Journal of Chemical Health Risks

www.jchr.org

JCHR (2025) 15(2), 403-411 | ISSN:2251-6727

Characterization of Hyaluronic Acid - Alginate - Divinyl Sulfone Scaffold for Cartilage Tissue Regeneration

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(Received: 16 January 2024 Revised: 20 February 2024 Accepted: 20 March 2025)

KEYWORDS

Hyaluronic acid, divinyl sulfone, alginate, cartilage, scaffold.

ABSTRACT:

Aim - Cartilage engineering holds tremendous potential for addressing cartilage defects and diseases. To facilitate successful cartilage formation, it is imperative to develop composite scaffolds that closely replicate the natural extracellular matrix of cartilage. This study delves into the characterization of a novel composite scaffold comprising hyaluronic acid, divinyl sulfone, and alginate for cartilage engineering.

Methodology - Hyaluronic acid (HA) solutions were incorporated with sodium alginate and later crosslinked with divinyl sulfone (DVS). The resulting HA, alginate, and DVS hydrogel was thoroughly characterized using Fourier Transform Infrared Spectroscopy (FTIR), X-ray diffraction (XRD), Field Emission Scanning Electron Microscopy (FESEM), Energy-Dispersive X-ray Analysis (EDAX), and in vitro cytotoxicity assays.

Results and Conclusion - This composite scaffold is tailored to provide an environment conducive to the growth and differentiation of chondrocytes or stem cells, ultimately leading to the regeneration of healthy cartilage.

1.INTRODUCTION

Natural polymer-based hydrogels, particularly hyaluronic acid (HA), have garnered attention in biomedicine for diagnostics. HA, found in all living organisms, retains water and maintains connective tissue elasticity.[1,2] It comprises repeating disaccharide units of α-1,4-D-glucuronic acid and β-1,3-N-acetyl-D-glucosamine, stabilized by hydrogen bonds.[3] Notable for its biocompatibility, non-immunogenicity, non-toxicity, and pain-relieving properties, HA is essential for wound healing.[4–6] These attributes make HA and its derivatives apt for various medical applications.[7] HA-based hydrogels are potential cell transplantation vehicles for tissue regeneration. Their properties depend on polymer-water interactions; increased water absorption enhances oxygen permeability but weakens the gel. High equilibrium swelling improves nutrient permeability and waste removal, maintaining the structural integrity necessary for tissue growth. HA hydrogel stability relies on crosslinks formed by chemical bonds and physical interactions.[8] However, HA degrades quickly in vivo and adheres poorly. These issues can be mitigated through chemical

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JCHR (2025) 15(2), 403-411 | ISSN:2251-6727

hybridization or crosslinking, enhancing viscosity, solubility, degradation resistance, and biological properties.[9] Crosslinking, often documented, is crucial for in vivo stability. Key functional groups for crosslinking HA are carboxyl and hydroxyl groups. Agents like DVS, which forms ether bonds via hydroxyl groups, have been tested.[10-11] DVS-crosslinked HA gels exhibit exceptional biocompatibility and non-immunogenicity.[12] Alginate, an anionic polymer from brown seaweed, is used in biomedicine due to its biocompatibility, low toxicity, affordability, and gelation with divalent cations like Ca2+.[13] Alginate hydrogels, formed through various crosslinking methods, resemble extracellular matrices and have numerous applications.[14] Though not a natural cartilage matrix component, alginate can be supplemented with extracellular matrix elements like HA.[15] Research focuses on scaffolds made of natural, synthetic, and hybrid materials. Synthetic materials often induce excessive cellular responses. This study emphasizes natural materials, such as alginate and HA, combined with Divinyl Sulfone (DVS) to create a natural-based scaffold enhancing biocompatibility and structural integrity. The innovation lies in developing a composite scaffold by crosslinking HA, alginate, and DVS, enhancing mechanical stability and biocompatibility. DVS crosslinking creates a stable network between polysaccharides, addressing the mechanical weaknesses of natural materials. This combination merges the high bioactivity and cell-friendliness of natural polymers with the structural integrity provided by synthetic crosslinking, suitable for regenerative medicine. This approach aims to balance bioactivity, degradation control, and mechanical strength.[16]

2.METHODOLOGY

The study protocol was approved by the institution's ethical committee.

2.1. Materials

75810, type 2 Hyaluronic acid sodium salt (1000-2000 kD) extra pure, 90% was purchased from Sisco Research Laboratories Private Limited, Mumbai (Figure 1a). Divinyl sulfone was procured from Sigma–Aldrich Chemicals, USA (Figure 1b). Alginate was procured from the NICE brand (Figure 1c).

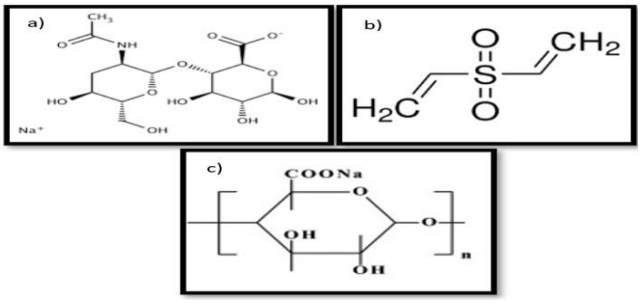

Figure 1 - a. Chemical structure of Hyaluronic acid, b. Chemical structure of Divinyl Sulfone, c. Chemical structure of Alginate

2.2 Formulation of the test sample (Biological membrane comprising HA, Alginate, and DVS)

2.2.1 Materials

Sodium alginate was used as a polymer matrix. HA and DVS were used as filler and crosslinker respectively. Sodium alginate ($(C_6H_7NaO_6)_n$, 216.121 g/mol) was purchased from the NICE brand. Hyaluronic acid ($(C_{14}H_{21}NO_{11})_n$, Type 2 (75810) Hyaluronic Acid Sodium Salt (1000-2000 KD) Mol. Wt. 5,000 to 20,000,000 Da) and DVS ($C_4H_6O_2S$, Mol wt. 118.154 g/mol.) were purchased from the Sisco Research Laboratories Private Limited, Mumbai.

2.2.2 Preparation of polymer composite

The polymer composite solution was synthesized by first dissolving 0.25 g of alginate in 5 ml of double-distilled water. Alginate was dissolved at room temperature (28 ℃) to obtain a clear solution of alginate polymer. To the prepared alginate polymer solution, 0.025 g of hyaluronic acid sodium salt was added and blended well. To obtain crosslinking of polymer with the hyaluronic acid sodium salt, 2.5 mM divinyl sulfone was added and mixed at room temperature at 700 rpm in a magnetic stirrer. HA is incorporated at 10 wt% of the alginate polymer, with DVS at 100 wt% of the alginate polymer. The stirring was allowed to continue for 3 hours to obtain the resultant biopolymer.

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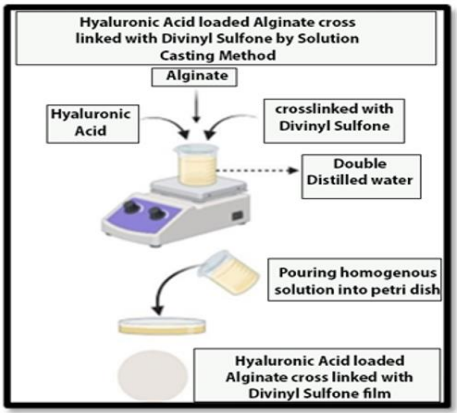

Figure 2- Fabrication process of the Scaffold

2.2.3 Fabrication of Alg-HA-DVS composite films

The Solution casting technique was used to fabricate Alginate-HA-DVS film, as shown in Figure 2. After constant stirring for around 3 hours, at 700 rpm, maintaining the room temperature, a uniform Alg-HA-DVS composite film was obtained with HA to DVS ratio of 1:0.25. Alg-HA-DVS composite was then transferred into a borosil Petri plate and left untroubled for 2 days, with a ceiling fan to aid in forced convection. Following the completion of the time, the film was peeled from the borosil Petri plate. Various analytical methods such as FESEM, EDAX, XRD, FTIR, and in-vitro cytotoxicity analysis were used for a thorough examination of the fabricated film to attain a detailed understanding of its characteristics and derive consensus on their potential applications.

2.3 Structural and In-vitro characterization

2.3.1 FTIR

To identify the presence of numerous functional groups in the Alginate, HA, and DVS thin biomembrane, FT-IR spectrometer analysis was performed. Spectral analysis was performed with a Nicolet 380 FTIR spectrophotometer, covering the range from 3000 cm^{-1} to 500 cm^{-1}. IR spectroscopy assessed the chemical cross-linking of HA and other components, and the peaks were compared with native HA.[1] The observed peaks confirmed the presence of different functional groups in the bio membrane comprising Alginate, HA, and DVS.

2.3.2 XRD

The crystalline properties and phase content of the biomembrane comprising Alginate, HA, and DVS were assessed utilizing an X-ray diffractometer, with the XRD pattern recorded over a 2Θ range between 10˚ and 80˚.[2]

2.3.3 FESEM and EDAX

The Alginate, HA, and DVS membrane's surface morphology and elemental composition were analysed using FESEM (Carl J Diss Supra 40e2007, Germany), functioning at 15 kV accelerating voltage, and equipped with EDAX.[2]

2.3.4 In vitro cytotoxicity: direct method

The Alginate, HA, and DVS membranes underwent in-vitro direct cytotoxicity testing. L929 cells from mice were cultured using MEM medium containing 10% Phosphate buffer saline (PBS), then trypsinized and a haemocytometer was used for counting. Approximately 10,000 L929 cells were seeded per well into a 96-well plate and incubated for 24 hours. Fresh medium was used to replace the existing medium and five different concentrations of the composite specimens were added in triplicate to the cells. Following an 18-hour incubation at 37 ± 1°C, MTT (1 mg/ml) was added and incubated for 4 hours. A 570 nm photometer was utilized to examine the cell morphology using Dimethyl Sulfoxide (DMSO).[3]

3. RESULTS AND DISCUSSION

3.1 FTIR survey of Hyaluronic acid, Divinyl Sulfone, and Sodium Alginate bio membrane

In the FTIR spectrum of HA, the wide peak at 3306 cm^{-1} represents O-H stretching vibrations, characteristic of carboxyl and hydroxyl groups.[4] The peak at 1601 cm^{-1} is attributed to the C=O stretching vibrations in the amide (amide I) and carboxylate (COO^-) groups, and the peak at 1054 cm^{-1} corresponds to the C–O–C bonds in the glucose ring of HA. These findings are consistent with the work of Chunhong Luo et al.[5]

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FTIR spectrum of sodium alginate reveals a peak at 3273 cm^{-1} which is associated with O-H stretching typical of alcohols, phenols, or carboxylic acids, while the 1576 cm^{-1} peak corresponds to asymmetric stretching of carboxylate groups (COO^-), and the 1050 cm^{-1} peak indicates C-O stretching, suggesting C-O-C bonds (Figure 3). The FTIR spectrum of divinyl sulfone indicates the presence of O-H stretching around 3296 cm^{-1}, C=C stretching for the vinyl groups at 1509 cm^{-1}, and C-S stretching around 1038 cm^{-1}. The peaks correspond well to the expected functional groups in divinyl sulfone.[6]

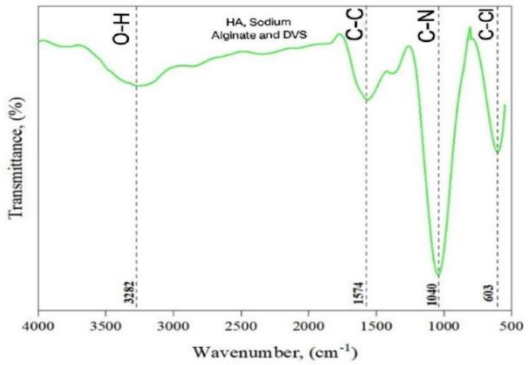

Figure 3-FTIR Graph

After the cross-linking process, a slight decrease in band intensity was observed. The FTIR spectrum of the bio membrane shows a glide in the peak from 3306 cm^{-1} to 3282 cm^{-1}, caused by hydrogen bonding between the -CO group of sodium alginate (SA) and the -NH group of HA. The C=O stretching peak is lower in the biomembrane (1574 cm^{-1}) than in HA (1601 cm^{-1}), suggesting an interaction between carboxyl groups and other functional groups in the biomembrane. During the cross-linking reaction with DVS, the hydroxyl groups in HA are consumed, accounting for the difference in band intensity.[7, 8] All frequency values slightly decreased due to the cross-linking of HA, sodium alginate, and DVS.

3.2 XRD analysis of Hyaluronic acid, Divinyl Sulfone, and Sodium Alginate biomembrane

The sodium salt of hyaluronic acid often shows amorphous behaviour in X-ray diffraction (XRD) due to its non-crystalline, polymeric structure.[9] A broad halo typically appears between 15° and 30° 2θ, reflecting the material's disordered nature, a characteristic of many hydrated polymers and salts. Sodium hyaluronate typically lacks the sharp diffraction peaks seen in crystalline substances.[10] Similarly, sodium alginate displays a broad peak or amorphous halo around 2θ = 13–23°, highlighting its disordered polymer structure and absence of long-range crystallinity.[11]

XRD analysis of the biomembrane revealed sharp peaks around 20°, 30°, and 40°, correlating to the crystallographic planes (111), (220), and (400), respectively. These peaks suggest a crystalline structure at these reflections, likely due to cross-linking with divinyl sulfone, which may be responsible for the observed crystallinity (Figure 4).[12]

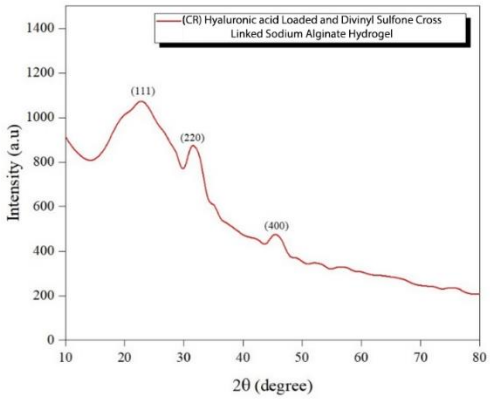

Figure 4- XRD Graph

3.3 SEM analysis of Hyaluronic acid, Divinyl Sulfone and Sodium Alginate biomembrane

- The surface morphology of Hyaluronic acid, Sodium alginate, and Divinyl sulfone was analysed through FESEM, as illustrated in Figure 5(A). The morphology demonstrated a uniform particle size distribution and an even spread across the surface. The surface appears densely packed with a granular structure, which contrasts with the typically smooth and featureless surface of non-crosslinked HA hydrogels.[13, 14] Evidence confirms that SEM images of hydrogels

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incorporating hyaluronic acid and sodium alginate reveal a smooth surface, much like other natural macromolecules.[15]

- The image in Figure 5(B) reveals the surface of the material at a higher level of magnification. The texture appears rough with an uneven distribution of small particles or irregular structures, potentially indicating a material with a crystalline or granulated surface.
- In Figure 5(C), at 10,000x magnification, the surface appears to have fine, irregular formations or particles, possibly indicating the presence of fibrous or crystalline structures. The texture is rough, with granular or uneven patterns distributed across the surface. Particles appear densely packed in clusters, and the surface shows more irregularities, indicating a transition toward a porous, textured structure ideal for enhanced interactions in a scaffold.
- In Figure 5(D), the image shows a much more detailed look at the surface, which appears rougher and more granular than the previous images. The brighter regions may correspond to denser or more conductive areas of the material, while the darker regions could represent areas of lower density or different material compositions. The texture appears irregular, with clusters of fine particles or fibrous formations, possibly indicating a composite material or a biological tissue with a high degree of surface complexity. The particles appear densely packed and are part of a more structured network, with many pores and valleys. This suggests that the scaffold's surface has evolved to accommodate higher particle density, likely improving cell attachment properties.
- In Figure 5(E), The image has a scale bar of 1 µm (micrometer), indicating the size reference for the structures seen in the image. The image seems to display a rough, irregular surface or a material with a granular or porous texture. The white and gray regions suggest differences in the topography or material composition. The particles are extremely close together and tightly packed, creating a well-connected network. The dispersion shows a clear transition to a highly porous scaffold, which would likely have enhanced properties for tissue engineering due to increased surface area.
- In Figure 5(F), Various regions of the sample have been measured, and their sizes are labelled in nanometers (nm). These include: D1 = 53.26 nm, D2 = 54.48 nm, D3 =38.83 nm, D4 = 31.32 nm, D5 = 44.76 nm, D6 = 43.35 nm and D7 = 47.58 nm.These measurements indicate the sizes of the different particles or surface features observed in the image. The size range is from 31 nm to 54 nm, suggesting that the features being measured are nanostructures.

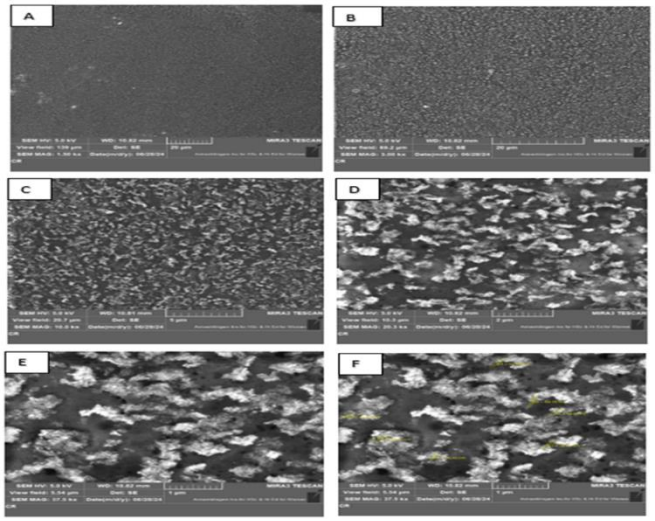

Figure 5- SEM Images A to F

Similar to the above images, this image also depicts a granular structure but with more detail about the size of the particles. Particles are densely packed and interspersed with deep pores. This high- magnification view highlights the intricate structure and precise distribution of particles within the scaffold, emphasizing the scaffold's potential for cellular integration due to the porous, interconnected framework.

3.4 EDAX analysis of Hyaluronic acid, Divinyl Sulfone,

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and Sodium Alginate biomembrane

The EDAX spectrum indicates a mixture of sodium alginate, hyaluronic acid, and divinyl sulfone. Hyaluronic acid is responsible for the strong oxygen and carbon peaks, while the presence of sodium, carbon, and oxygen identifies sodium alginate (Figure 6). The distinct sulfur peak points to divinyl sulfone, which acts as a crosslinker. This elemental composition suggests the material is a crosslinked hydrogel or blend of these compounds.

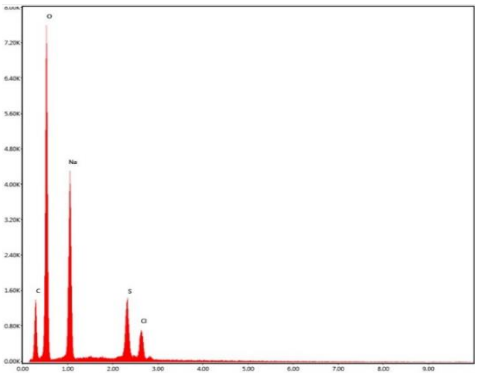

Figure 6- EDAX graph depicting the elemental composition

3.5 Cytotoxicity of Hyaluronic acid loaded Alginate cross-linked with Divinyl sulfone biomembrane

The graph (Figure 7a) illustrates the cytotoxicity of hyaluronic acid-loaded alginate films cross-linked with divinyl sulfone at five different concentrations of the composite specimens (5–100 µL). At 5 µL, the cytotoxicity was approximately 10%, increasing to around 15% at 25 µL, 27% at 50 µL, 35% at 75 µL, and reaching 40% at 100 µL. This trend shows a positive correlation between varying concentrations of the composite specimen and cell damage, with cytotoxicity rising from 10% at 5 µL to 40% at 100 µL. Lower concentrations (5–25 µL) exhibited cytotoxicity below 15%, indicating potential for safer biomedical applications, while higher concentrations (50–100 µL) displayed moderate to high cytotoxicity.

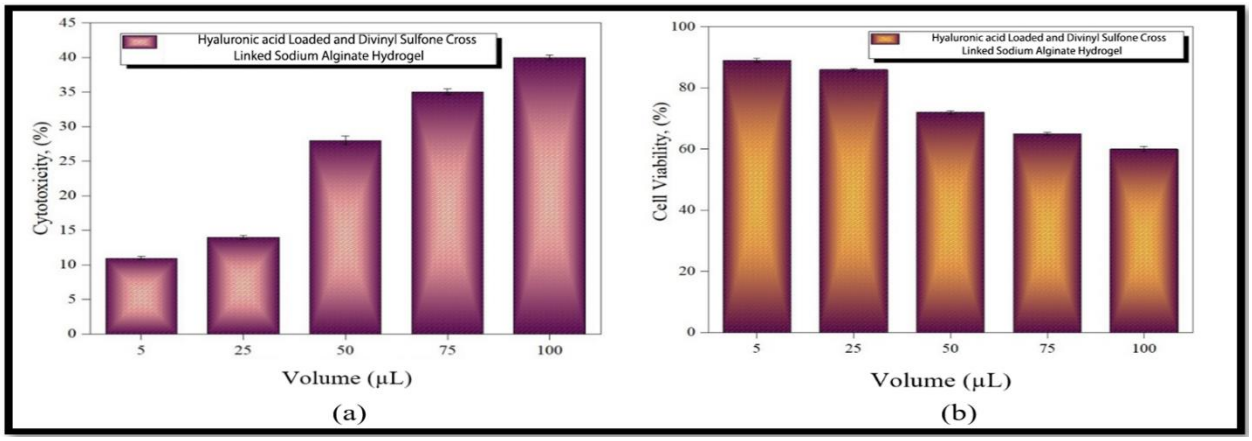

Figure 7 – Cytotoxicity (a), Cell viability (b) of Hyaluronic Acid-Loaded Alginate cross-linked with Divinyl Sulfone film at different volumes

3.6 Cell Viability of Hyaluronic acid loaded Alginate cross-linked with Divinyl sulfone biomembrane

The high sensitivity of L929 cells to toxic substances and their utility in cytotoxicity assays can be found in the ISO 10993-5 standard, which outlines the use of L929 cells in in vitro cytotoxicity testing. L929 cell lines are used widely in cytotoxicity assays and can aid in assessing the biocompatibility of various biomaterials.[16] With the aid of SEM, the morphology of the cultured L929 cells was

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observed (Figure 8). After1,7and14 days of proliferation, PBS was used to rinse the electrospun discs and then at 4°C, 4vol% glutaraldehyde (Scharlab) was used for fixing the electrospun discs for 30 min. After fixation, PBS was used to rinse the samples twice. A post-fixation procedure was applied: 2 vol% osmium tetraoxide was used to incubate the samples at room temperature for 2h, following four rinses with distilled water.[17]

The graph on cell viability (Figure 7 b) shows that with a 5 µL volume of the composite specimen, cell viability was around 85%, decreasing to approximately 80% at 25 µL, 65% at 50 µL, 60% at 75 µL, and about 55% at 100 µL. According to the UNE-EN-ISO 10993-5:2009 standard, a reduction in cell viability of more than 30% is considered cytotoxic.[18] Thus, this scaffold, comprising hyaluronic acid and alginate with DVS as a cross-linker, demonstrates biocompatibility, making it suitable for tissue engineering applications.

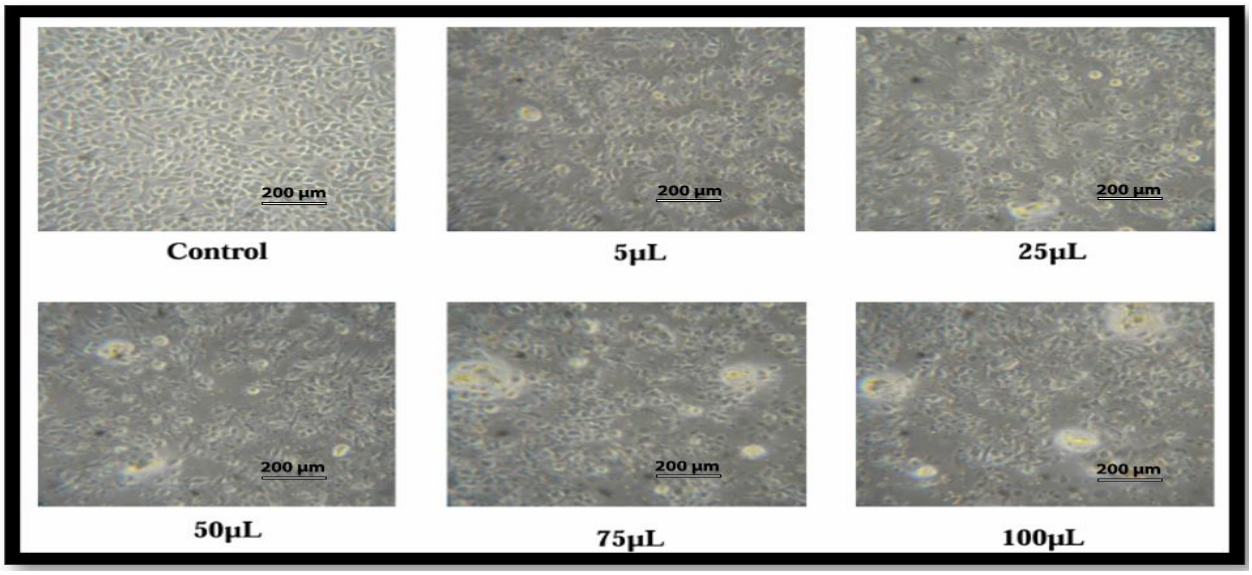

Figure 8-Images revealing cell morphology using Inverted phase contrast microscope (10x magnification)

4. SUMMARY AND CONCLUSION

The scaffold's design is crucial in determining the effectiveness of many void-filling agents, bioactive delivery techniques, and tissue constructs. This design is influenced by both the tissue type and its environmental context. Further progress in material development will likely play a prominent role in shaping the future of tissue engineering. With this approach in mind, a novel scaffold has been developed, capitalizing on the properties of hyaluronic acid and sodium alginate, along with divinyl sulfone as the cross-linking agent. Characterization of the scaffold demonstrated a well-structured and porous network with increased roughness. These enhanced surface properties could promote cell attachment and scaffold integration in tissue engineering applications. It also revealed low cytotoxicity with a reduced concentration of cross-linker. The scaffold needs to undergo testing to confirm its viability in tissue engineering. If proven successful, this development could be a promising one in the field of regenerative medicine.

CONFLICT OF INTEREST

There is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available upon request from the authors.

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JCHR (2025) 15(2), 403-411 | ISSN:2251-6727

FUNDING

Self-funded study.

Reference:

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