing time of just 1–2 days. Hence, chemical crosslinking of HA is very essential to increase the residence time of the compound (6).

Crosslinked HA has been recognized as an important biomaterial for tissue engineering and regenerative medical applications. Over the past decade, a wide variety of HA-based hydrogels have been synthesized and used for applications such as sustained cell expansion, facilitated cartilage repair, oriented bone regeneration etc. (7). HA hydrogels crosslinked via photo-polymerization and encapsulated with chondrocytes, can be useful in promoting cartilage regeneration (8). HA hydrogels has found application in cardiac repair and is used as substitute for heart valves (9). Additionally, HA hydrogels have been used for encapsulating mesenchymal stem cells and human embryonic stem cells and to direct stem cell behavior within a 3 D environment (10). As mentioned earlier, HA is water-soluble and exhibits a fast degradation profile and clearance within the body. Therefore, HA must be covalently crosslinked in order to provide a mechanically robust hydrogel. Several methodologies such as high energy irradiation, chemical reactions such as radical polymerization, photo-polymerization etc. have been reported for this purpose (11). Crosslinking widely involves chemical modification of HA by targeting either the hydroxyl or carboxylic acid functionalities of the sugar moieties (12, 13). In this study, HA has been crosslinked with divinyl sulfone (DVS) to form a three dimensional network of hydrogel. The reaction mechanism between hyaluronic acid and divinyl sulfone has been depicted in Scheme 1 (14).

The concentration of DVS has been varied in the hydrogels and the resulting viscoelastic properties have been compared. During cartilage repair, extracellular matrix formation dependents on the properties of the hydrogel such as swelling ratio, degradation rate and gel strength (15). The gel strength of the HA crosslinked hydrogel formed were studied by performing the rheology studies (16). Prepared hydrogels have been characterized using scanning electron microscopy (SEM). Thereafter, the prepared gels have been evaluated for swelling, enzymatic degradation, cytotoxicity evaluation and its interaction with adipose mesenchymal stem cells to study its potential therapeutic effect in the field of cartilage regeneration (17–19).

2. Experimental

2.1. Materials

Highly purified hyaluronic acid (HA) (Molecular weight: 1200 kDa) made by fermentation was purchased as a dry

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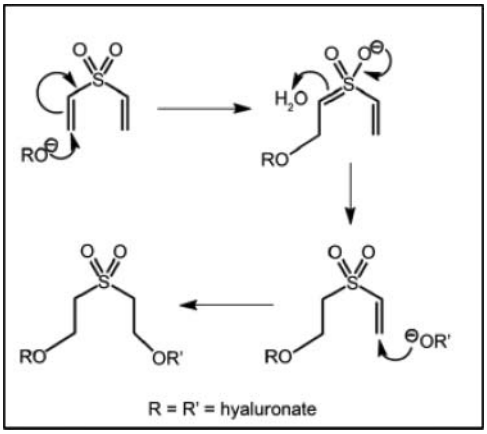

Scheme 1. The reaction mechanism between hyaluronic acid and divinyl sulfone.

powder from Kumar Organics Pvt. Ltd., India. Divinyl sulfone (DVS) and dialysis tubings were purchased from Sigma Aldrich, USA. Hyaluronidase enzyme (100U/mL) and phosphate buffer saline (PBS) were purchased from Sigma Aldrich (St. Louis, MO, USA). Deionized water used was purified with a milli-Q system (Millipore, Bedford, MA, USA). Mesenchymal stem cells (MSC), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay kit and other consumables were purchased from Himedia Laboratory, India.

2.2. Methods

2.2.1. Synthesis of hyaluronic acid crosslinked with divinyl sulfone using different concentrations

HA-DVS was synthesized by slightly modifying an already reported procedure (20). HA (200 mg) was dissolved in distilled water. 200 μL of 1 M sodium hydroxide was added for adjusting the pH. After the complete dissolution of HA; crosslinking agent was added. The amount of crosslinker added has an effect on the crosslinking property of the hydrogel and in turn it affects the viscoelasticity of the gel (21). Hence, we have tried three different concentrations i.e., 40 μl, 60 μl and 80 μl DVS to prepare hydrogel by continuous stirring at room temperature for 15 min. The hydrogel was purified by dialysis against H_2O for 12 h (22). We could observe that hydrogels made of 40 μl was a thick viscous liquid and the other two hydrogels had gel-like appearance.

2.2.2. Bioburden and sterility

Bioburden describes the population of viable microorganisms present on or in a product and the number is used to determine the most appropriate parameters for its final sterilization. The HA powder samples were analyzed for the bioburden test according to the ISO 11737–1:2006 and ISO 10993–12:2012 (E) and the sterility was checked as per ISO 11737–2:2009 (E).

For bioburden; the test item weighing 4.00 g (HA powder) was immersed in 20 mL of extracting fluid (1% Peptone solution with 3% polysorbate 80) separately which gives the extraction ratio of 0.2 g/mL under sterile condition. The prepared extract was manually shaken and allowed to pass through 0.20 μ membrane filter. For incubation, the membrane filters were placed on agar surface of the solid medium. The conditions applied for incubation are as follows; in Nutrient agar at 37°C for seven days to identify bacteria, in Sabouraud dextrose agar at 25°C for seven days to identify yeast and molds, and finally in Reinforced Clostridial media at 37°C for seven days to identify anaerobes. Colonies produced on the surface of the membrane filter was counted. For sterility studies, the test item weighing 2.00050 g was immersed in 10 mL of 1% peptone solution with an extraction ratio of 0.2 g/mL under sterile conditions. The prepared extract was aseptically inoculated in soybean casein digest broth and fluid thioglycollate broth. The inoculated broth was incubated to observe the macroscopic growth of aerobic bacteria and anaerobic bacteria and molds at 35°C for 14 days.

2.2.3. Morphology

Morphological analysis of HA-DVS hydrogels was conducted by SEM after gelation. Hydrogels were freeze-dried and then gold-coated using a Cressington 108 Auto (Cressington, Watford, UK). The surface and cross-sectional morphologies were viewed using a JSM-6330F SEM (JEOL, Peabody, MA) operated at 10 kV accelerating voltage (23).

2.2.4. Equilibrium swelling

The known weights of HA-DVS hydrogels were immersed in Dulbecco's modified Eagle's medium (DMEM) +10% fetal bovine serum (FBS) and PBS respectively, and kept at 37°C for 24 h until swelling equilibrium had been reached. The swollen hydrogels were removed and immediately weighed with a weighing balance excess of water lying on the surfaces was absorbed with a filter paper. The equilibrium swelling ratio (ESR) was calculated using the following equation:

$$\mathrm{ESR} = (\mathrm{Ws} - \mathrm{Wd})/\mathrm{Wd}$$

Where Ws and Wd are the weights of the hydrogels at the equilibrium swelling state and at the dry state (24).

2.2.5. Enzymatic degradation

Degradation of the known weight of HA-DVS hydrogels was examined with respect to weight loss under aqueous conditions in the presence of the enzyme hyaluronidase. Hyaluronidase was dissolved in PBS to get a 100U/mL enzyme solution. The lyophilized samples were weighed (W_0) and then immersed in 5 mL enzyme solution. The tubes were kept static at 37°C in an incubator. After soaking for 24 h, the samples were washed with distilled water three times. After lyophilization, the dry weights were measured (W_1). The rate of weight loss (WL) was calculated using the following equation (25).

$$\mathrm{WL} = W_1/W_0 \times 100\ \%$$

2.2.6. Rheology studies

The mechanical properties of HA-DVS hydrogels were characterized by oscillatory rheology studies using cone plate geometry at 37°C using Anton Parr Rheometer, MCR 302. The aim of the rheological measurement was to characterize the G' value and G" value - the elastic modulus and viscous modulus - for each HA-DVS hydrogels. Dynamic experiments were performed in the linear viscoelastic region where

G' and G" are independent of the stress applied at a given frequency. All the hydrogel formulations showed a significant difference in viscoelastic properties with increased divinyl sulfone concentrations (26).

2.2.7. Cytotoxicity studies

HA hydrogels were weighed and sterilized by autoclaving, and placed in a 24 well plate to which fresh culture medium was added at 0.1 g/mL. The MCF 7 cells were cultured at a density of 1.0×10^4 cells/mL on 96 well plates (100 μL/well) in a CO_2 (5%) incubator at 37°C. After incubation for 24 h, the extracts of the hydrogels were added to 96 well plates (100μL/well) in a CO_2 (5%) incubator at 37°C. Incubation was done for 24–48 h. 10 μL of MTT reagent (Roche, Version 18-Cell proliferation kit) was added to each well and was again incubated at 37°C for an additional 4 h. 100 μL of solubilization buffer was added to each well after 4 h and incubated overnight. Absorbency of the solution was measured at 570 nm using an enzyme linked immunosorbent assay (ELISA) Reader at 24 h and 48 h after the addition of the hydrogel extract. The relative cell growth (%) was calculated as:

$$\text{Relative cell growth} = \left\{(\text{OD})_{\text{test}}/(\text{OD})_{\text{control}}\right\} \times 100.$$

The plates were prepared and analyzed as duplicates to increase accuracy of results.

2.2.8. Mesenchymal stem cell interaction

Mesenchymal stem cells were isolated through enzymatic degradation and the pellet was re-suspended in DMEM/F12 with 10% FBS and 1% penicillin/streptomycin. The cells were seeded in tissue culture flasks and were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Adherent cells were expanded for a period of 5–8 days at 37°C and the medium was changed every other day until the cells achieved 80% confluence. Cell adhesion to the composite hydrogels was accessed. HA-DVS hydrogels was sterilized under ultraviolet (UV) radiation for 1 h and then dissolved in sterilized PBS to obtain 20 mg/mL. A total of 300 μL of HA-DVS solution at various volume ratios were injected into 24 well culture plates, mixed and incubated at 37°C for 10 min to form composite hydrogels. 1 mL DMEM/10% FBS solution containing 50,000 cells were added into each well. After 24 h, the number of stem cells attached to the composite hydrogels was quantified using MTT assay.

2.2.9. Quantitative determination of HA

The amount of HA in the hydrogel has been calculated by a new method for quantification of uronic acids (27). Here, 1 g of the hydrogel was dissolved in 100 mL of water. A different volume of the solutions were taken in empty test tubes and the tubes were refrigerated in crushed ice. To these test tubes, water is added in such a way that the total volume is 2 mL and shaken in a vortex mixer. To 1 mL of these solutions 6 mL sulfuric acid/tetraborate was added and again shaken in a vortex mixer. The tubes were then heated in a boiling water bath for 5 min. After cooling in an ice bath, 100 μL m-hydroxydiphenyl reagent was added. The tubes were shaken and within 5min, absorbance measurements were made at 520 nm using a UV spectrophotometer (UV PharmaSpec-1700, SHIMADZU). Carbohydrate produces a pinkish chromogen with sulfuric acid/tetraborate at 100°C. A blank was run without addition of the reagent, which was replaced by 100 μL of 0.5% NaOH.

3. Results and discussion

3.1. Bioburden and sterility

HA powder was tested for the presence of bioburden by incubating the membrane filters in Nutrient agar, Sabouraud dextrose agar and Reinforced Clostridial media for seven days. Based on the result shown in Table 1, HA powder is free from bacteria and yeast and molds. Also, the sterility test conducted for HA hydrogel after autoclaving for 5 min revealed a clear macroscopic evidence of microbial growth observed in the media inoculated with positive control whereas no evidence of microbial growth was observed in the media served as the negative control and in the media inoculated with HA hydrogel extract. Hence, based on the observation it is concluded that the HA hydrogel compiles sterility.

3.2. Hydrogel morphology

The HA-DVS gel was transparent and had better maintenance in shape. SEM images were obtained to characterize the microstructure morphologies of freeze-dried hydrogels. The surface images of 40 μL HA-DVS, 60μL HA-DVS and 80 μL HA-DVS hydrogels are presented in Figure 1. Figure 1(a, b and c) is that of 40 μL, 60 μL and 80 μL HA-DVS. Figure 1(a) and (b) shows interconnected microporous structure resembling other polymer rich hydrogel systems (28).Whereas, Figure 1(c) shows structures with closed pores, the SEM results demonstrated that a higher amount of crosslinking results in the formation of

Table 1. Bio-burden test of HA powder and HA hydrogel.

Test item	Colony count (cfu)						
	Acultative, non-fastidious, aerobic bacteria (nutrient agar)			Yeast and molds (Sabouraud dextrose agar)	Anaerobic bacteria (reinforced clostridial agar)		
	Cocci	Rod	Spores		Cocci	Rod	Spore
HA powder	Nil	Nil	Nil	Nil	Nil	Nil	Nil

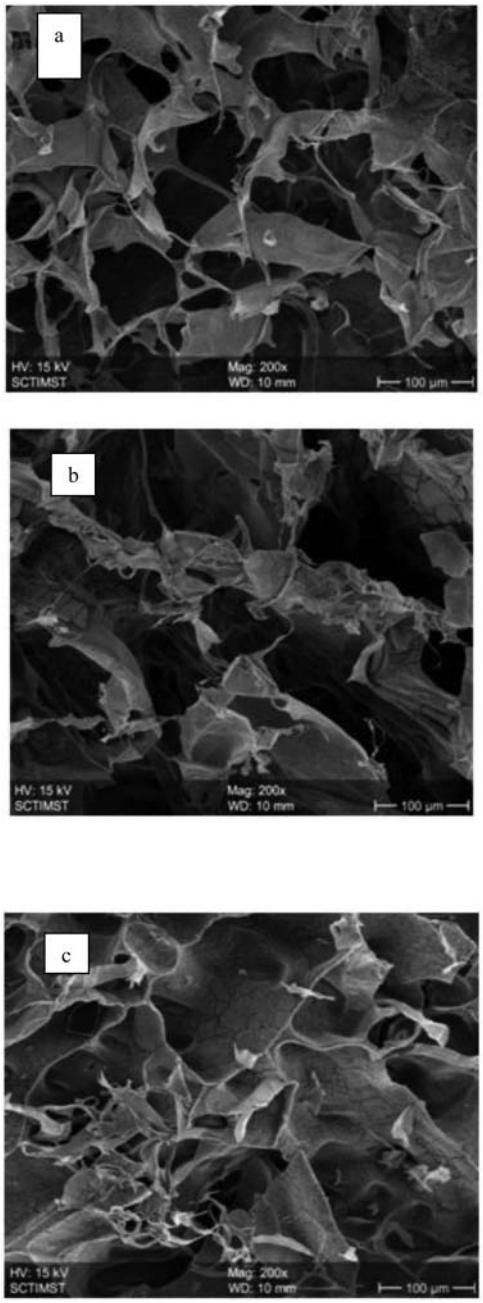

Figure 1. (a) HA hydrogel crosslinked with 40 μL DVS; (b) HA hydrogel crosslinked with 60 μL DVS; (c) HA hydrogel crosslinked with 80 μL DVS at magnification of 200×.

continuous and more compact network structure in HA hydrogels with no visible pores.

3.3. Equilibrium swelling

The equilibrium swelling has been studied in PBS and DMEM/10% FBS. PBS is a buffer solution commonly used in biological research. It is a water-based salt solution containing disodium hydrogen phosphate, potassium dihydrogen orthophosphate, sodium chloride and potassium chloride. The osmolarity and ion concentrations of PBS match that of the human body (isotonic). Hence, such solutions are used for the swelling studies.

DMEM is a modification of Basal Medium Eagle (BME) that contains a four-fold higher concentration of amino acids and vitamins, as well as additional supplementary components. DMEM contains no proteins, lipids, or growth factors. Therefore, DMEM requires supplementation, commonly with 10% Fetal Bovine Serum (FBS).

The equilibrium swelling ratio of all the three types of freeze-dried HA-DVS were performed in PBS and DMEM/10% FBS (Figure 2). Swelling ratio of chemically crosslinked HA hydrogels exhibited the ability to absorb large amounts of DMEM/10% FBS than PBS and reached equilibrium. Zhang et al. has summarized that the swelling ratio in DMEM/F12/10%FBS was slightly higher than those in PBS (29, 30). From Figure 2, it is clear that the swelling ratio in DMEM/10%FBS is higher than that of PBS for all the three DVS hydrogels. Here, in this case we may see that swelling is almost equal in the case of DMEM, whereas in the case of PBS swelling is increased with the amount of cross linking agent added.

3.4. Enzymatic degradation

Hyaluronidase is an enzyme with a mass of 50 kDa, that can decompose HA chains after being activated by the carboxyl acid of D-glucuronic acid, and cleave β 1,4-glucosaminidic bonds (31). HA is a promising biomaterial for the production of hydrogel carriers, since it is degradable under the action of hyaluronidase (32). Therefore, the *in vitro* degradation behavior of test samples was investigated by incubation at 37°C in PBS containing hyaluronidase (100U/mL).

Lu et al. has summarized that the resistance against enzymatic degradation of chemically crosslinked HA can be determined by immersing in a PBS solution containing hyaluronidase (33). In this case, degradation of HA-DVS hydrogels were examined with respect to weight loss under aqueous conditions in the presence of hyaluronidase. It has been found that the enzymatic degradation is completed within 24 h for all the three, 40 μL, 60 μL and 80 μL HA-DVS hydrogel.

3.5. Rheology studies

In Figure 3 (b and c); we can see that storage modulus of the hydrogels crosslinked using 60 μL DVS and 80 μL DVS are

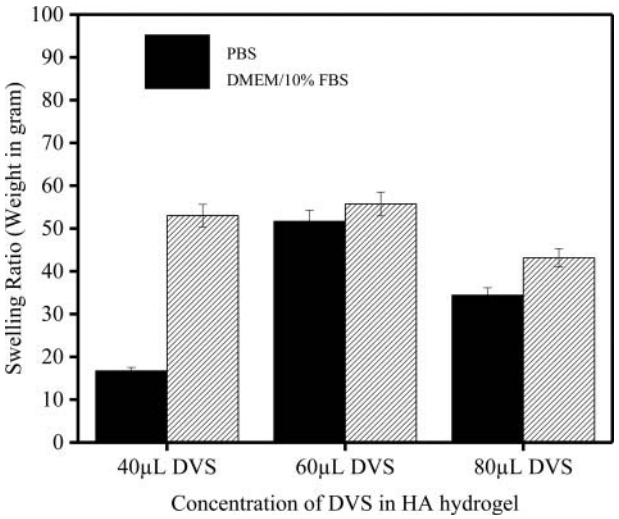

Figure 2. Equilibrium swelling ratio of HA-DVS (4 mM) hydrogels in PBS and DMEM/10% FBS at 37°C.

greater than the loss modulus indicating the prominent elastic nature of the hydrogel. But in the case of 40 μL DVS hydrogel loss modulus is higher at lower frequency and as the frequency increases both the moduli increases and at one point G' becomes equal to G". The frequency at which G' becomes equal to G" is called crossover frequency. This shows that 40μL DVS hydrogel is liquid like, whereas 60 μL DVS and 80 μL DVS are gel like. The tan delta is the ratio of viscous modulus (G") to elastic modulus (G') and it is useful in quantifying the presence and extent of elasticity in a fluid. Tan δ values of less than unity indicate elastic-dominant (i.e., solid-like) behavior and values greater than unity indicate viscous-dominant (i.e., liquid-like) behavior (34). Here, 60 μL DVS and 80 μl DVS hydrogels are showing tan δ value of less than one indicating a networked gel structure (Figure 3(e)). Rheological behavior of a gel in this case is due to the effective crosslinking happening due to the addition of DVS. These efficient crosslinks reduces the intrinsic mobility of the polymer chains and hence it is not able to release the stress. Consequently, the gel shows an elastic nature and behaves as a three dimensional network were the main mode of accommodation of the applied stress is by network formation (35).

Figure 3(e) shows shear thinning behavior for 60 μL DVS and 80 μL DVS. This again shows that a strong entangled polymer network for these two types of hydrogels. Here; the bond formed between hyaluronic acid molecules and divinyl sulfone is covalent in nature; but still there can be some weak forces such as hydrogen bonding and Vander Waal's interactions present between the

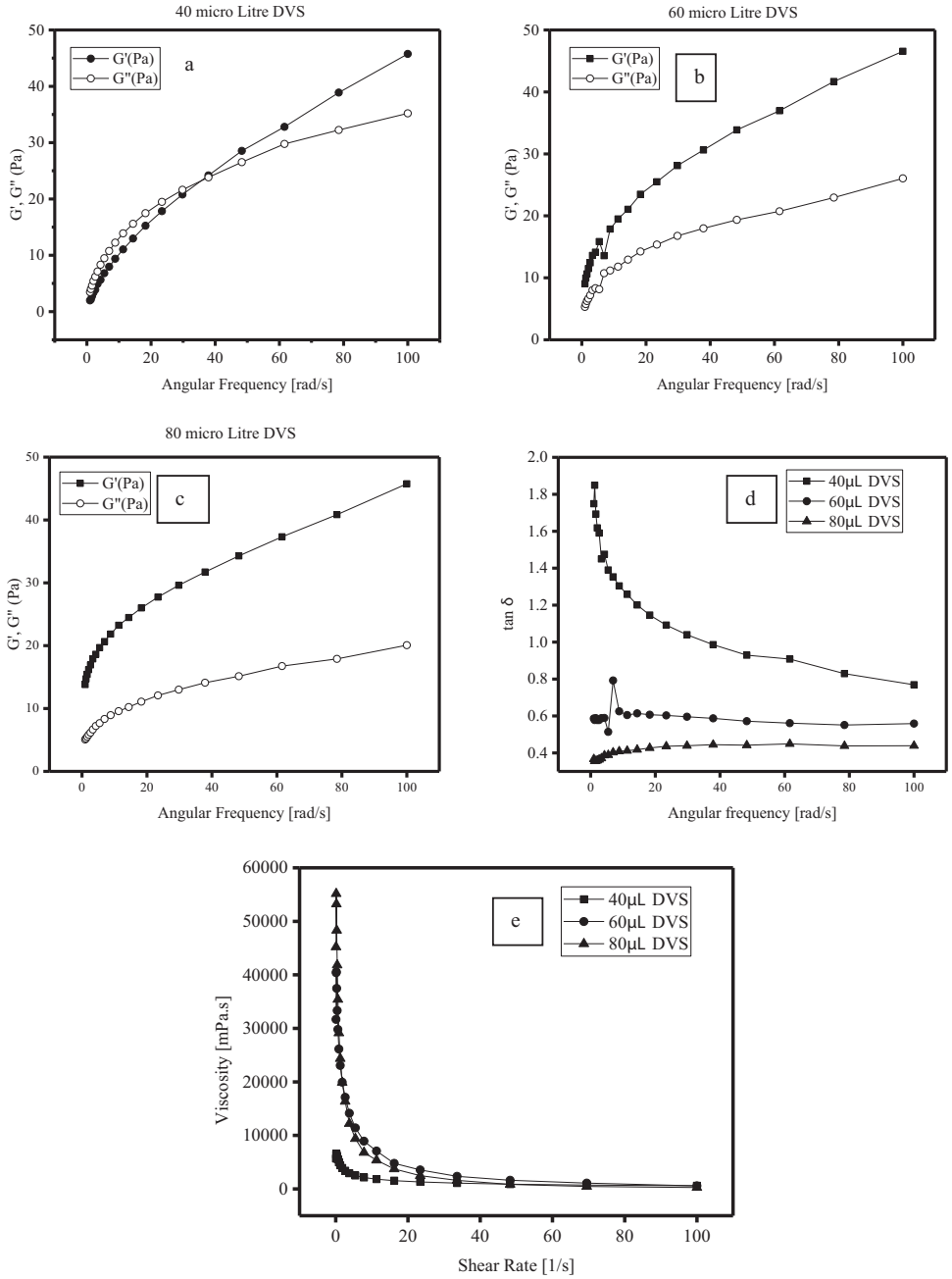

Figure 3. (a) HA hydrogel crosslinked with 40 μL DVS; (b) HA hydrogel crosslinked with 60 μL DVS; (c) HA hydrogel crosslinked with 80 μL DVS; (d) Tan δ value of three different concentrations; (e) shear thinning behavior for 60 μL DVS and 80 μL DVS.

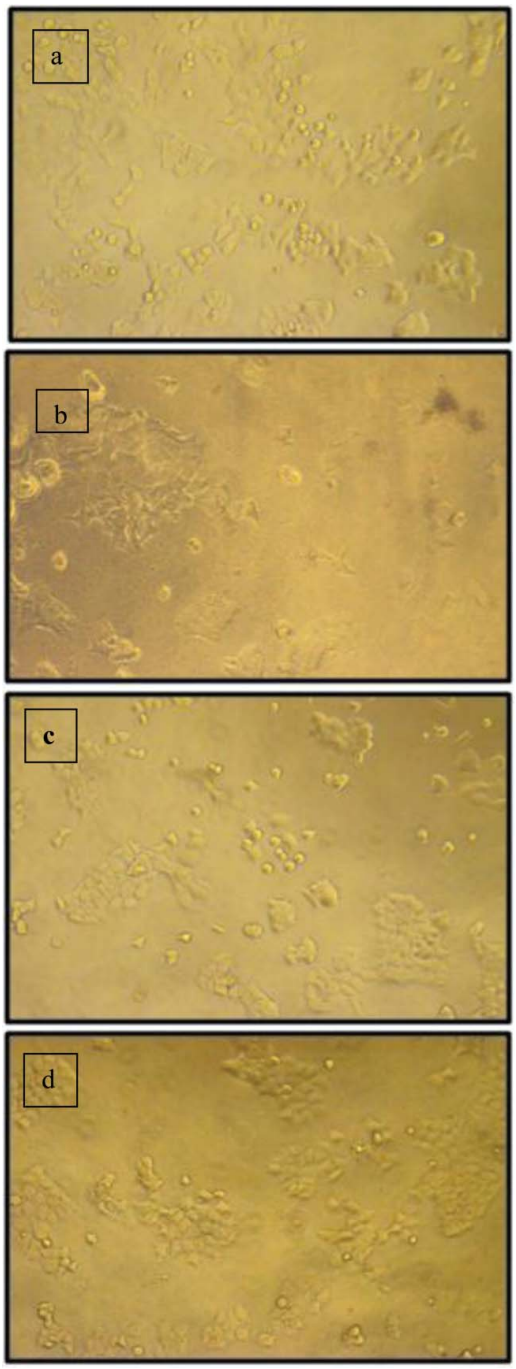

Figure 4. (a) Image of control of cell lines; (b) Cell proliferation of HA-DVS 40 μL; (c) Cell Proliferation of HA-DVS 60 μL; (d) Cell proliferation of HA-DVS 80 μL.

molecules. Due to the dynamic nature of such weak bonds; they may dissociate due to the applied shear and contribute to the shear thinning behavior. Once after removing the shear these dissociated bonds will re assemble again to form the hydrogel (36).

3.5.1. In vitro assay

Collins and Birkinshaw et al., has summarized in a recent paper about the modification and the crosslinking of the different concentrations HA-DVS hydrogels (37, 38). The hydrogel did not release any toxic products or produce adverse reactions. The MTT assay is a routine method for detecting the toxicity of tissue-filling materials. The study indicated had an influence on cell proliferation compared to the initial cells present. According to Ibrahim et al., the crosslinked HA gels containing a low concentration of DVS-HA are able to retain the biological characteristics of uncrosslinked HA, indicating that the DVS-HA does not compromise the biocompatibility of HA molecules (39).

Therefore, all the three 40 μL HA-DVS, 60 μL HA-DVS, 80 μL HA-DVS hydrogel did not restrain the proliferation of cells, indicating its potential as a biomaterial for tissue engineering applications.The percentage viability of the cells and relative cell growth varied in three different concentrations of hydrogel.

From the below Figures 4 and 5, it can be clearly seen that there is an increased cell proliferation in all the three hydrogels. Hence, it is proven that the HA hydrogel is a safe biomaterial for biological applications.

3.5.2. Mesenchymal stem cell interaction assay

HA plays a critical role in anchoring large proteoglycans in the cartilage extracellular matrix. Cells also interact directly with HA through CD44 receptors, and this interaction can modulate cell migration, proliferation, differentiation, and HA degradation. Interestingly, HA added to human MSCs increases cartilage matrix production, suggesting a direct biologic role for this molecule as well. Human MSCs possess abundant CD44 receptors and undergo tissue regeneration.

The three 40 μL HA-DVS, 60 μL HA-DVS, 80 μL HA-DVS hydrogels were observed for 24 h in mesenchymal stem cells. The cells remained rounded in all gels and no obvious spatial variations were observed between the perimeter of the gels and the central areas. Light staining of CS in hydrogels cultured in MSC's media was also observed. The MTT assay was used for detecting the interaction of hydrogels in MSC's. The percentage of the viability of the cells varied in three different concentrations of hydrogels. The relative mesenchymal stem cell growth also varied from 40 μL, 60 μL and 80 μL.

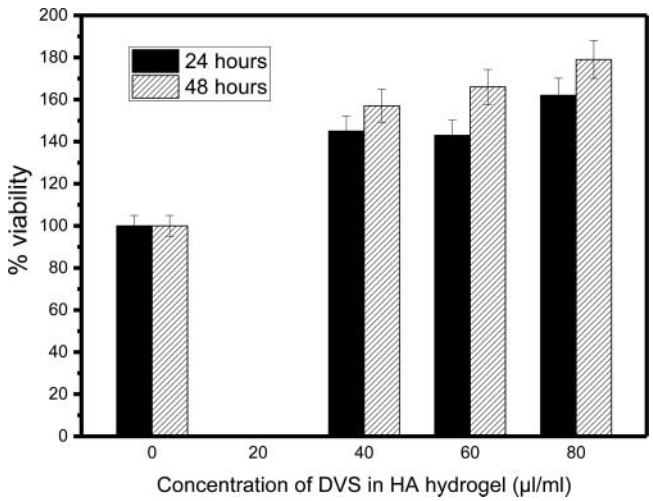

Figure 5. Percentage viability against concentration of DVS in HA hydrogel.

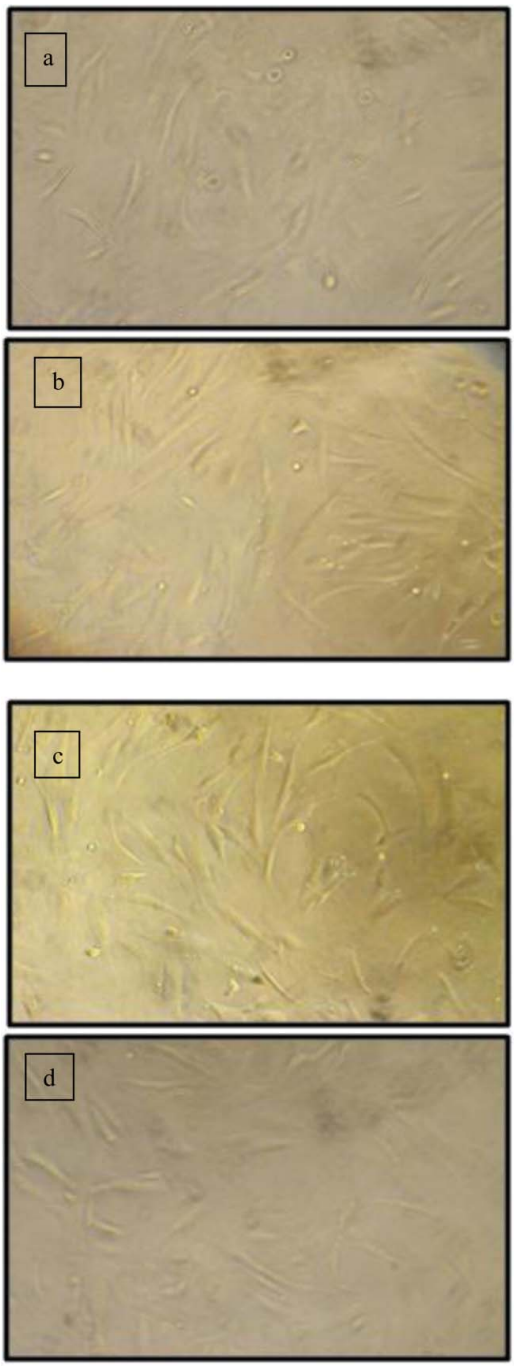

Figure 6. (a) Image of Control of mesenchymal stem cell lines; (b) Cell proliferation of HA-DVS 40 μL in MSC; (c) Cell proliferation of HA-DVS 60 μL in MSC; (d) Cell proliferation of HA-DVS 80 μL in MSC.

From the above Figures 6 and 7 it is seen that there is a continuous proliferation in mesenchymal stem cells and it is hypothesized that it may help in chondrogenesis. Hence, it is also proven that the HA hydrogel can be used in soft tissue regeneration (40–42).

Based on the studies conducted it can be concluded that HA 60 μL DVS is found to be a stable and compatible hydrogel for cartilage regenearation. The modified method of uronic acid determination shows that the injectable hydrogel contains 9.1693 mg/mL active content of HA.

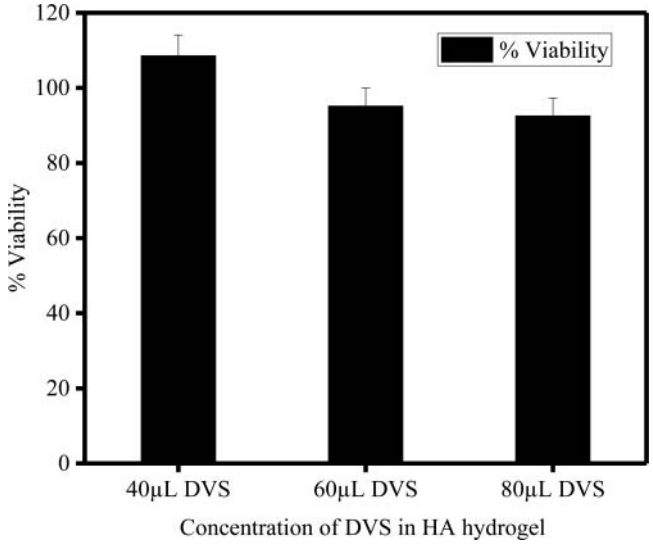

Figure 7. Percentage viability against concentration of DVS in HA hydrogel.

4. Conclusions

HA has been crosslinked with three different concentrations of divinyl sulfone. As prepared hydrogels were investigated using SEM analysis, swelling studies, enzymatic degredation and rheology analysis. 40 μL hydrogel was liquid like wereas 60 and 80 μL hydrogels were gel like in nature. SEM analysis shows a porous sturucure for 40 and 60 μL hydrogel whereas, for 80 μL it shows a more compact morphology. All the hydrogels showed swelling behavior in PBS and DMEM. Enzymatic degredation studies shows that all the hydrogels are degrading within 24 h. HA content/mL was found out by the modified uronic acid determination method. The *in vitro* cytotoxicity analysis demonstrated all the three hydrogels were without cytotoxicity and can be used for delivering of adipose tissue-derived mesenchymal stem cells (AMSCs). It was found that hydrogels helps in the induction of AMSC differentiation into chondrocytes and hence can find a potential aid in articular cartilage repair for osteoarthritis therapy.

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