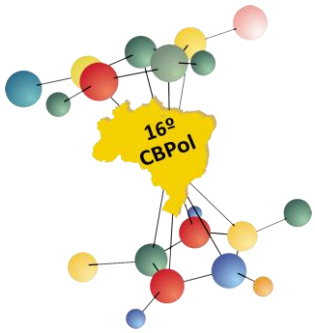

BLOW-SPINNING OF AQUEOUS POLYMER SOLUTIONS AND ITS PRELIMINARY BIOLOGICAL EVALUATION

André O. Arruda[1], Leonardo Engler[2], Lucas Dall Agnol[2], Natália F. Nicoletti[1], Fernanda T. G. Dias[3], Janete E. Zorzi[1,2], Otávio Bianchi[4], Asdrubal Falavigna[1]

1 – Postgraduate Program in Health Science, University of Caxias do Sul, Caxias do Sul, RS, Brazil
2 – Postgraduate Program in Materials Science and Engineering (PPGMAT), University of Caxias do Sul, Caxias do Sul, RS, Brazil
3 – Postgraduate Program in Technology and Materials Engineering (PPG-TEM), Federal Institute of Rio Grande do Sul (IFRS), Feliz, RS, Brazil fernanda.dias@feliz.ifrs.edu.br
4 – Postgraduate Program in Mining, Metallurgical and Materials Engineering (PPGE3M), Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil

Abstract - Here, we used solution blow-spinning (SBS) to produce fibers from aqueous solutions of poly(vinyl alcohol) (PVA) utilizing a commercial airbrush. This portable device allows for rapid fiber deposition onto any target, exhibiting promising future applications in clinical and surgical therapeutic models. However, to translate SBS into an operating room, safety considerations about the starting polymer-solvent system and fibers' biological toxicity are pivotal. The main goal of this work was to produce fibers from water successfully, which increases SBS use in living tissues. This was achieved by forced water evaporation. When in contact with two cell lines, the nanofibers caused an initial decrease in their metabolism and dynamics until 48 h exposure, which were recovered after this time. Further investigation is needed to elucidate the biological mechanism associated with nanofibers and how this initial apoptotic event can be modulated according to the therapeutic model of interest.

Keywords: *PVA/water blow-spinning; PVA nanofibers; in vitro cytotoxicity; DAPI staining assays; regenerative medicine*

Introduction

This work is part of a study about translating the solution blow-spinning (SBS) technique to clinical and surgical environments through in-situ fibers deposition onto living tissues. SBS is a safe, portable, and low-cost apparatus that provides personalized fabrics capable of carrying therapeutic agents locally delivered and supporting cell survival and growth [1]. A simplified SBS device consists of a syringe pump, a customized nozzle, a high-velocity gas source (typically compressed air), and a target [2]. A polymer solution prepared from a volatile solvent is pumped through a nozzle and carried by the pressurized gas to a target positioned at a certain distance from the nozzle. The solvent evaporates during the solution jet path, and solidified micro/nanofibers are accumulated at the target. Basically, three factors affect the quality of blow-spun fibers: the solvent type, the polymer concentration in solution, and the set of SBS operational parameters (nozzle diameter, gas pressure, and nozzle-target distance). Considering a biomedical context, the solvent choice is the critical parameter for SBS clinical application since most organic solvents impair biological activity [3]. Thus, the use of less volatile solvents in SBS, such as water, becomes a significant challenge for a safe application on living tissues. Few studies report the pilot use of SBS in minimally invasive surgeries, and none of them use water but other solvents such as acetone, anhydrous ethanol, and dimethylformamide [1,4]. This solvent limitation is what we hope to overcome in this work. A strategy for dealing with low volatile solvents in SBS is using heat

blowers to accelerate solvent's evaporation during fiber deposition [5]. Poly(vinyl alcohol) (PVA) was the polymer chosen for this exploratory investigation due to its water solubility and proven efficacy as a biomaterial in different contexts [6,7]. Besides successfully obtaining blow-spun fibers from aqueous PVA solutions, another objective of this study was to demonstrate the first signs of safe biological use of the produced PVA mats. Thus, *in vitro* cytotoxicity and DAPI staining assays were performed on PVA nanofibers to predict their feasibility for a possible future translational therapeutic strategy.

Experimental

Solution blow-spun fibers production and characterization

Aqueous poly(vinyl alcohol) (PVA) solutions (1−20wt%) were prepared at 90 °C and 300 rpm for 3 h. PVAs with different molar masses and hydrolysis degree were tested: PVA1 (104,500 $g.mol^{-1}$ and 87–89% hydrolysis degree, Neon Comercial, Brazil), PVA2 (31,000–50,000 $g.mol^{-1}$ and 98–99% hydrolysis degree, Sigma-Aldrich, Germany), and PVA3 (89,000–98,000 $g.mol^{-1}$ and >99% hydrolysis degree, Sigma-Aldrich, Germany). The rheology of these solutions was investigated at 50 ºC, using an Anton Paar MCR 502 Rheometer equipped with a cone-plate geometry (50 mm and 1°) and shear rates at 1–1000 s^{-1}. The fibers were produced from PVA solutions in a commercial airbrush (model BD-134K) at different experimental conditions: air pressure (1 – 7 bar), outer nozzle diameter (0.2–0.5 mm), solution feeding rate (0.3–5 $mL.min^{-1}$), nozzle-to-collector distance (15–90 cm), and the number of thermal blowers coupled to the SBS system (1 or 2). The thermal blowers (each with 12.2 $m.s^{-1}$ and maximum temperature of 120 ºC) were positioned 30º to the nozzle outlet to assist solvent's evaporation. The morphology and mean diameter of the fibers were investigated by field emission gun scanning electron microscopy (FEG-SEM Mira 3 Tescan, plasma coating, 10 kV magnification images). The fibers' mean diameter was calculated at Image J analyzer software (National Institutes of Health, Bethesda, MD) using at least 100 random measurements for each sample.

In vitro cytotoxicity

VERO (kidney epithelial) and 3T3 (murine fibroblast) cells were cultured in Dulbecco's Modified Eagle Medium (10% fetal bovine serum, 37 °C, 95% minimum relative humidity, 5% CO_2 air atmosphere) and incubated by direct contact with blow-spun fibers (3 cm^2 samples, N=3 per group per experiment, ISO 10993 [8]) produced from the 18wt% PVA2 solution. The cell lines were seeded at $3–5\times10^3$ cells per well in 96-well plates or $15–20\times10^3$ cells per well in 24-well plates, depending on the experimental protocol. All experiments were performed three times in triplicate. Metabolically active mitochondria assessed cell viability through MTT assay at 24 h, 48 h, 72 h, and 7 days.

Nuclear morphology and mitotic index (DAPI)

The 40,6-diamino-2-phenylindole (DAPI) staining was carried out to establish the cells' nuclear morphology – seeded in 24-well plates and incubated with fixed PVA2 fibers (blow-spun from PVA2 18wt% solution) elution for 24, 48, 72 hours, and 7 days. After incubation, the cells were fixed with 4% formaldehyde at room temperature for 15 min and stained with a 300 nM DAPI solution (Santa Cruz, CA) for 10 min. The cell's nuclear morphology was examined under a fluorescent microscope (Carl Zeiss MicroImaging GmbH, Germany). DAPI staining delineates mitotic figures and the mitotic index was calculated as the number of mitotic events in 10 fields per well, three times in triplicate. DAPI staining also delineates the nuclear morphology that quantifies the nuclear roundness and solidity measurements by Image J Software. Data from control cells (untreated) are used to set the parameters of the normal population. The morphometric parameters were calculated considering 100 events, three times in triplicate.

Results and Discussion

Three distinct grades of PVA and different PVA/water solution concentrations (1-20wt%) were initially tested to investigate their rheological behavior, particularly the critical concentration (c*) parameter. Only solutions prepared from PVA2 showed rheological data and signs of spinnability relevant for the production of fibers. PVA1 solutions were analyzed for their rheology but did not show good spinnability when tested by SBS. The PVA3 solutions, on the other hand, proved to be insoluble in all conditions explored. Fig. 1(a) shows the viscosity profile for PVA2 solutions as a function of concentration. The dilute, semi-dilute, and concentrated rheological regimes in PVA2 solutions are well characterized. The critical concentration (c*) for the PVA2/water system was observed at approximately 7.5wt% PVA2, above which there is an overlapping of macromolecules responsible for solution jet stretching and fiber formation in the SBS process [9]. However, the processing variables chosen for SBS also influence spinnability and fiber morphology. Different processing conditions (as described in the experimental section) were tested for PVA solutions blow-spinning, but the most efficient for producing uniform fibers were: 7 bar air pressure, 0.5 mm outer nozzle diameter, 30 cm nozzle-to-collector distance, and two thermal blowers coupled to the SBS apparatus. Although c* occurred for 7.5wt% PVA2, only systems with 15-20wt% PVA2 produced fibers. This concentration range is in the transition between semi-diluted and concentrated regimes, where a high topological interaction of polymer chains is observed. Fig.1(b) e Fig.1(c) illustrate the influence of PVA2 solution concentration on the morphology and average diameter of fibers. In Fig. 1(b), few solvent droplets were detected for the blow-spun 16wt% PVA2 solution, but poor chain entanglement was denounced by fibers with a broad dispersion of diameters. Fig. 1(c) represents the system obtained from the 18wt% PVA2 solution blow-spinning, composed of fibers with better quality and the most uniform diameters distribution. The fibers produced from the 18wt% PVA2 solution were adopted for all biological investigations. These fibers presented an average diameter of 247.4 nm, which is quite interesting for mimicking the extracellular matrix (ECM) environment (collagen fibers of 50 - 500 nm) [10, 11].

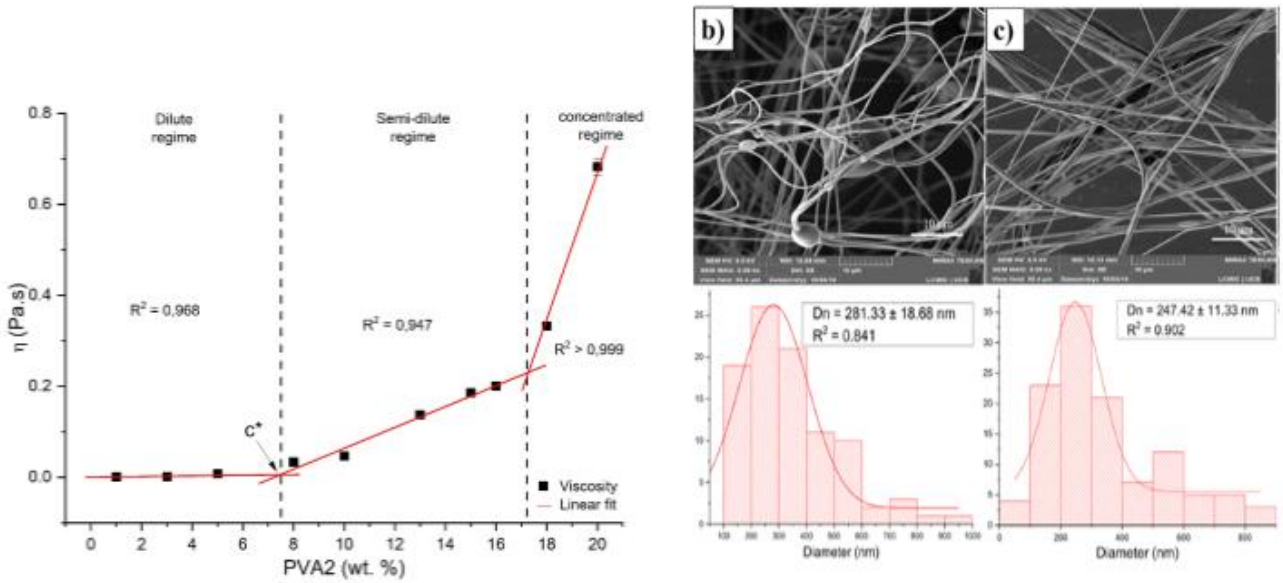

Fig. 1: (a) Viscosity as a function of the PVA2 concentration; FEG-SEM image (and the respective histograms) of blow-spun fibers produced by PVA2 solutions at 16wt% (b), and 18w% (c).

Fig. 2 shows the *in vitro* cytotoxicity of the PVA2 in contact with 3T3 and VERO cells for 24 h, 48 h, 72 h, and 7 days. Both cell lines showed a significant reduction in viability related to the control after 24 h and 48 h time exposure ($p < 0.01$). However, an increase in cell viability occurred for incubation times of 72 h and 7 days ($p > 0.01$), being restored to the control level. This behavior

is compatible with the ability of cells adapt to grow and proliferate the structure of the PVA2 mats [10].

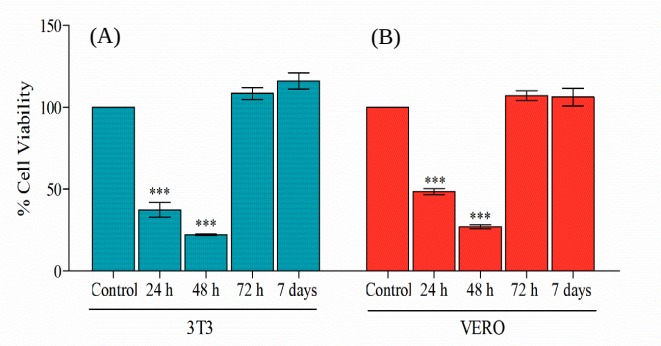

Fig. 2: 3T3 murine fibroblast (A) and VERO (B) cells viability by direct contact with PVA2 mats

To support the biological compatibility observed after 72 h, a proliferative cell profile by the mitotic index was also determined (Fig. 3A e Fig. 3B). A similar MTT pattern was seen, pointing out a firstly negative impact of PVA2 nanofibers on cell proliferation (48h, 3T3 and VERO, $p < 0.05$ vs control). However, this cell behavior could be related to a downregulation on cell-cycle progression (stopped at G0/G1 phases), since quickly after 72 h post-exposure, the frequency of mitotic index in both 3T3 and VERO cells was clearly restored ($p > 0.05$ vs control), up to 7 days exposure.

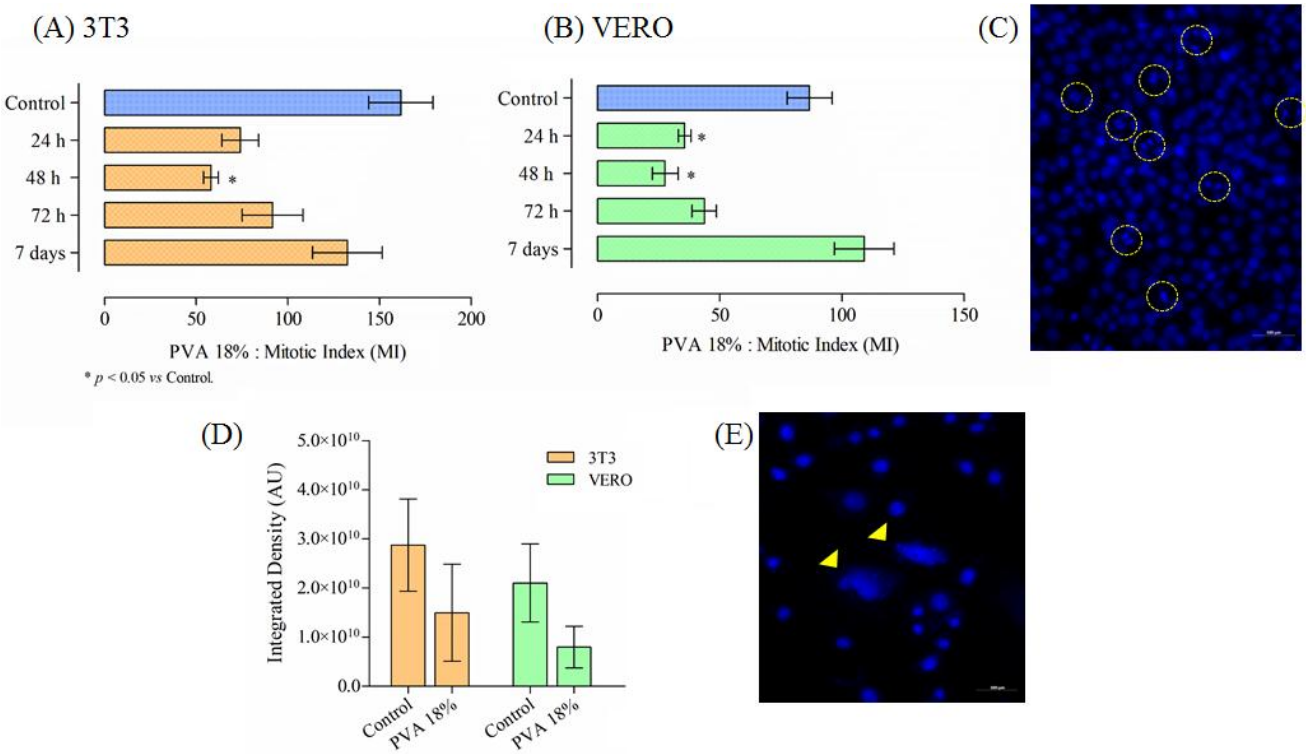

Fig. 3: Mitotic index after PVA2 mats exposure to (A) 3T3 and (B) VERO cells; (C) VERO cells showing mitotic figures; (D) DAPI Integrated density (AU) rates and (E) DAPI image (100 μm scale bar) of VERO cells, after 72 h exposure to PVA2 nanofibers

A representative image of VERO cells denotes mitotic events in Fig. 3C. In parallel to the mitotic index, a study of nuclear morphology was conducted to evaluate the nuclear morphological features related to mechanisms that affect cell survival processes [12]. 3T3 and VERO cells

presented morphological signs of nuclear irregularities (Fig. 3E), with a trend to decrease their integrated density (AU) rates by morphometric analysis (Fig. 3D), even up to 72 h post-PVA2 nanofibers exposure. Because PVA itself is not cytotoxic, we can hypothesize that heating process for water evaporantion may be mildly degrading it to generate low-molecular-weight cytotoxic ketones and aldehydes. Electrospinning of PVA already resulted in small-molecular weight fractions cytotoxic to cells [13]. Non-electrospun PVA solutions were completely non-toxic for cells before electrospinning at all the concentrations tested, but electrospun PVA negatively affected human coronary artery smooth muscle cells (HCASMCs) and human coronary artery endothelial cells (HCAECs). This adverse effect was dependent on the molar mass of the starting polymer. In our study, this initial cytotoxicity was not permanent, being reversible without medium supplementation. The 3T3 and VERO cells regained their metabolism and continued to proliferate after 72 h exposure to PVA2 nanofibers. Future spectrophotometric measurements are needed to understand this initial decrease in cell viability and how it is possible to modulate this effect in favor of the scaffold's desired application.

Conclusions

In this work, blow-spun poly(vinyl alcohol) (PVA) nanofibers were successfully obtained by SBS from aqueous solutions aided by forced water evaporation. The ease of use of SBS increases the possibilities of application in tissue engineering. Another essential requirement for SBS to be a translational therapeutic prototype is that nanofibers are not cytotoxic. When in contact with two cell lines, the nanofibers caused an initial decrease in their biological activity until 48 h exposure, which was recovered afterward. This denoted a favorable microenvironment for regeneration. Subsequent investigations using *ex-vivo* analysis is necessary to elucidate PVA nanofibers' biological behavior and modulate the preliminary results according to the therapeutic model of interest.

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