PHYSICAL STABILIZATION OF WATER-SOLUBLE PVA NANOFIBROUS MATERIALS FUNCTIONALIZED WITH BIOLOGICALLY ACTIVE SUBSTANCES

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Abstract

Tissue engineering aims to develop materials that enhance biological activity and promote tissue healing and regeneration. One promising approach is to functionalize nanofibrous materials with antimicrobial substances, such as lipophosphonoxin (LPPO), and use water-soluble polymers like polyvinyl alcohol (PVA) to incorporate bioactive molecules into fibers. However, water-soluble materials often face the issue of "burst release," releasing over 90% of the active substances within the initial 24 hours. This research focuses on preparing functionalized nanofibrous materials based on PVA containing the experimental antimicrobial compound LPPO and subsequent physical stabilization of the materials using the "Heat treatment" method. The applied stabilization successfully reduced the incorporated substance's release rate by up to 50%. The resulting materials have the potential to provide functional cross-linked PVA nanofiber scaffolds for regenerative medicine applications in large and chronic skin injuries.

Keywords: Nanofibrous materials, polyvinyl alcohol, functionalization, heat treatment, tissue engineering

1.   INTRODUCTION

In recent years, polyvinyl alcohol (PVA) has been widely used in the textile, cosmetic, and paper industries. It represents an interesting area of research in polymeric materials for various biomedical applications: the production of the artificial meniscus, hemodialysis membrane, heart valve, cardiac patch with drug delivery capability, wound dressings, and artificial vitreous [1], [2].

Due to its biocompatibility, adhesiveness, absence of toxic and carcinogenic effects, and water solubility, PVA is becoming a promising candidate for biomaterials production. In addition, it should be mentioned that the hydroxyl groups present in the PVA macromolecule are suitable objects for both chemical covalent crosslinking and non-covalent physical crosslinking due to the formation of hydrogen bridges between the side groups of the chain. Therefore, additional chemical or physical crosslinking is often applied to potentially reduce the solubility and increase the stability (mechanical properties) of the resulting PVA material [3].

Chemical cross-linking often involves toxic compounds such as glutaraldehyde, isocyanates, some epoxides, or methanol, which affect the efficacy of biologically active compounds [4]. Therefore, chemical cross-linking may be a less suitable method for tissue engineering and represents a health risk to the resulting material. On the other hand, physical crosslinking methods (thermal stabilization, electromagnetic radiation, plasma action) tend to be more biocompatible but provide a lower degree of crosslinking [5].

Although many specialized studies deal with the analysis of PVA for biomedical applications, there is a lack of sufficient investigation of the properties of physically crosslinked nanofibrous PVA materials. The aim of this

research was to develop functional PVA nanofibrous materials with incorporated bioactive agents and to investigate the effect of applied physical stabilization on the properties of the final material, which could serve as a composite scaffold functionalized with the experimental thermostable antibiotic lipophosphonoxine (LPPO) [6], provided by the Institute of Organic Chemistry and Biochemistry, CAS.

2. MATERIALS AND METHODS

2.1 Preparation of Nanofibrous Materials by DC Electrospinning

The DC electrospinning process was carried out on a Nanospider™ (model NS 1WS500U) from Elmarco, Czech Republic. The spinning of the prepared solutions was carried out sequentially. First, a pure 10 wt% PVA solution (98 % hydrolysis, Mw 125 000, Merck, Germany) dissolved in a 9:1 water and ethanol mixture. Then, after cleaning the cartridge and string, electrospinning of a 10 wt% PVA solution with lipophosphonoxine DR6180 (LPPO, Institute of Organic Chemistry and Biochemistry of the CAS) followed. The amount of antibiotic added to the solution corresponded to 5% of the LPPO content in the final spun material. The spinning parameters were as follows: spinneret voltage 40 kV, collector voltage -10 kV, electrode distance 170-190 mm, fabric removal 3-8 mm/min, string removal 10 mm/s, cartridge speed 2-2.5 s, temperature 20 °C, humidity 20%.

2.2 Thermal Stabilization of Nanofibers by "Heat Treatment"

For the high-temperature thermal stabilization, the prepared samples were transferred to glass Petri dishes and placed in a laboratory oven (TCF 120 Plus, Chromservis, Czech Republic) preheated to the desired temperature. The temperatures selected for the "Heat treatment" heat stabilization were 90, 120, and 150 °C and times of 1, 4, 8, and 16. Once stabilization was achieved, the samples were stored at room temperature until further analysis.

2.4 Sample Preparation for Testing

Each sample was prepared to weigh precisely 10 ± 0.1 mg. Three samples of each material were prepared and pre-sterilized with ethylene oxide for 12 hours at room temperature. Subsequently, all samples were placed in 1.5 ml microtubes, and 1 ml of PBS solution (prepared at the Technical University of Liberec, Department of Bioengineering) was added. The samples were then placed in an incubator (Q-cell 140/40 Basic, POL-LAB, Poland) at 37 °C, and aliquots of 200 μl were taken at specified time intervals. An equal volume of buffer was returned to the tube at each collection. Furthermore, the collected samples were immediately frozen at -20 °C for later analyses focusing on the kinetics of active substance release and monitoring the dissolution of polyvinyl alcohol.

2.5 Testing Methods of Prepared Nanofibrous Materials

The morphology of the prepared materials was analyzed using a scanning electron microscope (SEM, Tescan Vega 3, Tescan Orsay Holding, Czech Republic) at different magnifications. The fiber diameters were then measured using image analysis in ImageJ (National Institute of Health, USA). For each material, 300 fiber diameter values were randomly measured at a magnification of 5000×. The data were further evaluated as mean ± standard deviation (SD) and graphed using GraphPad Prism 9.2.0 (GraphPad Software Inc., USA).

Fourier transform infrared spectroscopy (FTIR) was used to perform structural characterization of materials on a Nicolet iZ10 instrument from Thermo Fisher Scientific, USA. For the analysis, the samples were placed on a diamond crystal (attenuated total reflection), and then the identification of organic compounds in the spectral range between 400 and 4000 cm^{-1} was performed.

For UV-VIS spectroscopy on a Spark spectrometer (Tecan Group Ltd., Switzerland), calibration solutions of polyvinyl alcohol (PVA) of known concentrations were first prepared. Determination of PVA concentration in the aliquots collected by spectrophotometry was performed according to the method of Pritchard and Akintola [7]. After thoroughly preparing all samples, the absorbance at 630 nm was measured.

The lipophosphoroxine molecules used in this work exhibit absorption of ultraviolet radiation at a wavelength of 260 nm. First, a calibration curve was generated where each sample of the calibration series was measured in triplicate. Furthermore, the samples were measured on a spectrophotometer (Spark, Tecan Group Ltd., Switzerland).

For differential scanning calorimetry (DSC), 10 mg samples were used and spread on the bottom of a 25 µl aluminum crucible. The samples were measured on a DSC 1/700 instrument from Mettler Toledo, Switzerland. The materials were heated over a temperature range from 0 °C to 300 °C in an inert nitrogen atmosphere with a gas flow rate of 50 ml/min and a heating rate of 10 °C/min.

3. RESULTS AND DISCUSSION

This work focuses on the fabrication and subsequent thermal stabilization of functionalized nanofibrous scaffolds using "Heat treatment" methods. The principle of this physical stabilization is the gradual heat treatment of the fabricated PVA materials at given temperatures and times to reduce their solubility. Due to the significant instability of the nanofibrous material in aqueous media, rapid dissolution of the PVA material occurs, which is closely linked to the process of rapid initial release of bioactive substances. [8] Due to the higher hydrolysis and molecular weight, hydrogen bridges are more frequently formed between the hydroxyl groups of the individual chains, as well as the formation of Van der Waals forces between the hydrocarbon chains of PVA [9]. The high number of non-covalent interactions will contribute to reducing PVA solubility in aqueous media and the controlled release of active species during physical-thermal stabilization [10]. After the preparation of PVA, PVA + LPPO, and their subsequent stabilization, a series of analyses were performed.

3.1. Analysis of the morphology of manufactured materials before and after thermal stabilization

After the spinning process was completed, measurements of the materials' surface weights and fiber diameters were made. The PVA material showed an area weight of 5.9 ± 0.6 g/m2, and the mean fiber diameter was around 432 ± 131 nm. For the PVA + LPPO material, an area weight of 9.1 ± 0.3 g/m2 was measured, and the mean fiber diameter was 295 ± 78 nm. Images of the input materials are presented in **Figure 1** along with histograms of the fiber diameter distribution. A significantly smaller fiber diameter is visible when comparing the PVA nanofiber material containing LPPO with PVA nanofibers without LPPO. This phenomenon can be explained by the fact that LPPO (an amphiphilic molecule) acts as a surfactant, and molecular interactions between PVA and LPPO can lead to changes in the structure of the nanofibers. Due to the reduction of the solution's surface tension, the solvent's evaporation in the softening process is sufficiently fast, reducing the likelihood of droplet defects. A similar trend was observed for polycaprolactone nanofibrous materials containing LPPO in the work of Do Pham [6].

After heat treatment of the materials, no significant change in fiber diameters was observed in the morphology analysis under any applied stabilization conditions. All PVA materials after thermal stabilization had mean fiber diameters ranging from 419 to 460 nm, while for PVA + LPPO materials the fiber diameter ranged from 296 to 310 nm. This is probably due to the natural inhomogeneity of electrostatically spinning nanofibers [11], which explains the variation in fiber diameters. **Figure 1** shows selected images of PVA and PVA + LPPO samples representing the extremes of stabilization along with the corresponding histograms of fiber diameter distributions.
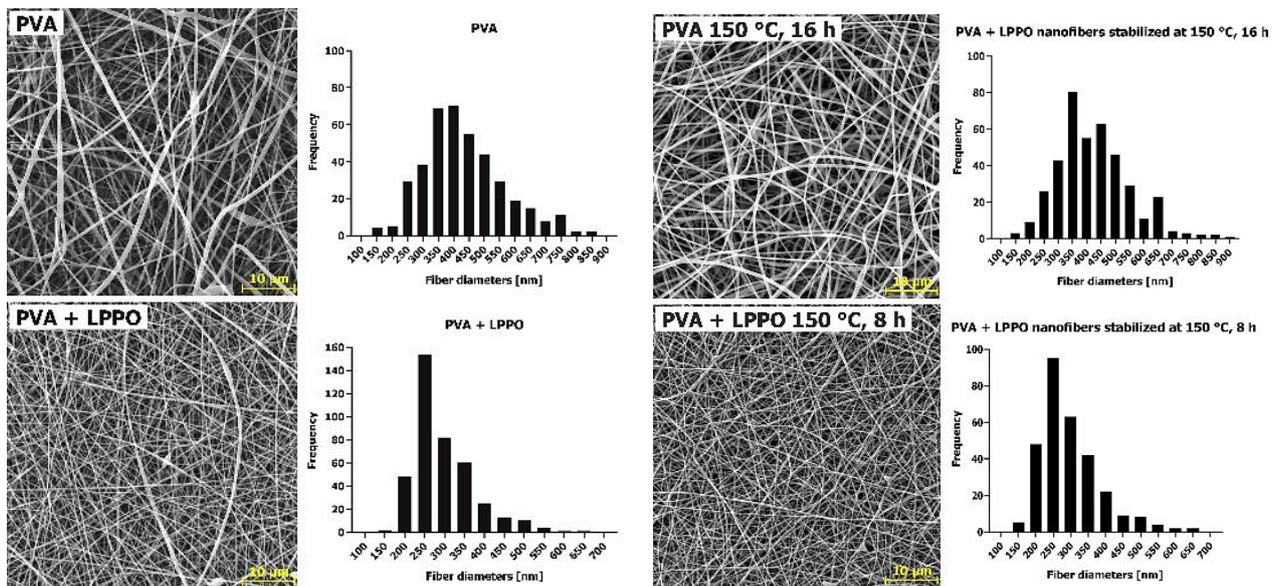
Figure 1 Images of the original PVA and PVA + LPPO nanofibrous materials and images of the same materials after extreme "Heat treatment" conditions with corresponding histograms of fiber diameter distributions (10 μm scale)

3.2 Solubility analysis of PVA materials after stabilization

The samples were further analyzed for PVA solubility by spectroscopy. **Figure 2** presents graphs showing the effect of different stabilization times and temperatures on the solubility of PVA nanofibers. The results show that thermal stabilization at 90 °C and 120 °C did not cause a decrease in the solubility of PVA nanofibers, and no clear correlation between material stability and stabilization time was observed. On the contrary, the materials stabilized at 90 °C (all times) and 120 °C (all times except 16 hours) showed higher solubility rates (75 to 81 % and 62 to 69 % depending on the stabilization time) than the unstabilized material (60 %) after 7 days of testing. The reason for this may be that water is not released from the materials at these temperatures because there is not enough energy or time to do so, but rather when water molecules try to evaporate, they disrupt the existing hydrogen bonds in the PVA, causing the materials to be more soluble. Significant reductions in the solubility of PVA nanofibers were observed only at 150 °C. After 7 days of testing, between 4 and 13 % of the PVA was dissolved out of the total sample weight (the decrease in PVA solubility was observed with increasing heat treatment time). Hence, 150 °C appears to be the most suitable temperature for heat stabilization of PVA.

Furthermore, **Figure 2** shows the effect of physical crosslinking at different temperatures and times on the solubility of PVA + LPPO nanofibers in phosphate buffer at 37 °C. The analysis results show that the materials stabilized at 90 and 120 °C showed higher solubility (ranging from 70 to 80% and 63 to 73% depending on the stabilization time) compared to the unstabilized material (60% after 7 days of testing). This observed change in solubility corresponds to the results of the release analysis of pure PVA nanofibres. A significant decrease in the solubility of PVA + LPPO nanofibers was observed only at 150 °C (same as for the pure PVA nanofibrous material). The solubility of these materials gradually decreased with a longer stabilization time. However, it was found that the temperature of 150 °C had a higher stabilization effect for the pure PVA nanofibrous material than for the PVA + LPPO nanofibrous material. This effect is probably due to the presence of LPPO molecules in the nanofiber structure, which prevents the formation of hydrogen bridges between the PVA chains and affects the formation of the crystalline phase in the material. A similar phenomenon of preventing the formation of hydrogen bonds between PVA chains due to the incorporation of bioactive agents is described by Bernal-Ballen [12] in the functionalization of PVA with the antibiotic ampicillin and Chen [13] in the functionalization of PVA with tannic acid.
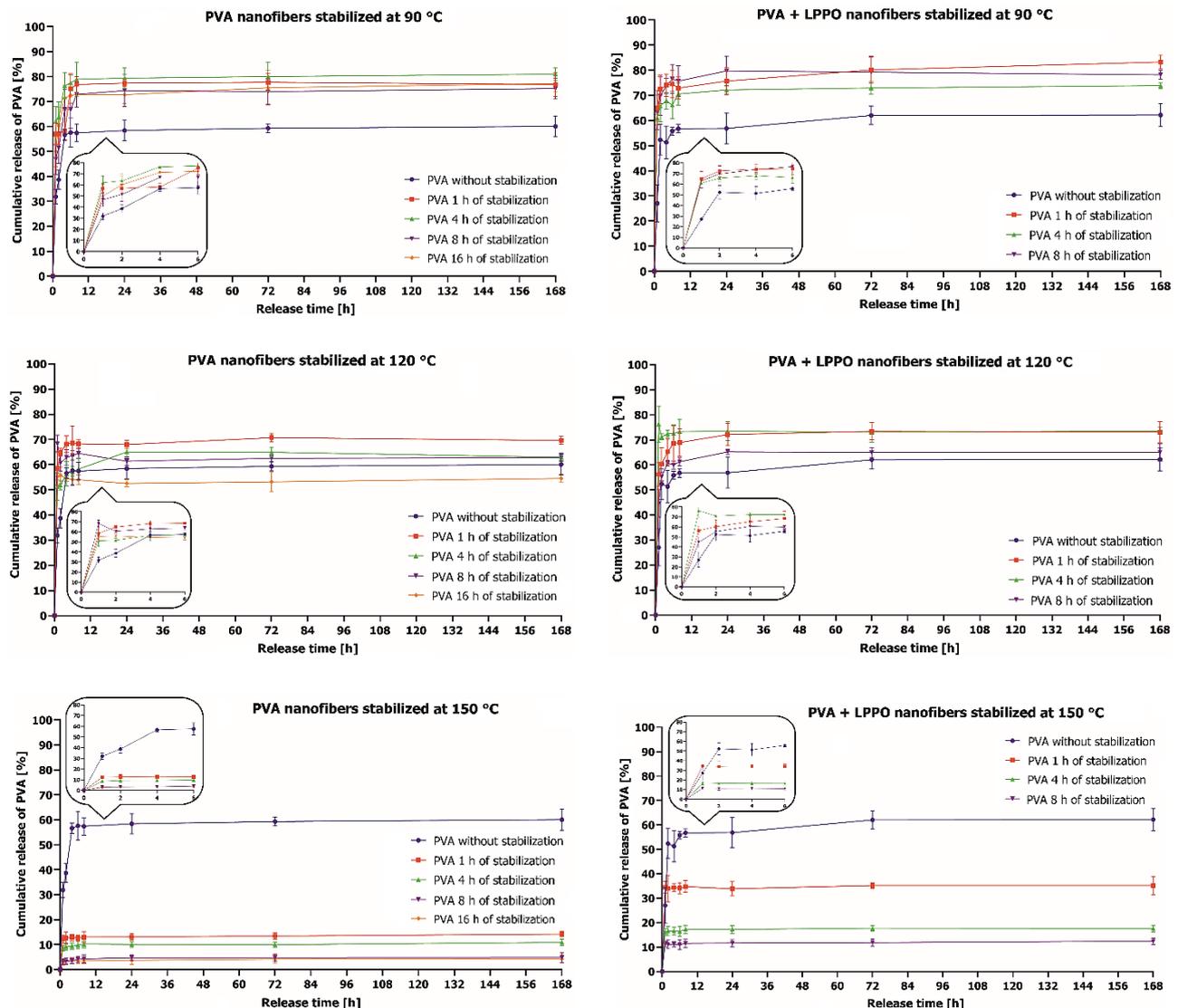
Figure 2 Plots of the cumulative release of polyvinyl alcohol (expressed as [%] of total sample weight) from PVA and PVA + LPPO nanofibres versus time for stabilization temperatures of 90 °C, 120 °C and 150 °C

3.3.   Analysis of active substance release from stabilized materials

The analysis of the release of the incorporated active substance (LPPO) and the effect of thermal stabilization was performed by spectrophotometry. The graphs in **Figure 3** compare the effect of heat treatment time on the release of LPPO at a specific stabilization temperature. The amount of cumulative LPPO released is expressed as a percentage of the maximum theoretical mass of the incorporated substance. In the case of a 10 mg nanofibrous sample containing 5% LPPO in dry weight, the maximum theoretical mass of the incorporated substance is 0.5 mg. From the results of the analysis, it can be seen that the total LPPO released from PVA nanofibers gradually decreases with increasing stabilization temperature. While almost 100 % of LPPO was released in the unstabilized material, 80 %, 70 %, and only 50 % of the LPPO contained in the nanofibres were released in the samples after thermal stabilization at 90 °C, 120 °C and 150 °C, respectively. The curves are typical for the functionalization of nanofibrous materials by the "wetting from blend" method, including the release by the "burst release" mechanism for all materials [14]. There is an initial significant release of the active substance, followed by a slower release.
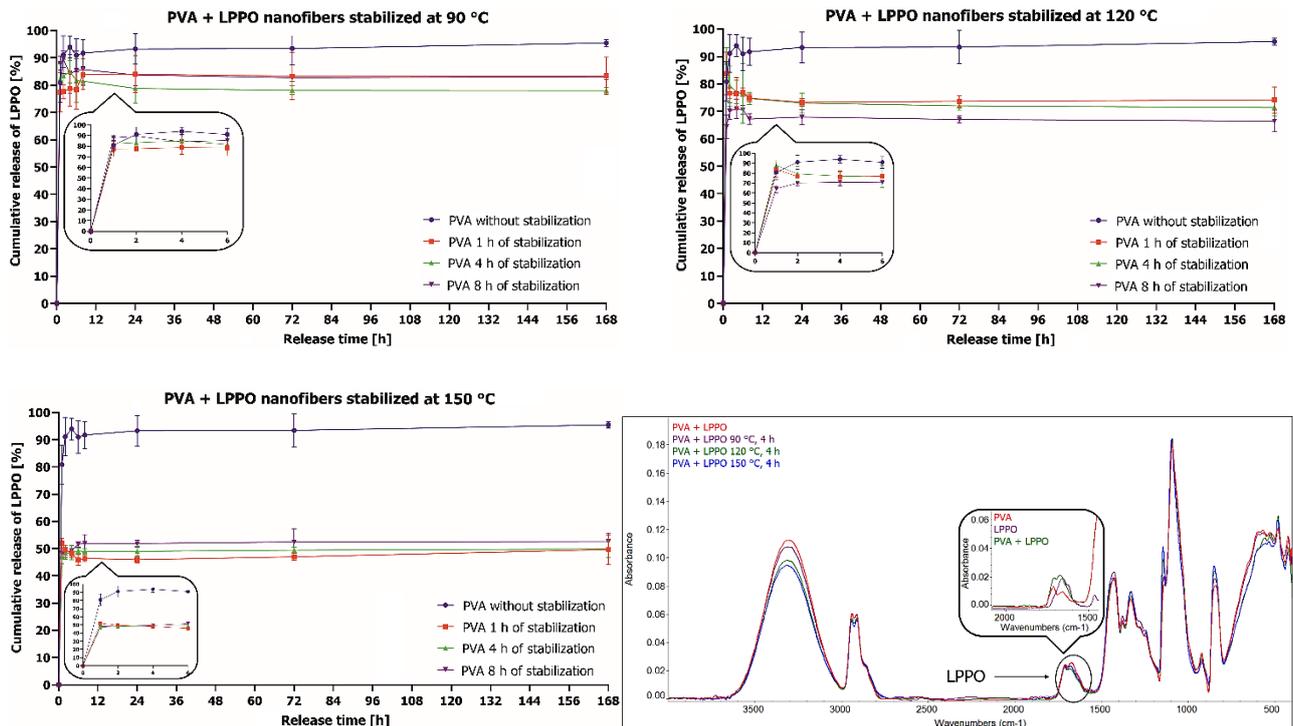
Figure 3 Plots of cumulative LPPO release (expressed in [%] of theoretical maximum mass) from PVA +
LPPO nanofibers versus release time for stabilization temperatures of 90 °C, 120 °C and 150 °C;
Infrared spectra of PVA + LPPO nanofibers subjected to thermal stabilization for 4 hours at different
temperatures; the cutout represents infrared spectra of unstabilized PVA, PVA + LPPO nanofibers
and used LPPO

Furthermore, the PVA + LPPO nanofibrous materials were subjected to FTIR analysis after thermal
stabilization. To compare the effect of increasing stabilization temperature on chemical and structural changes,
spectra of materials stabilized for 4 h were selected, as shown in **Figure 3**. The analysis results indicate that
a regular trend is observed in the spectra of PVA + LPPO, which is similar to PVA. As the temperature and
thermal stabilization time increase, the absorbance in the 3100 cm^{-1} to 3500 cm^{-1} wavenumber region
decreases (higher band intensity is an indicator of higher water content in the material), while the absorbance
in the 1140 cm^{-1} wavenumber region increases (Wong [15] suggests that this peak is very sensitive to the
crystalline phase content in PVA, the polymer with higher crystallinity shows higher IR absorbance).
No significant changes were observed in the spectra of the absorption band from 1650 cm^{-1} to 1750 cm^{-1}. This
PVA absorption band is overlain by the LPPO absorption band, which may mask any changes in this region.

3.4. Crystallinity analysis of PVA materials by DSC

From the results of the DSC analysis, it is evident that the PVA nanofibers without LPPO after "Heat treatment"
showed an increase in crystallinity as a function of the length of heat stabilization application. The graph
in **Figure 4** (left) shows that at 120 °C and 150 °C, there is a deviation from the emerging trend for samples
after 4 h and 1 h of stabilization. For PVA + LPPO samples, the trend of increasing crystallinity with longer
stabilization times was only observed at 90 °C (except for 1 h) and 120 °C. **Figure 4** (right) shows some
samples' irregular crystallinity decrease. Although some increase in crystallinity was observed at 90 °C
and 120 °C after 8 h of stabilization, the material stabilized at 150 °C after 8 h had the same crystallinity as the
unstabilized material according to the analysis.

Change in crystallinity of PVA nanofibres

Change in crystallinity of PVA + LPPO nanofibres
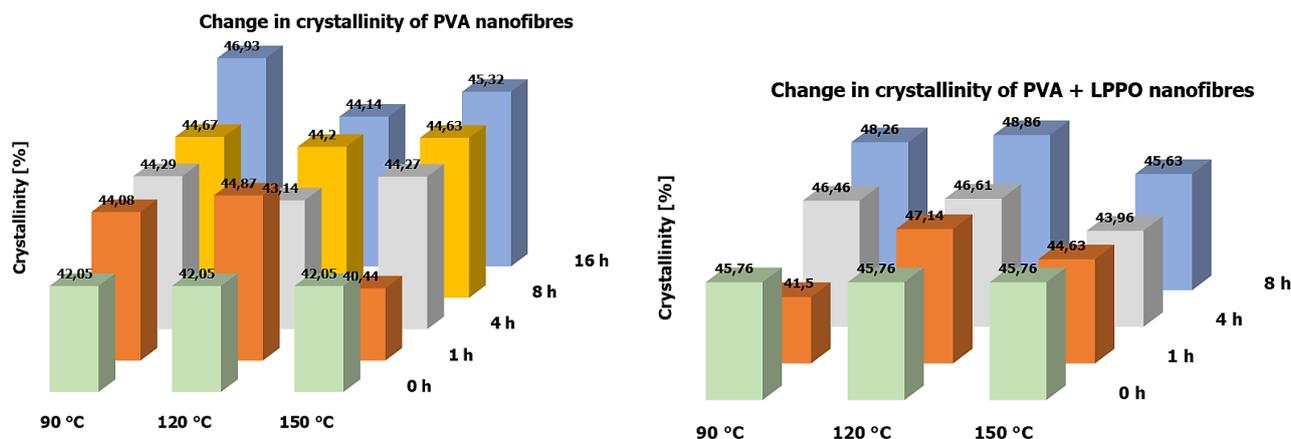
Figure 4 Change in crystallinity of PVA (left) and PVA + LPPO (right) nanofibrous materials as a function of time for a given stabilization temperature (90 °C, 120 °C and 150 °C)

4. CONCLUSION

Based on the results obtained in this study, it can be concluded that the "Heat treatment" method leads to stabilizing PVA nanofibrous materials in an aqueous environment. This process leads to a reduction in their solubility and to a reduction in the release rate of the incorporated bioactive substances. It is foreseeable that this strategy will provide functionalized physically cross-linked PVA nanofibrous layers that will meet the requirements of regenerative medicine and become an indispensable tool in, for example, the healing of large and chronic skin wounds. In further experiments, it would be useful to investigate more thoroughly the temperature range of 120 to 150 °C for "Heat treatment," where the most significant changes in the solubility of PVA nanofibrous materials were observed.

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