PHYSICAL PRINCIPLES OF CROSSLINKING OF WATER-SOLUBLE POLYMERS FOR TISSUE ENGINEERING

Kateřina STRNADOVÁ[1], Maxim LISNENKO[1], Ashot AGAZARIAN[1], David CHVÁTIL[2], Štěpán MATĚJŮ[1], Jaroslav MIKULE[1], Věra JENČOVÁ[1]

[1]*Technical University of Liberec, Faculty of Science, Humanities and Education, Liberec, Czech Republic, EU, katerina.strnadova@tul.cz*

[2]*Czech Academy of Sciences, Nuclear Physics Institute, Prague, Czech Republic, EU, chvatil@ujf.cas.cz*

https://doi.org/10.37904/nanocon.2025.5173

Abstract

Physical crosslinking of water-soluble polymers is a promising route for creating highly pure and biocompatible hydrogel scaffolds, offering an advantage over methods that use potentially cytotoxic chemical reagents. This study investigated the effectiveness of heat treatment and β-electron irradiation for the physical crosslinking of electrospun nanofibrous scaffolds / hydrogel disks derived from synthetic polymer - polyvinyl alcohol (PVA) and natural polymer - gelatin (GEL), focusing on material stability and cytocompatibility. PVA and GEL samples were fabricated and subsequently subjected to either heat treatment or β-electron irradiation before being characterized for morphology, water-solubility, and cytotoxicity (ISO 10993). Both physical methods were found not to significantly alter the nanofiber morphology. Crucially, β-electron irradiation failed to crosslink dry samples, which immediately dissolved, revealing that the presence of water is essential for this crosslinking mechanism. Biologically, physically crosslinked GEL nanofibers demonstrated excellent cell viability, while irradiated PVA nanofibers exhibited unexpected cytotoxicity. Otherwise, cell proliferation with PVA samples was unaffected, compared to GEL samples. Cell adhesion assays were inconclusive and require further investigation. These findings highlight that while physically crosslinked water-soluble polymers are good scaffold candidates, optimizing irradiation conditions, particularly by ensuring proper hydration, is critical for developing stable and functional scaffolds for tissue engineering applications.

Keywords: Polyvinyl alcohol, gelatin, water-soluble polymers, physical crosslinking

1. INTRODUCTION

Tissue engineering is a rapidly developing scientific field with significant potential for replacing or restoring damaged tissues. Central to this field is the use of hydrogels, which are three-dimensional networks of water-soluble polymers. Hydrogels are favored because their high-water content and mechanical properties closely mimic the native extracellular matrix, supporting nutrient supply, metabolite disposal, and cell viability [1-4].

From a clinical perspective, biocompatibility is paramount. Traditional methods for stabilizing water-soluble polymer scaffolds often rely on chemical cross-linking, which may involve cytotoxic reagents like glutaraldehyde, isocyanates, or epoxides [5]. These agents pose a health risk to the resulting material and can negatively affect the efficacy of biologically active substances.

To create safe, highly pure scaffolds - termed "green scaffolds" - physical cross-linking methods (e.g., thermal stabilization or irradiation) are preferred, as they avoid the introduction of undesirable chemicals [6]. While physical methods may offer a lower degree of cross-linking than chemical methods, they allow for precise control of the stabilization process. For example, heat treatment stabilizes PVA by increasing crystallinity and hydrogen bonding [7,8], while accelerated electron beam irradiation is a less common physical method that offers potential for precise control without chemical contamination [9].

This study focuses on two representative water-soluble polymers: the synthetic polymer polyvinyl alcohol (PVA), known for its mechanical strength and FDA approval for biomedical use [10, 11], and the natural polymer Gelatin (GEL), a denatured form of collagen that offers excellent biocompatibility and cell binding sites [12, 13].

Despite the importance of physical stabilization for producing safe tissue scaffolds, the comparative efficacy and biological impact of different physical cross-linking methods - specifically heat treatment versus β-electron irradiation - on PVA and GEL nanofiber scaffolds / hydrogels remain underexplored.

The primary motivation of this study was to investigate and compare the effect of heat treatment and β-electron irradiation on the physiochemical stability (morphology and water solubility) and cytocompatibility (cell viability and adhesion) of electrospun PVA and GEL nanofibrous scaffolds.

2. MATERIALS AND METHODS

2.1 Preparation of the polymeric materials

The water-soluble polymers, polyvinyl alcohol (PVA; 98 % hydrolysis, Mw 125,000, Merck, Germany) and gelatine (GEL; from porcine skin, Bloom 300, Sigma Aldrich), were used in these experiments. These polymers were either electrospun by NanospiderTM (model NS 1WS500U, Elmarco) or were prepared in the form of hydrogel disks (GEL only).

For electrospinning we used 10 % solution (w/w) of PVA in the solvent system of water : ethanol in the ration 9:1; and 18 % solution (w/v) of GEL in 30 % of acetic acid. The GEL disks were prepared from 8 % solution of GEL dissolved in distilled water, heated to 40 °C, poured into the silicone molds and let to solidify at room temperature.

Further, the samples were physically crosslinked. GEL disks were crosslinked by irradiation by β-electrons (Microtron MT25, NPI CAS, dose of 25 kGy) or by 0.5 % of glutaraldehyde (GTA; followed by glycine wash for two hours). The fibrous PVA and GEL samples were crosslinked by irradiation (50 and 100 kGy), or by heat treatment. PVA was stabilized by heat at 150 °C for 1 hour, whereas GEL was stabilized at 150°C for 72 hours in the vacuum oven. Once stabilization was achieved, the samples were stored at room temperature until further analysis.

2.2 Morphology of the materials

The morphology of the fibers was evaluated prior and after crosslinking by the scanning electron microcopy (Vega3 SB EasyProbe, Tescan), followed by the stereological evaluation of the fiber diameter (ImageJ), which was calculated from at least 100 measurements.

2.3 Solubility of the materials

The solubility for PVA was done and published previously [14]. Briefly, the heat-treated PVA samples were cut into pieces, weighted and incubated in sterile PBS at 37 °C. The samples (PBS with dissolved PVA) were collected at given time points and measured according to Pritchard and Akintola [15] at 630 nm using spectrophotometer (Spark, Tecan Group Ltd., Switzerland). The concentration values were calculated using the PVA calibration curve and its regression equation. Based on this concentration, the cumulative mass of dissolved PVA was subsequently determined.

For GEL, the electrospun heat-treated samples were cut into pieces, weighted and incubated in sterile HPLC dH_2O at 37 °C. The GEL samples were collected at given timepoints and were let to dry completely and weighted. For the crosslinked GEL disks, for each material, 5 samples were prepared for each sampling day. Samples were frozen at -80 °C and lyophilized at 0.03 mBar for a minimum of 48 hours or until completely lyophilized. After lyophilization, the individual samples were weighed, placed into 24-well plates, and immersed

in 1.5 ml of sterile PBS buffer containing 0.02% sodium azide (pH 7.4). The plate was placed in a sealable box. The entire box was then transferred to an incubator for 7 and 14 days at 37 °C. After incubation, the samples were removed from the PBS, placed into a 12-well culture plate with parafilm, gently dried with filter paper if necessary, and frozen at -80 °C, lyophilized, and finally weighed. The weights were compared with the original measured weight.

2.4 Cytotoxicity measurement

The cytotoxicity was measured according to the ISO 10993 with NIH-3T3 mice fibroblasts (ATCC). Briefly, the cells are seeded on a 96-well plated in the concentration of 10^4 per well (min. 8 wells for each condition) one day prior to the experiment. At the same time, the crosslinked materials (heat treatment, irradiation) were immersed in the complete DMEM medium (10 mg/ml) for 24 hours at 37 °C. The negative control (NC) is the pristine medium, whereas the positive control (PC, toxic) is medium with 0.1 % Triton X-100 incubated the same way. The next day, the eluates are added to the adhered cells and incubated for 24 hours at 37 °C. After incubation, the metabolic activity is measured spectrophotometrically via CCK-8 (Sigma Aldrich) according to the manufacturers protocol.

To better understand the cell behavior, live-cell imaging was conducted using the eluate from the irradiated or heat treated electrospun samples. The cells (mice fibroblasts) were seeded in the concentration of 10^5 per well in 24 well-plate using 500 µl of eluate as medium. The well-plate was placed into the microscope (Zeiss Axio Observer) equipped with the incubator and incubated for 72 hours at 37 °C and 5 % CO_2. The images of the selected area were taken every 10 minutes. From the time-lapse microscopy we calculated the cell culture spreading and doubling time and compared it to the values of the control (pure medium).

2.5 Data management

Processing of the data was done in Microsoft Excel; statistical analysis was made using GraphPad Prism (version 10.6.1). Data were statistically analyzed by one-way ANOVA with Tukey's or Sidak's post-hoc analysis, or by unpaired t-test with Welch's correction. Error bars represent standard deviations.

3. RESULTS AND DISCUSSION

3.1 Fiber morphology

Fiber diameter for GEL before and after DHT or irradiation was 170 nm (SD ±40 nm), for GEL after heat treatment the fiber diameter was 150 nm (SD ±30 nm) and for the irradiated GEL by 50 kGy 170 nm (SD ± 50 nm) and by 100 kGy 170 nm (SD ± 70 nm). For PVA the data were published previously [14]. Neither after heat treatment of the materials, nor after the irradiation, no significant change in fiber diameters was observed in the morphology analysis. From the data it is evident, that the heat treatment or irradiation has no effect on the fiber diameter (**Figure 1**).

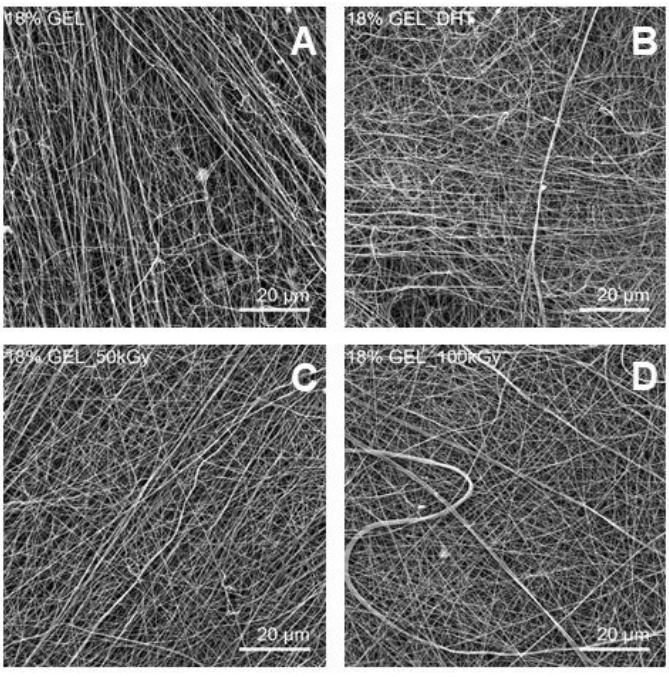

Figure 1 Images from the scanning electron microscopy showing the fibrous structure of GEL samples with no treatment (A) or after heat treatment (B) or irradiation (C: 50 kGy and 100 kGy: D). Scale bar: 20 µm.

3.2 Solubility

Solubility for PVA stabilized by heat treatment was published previously [14] and the data show that after 7 days of testing, between 4 and 13 % of the PVA was dissolved out of the total sample weight. For gelatin the data after 3 days of testing show that around 17 % of the GEL was dissolved out of the total sample weight (**Figure 2**).

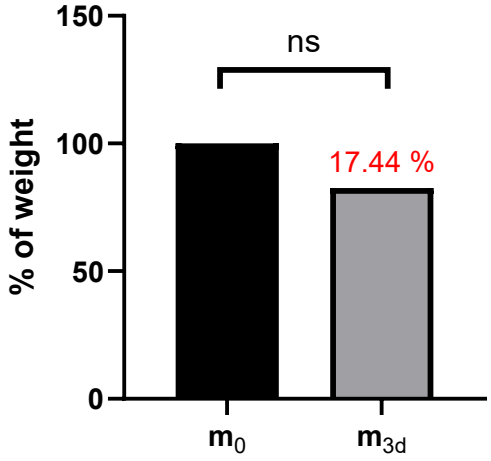

Figure 2 Solubility of the gelatin electrospun samples after heat treatment after 3 days of incubation with a 17.44 % of difference in weight of samples.

When we compare the solubility of the irradiated GEL disks to the glutaraldehyde crosslinked samples (**Figure 3**), it is evident, that the crosslinking efficiency is higher when using glutaraldehyde. Crosslinking by accelerated β-electrons results in approximately 20 % difference of weight after 7 days of incubation. On the other hand, after that period the samples remain stable.

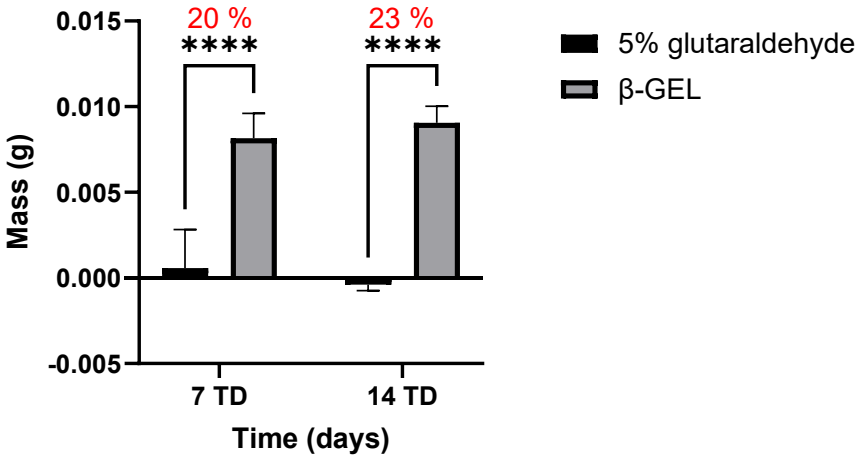

Figure 3 Comparison of the mass loss of the irradiated GEL disks to the glutaraldehyde crosslinked samples. Asterisks denote the p-value below 0.0001.

Unfortunately, the solubility data for the irradiated samples were not obtained, since the accelerated β-electrons do not crosslink the dry fibrous samples. Apparently, for the crosslinking by irradiation, the water plays a key role [16] by producing free radicals after irradiation and further crosslinking the macromolecules. The side chains of the macromolecules itself when irradiated are not sufficient to crosslink the polymer and the irradiation rather causes degradation of the dry samples.

3.3 Cytotoxicity

When testing the cytotoxicity of the irradiated hydrogel-like GEL materials, the viability is overall very good. Moreover, when you compare the viability of these samples to the ones crosslinked by glutaraldehyde, the most commonly used crosslinking chemical, where the viability decreases dramatically below 70 % threshold (**Figure 4**).

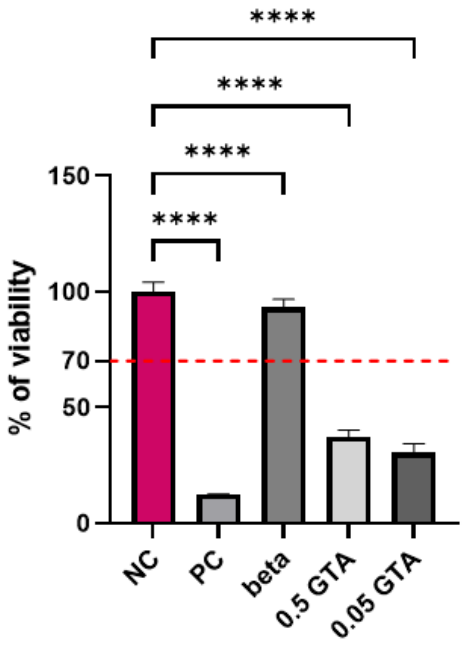

Figure 4 Cytotoxicity measurement of the irradiatiated hydrogel-like materials in comparison to glutaraldehyde crosslinking. The NC represents the negative control of cells in complete medium, PC represents positive control of cells in medium with Triton-X100 (toxic), beta are samples irradiated with β-electrons, GTA are samples crosslinked by different concentrations of glutaraldehyde. The red dashed line represents the limit for cytotoxicity according to the ISO 10993. Asterisks denote p-value (**** $p < 0.0001$).

Although the irradiated materials dissolve in the medium almost immediately, the cytotoxicity test was done to show that the irradiation does not cause any other unwanted chemical changes to influence the cell viability. From the graph (**Figure 5**) it is evident, that for GEL neither the heat treatment, nor the irradiation affects the overall cell viability. Whereas the irradiation of PVA has negative influence on the cell viability, which is below the threshold of 70 % viability of the control culture.

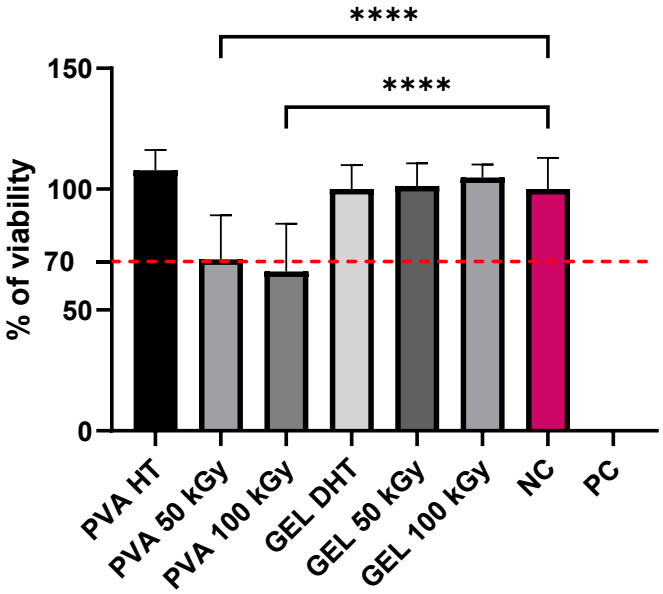

Figure 5 Cytotoxicity measurement of the crosslinked (DHT) fibrous materials. The NC represents the negative control of cells in complete medium, PC represents positive control of cells in medium with Triton-X100 (toxic), HT and DHT represents samples crosslinked by heat / dehydrothermal treatment, kGy are samples irradiated with β-electrons. The red dashed line represents the limit for cytotoxicity according to the ISO 10993. Asterisks denote p-value (**** $p < 0.0001$).

On the other hand, when we evaluated the doubling time of cells for the electrospun samples, which is between 20 to 26 hours for 3T3 mice fibroblasts, there was no significant change in the values for all types of samples (**Figure 6**).

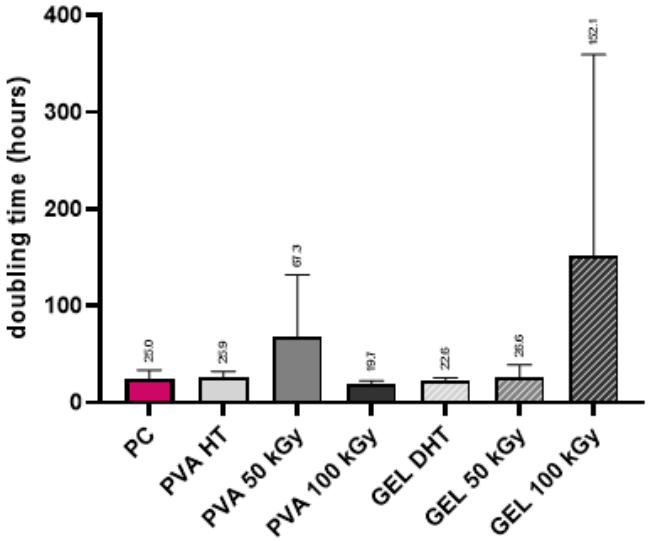

Figure 6 Doubling time of the cell culture treated with specific eluents from tested materials. The PC represents the cells seeded in pure complete medium, HT and DHT represents samples crosslinked by heat / dehydrothermal treatment, kGy are samples irradiated with β-electrons. The differences between columns are not significant.

The spreading time shows, how much time the cell culture needs to be adhered. For the normal cell culture, usually around 95 % of cells adhere in 1 hour. When evaluating the effects of eluates on the cell culture, it is evident, that the crosslinked gelatin samples negatively influence the cell spreading, where only maximum of around 30 % of cells are adhered (GEL DHT) after one hour incubation. On the other hand, also the control cells do not adhere as expected. Only around 70 % of cells are adhered under control conditions and that is also why these analyses need further investigation (**Figure 7**).

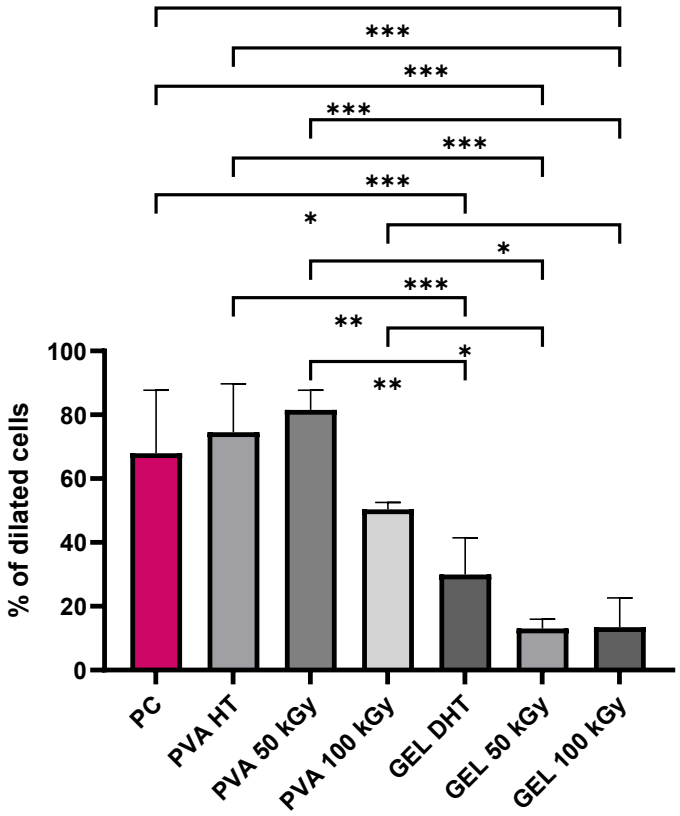

Figure 7 Spreading of cells treated with specific eluents from tested materials after 60 minutes of incubation period. Asterisks denote p-value (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$).

Apparently, the crosslinking of gelatin negatively influences other biological aspects of the cell culture, which define the fitness of the cells. Thus, the viability, doubling time and spreading of the samples depending on the crosslinking method need to be further and more deeply investigated and described.

4. CONCLUSION

This study investigated the efficacy of physical crosslinking methods - specifically heat treatment (DHT) and β-electron irradiation - on the morphological, physical, and biological properties of gelatin (GEL) and PVA nanofibrous and hydrogel-like scaffolds, positioning them as potential alternatives to chemical crosslinking for tissue engineering.

Our findings indicate that these physical methods are gentle on the material's nanostructure, as no significant changes in fiber diameter were observed after either heat treatment or irradiation. However, the study revealed significant challenges in achieving functional crosslinking. A critical finding was the complete failure of β-electron irradiation to crosslink dry fibrous samples, which dissolved almost immediately. This strongly suggests that the presence of water is indispensable for a successful irradiation-based crosslinking, likely by mediating the formation of free radicals. In the absence of water, polymer degradation appears to be the dominant outcome. Consequently, while glutaraldehyde (chemical) crosslinking remains more efficient, the stability of irradiated samples (when crosslinked) after an initial weight loss warrants further study. On the other hand, the results from cytotoxicity show that glutaraldehyde is highly toxic to cells.

The biological evaluation of the nanofibrous samples yielded mixed results. Physically crosslinked GEL scaffolds demonstrated excellent biocompatibility, maintaining high cell viability comparable to controls. Conversely, irradiated PVA showed a negative influence on cell viability, dropping below the 70% threshold. While cell proliferation (doubling time) was unaffected by any treatment, a significant limitation was observed in the cell spreading assay. All crosslinked gelatin samples negatively impacted cell adhesion. However, the compromised performance of the control group itself renders these specific adhesion data inconclusive and necessitates a thorough methodological re-evaluation in future work.

ACKNOWLEDGEMENTS

This research is funded by the project SGS-2025-4519 of Technical University of Liberec.

REFERENCES

[1] CHYZY, A., PLONSKA-BRZEZINSKA, M. E. Hydrogel Properties and Their Impact on Regenerative Medicine and Tissue Engineering. *Molecules*. 2020, vol. 25, no. 24, doi: 10.3390/MOLECULES25245795.

[2] RATNER, B. D., HOFFMAN, A. S., SCHOEN, F. J., LEMONS, J. E. *Biomaterials science: An Introduction to Materials in Medicine*, 3rd edition. Amsterdam: Academic Press, 2013.

[3] SLAUGHTER, B. V., KHURSHID, S. S., FISHER, O. Z., KHADEMHOSSEINI, A., PEPPAS, N. A. Hydrogels in Regenerative Medicine. *Adv Mater.* 2009, vol. 21, no. 0, p. 3307, doi: 10.1002/ADMA.200802106.

[4] GRIJALVO, S., NIETO-DÍAZ, M., MAZA, R. M., ERITJA, R., DÍAZ, D. D. Alginate Hydrogels as Scaffolds and Delivery Systems to Repair the Damaged Spinal Cord. *Biotechnol J*. 2019, vol. 14, no. 12, pp. 1–8, doi: 10.1002/biot.201900275.

[5] CAMPIGLIO, C. E., NEGRINI, N. C., FARÈ, S., DRAGHI, L. Cross-Linking Strategies for Electrospun Gelatin Scaffolds. *Materials* 2019, Vol. 12, Page 2476, vol. 12, no. 15, p. 2476, doi: 10.3390/MA12152476.

[6] OTSUKA, E., SUZUKI, A. A simple method to obtain a swollen PVA gel crosslinked by hydrogen bonds. *J Appl Polym Sci* 2009, vol. 114, no. 1, pp. 10–16, doi: 10.1002/APP.30546.

[7] HASIMI, A., STAVROPOULOU, A., PAPADOKOSTAKI, K. G., SANOPOULOU, M. Transport of water in polyvinyl alcohol films: Effect of thermal treatment and chemical crosslinking. *Eur Polym J* 2008, vol. 44, no. 12, pp. 4098–4107, doi: 10.1016/J.EURPOLYMJ.2008.09.011.

[8] WANG, Y. L., YANG, H., XU, Z. L. Influence of post-treatments on the properties of porous poly(vinyl alcohol) membranes. *J Appl Polym Sci* 2008, vol. 107, no. 3, pp. 1423–1429, doi: 10.1002/APP.24712.

[9] TERAO, K., NAGASAWA, N., NISHIDA, H., FURUSAWA, K., MORI, Y., YOSHII, F., DOBASHI, T. Reagent-free crosslinking of aqueous gelatin: manufacture and characteristics of gelatin gels irradiated with gamma-ray and electron beam. *J Biomater Sci Polym Ed* 2003, vol. 14, no. 11, pp. 1197–1208, doi: 10.1163/156856203322553437.

[10] BEN HALIMA, N. Poly(vinyl alcohol): review of its promising applications and insights into biodegradation. *RSC Adv* 2016, vol. 6, no. 46, pp. 39823–39832, doi: 10.1039/C6RA05742J.

[11] KOPRIVOVA, B., LISNENKO, M., SOLARSKA-SCIUK, K., PROCHAZKOVA, R., NOVOTNY, V., MULLEROVA, J., MIKES, P., JENCOVA, V. Large-scale electrospinning of poly (vinylalcohol) nanofibers incorporated with platelet-derived growth factors. *Express Polym Lett* 2020, vol. 14, no. 10, pp. 987–1000, doi: 10.3144/EXPRESSPOLYMLETT.2020.80.

[12] A. B. BELLO, D. KIM, D. KIM, H. PARK, and S. H. LEE. “Engineering and Functionalization of Gelatin Biomaterials: From Cell Culture to Medical Applications,” Tissue Eng Part B Rev, vol. 26, no. 2, pp. 164–180, Apr. 2020, doi: 10.1089/TEN.TEB.2019.0256.

[13] YANG, G., XIAO, Z., MA, K., ZHANG, J., REN, X., ZHANG, J. Assessment of the characteristics and biocompatibility of gelatin sponge scaffolds prepared by various crosslinking methods. *Sci Rep* 2018, vol. 8, no. 1, doi: 10.1038/S41598-018-20006-Y.

[14] LISNENKO, M., HOLEČEK, M., KUŽELOVÁ KOŠŤÁKOVÁ, E., VALTERA, J., REJMAN, D., MÜLLEROVÁ, J., BĚHÁLEK, L., JENČOVÁ, V. Physical stabilization of water-soluble PVA nanofibrous materials functionalized with biologically active substances,” *In: NANOCON Conference Proceedings - International Conference on Nanomaterials*, pp. 319–326, 2024, doi: 10.37904/NANOCON.2023.4793.

[15] PRITCHARD, J. G., AKINTOLA, D. A. Complexation of polyvinyl alcohol with iodine: Analytical precision and mechanism. *Talanta* 1972, vol. 19, no. 7, pp. 877–888, doi: 10.1016/0039-9140(72)80256-X.

[16] HAEMA, K., OYAMA, T. G., KIMURA, A, TAGUCHI, M. Radiation stability and modification of gelatin for biological and medical applications. *Radiation Physics and Chemistry* 2017, vol. 103, pp. 126–130, doi: 10.1016/J.RADPHYSCHEM.2014.05.056.