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List of Publications and Papers Presented in Conferences

Referred Journal Publications:

1. Kumari, S., Singh, B. N., & Srivastava, P. (2019). Effect of copper nanoparticles on physico-chemical properties of chitosan and gelatin-based scaffold developed for skin tissue engineering application. 3 Biotech, 9(3), 1-14. [I.F.-2.5]
2. Kumari, S., Singh, D., Srivastava, P., Singh, B. N., & Mishra, A. (2022). Generation of graphene oxide and nano-bioglass based scaffold for bone tissue regeneration. Biomedical Materials, 17(6), 065012. [I.F.- 3.5]
3. Kumari, S., Singh, D., Mishra, A., Li, C. & Srivastava, P. (2022). In-vitro studies on Copper nanoparticles and Nano-hydroxyapatite infused Chitosan and Gelatin based composite scaffolds for Bone Bioengineering applications. Biotechnology and Bioprocess Engineering, Accepted. [I.F.-3.386]
4. Kumari, S., Srivastava, P. & Mishra, A. (2022). Generation of bioactive porous chitosan/gelatin based scaffold modified with Tri-calcium phosphate /nano-bioglass for Bone Tissue engineering applications. Journal of Porous Materials [I.F.-2.523]
5. Kumari, S., Katiyar, S., Darshna, Anand, A., Singh, D., Singh, B. N., Mallick, S.P., Mishra, A. & Srivastava, P. (2022). Design Strategies for Composite Matrix and multifunctional polymeric scaffolds with enhanced Bioactivity for Bone Tissue Engineering. Frontiers in Chemistry – Submitted [I.F.-5.545]
6. Kumari, S., Singh, D., Katiyar, S., Darshna, Singh, B. N., Srivastava, P. & Mishra, A. (2022). Hydrogels: The future of Tissue engineering and Regenerative Medicine. 3 Biotech-Under Review [I.F.-3.446]
7. Anand, A., Mallick, S. P., Singh, B. N., Kumari, S., Suman, D. K., Tripathi, S., ... & Srivastava, P. (2022). A Critical Aspect of Bioreactor Designing and Its Application for the Generation of Tissue Engineered Construct: Emphasis on Clinical Translation of Bioreactor. Biotechnology and Bioprocess Engineering, 1-21. [I.F.-3.386]
8. Katiyar, S., Singh, D., Kumari, S., Srivastava, P., & Mishra, A. (2022). Novel strategies for designing regenerative skin products for accelerated wound healing. 3 Biotech, 12(11), 1-30. [I.F.-3.446]
9. Liu, C., Kumari, S., Singh, B. N., Singh, D., Srivastava, P. & Li, C. (2022). Characterization and Monitoring 3D Tissue Engineering on a Chip. Biosensors and Bioelectronics – Submitted [I.F.-10.545]

List of Book Chapters:

1. Advances of the Nanobiosensor in Tissue Engineering (Springer)- Revised and submitted

Paper/ Poster Presented at Conferences

- International Conference BIOSANGAM-2022, organized by MNNIT Allahabad (First prize in Poster presentation)
- International Conference on Bioengineering and Regenerative Medicine (ICBR- 2020), organized by IIT BHU, Varanasi (Poster presentation)
- International Conference on Recent Advances in Composite materials (ICRACM-2019) organized by IIT BHU, Varanasi (Oral talk)

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Abstract: Hyaluronic acid (HA) is one of the most attractive natural polymers employed in biomaterials with biological applications. This polysaccharide is found in different tissues of the body because it is a natural component of the extracellular matrix; furthermore, it has crucial functions in cell growth, migration, and differentiation. Since its biological characteristics, HA has been utilized for the new biomaterial's development for tissue engineering, such as hydrogels. These hydrophilic macromolecular networks have gained significant attention due to their unique properties, making them potential candidates to be applied in biomedical fields. Different mechanisms to obtain hydrogels have been described. However, the research of new non-toxic methods has been growing in recent years. In this study, we prepared a new hydrogel of HA and polyvinyl alcohol by the cost-effective technique of cross-linking by gamma irradiation. The hydrogel was elaborated for the first time and was characterized by several methods such as Fourier Transform Infrared Spectroscopy, Differential Scanning Calorimetry, Thermogravimetric Analysis, and Scanning Electron Microscopy. Likewise, we evaluated the cytotoxicity of the biomaterial and its influence on cell migration in human fibroblasts. Furthermore, we provide preliminary evidence of the wound closure effect in a cellular wound model. The novel hydrogel offers an increase of HA stability with the potential to expand the useful life of HA in its different medical applications.

Key words: Biomaterial; Hyaluronic acid; Poly(vinyl) alcohol; Hydrogel; Gamma irradiation; Polymers.

Introduction

Hydrogels are semi-solid systems formed by the cross-linking of natural and synthetic polymers (1,2). They have a three-dimensional network structure under covalent or non-covalent bonds in the liquid medium that can retain a large amount of water in its swollen state (3). The properties of hydrogels resemble those of biological tissues, resulting in excellent biocompatibility (4). In this regard, hyaluronic acid (HA) is a natural polymer widely studied and applied as a hydrogel constituent for biomedical applications (5). It is a component of the Extracellular Matrix (ECM), which is involved in regulating diverse cellular responses, mainly in fibroblasts and smooth cells (6,7). These functions are primarily mediated by interactions with three classes of cell surface receptors: a) CD44 (a membrane glycoprotein), b) receptor for hyaluronate-mediated motility (RHAMM), and c) Intercellular adhesion molecule 1 (ICAM- 1). The binding to these receptors triggers different intracellular signaling pathways that control cellular biological processes such as angiogenesis, cell migration, cell proliferation, cell aggregation, and cell adhesion to ECM components (8,9). On the other hand, polyvinyl

alcohol (PVA) is a linear synthetic polymer that presents suitable biological properties. It is used frequently as a polymeric membrane material for wound dressings and coverings (10). However, its use is restricted due to its limited hydrophilicity, resulting in insufficient elasticity and rigid structure (11). Therefore, to improve its mechanical characteristics and biological properties, it has been merged with some natural polymers (12).

In this study, HA was cross-linked with PVA through gamma irradiation. This cross-linking method is considered a cost-effective, safe, and straightforward process because no toxic chemical agents are employed (13). The cross-linking by gamma irradiation of aqueous polymer solutions is carried out through the linear carbon radicals formation along the skeleton and the construction of a network by the recombination of polymer radicals (14,15). The obtained biomaterial was characterized to evaluate its chemical interactions, thermal behavior, and morphology. The cytotoxicity of the biomaterial and its influence on cell migration was assessed in human dermal fibroblasts. According to the results, the new and versatile hydrogel could be applied in different fields such as tissue engineering or drug delivery systems.

Materials and Methods

Materials

HA was purchased from Sigma-Aldrich® Chemical Co. (St. Louis, MO, USA) (Mw reported ~30,000 Dalton). PVA was obtained from Merck (Mowiol® 4-88, CAS 9002-89-5). The LIVE/DEAD Viability/Cytotoxicity for Mammalian Cells kit was acquired from ThermoFisher Scientific® (Carlsbad, CA, USA).

Sample preparation

Preparation of the Hyaluronic Acid + PVA solution (HA+PVA)

The PVA solution at 5% (w/v) was prepared in water at 90 °C by magnetic stirring. This solution (200 mL) was added to 50 mL of HA (0.02% w/v) in a glass container to obtain a homogeneous mixture.

Synthesis of Hyaluronic Acid-PVA hydrogel (HA–PVA)

The solution of HA and PVA obtained was subjected to gamma irradiation with an absorbed dose of 25 kGy, using a ^{60}Co source in the presence of air. This procedure was carried out through Fricke dosimeter equipment, with a dose ratio of 8.5 kGy h^{-1}.

Physicochemical characterization

In order to evaluate the structural changes triggered by the radiation, the lyophilized samples were characterized by Fourier Transform Infrared Spectroscopy (FT-IR), Differential Scanning Calorimetry (DSC), Thermogravimetric Analysis (TGA), and Scanning Electron Microscopy (SEM). FT-IR spectra were recorded on an FTIR Nicolet 6700 spectrophotometer (Thermo Fisher Scientific®, USA), and the samples were scanned in the range of 4,000 and 400 cm^{-1}. The TGA tests were carried out on a Hi-Res TGA 2950 Thermogravimetric Analyzer (Modulated TA Instruments®, DE, USA). The samples were analyzed from room temperature to 500 °C. The DSC tests were performed from -50 °C to 350 °C with the DSC 2910 (Modulated TA Instruments®, DE, USA). The heating ratio of 10 °C/min under a nitrogen atmosphere was utilized for both techniques. The morphology was analyzed using an EVO MA10 Microscopy (Zeiss®, Germany).

Biological characterization

The biocompatibility of the hydrogel and its performance in a wound model was evaluated to analyze changes in its biological behavior due to the radiation process. Dermal fibroblasts were isolated from human skin after aesthetic surgeries and obtaining the signed informed consent. We used calcein and Ethidium homodimer 1 (EthD-1) to determine cell viability through the LIVE/DEAD Viability/Cytotoxicity for Mammalian Cells kit. Photographs were taken using an epifluorescence microscope AxioObserver (Zeiss®, Germany). The number of live (calcein-positive) and dead (EthD-1-positive) cells were counted employing ImageJ software. For the wound-healing assay, fibroblasts were cultured until confluence in 24-well culture dishes (Corning®, USA). Then, a scratch was performed using a 1-mL micropipette tip. Cells were cultured with the hydrogel (1, 2, 4, and 8%) in addition to the control condition (culture medium) and the vehicle condition (PBS, GIBCO®, USA). The closure of the wound was analyzed for two days, and the distance between the cells was calculated using ImageJ software.

Statistical analysis

Statistical analysis was performed by ANOVA with a Tukey posthoc test, and $p < 0.05$ was considered significant. All data were analyzed using Graph Pad Prism Software version 7.05 (San Diego, CA, USA).

Results

Physicochemical Characterization

The FTIR spectra for HA and PVA (Fig. 1a and 1b, respectively) presented the characteristic bands of these excipients (11,16,17). For the HA spectrum (line a), the bending and tension bond of the N-H group were observed at 3,291 cm^{-1} and 2,940 cm^{-1}, respectively. Furthermore, the carbonyl group (C=O) of HA was visible at 1,600 cm^{-1}. Simultaneously, the bands of acid were presented with the asymmetric stretching and symmetric vibrations of the COO– group at 1,406 cm^{-1} coupled with the CO- bridge and the flexion of the group COH, observed at 1,149 cm^{-1} and 1,034 cm^{-1}, respectively (17,18).

Concerning the PVA (line b), the characteristic large band of absorption corresponding to the tension of the intermolecular and intramolecular hydrogen bonds of the hydroxyl group (O-H) was observed at 3,300 cm^{-1}. Likewise, the stretching of the carbonyl group of the non-hydrolyzed acetate ion was observed at 1,730 cm^{-1} (16). The HA+PVA and HA–PVA spectra presented mainly the PVA behavior.

The thermogravimetric analysis of the samples is depicted in Fig. 2. For the excipients (line a and line b), the characteristic thermal behavior was observed (16,17). In thermograms of PVA, HA+PVA, and HA–PVA (lines b, c, and d, respectively), a weight loss in the range of 300 °C to 500 °C was found. The drastic drop was

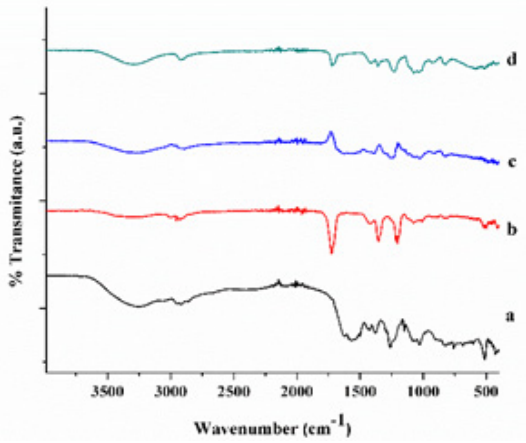

Figure 1. FT-IR spectra of the components of the biopolymer: (a) Hyaluronic Acid (HA); (b) Polyvinyl Alcohol (PVA); (c) HA+PVA, and (d) HA–PVA.

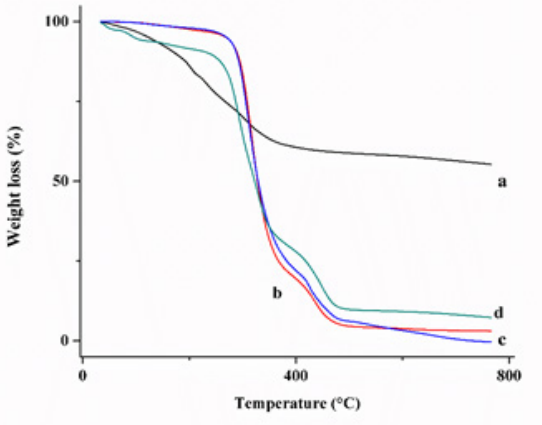

Figure 2. TGA of the components of the biopolymer: (a) Hyaluronic Acid (HA); (b) Polyvinyl Alcohol (PVA); (c) HA+PVA, and (d) HA–PVA.

Table 1. Differential Scanning Calorimetry (DSC) results.

Sample	Tm1 (°C)	Tm2 (°C)	Tm3 (°C)
HA	46.0	136.8	210.7
PVA	51.8	192.4	324.6
HA+PVA	51.8	194.5	326.0
HA–PVA	58.6	181.4	298.9

mainly due to the decomposition of the organic components, as previously described for HA hydrogels (18). A difference of approximately 7% in weight was observed at the test end between HA+PVA and HA–PVA (lines c and d, respectively). Moreover, Table 1 details the DSC results; a substantial left peak shift is seen on Tm2 and Tm3 in HA–PVA analysis compared to the excipients and the HA+PVA behavior, suggesting a chemical interaction between the components triggered by the irradiation.

The morphology of the biomaterial was evaluated by SEM analysis (Fig. 3). As can be observed, the hydrogel presented a porous microstructure. The interconnected flat pores had a diameter between 100 and 200 µm.

Biological characterization

In this study evaluated the viability of dermal fibroblasts in the presence of different concentrations of the hydrogel (Fig. 4.A). All treatments were not cytotoxic due to the percentage of viability in all cases was very close to 100% (Fig 4.C). Statistical analysis revealed that none treatment with HA–PVA samples affected cell viability. Remarkably, in all conditions, dermal fibroblasts presented their classical morphology, suggesting that the hydrogel did not affect cell adhesion.

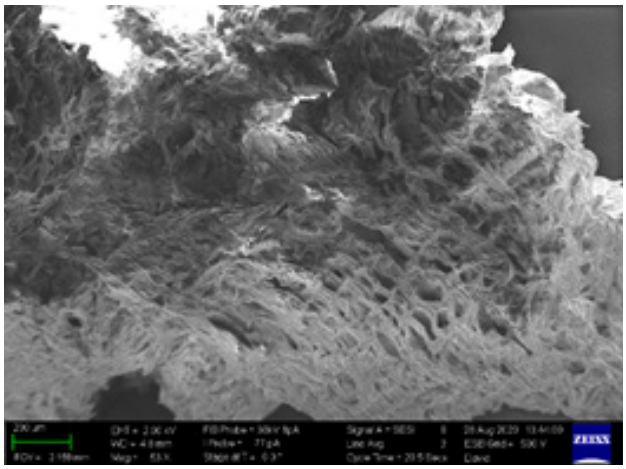

Figure 4. SEM image of the HA–PVA hydrogel microstructure. Scale bar: 200 µm.

In the wound-healing assay (Fig 4.B), the cell migration velocity was nearly the same in all the treatments (1%, 2%, 4, and 8%), even in control. The vehicle (Vh) showed a slight delay in closing; however, it was not significant (Fig. 4D). On the first day of analysis, the treatment of 1% of the biomaterial presented a slight increase in migration; however, it was not significant. On the second day of the analysis, all the experiments showed a close wound.

Discussion

Physicochemical characterization

The FTIR spectra revealed an abundance of intermolecular and intramolecular bonds of O-H in the band at 3,300 cm^{-1} on the spectra of HA+PVA and HA–PVA (Figure 1 line c and line d, respectively), suggesting a high affinity for an aquatic environment and the formation of free radicals. Furthermore, these bonds could provide high hydrophobicity to the new biomaterial, obtaining a material that retains a large amount of liquid. This characteristic is required in terms of compatibility with blood and biological fluids (19). Therefore, an interaction between HA and PVA was exhibited due to the presence of covalent bonds between the polymers, and we observed the decrease in the intensity of the C=O. Besides, in HA–PVA spectrum, the amine bands are present; however, the bands related to PVA revealed a decrement in intensity, indicating an interaction between HA and PVA. The last could be supported by expecting that the dominant interactions were the hydrogen bonds between the hydroxyl groups of PVA and the amino groups of HA. This conclusion generated an idea of the possible mechanism of interaction between the polymers presented in Figure 5.

To understand the formation of the covalent bond in the biomaterial and obtain information on the new material obtained, we propose the mechanism of the synthe-

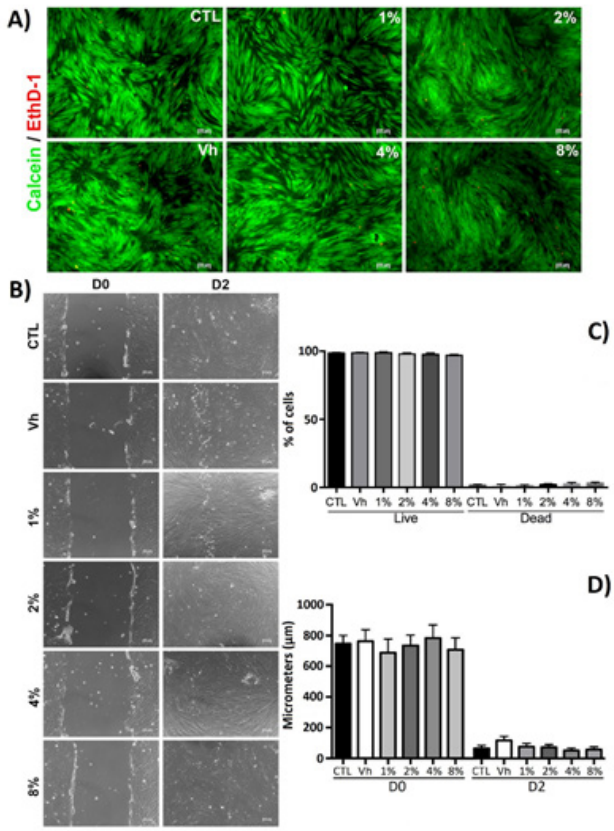

Figure 4. Biological characterization. A) Viability assay of HA–PVA (1, 2, 4, and 8%), control cells, and the vehicle (Vh, PBS) after 24 h of treatment. Scale bar: 100 µm. B) Cell migration. The figure exhibits the migration on day 0 and day 2 with the different treatments of HA-PVA (1, 2, 4, and 8%), control (medium), and vehicle (Vh, PBS). Scale bar: 100 µm. C) Graph indicates the percentage of live cells (calcein-positive) at the left and dead cells (EthD-1-positive) at the right. We did not find significant differences under any condition. D) Wound close. Graph bars compare the micrometers of the close wound on day 0 and day 2. In all conditions, the wounds were fully closed on the third day. There were no significant differences in any condition.

sis of the HA–PVA hydrogel with the cross-linking process by gamma irradiation (Fig. 5). First, the primary radicals of water (species 2−8) are obtained. The high energy radiation produces the radiolysis of water as the key step leading to cross-linking (15,20). The irradiation of PVA (see compound 1) led to the formation of three combinations of PVA radicals (precursors 10−12), and a carbonyl radical (see precursor 13). The irradiation of HA also yielded diverse types of radicals. A red-point depicts a typical backbone HA radical, while the arrows display the possible sites where other radicals can be formed. The HA has sites for various chemical reactions on its groups, including carboxyl, hydroxyl, and acetamido (21). Blue and light- and dark-green arrows denote that other Carbon radical (C•, precursors 15−25), hydroxyl (OH•, precursors 26−30), and amine groups (R1R2NH•, precursor 31) are likely to be achieved, respectively. Second, the absence of a monomer partially overrode the initiation and propagation steps. However, we considered the possibility of free radical transfer among PVA molecules in gamma irradiation (precursor 32). Finally, the termination reactions involved all possible deactivation reactions between PVA and HA precursors (compounds 33−49) and the recombination reaction between two PVA radicals to yield a PVA growing chain (compound 50). This occurred because the polymers that contain ternary amide groups and electronegative oxygen, such as HA, are potentially proton acceptors due to the fundamental nature of the functional groups (22). Interestingly, the deactivation of PVA radicals by the HA hydroxyl of amine groups yielded the associated ester and amide groups, as confirmed by FTIR. This group formation could indicate inhibition of HA degradation by modifying a hyaluronidase binding site (9). By inhibiting the enzymatic degradation of HA, indicates that the material obtained could present a longer degradation time.

As it is known, the cross-linking process implicates the creation of covalent bonds. The thermogram of HA–PVA (Fig. 2, line d) showed that the weight percentage remains compared with one of HA+PVA (Figure 2-line c). This result suggested that the irradiation process could promote the creation of new covalent bonds, indicating that the new HA–PVA hydrogel is more resistant to thermal energy. We observed slight variations at the end of the lines, which could be related to modifications in the material's structure caused by irradiation. We also found this behavior in the DSC study, due to the hydrogel presented a different thermal pattern from HA and PVA related to the cross-linking process (23). In Table 1, the treatments displayed two endothermic peaks that can be attributed to the loss of moisture or the melting of crystallites due to the strong hydrogen bonding between PVA-water and PVA chains themselves, aforementioned conferred the capacity of the biomaterial to absorbing water (24).

Another critical characteristic that regulates the hydrogel applications is the morphology (25,26). In this respect, we observed that the new biomaterial presented an interconnected porous network with elongated pores distributed into the structure (Fig. 3). This porous structure was owing to the gradual polymerization reaction

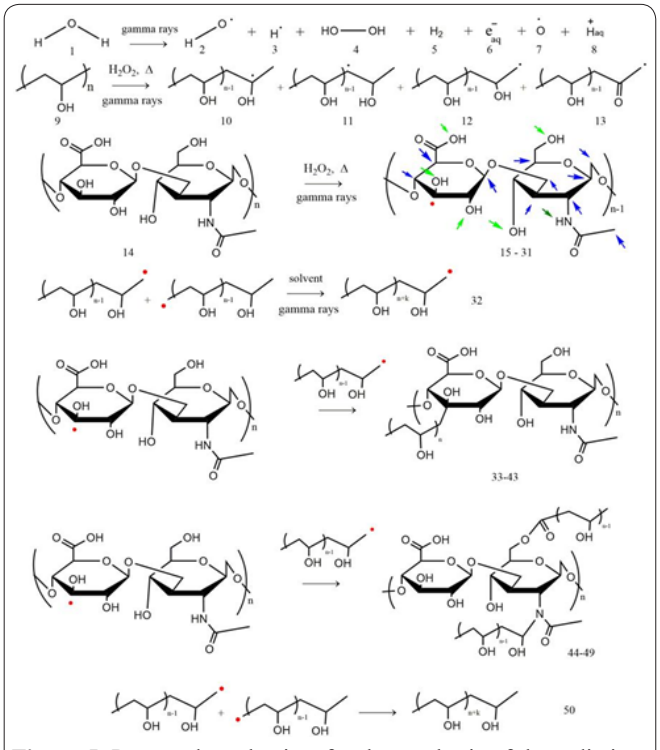

Figure 5. Proposed mechanism for the synthesis of the radiation-induced HA–PVA hydrogel (See text for details).

and the cross-linking density of the hydrogels (27), allowing an ordered matrix that helps the proper infiltration of living cells, gases, nutrients, cell factors, and waste products diffusion (28). Hence, this hydrogel could be applied as a scaffold in tissue engineering and as a wound dressing

Biological characterization

We evaluated cell viability and migration to analyze the effect of different concentrations of the new material (Fig. 4). The calcein/EthD-1 assays were performed at different concentrations of HA–PVA samples to analyze cell viability, evaluating the fluorescence of the cells. We found abundant living cells under all conditions. Furthermore, the dermal fibroblasts exhibited their classical morphology in the biomaterial presence. The morphology is related to the cell adhesion, which is reported as meaningful communication with the surrounding environment, indicating strong vitality (18). Thus, the mentioned above suggests that the treatments did not affect cell viability; therefore, they did not affect cell adhesion (29). Based on these results, the hydrogels may be considered a non-toxic biomaterial that does not affect the morphology and physiology of the cells; thus, these could be used in future models because they allow cell viability.

The wound-healing assay, an *in vitro* standard test representative of separation in a wound, was carried out to study the behavior that exists regarding the migration of cells in the presence of the biomaterial. After creating a linear scratch on the dermal fibroblast cell monolayer, the cells started to recruit from the edges of the space to close the gap at a measurable rate from an area with higher cell density to a region with lower cell density (30,31).

It is well-known that the HA is implicated in cell migration, which is essential for wound healing (35,36) and scar-free healing (37). Furthermore, it has been reported that the treatment of dermal wounds with HA hydrogels promotes cellular migration and proliferation, indicating the pathway of interaction with the other cells (32,33). We found a complete migration of cells for all HA–PVA concentrations, revealing that the hydrogel provides adequate cell migration (Figures 4B and 4D). Also, the 4% HA–PVA treatment demonstrated a slight increase in fibroblast migration, accelerating the closure of the wound model. These findings suggest that the biochemical interaction of the HA–PVA hydrogel could activate signaling pathways similar to HA when bonded to CD44 and RHAMM receptors (34). Those properties suggest the potential biomedical application of the new biomaterial as a wound dressing that could help in the re-epithelialization and healing of wounds to optimize the repair skin process (17).

Therefore, we developed a unique and multipurpose new material based on HA and PVA, which exhibits intrinsic characteristics that make it a versatile biomaterial with the same biological properties and higher HA stability; this is attractive because it can increase the shelf life of HA applications. Moreover, the HA–PVA hydrogel increases the potential applications of HA in different biomedical and pharmaceutical fields.

A new HA- and PVA-based hydrogel was successfully obtained by gamma irradiation. Additionally, this safe and effective technique provides sterility to the irradiated material. The novel hydrogel presented a porous network structure and possessed a synergistic mixture of the attractive characteristics of the individual materials. Both the biological activity of the HA and the well-studied features of PVA were maintained. The biological properties were evaluated in cell cultures of human dermal fibroblasts, obtaining a non-toxic material that promotes cell viability and cell migration. The biocompatibility, high porosity, and thermal stability characteristics render this new hydrogel an alternative biomaterial to be applied in the tissue engineering field with excellent stability and the potential to increase the useful life of HA in its different medical applications.

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Conflicts of interest

There are no conflicts to declare.

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