LONG-TERM COMPARATIVE STUDY OF THE STABILITY OF NANOFIBROUS MATERIALS FROM BIODEGRADABLE POLYCAPROLACTONE FOR SKIN WOUND DRESSING

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Abstract

This study deals with a long-term stability study of biodegradable polycaprolactone (PCL) nanofibrous dressings for skin applications, an important parameter for the storage of materials prior to actual application. Nanofibrous materials generally have suitable properties for regenerative medicine and tissue engineering, such as high porosity with small pore size, high areal density of fibers, large specific surface area, and these materials are able to mimic natural skin extracellular matrix with their fibrous structure. These properties allow nanofibrous dressings to provide the required protection to damaged tissues while promoting the wound-healing process. However, the issue of their long-term stability and degradation behaviour is still under investigation. The aim of this study was to observe and compare the long-term stability of electrospun PCL nanofibrous dressings exposed to different storage conditions. The study focused on the effect of nanofibrous material parameters, which included the surface weight; the solvent system used during the preparation of the polymeric spinning solutions and last but not least, the effect of ethylene oxide sterilization on the stability of the nanofibrous planar dressing over a period of 6-12 months.

Keywords: Electrospinning, polycaprolactone, long-term stability, degradation, wound dressing

1. INTRODUCTION

Nanofibrous materials are becoming increasingly important in tissue engineering (TI) and medical applications, especially structures mimicking native extracellular matrix (ECM). [1], [2] The materials exhibit sub-micron fiber diameters, large specific surface area, small pore size, which can be tuned according to application requirements. One of the methods used to prepare these materials is electrospinning, which allows the preparation of structures mimicking native extracellular matrix (ECM). [3] These materials then represent key components for the creation of scaffolds [4] (e.g. skin dressings) [5], [6], which play an important role in promoting cell growth, tissue regeneration and wound healing. [7] By combining the unique properties of nanofibrous materials with the selection of the appropriate polymer, innovative and functional materials can be created that bring many benefits to TI and regenerative medicine. [8]

Often used polymers are biodegradable polymers that eliminate or minimize the need for reoperations and other post-implantation procedures. [9] Scaffolds made of biodegradable polymers provide temporary support and space for cell growth, allowing new tissue to form. [10] At the same time, they are degraded over time during tissue regeneration. Monitoring and trying to understand the degradation behavior of the described structures is essential for the successful application of fiber scaffolds in the field of TI.

To monitor and optimize the degradation of nanofiber scaffolds, several factors need to be considered. [11] The first factor is the choice of the polymer used to make the scaffold. There are a number of biodegradable polymers, aliphatic polyesters such as polyglycolide (PGA), polylactide (PLA) and polycaprolactone (PCL) and their copolymers are often used. They differ in their chemical structure, which affects the rate of degradation of the material. [12], [13] The nanostructure and morphology of nanofibrous scaffolds is also an important aspect, which can be influenced not only by the choice of polymer, but also by technological and process parameters [14]-[16], and the applied sterilization can also have an influence. [17], [18] The surface structure can affect the degree of interaction with the medium in which they are incubated, depending on the model (in vitro/in vivo), which in turn affects the course and rate of degradation. The medium itself and the environment in which the structure is incubated (temperature, pH, enzymatic activity) have a major influence. [19]-[21]

The present study deals with the long-term monitoring of nanofibrous planar layers made of PCL. The motivation for the presented research was to evaluate the behavior of PCL nanofibrous materials over time and thus determine the "safe" time and suitable conditions for storing the materials before direct application in clinical practice. In view of the above aspects, it is also necessary to consider in terms of application potential how long the materials are stable not only non-sterile, but also after applied sterilization, which is an inevitable step for use in TI. The aim of the study was to observe the tested materials, evaluate their properties, stability and degradation behaviour over a period of 6 to 12 months. The experiment evaluated several aspects that can significantly influence the behavior of the fiber materials over time - the influence of the input parameters of the submitted layers, the influence of the applied ethylene oxide sterilization and the influence of the environment in which the materials were stored during the experiment. The above factors were evaluated in an attempt to contribute to the knowledge about the behaviour of fibrous materials during long-term storage and to monitor their stability over time, while understanding the interactions during the degradation process in vitro and thus contributing to a possible, at least partial prediction of the degradation process of PCL nanofibrous layers in in vivo models. At the same time, the aim was to provide insights into the stability of PCL materials in the longer term.

2. MATERIALS AND METHODS

2.1 Materials

PCL45 (Mw 45 000 g·mol^{-1}; Merck, Germany) was used for the preparation of micro/nanofiber layers. Solvent systems for preparing polymer solutions consist of chloroform, ethanol and acetic acid (Penta, Czech Republic). Tetrahydrofuran (Penta, Czech Republic) was used for gel permeation chromatography.

2.2 Electrospinning process

Fibrous materials were prepared from PCL 45, granules of the polymer were dissolved in a solvent system composed of: i) chloroform/ ethanol/ acetic acid (8/1/1, v/v/v); ii) chloroform/ethanol (8/2, v/v) in a final concentration of 16 wt %. The solutions were stirred at room temperature until complete dissolution was achieved. The solutions were electrospun using a Nanospider 1WS500U (Elmarco, Czech Republic) device. The conditions had been optimized previously in the preliminary experiments that led to the production of a macroscopically homogeneous nanofiber layer. The parameters of polymer solutions and conditions applied to the needleless electrospinning of the PCL are listed in Tables 1 and 2. The resulting fibrous layer was collected on a nonwoven textile layer (polypropylene spunbond - 40 g/m2).

Table 1 The marking of samples, parameters of polymer solution A) and the conditions applied at electrospinnig process B) of polymer solution at preparation PCL fibrous materials.

A)

sample	solvent system	rewinding speed [mm/min]
A/AS	8/1/1	40
B/BS	8/2	20
C/CS	8/1/1	20

B)

electrospinnig parametr	
distance between electrodes [mm]	180
high voltage [kV]	-10/+40
rewinding speed [mm/min]	40/20
steel orifice diameter [mm]	0.6
temperature [°C]	22
relative humidity [%]	50

2.3 Stability study experiment

The stability study was aimed at monitoring the degradation behaviour of PCL 45 fibrous layers over 6-12 months. During the study, several factors of the material were monitored: the effect of the solvent system for the preparation of the polymer solutions, the effect of the area weight of the fibre layers, the effect of ethylene oxide sterilization and last but not least the effect of the sample storage environment during the study. Non-sterile materials are labelled PCL45_A/B/C, ethylene oxide sterilised materials are labelled PCL45_AS/BS/CS. Fibre layers were cut into 50 ± 5 mg samples, placed in plastic tubes and put in 3 different environments: at room temperature 22°C (RT), in fridge at 4°C (F) and in incubator at 37°C and in aquenous environment (I). Samples were removed periodically every month. Subsequently, weight loss, molecular weight change and crystallinity were analyzed and morphological changes were also monitored.

2.4 Sterilization of fibrous material

Sterilization performed using ethylene oxide was done according to the ČSN EN ISO 11135-1 standard “Sterilization of products for health care - Sterilization with ethylene oxide - part 1: Requirements for the development, validation and continuous control of the sterilization procedure for medical devices”. The samples were sterilized at RT for 12 hours in an Anprolene steriliser device. The materials were then vented at room temperature for more than two weeks.

2.5 Morphological analysis

The electrospun samples were sputtered with a thin layer of gold and imaged by a Vega 3SB Easy Probe (TESCAN, CZ) scanning electron microscope. The fiber diameter characteristics were determined for each material by measuring fiber diameters (n=300) from SEM images The fiber diameters (about 100 measurements for each sample) were measured using the FIJI/ImageJ software (NIH). The areal weights of the sheets were determined by weighing samples (n=10) of 100x100 mm.

2.6 Weight loss analysis

Dried samples were immediately weighed using a digital balance (PA224C four-range analytical balance, Ohaus, Switzerland). To determine the percentage weight loss, weights of samples before and after incubation were determined and then subjected to calculation by Eq. (1). Three replicate samples were prepared and the values were averaged.

$$w_{loss} = \frac{w_b - w_a}{w_b} \cdot 100 \quad (1)$$

where:

w_{loss} - weight loss of samples during the degradation (%)

w_b - dry weight before degradation (mg)

w_a - dry weight after degradation (mg)

2.7 Gel permeation chromatography

Changes in molecular weight were assessed by means of gel permeation chromatography (GPC-ELSD). The samples were dissolved in tetrahydrofuran (THF) prior to analysis so as a final concentration of 1 mg/mL and mixed by authomatic vortex machine. Solutions were filtrated by using a 13 mm PTFE syringe microfilter (diametr 13 mm, 0.45 µm pore size). The system used was Dionex Ultimate 3000 with a Varian 385-LC ELSD detector. A Phenomenex Phenogel 5 µm 10E4 Å LC column with a length of 300 mm and an internal diameter of 7.8 mm was used. The temperature of the column compartment was set to 35°C during analysis. Pure THF was used as the mobile phase with a flow rate of 1 ml/min. The volume of the samples injected was 30 µl. The ELSD detector operated at a nebuliser temperature of 90°C, evaporator tube temperature of 80°C and the nitrogen flow rate was set to 1 l/min. The light source intensity was set to 10% and a sampling frequency of 1 Hz was used. Each chromatogram was recorded for 14.2 min. The change in the molecular weight was evaluated from the shift of the maximum peak of the chromatogram representing the most numerous molecular weight in the sample.

2.8 Differential scanning calorimetry

Differential scanning calorimetry (DSC) was used for the determination of changes in the thermal properties of the materials by DSC 1/700 METTLER TOLEDO (Metler-Toledo Greifensee, Switzerland) device. Samples aptoximetly 10 mg were prepared from nanofibres materials and placed in an aluminium pan, then in the DSC chamber. The materials were first tempered for 120 s and then heated in two cycles (the rate of temperature change was set at 10 °C/min). Measurements were taken in an inert nitrogen atmosphere. The PCL samples were analyzed within a temperature range of −20 to 100 °C. The resulting values were taken from the first heating cycle. The thermal properties of the PCL such as the melting temperature (Tm) and the enthalpy of fusion (ΔHm) were determined from the DSC curves. The crystallinity of polycaprolactone was determined by comparison of the ΔHm of 100% polymer (135.44 J g-1) as measured by [22]

3. RESULTS AND DISCUSSION

3.1 Electrospinning of PCL 45 and morphological analysis

PCL 45 was successfully electrospunun into micro/nanofibrous sheets. The electrospinning process was carried out under the conditions mentioned above, resulting in homogeneous fibrous layers. The above characteristics are summarized in **Table 2**. Statistical comparison of fiber diameter of each layer is shown in **Figure 1G** in form of box plot graph, where is no statistically significant differences between materials. The structure of the materials before and after ethylene oxide sterilization, supplemented by histograms of fiber diameters are shown in **Figures 1 A-F**, also in this case no significant morphological changes in the structure of the fibrous layers are observed.

Table 2 Characteristic of fibrous sheets used for the stability study.

sample	areal weight [g/m2]	fiber diameter [µm]
A	9.7 ± 0.2	0.36 ± 0.38
AS		0.37 ± 0.44
B	24.5 ± 3.1	0.39 ± 0.58
BS		0.37 ± 0.38
C	26.7 ± 1.1	0.36 ± 0.32
CS		0.38 ± 0.33

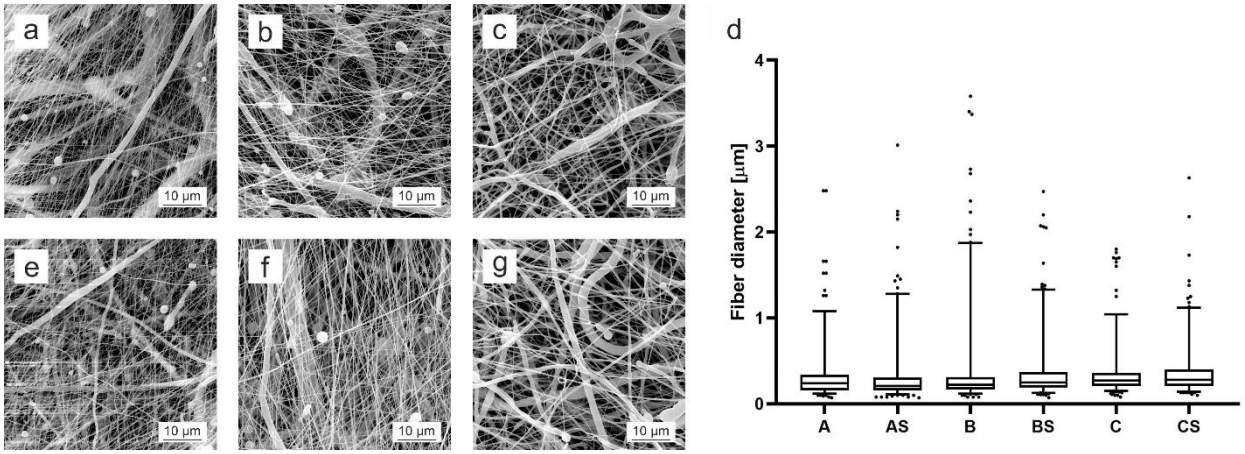

Figure 1 SEM images of nonsterile electrospuned fibrous layers of PCL45: sample A (a), sample B (b), sample C (c) and fibrous layers after ethylene oxide sterilization of sample AS (e), sample BS (f), sample CS (gf). Box plot graph comparing fiber diameters of each fiber layer (d)**.**

3.2 Study of the stability of nanofibrous materials

The stability study was evaluated by mass loss analysis, change in molecular weight (GPC), change in crystallinity (DSC) and finally by morphological analysis (SEM).

3.2.1. Morphological analysis (SEM)

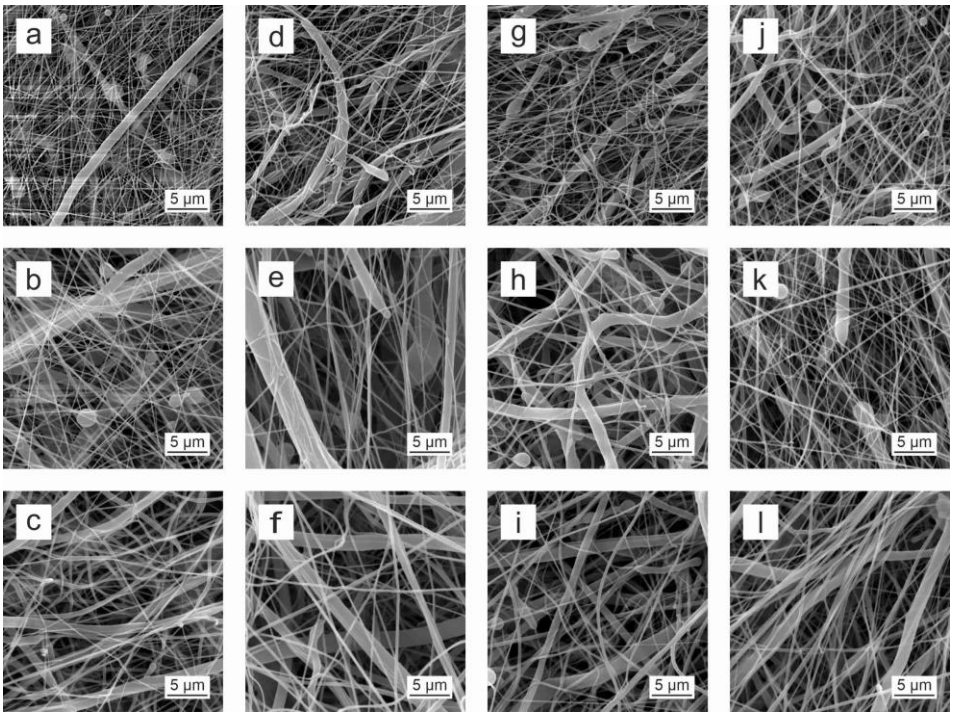

Figure 2 SEM images of nonsterile PCL45 electrospun fibrous layers: negative control NC - samples A (a), B (b), C (c); nonsterile PCL45 fibrous layers after 12 months: stored at 4 °C - sample A_F (d), B_F (e), C_F (f); fibrous layers stored in PBS at 37 °C - sample A_I (g), B_I (h), C_I (i); fibrous layers stored at room temperature 22 °C - sample A_RT (j), B_RT (k), C_RT (l). Scale bar 5 µm.

The morphological changes during the stability study were monitored by SEM, and the images obtained were compared in terms of changes in structure, defect formation or degradation phenomena characteristic of PCL

(fibre restructuring and disintegration). The tested nanofibrous layers did not show significant morphological changes after 6 months (sterile samples) and 12 months (non-sterile causes) for all storage environments (see **Figure 2**). Compared to the negative controls, no visible signs of degradation were observed. If we consider the commonly reported length of PCL degradability (2-3 years), then changes in the morphology of the materials without acceleration of degradation will manifest themselves in a longer time horizon. These results are consistent with the work of Horakova et al. [18], Dias et al. [23] and Dulnik et al..[24]

3.2.2. Weight loss analysis

The stability of the tested materials was further monitored by weight loss analysis. The results, summarized in **Figure 3**, show that the tested materials exhibit minimal weight loss (in most cases below 1 %) over a period of 6-12 months. Comparable results were obtained by Larrañaga el al. [25] More pronounced losses, but still minimal (around 2-3%) were observed in samples marked I, incubated in PBS at 37 °C, which is caused by the penetration of the aqueous medium (PBS) into the material with subsequent hydrolytic splitting of polymer chains and also due to increased temperature (37°C). Comparable results were achieved by Liu and Leonas [26], Dias et al. [23]. There is also a trend where the lower basis weight A_I and AS_I (PCL 45, 8:1:1) materials incubated in PBS at 37 °C show higher losses (up to 4%) compared to the other materials after 6 and 12 months. This result can be reasoned by the higher available area of samples with a lower surface weight (9.7 +- 0.2) while maintaining a uniform weight (50 mg) for all groups of materials. [27] The above findings would probably be more pronounced in a longer-term degradation study. Furthermore, we observe that the samples after the application of ethylene oxide have comparable weight loss as samples without sterilization, the applied EO sterilization has no impact on material degradation.

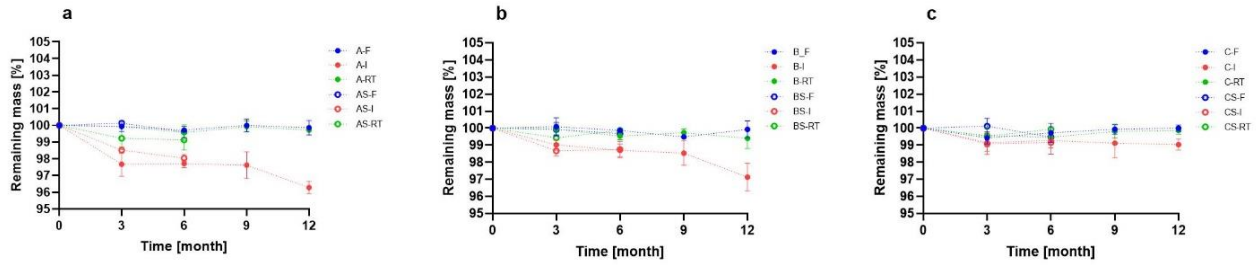

Figure 3 Graphs representing weight loss of PCL45 fibrous layers after 6 -12 months of stability study for samples: A/AS (a), B/BS (b); C/CS (c).

3.2.3. Molecular weight change analysis (GPC)

The stability of the materials was also evaluated in terms of the change in molecular weight of the polymer (Mn) fiber structure by GPC. Fiber layers PCL45_A, PCL45_B and PCL45_C show comparable trends in Mn change over a period of 6-12 months, see **Figure 4**. Also, no effect of ethylene oxide sterilization is observed. The results correspond with the study of Horakova et al. [18]. For materials incubated in F (4 °C) and RT (22 °C), minimal changes in molecular weights are observed over 6-12 months of storage compared to the negative control. More pronounced changes in Mn compared to the control are observed for all materials (A, B and C) in environment I (37 °C, PBS). From 6 months onwards, a more pronounced shift of the curves in the chromatograms to the right is observed in these samples, indicating a decrease in the molecular weight of the polymer. An important factor is the presence of an aqueous environment (I - 37 °C, PBS), which gradually penetrates the material and initiates a gradual hydrolytic cleavage of the polymer chains in the fibers, resulting in a decrease in the Mn of the polymer over time. These results are consistent with the study of Dong et al. [11] and Larrañaga el al. [25] Another factor that probably contributes to the more pronounced degradation of materials incubated in environment I compared to the others is the elevated temperature (37°C). This trend is pointed out by Xu et al. in their work. [28] In relation to the change in Mn for samples incubated in PBS at 37 °C,

no change in mechanical properties during sample handling was observed after 6 months. From 9 months onwards, the materials became slightly brittle but still retained their initial shape.

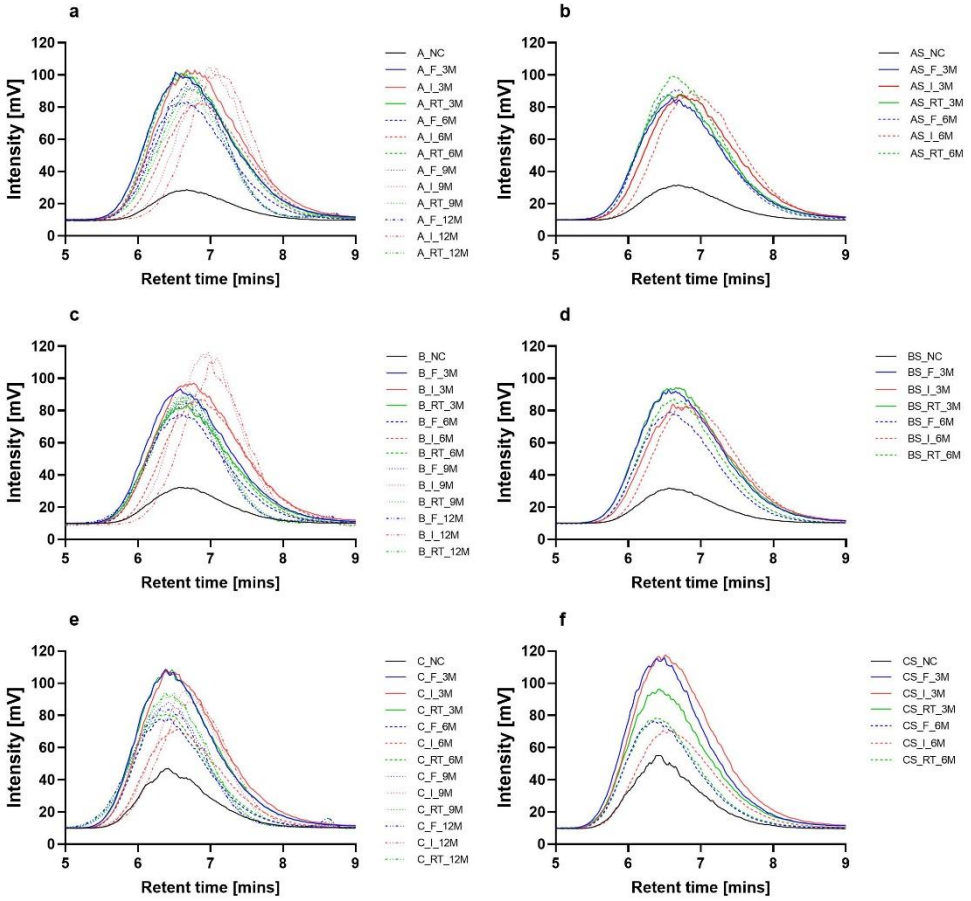

Figure 4 Graphs representing chromatograms of PCL45 fibrous layers during stability study in different enviroments. Sample A (a), AS (b), B (c), BS (d), C (e), CS (f).

3.2.4. Crystalinity change analysis (DSC)

The monitoring of the stability of the tested materials was complemented by the analysis of the change in crystallinity by DSC. The results are presented in the graphs in **Figure 5**. The graphs show an increase in crystalline phase over time for all materials (A, B, C) under all storage conditions (RT, I, F). At the same time, we observe a trend towards a more pronounced increase in crystalline phase for all materials in storage environment I. The increase in crystallinity during degradation is probably due to two mechanisms: (1) an increase in the flexibility of the polymer chains in the amorphous domains due to the penetration of water into the polymer and increased temperature [29], [30], [31] (2) crystallization of degraded fragments (oligomers) in the previously undegraded polymer material. [32] The increase in the crystalline phase is probably related to post-process crystallization of the material and rearrangement of the polymer chains over time. In this case, changes in crystallinity are not sufficient to be noted by SEM analysis. These are changes in the internal arrangement of macromolecules, at the same time the percentage changes in crystallinity detected in the materials are too low to cause structural changes in the material. Morphological changes can be observed in the later stages of polymer degradation (2-3 years depending on the type of PCL) or in the case of accelerated degradation (enzymatically catalysed degradation of PCL). [23] The growth of crystalline phases of polymer materials without morphological changes is reported by [23], [24], [33] At the same time, we observe a trend towards a more pronounced increase in crystalline phase for all materials when stored in environment I. We

also observe that the evolution of the crystallinity of a material is related to the temperature at which the material is stored. At the lower temperature in environment F (4 °C) we observe a lower growth of the crystalline phase of the material compared to materials stored in environment I and RT. A similar trend is described by Lopez et al. [34]

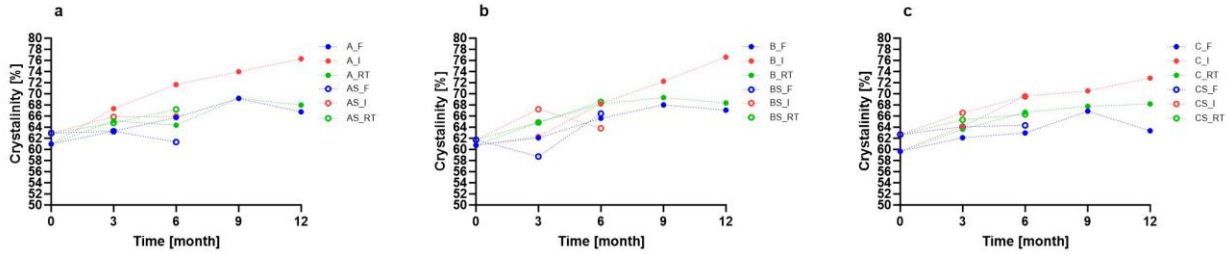

Figure 5 Graphs representing process of crystallinity changes in 6-12 months for PCL 45 fibrous layers - samples A/As (a), B/BS (b), C/CS (c).

4. CONCLUSION

In the presented study, the long-term stability of biodegradable PCL nanofibrous layers was monitored with regard to the length of storage, different types of storage conditions and the effect of applied sterilization with ethylene oxide. Morphological changes of the materials were not observed during the experiment, however, GPC and DSC analyzes show that storage conditions (especially aqueous environment and temperature) significantly affect the molecular weight (reduction of Mn) and the crystalline arrangement of the polymer (increase in the proportion of the crystalline phase) in nanofibrous materials. No effect of the ethyl oxide sterilization method on the stability of the materials was observed. Based on the results, we can state that the materials stored at room temperature (22°C) and in a refrigerator (4°C) are stable for 6 months (ethylene oxide sterilized cases) and 12 months (non-sterile samples) and no degradation manifestations that would affect the behavior of nanofibrous layers. These conditions are therefore suitable for long-term storage of nanofibrous PCL after the production of the materials before further use.

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