INFLUENCE OF STERILIZATION METHODS ON NANOFIBROUS LAYERS MADE FROM PVDF

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Abstract

Polyvinylidenefluoride (PVDF) is a biocompatible, nondegradable material commonly used in medicine as a surgical suture. However, the use of PVDF in the field of tissue engineering has more significant potential. One is the possibility of using nanofibrous PVDF layers for applications such as hernia meshes, glaucoma drainage implants, or nondegradable vascular grafts and verifying many properties, such as sterilization, precedes using such biomaterial in clinical practice. An inappropriately chosen method tends to disrupt the fibrous structure or affect the behavior of cells in contact with the material. Current literature does not provide a comparison of different sterilization methods on PVDF. The study is devoted to the influence of the most commonly used sterilization and disinfection methods on the nanofibrous PVDF layer prepared by the needleless electrospinning technique. Changes in fiber morphology, molecular weight, macroscopic changes, and behavior of cells on the material surface were monitored. It was discovered that the chosen methods do not affect fiber morphology or changes in molecular weight. However, the behavior of the cells on the materials sterilized by plasma was different.

Keywords: Polyvinylidenefluoride, sterilization, nanofibers, electrospinning

1. INTRODUCTION

Sterilization can be defined as a physical or chemical process that eliminates the action of microbial life on the surfaces and in the structure of materials [1]. The most common sterilization methods include autoclaving, ethylene oxide, gamma radiation, peracetic acid, or plasma [2,3]. It is also a common practice to use disinfection techniques, such as alcohol exposure with ethanol or UV radiation, which, however, is only superficial [4,5]. For PVDF, it is theoretically possible to use all the mentioned methods. Autoclaving is the most commonly used method of sterilization in general. The advantage is primarily its availability and price, disadvantage seem to be the necessity of temperature stability of the sterilized material [6]. More expensive methods include ethylene oxide sterilization and gamma irradiation. However, both methods are widely used, despite the potential danger to the service staff (ethylene oxide is a carcinogen, which must be provided with a sufficient period of aeration). In the case of gamma it is necessary to control the process according to the each specific material [7,8]. Gas plasma has an advantage in the residue aeration process, because the byproducts are nontoxic. Disinfection method by soaking of material to ethanol possess bactericidal properties [1]. Current literature does not provide information on the effect of different sterilization methods on the morphology or changes in biocompatibility of PVDF. For example, Chu reports the common use of conventional gamma radiation to sterilize PVDF surgical sutures [7]. Laroche investigated the effect of ethylene oxide sterilization on PVDF monofilaments in vascular sutures. He found that this method did not affect the morphological or mechanical properties of PVDF [9]. Considering the potential use of the PVDF implant in human medicine, it is good to compare the individual techniques of sterilization and whether they can influence the morphology or structure of the material. This study follows the author's previous study on the Impact of various sterilization and disinfection techniques on electrospun poly-ε-caprolactone [10].

2. Materials and methods

2.1 Preparation of the materials

Fibrous materials were prepared from polyvinylidenefluoride (PVDF, Mn: 180.000 g/mol; Merck, Germany). The polymer was dissolved in a solvent system composed of dimethylacetamid/acetone (8/2, v/v, Penta Chemicals, Czech Republic) in a final concentration of 26 wt %. The solution was stirred at 60 °C in dimethylacetamide until complete dissolution, then cooled to room temperature, followed by the addition of acetone. Such a prepared solution was immediately electrospun via Nanospider NS1WS500U (Elmarco, Czech Republic). The device is demonstrated in **Figure 1**.

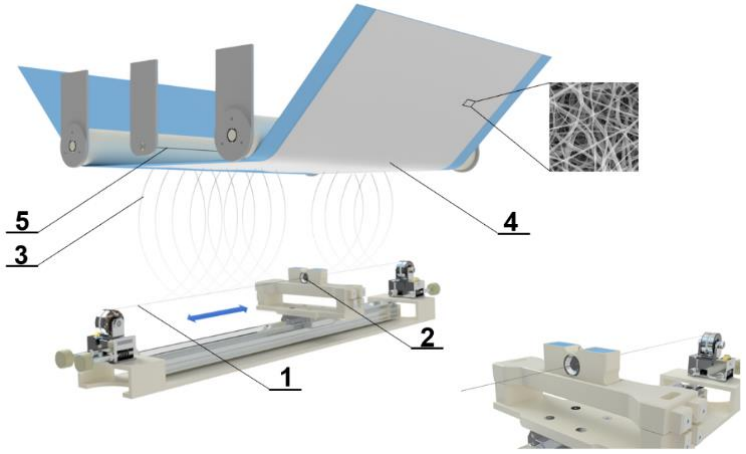


Figure 1 Scheme of Nanospider device. 1) String electrode, 2) die, 3) polymer jets, 4) nanofibers layer, 5) string counter electrode.

Climatic conditions (temperature and relative air humidity) were regulated by a precisely controlled air conditioning system NS AC150 (Elmarco, Czech Republic). The polymer solution was applied to a wire electrode with a diameter of 0.2 mm. Results: the fibrous layer was caught on a base substrate consisting of polypropylene spunbond with an area weight of 20 g/m2 (PF Nonwovens, Czech Republic). The surface weight of the resulting layer was regulated by the withdrawal of the substrate (REW) and the speed of the reservoir with the polymer solution (EMW). During the experiment, the highest cleanliness was observed to prevent contamination of the samples. Electrospinning conditions are shown in **Table 1**.

Table 1 Electrospinning conditions of PVDF nanofibrous layers

Distance between electrodes [mm]	207	
Voltage [kV]	Electrode 1	Electrode 2
	-22	+46
EMW [mm/s]	287	
REW [mm/min]	18	
Temperature [°C]	22	
Relative air humidity [%]	32	
Area weight [g/m^2]	20.51	

2.2 Sterilization of the materials

Various sterilization and disinfection techniques were successfully applied to PVDF nanofibrous layers with an area weight of 20.51 g/m^2 and compared with a non-sterile PVDF nanofibrous layer (control). Autoclaving was

carried out in the standard method at a temperature of 121 °C and a pressure of 103 kPa for 15 minutes using the Icanclave E118 device (class N; China). Ethylene oxide (EtOx) was applied at a temperature of 22 °C for 12 hours by an Anprolene An-74i device (Andersen sterilizers, USA). Gamma radiation (Gamma) was performed by Bioster Ltd. (Czech Republic). The samples were irradiated once with a dose of 29 ± 3.4 kGy. Plasma sterilization (Plasma) with hydrogen peroxide was performed with a STERRAD 100S device (Advanced Sterilization Products, Johnson & Johnson, USA) at a temperature of 47-56 °C for 47 min. Disinfection was performed with a 70% ethanol solution (EtOh; Penta Chemicals, Czech Republic) for 30 min. Followed by two rinses in PBS.

2.3 Morphology assessment

Morphological changes in fibrous layers were analyzed by means of a scanning electron microscope (Tescan Vega 3SB Easy Probe, Czech Republic). Prior to analysis, the materials were coated with a 10 nm layer of gold. Morphological evaluation was performed using the NIS Elements image analysis software (LIM s.r.o., Czech Republic). In the case of fiber diameters, 200 values were measured. Statistical evaluation was performed with GraphPad Prism v7 software (GraphPad Software, USA). Data did not show a normal distribution. Therefore, the Kruskal-Wallis test was used for comparison. Statistically significant changes were accepted at a significance level of $p \leq 0.05$. The results are presented in the form of graphs as the mean value ± 95% confidence interval from the standard deviation (95% CI).

2.4 Analysis of molecular weight

PVDF nanofibrous layers before and after sterilization were subjected to molecular weight analysis by gel permeation chromatography (GPC). Samples of pure granulate and nanofibrous layers before and after sterilizations were compared with respect to changes in molecular weight. The samples were dissolved in DMAC at a final concentration of 1 mg/ml before analysis. Analysis was performed using an HPLC system (Dionex UltiMate 3000; Varian LC-385 ELSD detector; Phenomenex Phenogel 1E5 polymer column, 300 mm long, 4.6 mm internal diameter, and 5 µm particle size). The mobile phase was DMAC (Sigma Aldrich; ≥ 99.9% for HPLC) with a 1 ml/min flow rate. Chromatographs were recorded for 23 minutes. The temperature of the nebulizer and vaporizer was 80°C, and the nitrogen flow rate was 1.1 ml/min. The volume of the polymers was 30 µl. The change in molecular weight was evaluated from the shift of the chromatogram's maximum peak, representing the sample's most abundant molecular weight. The peak shift towards longer elution times corresponded to decreasing molecular weight.

2.5 *In vitro* study

Materials after sterilization were cut into shape spheres with a diameter of 15.6 mm and seeded with 3T3 mouse fibroblasts (ATCC, USA). Dulbecco's modified Eagle medium (Lonza Biotec s.r.o., Czech Republic) supplemented with 10% fetal bovine serum (Lonza Biotec s.r.o., Czech Republic), 1% glutamine (Biosera, Czech Republic), and 1% penicillin/streptomycin/amphotericin B (Lonza Biotec s.r.o., Czech Republic) was used to culture of the cells. Adhesion and proliferation of the cells were evaluated after 1 and 7 days utilizing metabolic MTT assay by standard methods such as that shown in the publication [11].

3. RESULTS AND DISCUSSION

3.1. Morphology

The nanofibrous PVDF layer was successfully electrospun based on the team's previous experiences with this type of polymer. SEM images with morphology of non-sterile material and materials after individual types of sterilization are depicted in **Figure 2A**. The fiber diameters before and after sterilization of the layers are shown in **Figure 2B**. This graph compares the fiber diameters of variously sterilized layers to a non-sterile layer with

a fiber diameter of 261 ± 17 nm. The largest fiber diameter value was noted at the ETOX sterilized layer (334 ± 14 nm), the smallest for the gamma radiation sterilized layer (282 ± 9 nm). However, in all cases, very fine fibers with values about 110 nm and fibers attacking or slightly exceeding the limit of one micrometer were observed. The different values in fiber diameters are caused by the common variability of the fiber layer during production. Analysis of the obtained images of the created materials shows that all the mentioned sterilization methods are appropriate without a demonstrable influence on the fiber morphology. A layer containing a large number of beads defects, loss of fibrous morphology or creation of foil structure would be considered as a significant changes on the fiber morphology. None of these changes were present in the sterilized layers.

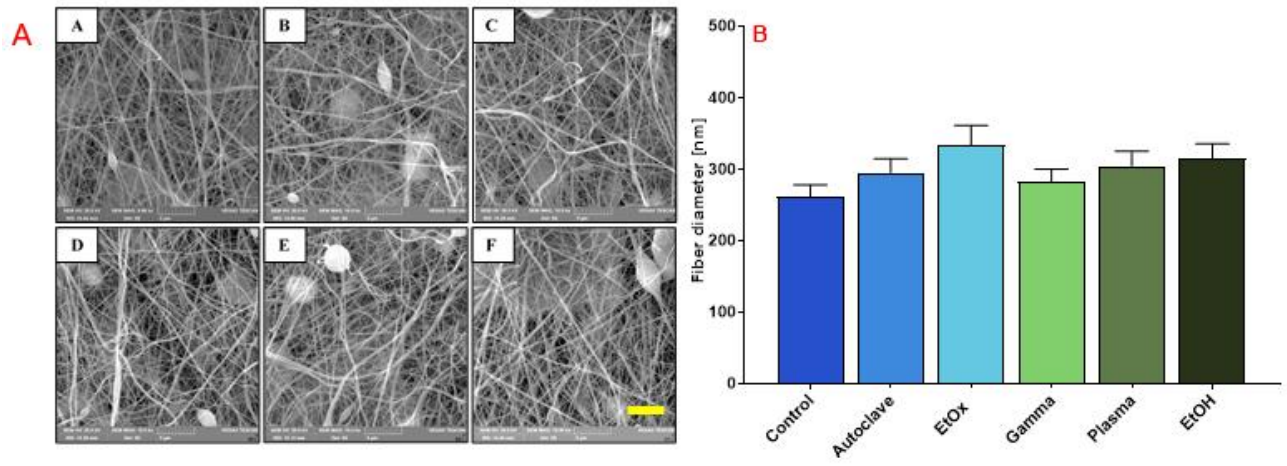
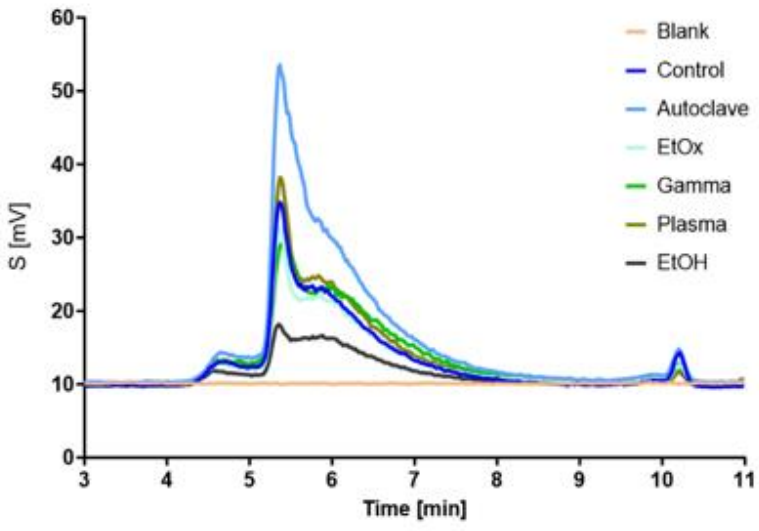


Figure 2 A) SEM images of fibrous layers after different sterilization methods. A: control, B: autoclave, C: EtOx, D: Gamma, E: Plasma, F: EtOH; scale bar 5 μm; B) Fiber diameter after sterilization by different methods; n = 200; mean ± 95% CI.

3.2 Molecular weight analysis

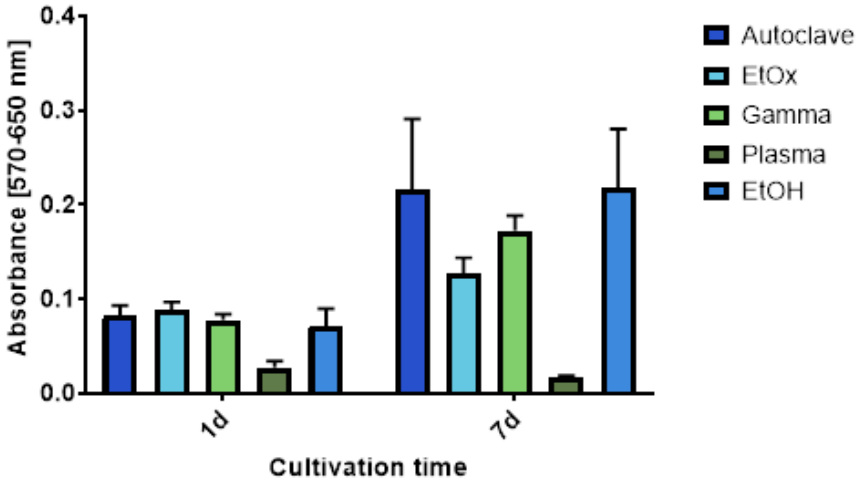
The changes in molecular weight after the application of sterilization methods were evaluated using GPC. Graph 1 compares the curves of measured data. All tested materials were eluted at the same time as the elution of the control sample, i.e. 5.37 min. This fact means that the molecular weight of the polymer does not change due to the sterilization methods used.



Graph 1 GPC chromatograms of the PVDF nanofibrous layers after sterilization: Blank (pure solvent), control (prior to sterilization), following Autoclave, EtOx, Gamma, Plasma, EtOH

3.3 *In vitro* study

The results of metabolic activity assessed by MTT assay are shown in Graph 2. Cell adhesion measured after 1 day was comparable on all materials except PVDF sterilized by plasma. After 7 days of culturing, the metabolic activity of cells on the plasma treated sample remained at low levels. Other materials supported cell proliferation; after a week of culturing, cell metabolic activity increased compared to the first testing day. Autoclave, EtOx, Gamma, and EtOH detected the highest cell viability on samples. In the case of using plasma sterilization, the fiber surface modification probably occurs, which become "inhospitable" for the cells. As is known, plasma treatments affect the surface structure of fibers. Dai evaluated the surface wettability of the polycaprolactone nanofibrous layer and discovered that it decreased after plasma treatment [12]. A similar case, where the change in the surface morphology of the PVDF fibers together with the reduction of its wettability led to suppression of the growth of the fibroblast cell line, was reported by Klápšťová [11]. Fraenklin concluded that plasma sterilization of PCL scaffolds resulted in significant cytotoxicity to canine chondrocytes [13].



Graph 2 Graphs of fibroblast metabolic activity measured by MTT assay

4. CONCLUSION

This study subjected nanofibrous materials from nondegradable polymer PVDF to the most commonly used sterilization and disinfection methods. The main goal was to compare the influence of these techniques primarily to significant morphological properties such as the loss of fiber morphology, the formation of a foil structure, or the appearance of a large number of beads defects. The fiber diameter of the selected PVDF layer was around 280 nm with the simultaneous occurrence of very fine fibers reaching the 110 nm as well as very thick fibers around 1 μm. As is known, nanofibrous layers produced by the needleless electrospinning method contain variable fiber diameters [14]. The mentioned differences in fiber diameters are completely consistent with the technological production possibilities and the methodology of their determination. Simultaneously, an analysis of the change in the molecular weight of the polymer due to the sterilization process was performed. PVDF is an inert material with common use in medicine, including the assumption of absolute non-degradability. As expected, there was no change in molecular weight due to the sterilization process. The metabolic MTT test verified the material's behavior in contact with cells. Autoclave, EtOx, Gamma and EtOH showed good cell adhesion and proliferation and appear to be suitable serilization methods for possible biological applications. Plasma sterilization did not support the growth of the fibroblast cell line. This fact was apparently caused by the fiber surface modification as a result by the effect of the plasma. In the case of producing an antifibrotic material resistant to cell growth, this sterilization method could benefit the intended application. In the current literature, there is no similar comparative study. In this regard, the obtained results

could help solve various medical problems. Extension of study, including detailed explanation, images from fluorescence microscopy, determination of the number of cells, etc. are subject to follow-up publication.

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