Tailoring electrospun poly(l-lactic acid) nanofibers as substrates for microfluidic applications

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Abstract: Novel microfluidic substrates based on electrospun poly(l-lactic acid) (PLLA) membranes were developed to increase the limited range of commercially available paper substrates, commonly used for the fabrication of microfluidic paper-based analytical devices (μPADs). PLLA advantageous properties include being biodegradable, biocompatible, easily processed in various tailored morphologies, and cost effective, among others. Oriented and non-oriented electrospun PLLA membranes were fabricated using electrospinning and the influence of fibre orientation, addition of hydrophilic additives and plasma treatments on the morphology, physicochemical properties and capillary flow rate were evaluated and compared with commercial *Whatman* paper. In addition, a proof of concept application based on the colorimetric detection of glucose in printed PLLA and paper-based microfluidic systems was also performed. The results show the potential of PLLA substrates for the fabrication of portable, disposable, eco-friendly and cost-effective microfluidic systems with controllable properties that can be tailored according to specific biotechnological application requirements, being a suitable alternative to conventional paper-based substrates.

Keywords: Microfluidic paper-based analytical devices, poly(l-lactic acid), electroactive polymers, microfluidic, hydrophilicity, capillary flow, electrospun membranes.

1. Introduction

Microfluidic paper-based analytical devices (µPADs) represent a promising platform for the development of fast, portable, disposable and cost-effective analytical tools in clinical diagnostic, environmental monitoring and food safety, among others[1]. This motivation is supported by the potential of µPADs to address the ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Deliverable to end-users) criteria for the development of point-of-care (POCs) devices defined by the World Health Organization (WHO), enabling local communities in developing regions to improve healthcare, environmental safety, animal health and food quality[2]. They were first introduced in 2007 as alternative to silicon, glass and polydimethylsiloxane (PDMS) materials, commonly used for prototyping and proof-of-concept studies[3]. Paper is typically made of cellulose fibres and shows the advantages of being inexpensive, ubiquitous, biocompatible and scalable[4]. In addition, cellulose's hydrophilicity, combined with patterned hydrophobic microfluidic barriers to create channels, generate sample flow via capillary action from an inlet to a defined reaction place for subsequent analysis[5]. Although with extensive academic developments, the entry of µPADs into real-life applications is still very limited[6]. This discrepancy may be related to the limited grade and properties of commercially available papers with low storage stability, poor wet strength, and limited control of fluid flow[7]. In this sense, various strategies have been developed for programmable and analyte manipulation (e.g. filtering, mixing, heating, separation, concentration) by the integration of functional components into µPADs, including diode valves, dissolvable sugar barriers and external electromagnets[8]. Moreover, there have been attempts to control the flow by tailoring the channels design, guiding the fluid to transport vertically and laterally through paper sheets and using

materials such as threads and yarns[9]. However, the reported strategies have limitation for rapid and easy production.

Thus, as an attempt to address the limitations with the existing µPADs, alternative microfluidic substrates to complement paper have been developed in the present work. Poly(l-lactic acid) (PLLA), a aliphatic semi-crystalline polyester, constitutes a promising candidate for this purpose. In fact, PLLA, which is a FDA approved biomaterial, has attracted wide interests, from academic research to industry, due to its biocompatibility and biodegradability, as well as environmental friendliness and low-cost[10]. In addition, PLLA is also piezoelectric, allowing to convert mechanical energy into electrical and vice versa, which has been successfully used in tissue engineering and related biomedical applications[11]. A number of methods have been reported for the production of different types of PLLA structures such as films[12], membranes[13], fibres[14] and spheres[15], among others. Fibrous membranes are particularly interesting for microfluidic applications based on the similar morphology with commercial *Whatman* cellulose filter paper grades, commonly used for the manufacture of µPADs[16]. They have been produced by a variety of methods such as template synthesis, self-assembly, phase separation, wet spinning, microfluidic spinning and electrospinning[17]. Within these techniques, electrospinning is currently the only one that allows the simple and reproducible fabrication of functional fibres to be applied in microfluidics[18] with diameters from few nanometres to several micrometres by applying a high electrical field to a droplet of polymer or composite solution from natural or synthetic polymers[18-19]. By properly controlling the composition of the polymer-based solution and processing parameters, a stable process can be achieved allowing to obtain structures ranging from single fibre to ordered arrangement of fibres with tailored fibre dimension and orientation[20]. The major drawback of PLLA-based electrospun membranes is associated to its inherent hydrophobicity, poor

wettability and low surface energy that must be overcome so that it can be used as substrate, similar to paper, for the fabrication of microfluidic systems. Various strategies can be used to modify the surface properties and thus tailor the polymer wettability, such as chemical grafting, self-assembly and surface hydrolysis[21]. Another reported strategy involves the integration of specific fillers (such as polyethylene glycol[22], hydrophilic zeolites[23], superhydrophilic metal-organic frameworks[24], wet-chemistry treatment[25], among others) on the polymer matrix in order to reduce the hydrophobic behaviour of polymers[26].

Nevertheless, plasma treatment is one of the most extensively used methods to tailor surface adhesion and wetting properties, by the insertion of chemically reactive functional groups on the polymer surface, modifying its composition without affecting their bulk characteristics. A proper selection of plasma atmosphere (oxygen O_2, argon Ar, hydrogen H_2, or nitrogen N_2, among others), power and time is the key to ensure the success of the plasma treatment[27].This approach has been used to promote surface modification in PLLA[28]. Thus, the surface modification of melt-extruded sheets of PLLA under O_2, H_2 and N_2 plasmas has been investigated, and the obtained results demonstrated a significant increase of the surface wetting, by the incorporation of polar groups composed of carboxylic (–COOH) and hydroxyl (–OH), along with a pronounced modification of its morphology, which depends much on the type of plasma[29]. Poly(glycolic acid) (PGA), poly(lactic-co-glycolic acid) (PLGA) and PLLA have been processed by electrospinning to obtain nanofibrous membranes, followed by a post-treatment by O_2 plasma and *in situ* grafting of hydrophilic acrylic acid (AA), which results in higher ratios of oxygen to carbon, lower contact angles and the presence of –COOH groups[30]. The influence of Ar-plasma treatments on flat rigid PLLA substrates, obtained by melting of PLLA powder on glass slide[31], shows that different times of this type of plasma treatment allows to

achieve controlled water contact angles, down to the superhydrophilic regime. Similarly, the suitability of carbon dioxide (CO_2) plasma treatment has been proved to decrease the hydrophobicity of PLLA surface, by the introduction of O_2-containing functional groups[32]. The hydrophobicity of PLLA has been also reduced by helium (He) atmospheric pressure plasma treatment[28a], Ar and O_2 plasma treatments[33]. It is to notice that most of the aforementioned studies have been tested and applied, mostly on tissue engineering applications, in order to tailor cell-biomaterial interfaces. On the other hand, only few studies report on the stability of plasma treatment over time. To the best of our knowledge, there is no work reporting on the processing of PLLA-based materials with stable superhydrophilic behavior, to be used as substrate, instead of paper, for the fabrication of microfluidic systems.

In this work, the effect of hydrophilic additives (NaY zeolites) and plasma treatments (using O_2 and Ar atmospheres) on the surface wettability and others relevant physicochemical properties of electrospun PLLA-based membranes are investigated. Randomly oriented electrospun PLLA-based membranes were first produced in an attempt to mimic the structure of commercial *Whatman no.1* papers, commonly used in the fabrication of μPADs. Oriented electrospun PLLA membranes were also manufactured to study the effect of fibres orientation. Thus, microfluidic substrates based on biodegradable and biocompatible PLLA material with superhydrophilic behaviour, high storage stability, high wet strength and controlled fluid flow are obtained.

2. Experimental procedures

2.1. Materials

PLLA with an average molecular weight of 217.000-225.000 $g.mol^{-1}$ (*Purasorb PL18*) and NaY zeolites (*CBV 100*: SiO_2/Al_2O_3 mole ratio: 2.83; nominal cation form: sodium;

Na_2O weight: 13 %; unit cell size: 24.65 Å; surface area: 900 $m^2.g^{-1}$) were supplied by *Purac* and *Zeolyste International*, respectively. N,N-dimethylformamide (DMF) and dichloromethane (DCM) were obtained from *Merck* and *Sigma-Aldrich*, respectively. *Whatman* qualitative filter paper, grade 1, was purchased from *Sigma-Aldrich*. All chemicals and solvents were used as received.

2.2. Sample preparation

2.2.1. Randomly oriented electrospun substrates

Randomly oriented electrospun PLLA-based membranes were produced in an attempt to mimic the structure of commercial *Whatman no.1* papers, commonly used in the fabrication of µPADs, whose microstructure consist on flat randomly oriented fibres. A 10 wt% solution of PLLA in 3/7 vol/vol DMF/DMC mixture was prepared under magnetic stirring (*IKA C-MAG HS7*) at room temperature, until complete dissolution of the polymer. In the preparation of polymer solutions with zeolites, NaY was previously dispersed in the solvents under ultrasound bath for 1 h, followed by the addition and dissolution of PLLA by magnetic stirring. NaY percentages of 0, 5, 10 and 20 % relative to the polymer mass were studied. Then, the polymer solution was placed in a 10 mL plastic syringe fitted with a steel needle with inner diameter of 500 µm and introduced into a syringe pump (*Syringepump*). The electrospinning procedure was conducted at 17 kV by means of a high voltage power supply (*Glassman PS/FC30P04*) at a solution feed rate of 0.5 $mL.h^{-1}$. Finally, randomly oriented electrospun membranes were collected on a grounded 20 cm x 15 cm static plate collector placed 15 cm away from the needle.

2.2.2. Oriented electrospun substrates

In addition, oriented electrospun PLLA-based membranes were processed to increase the range of microfluidic substrate structures, as well as to study the influence of fibre orientation on the capillary flow rate. The sample preparation is similar to the one described in section 2.2.1, except for the use of a rotating collector. Thus, oriented PLLA-based fibres were produced using a grounded rotating drum collector at a velocity of 1500 rpm. In this case, the addition of NaY zeolites was also performed.

2.3. Surface modification

As previously exposed, a limitation of PLLA comparatively to cellulose-based microfluidic substrates arises from their high hydrophobicity. Plasma treatments were performed, which is a technique commonly used to alter the hydrophobicity of polymers[34]. The success of a plasma treatment is associated with the selection of the plasma atmosphere (O_2, Ar, N_2, among others), treatment time and applied power. Oxygen is one of the most reactive elements and can generate carboxyl groups on the surface of polymers due to the incorporation of hydrophilic functional groups. Argon, in turn, tends to lead to relevant changes in surface morphology[21a, 27c].

Surface treatments were conducted in a plasma chamber (*Zepto, Diener Electronics*), equipped with a 40 kHz radio frequency plasma generator. The base pressure, before plasma ignition, was 20 Pa. Plasma treatments were performed independently with O_2 and Ar as working gases, for 10 min. A plasma power of 100 W under a total pressure of 80 Pa was applied. This procedure was performed on both surfaces of the processed PLLA-based substrates.

2.4. Samples characterization

2.4.1. Physicochemical characterization

A scanning electron microscope (SEM) *Quanta 650 FEG* from *FEI* was employed to characterize the morphology of the electrospun PLLA-based membranes. The samples were previously coated with a thin gold layer using a sputter coater *Polaron SC502*. The mean fibre diameter and corresponding standard deviation were calculated measuring approximately 50 fibres by means of *ImageJ* software.

Surface wettability of the samples was evaluated by measuring the contact angle of 3 µL ultrapure water drop using a contact angle analyser *Data-Physics OCA20*. Six measurements were carried out in each membrane at different locations, the contact angles being reported as the average and standard deviation.

Fourier transformed infrared spectroscopy in attenuated total reflectance mode (FTIR-ATR) measurements were performed at room temperature using a *Spectrum Two*™ from *Perkin-Elmer*, with 64 scans in the range between 400 and 4000 cm^{-1} and a resolution of 4 cm^{-1}.

Differential scanning calorimetry (DSC) was performed with a *DSC 6000* from *Perkin-Elmer*. Pieces of approximately 6 mg were cut and placed into 40 µL aluminium pans. The samples were heated between 30 and 200 ºC at a scanning rate of 10 $ºC.min.^{-1}$. The degree of crystallinity (ΔX_C) was calculated using the following equation $\Delta X_C=(\Delta H/\Delta H_m^0)\times 100$, where ΔH is the area under the thermogram between 65 and 160 ºC and ΔH_m^0 is the melting enthalpy for fully crystallized PLLA samples (93.1 $J.g^{-1}$)[35].

The mechanical properties were studied by means of a Shimadzu AD-IS with a load cell of 50 N. Samples with length and width of 15 and 10 mm, respectively, were prepared and stretched at a rate of 1 $mm.min^{-1}$. The oriented fibres were evaluated along the fiber direction. The thickness of the samples ranged between ~30 and ~100 µm. The essays

were performed on dry and wet samples using in this last case 40 µL of water. Three tests were performed for each sample.

2.4.2. Cytotoxicity assay

Indirect cytotoxicity evaluation of the processed electrospun PLLA-based membranes was performed adapting the ISO 10993-5 standard test method. MC3T3-E1 pre-osteoblast cells (Riken cell bank, Japan) were cultured in 75 cm^2 cell culture flask at 37 ºC, 5 % CO_2, in humidified environment using Dulbecco's modified Eagle's medium (DMEM, *Gibco*) containing 1 $g.L^{-1}$ glucose, 10 % fetal bovine serum (FBS, *Biochrom*) and 1 % (v/v) penicillin/streptomycin solution (P/S, *Biochrom*). Sterilization of the samples (13 mm diameter) were carried out by multiple immersion into 70 % ethanol for 30 min each, washing with sterile phosphate-buffered saline solution (PBS; 137 mM NaCl; 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 at pH 7.4) for 5 min and exposition to ultraviolet radiation for 1 h each side. Thus, a suspension of 2×10^4 $cell.mL^{-1}$ was seeded in 96-well tissue culture polystyrene plates and incubated for 24 h at 37 ºC with 5 % CO_2 in humidified environment to ensure cell attachment on the plate. Simultaneously, each sample was incubated for 24 h in 24-well tissue culture polystyrene plate under the conditions described above. After the incubation time, the cell culture medium in the 96-well plates was removed and 100 µL of culture medium that was in contact with the different samples was added to each well, individually. Cell viability was then evaluated after 72 h of incubation using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay according to the manufacturer's instructions (*Sigma-Aldrich*). Briefly, the medium of every well was removed and fresh medium containing MTT solution (5 $mg.mL^{-1}$ of MTT dissolved in DMEM in a 1:10 ratio) was added to the cells and incubated for 2 h at 37 ºC in the dark. Thus, the MTT

solution was removed and the precipitated formazan was dissolved with 100 µL dimethyl sulfoxide (DMSO)/well followed by measuring the optical density at 570 nm. Cells cultured in 20 % DMSO (*Sigma-Aldrich*) and standard culture medium were used as negative and positive controls, respectively. All quantitative results were obtained from four replicate samples and controls and presented as the average of viability ± standard deviation. The percentage of cell viability was calculated according to the following equation

Cell viability (%)=(Absorbance of sample/Absorbance of negative control)×100 [36].

2.4.3. Capillary flow rate tests

Capillary flow rate represents the speed at which a liquid sample moves along a membrane strip in a diagnostic test strip or application. A proper control of this flow will enable to select the appropriate membrane for a specific application[37]. Thus, capillary tests were performed on all samples without and with plasma treatments. Strips with dimensions of 2.5 cm length and 1 cm width were cut and placed vertically in contact with a water solution containing food colouring. The sample portion immersed in the solution was 0.5 cm and the time taken for the dye to travel across the sample in the "antigravity" direction was measured. These tests allow to evaluate the effect of the fibre orientation, presence of zeolites and plasma treatments with Ar and O_2, in the capillary flow rate and ultimately on the ability of the processed materials to generate passive flows.

2.4.4. Proof-of-concept

Glucose essays based on colorimetric detection were performed using a glucose kit from *BioLabo Reagents*^{TM}. During the reaction, glucose is oxidised by the enzyme glucose

oxidase to gluconic and hydrogen peroxide, which in conjunction with peroxidase, reacts with chloro-4-phenol and 4-amino-antipyrine to form a red quinoneimine. The intensity of the coloured complex is proportional to the concentration of glucose. As a simple proof-of-concept, calibration curves were built using the following glucose concentrations: 10, 25, 75, 150 and 500 $mg.dL^{-1}$, for both commercial *Whatman no.1* paper and non-oriented electrospun PLLA membranes with 10 % NaY (as representative example). This range of concentrations was selected according to the datasheet, comprising very low concentrations of glucose, allusive to hypoglycaemia, to values corresponding to hyperglycaemia. A microfluidic system was designed using a computer aided design software and printed on both substrates using a *Xerox ColorQube 8580* printer, as illustrated in Figure 1. After printing, the samples were placed on a heating plate at 60 ºC during 10 min so that the wax penetrates the entire cross-section of the membrane and thus creating hydrophobic barriers that will contain the fluid. This temperature is lower than the commonly used in paper (100 ºC for 2 min) to maintain the integrity of the PLLA samples that undergo mechanical deformations when subjected to higher temperatures.

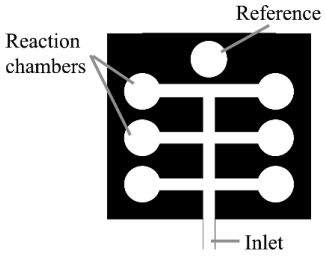


Figure 1: Microfluidic system design used for the quantification of glucose. Each reaction chambers and reference were first functionalized with reagent. The glucose oxidase flow by capillarity from the inlet to the reaction chamber to form a red colour with intensity proportional to the concentration of glucose. The reaction chamber features an area of ~80 mm^2 and the channels a width of 1.2 mm.

Each microfluidic system comprises six reaction chambers for the reproducibility of the study. A total a five microfluidic systems corresponding to the five studied glucose

concentrations were printed in each substrate. The reagent chambers were first functionalized using 15 µL of reagent. After approximately 5 min, the inlet of the microfluidic system was introduced in the glucose solution under study, reaching the reaction chamber by capillarity. After 10 min of reaction, a red colour with intensity proportional to the concentration of the glucose was formed. Image analysis was performed by using *ImageJ* software in each microfluidic system. For each reaction chamber, the grey value and corresponding standard deviation were measured and the calibration curve built.

3. Experimental Results and Discussion

Microfluidic substrates based on electrospun PLLA-based membranes were processed as alternative to paper filters, such as the *Whatman* cellulose filter paper grades, commonly used for the fabrication of µPADs[16]. The effect of PLLA fibre orientation, introduction of NaY zeolites in the polymer matrix and plasma treatments with Ar and O_2 on the morphology, physicochemical properties and capillary flow rate were properly studied and compared with commercial *Whatman no.1*.

3.1. Physicochemical characterization

Representative SEM images of commercial *Whatman no.1* and non-oriented and oriented electrospun PLLA-based membranes are shown in Figure 2. The respective mean fibre diameter and standard deviation are presented in Figure 3.

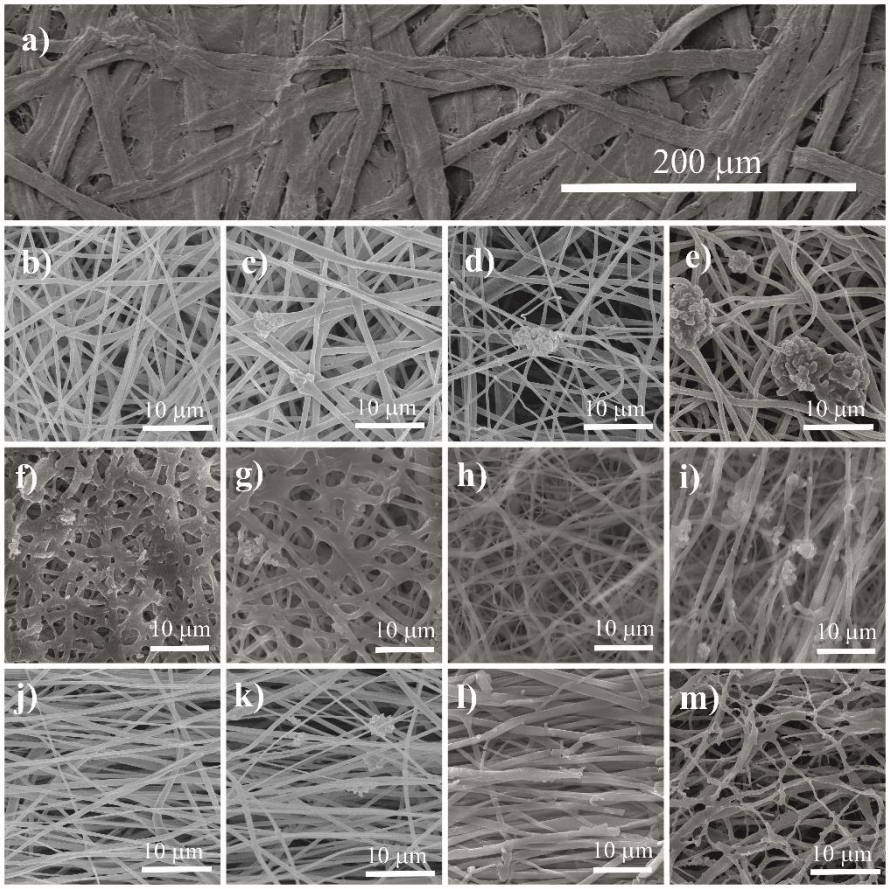


Figure 2: Representative SEM images of a) commercial *Whatman no.1*; non-oriented PLLA fibres with NaY filler content of b) 0 %, c) 5 %, d) 10 %, e) 20 %, f) 0 % and Ar plasma treatment, g) 20 % and Ar plasma treatment, h) 0 % and O_2 plasma treatment, i) 20 % and O_2 plasma treatment; oriented PLLA fibres with NaY filler content of j) 0 %, k) 20 %, l) 0 % and Ar plasma treatment, m) 0 % and O_2 plasma treatment.

Whatman no.1 membranes consist on cellulose fibres with a flat microstructure associated with the pressing process generally employed in the manufacture of paper (Figure 2a). The fibres show sizes in the micrometre scale, with average width of ~16±7 µm. In turn, untreated electrospun PLLA-based membranes are characterized by finer and rounded fibres with smooth surface, spatially distributed both longitudinally and transversally (Figures 2b-e and Figures 2j-k). The dimension, orientation and distribution of the fibres are not affected by the introduction of zeolites, whose presence is confirmed by some clusters in the corresponding SEM images.

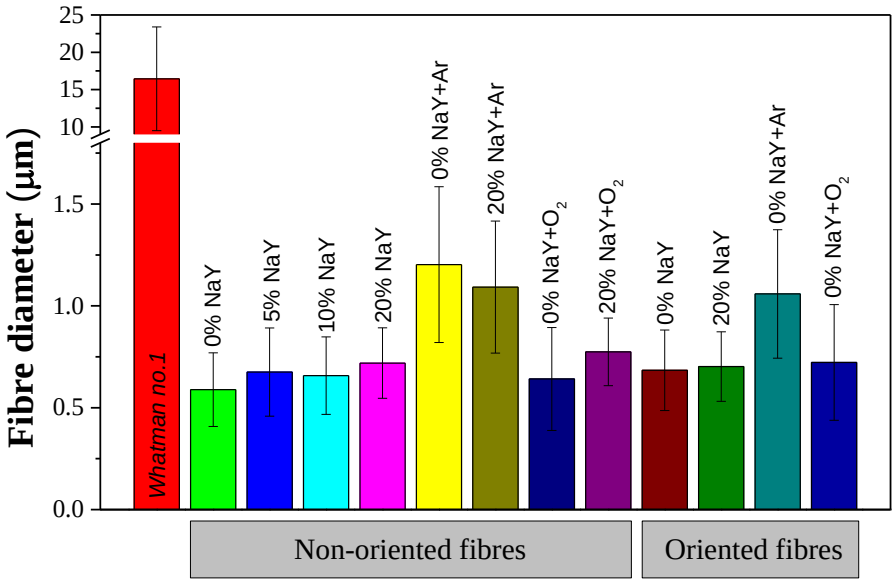


Figure 3: Fibre diameter of commercial *Whatman no.1* and representative electrospun PLLA-based membranes.

Regardless the orientation of the fibres or presence of zeolites, fibres with average diameter of 650±200 nm are obtained. After plasma treatments, important fibre modifications were observed, being more evident using Ar as working gas. This result is consistent with the literature[21a, 33]. Surface modification by plasma treatment can cause the cleavage of polymer molecular chains promoting, therefore, the formation of free radicals that activate the surface[13]. While O_2 is one of the most reactive elements and can generate carboxyl group on the polymer surface due to the incorporation of hydrophilic functional groups, Ar typically leads to relevant changes in surface morphology[13, 21a]. Thus, it is observed that the diameter of the PLLA fibres remains approximately the same after O_2 plasma treatment, whereas an increase of this value occurs when Ar plasma treatment is performed, showing flat fibres with average surface width of 1.10±0.40 µm. In addition, Ar plasma treatment leads to the fusion of non-oriented fibres. On the other hand, Ar plasma treated PLLA oriented fibres also show significant morphological

variations, but the fibres remain intact and with defined orientation. After O_2 plasma treatment, PLLA fibres show some fusion between them and the orientation appears to be compromised in the case of initially oriented fibres. Surface polymer melting associated with the high power (100 W) and with the high exposure time (10 min) used during plasma treatment in electrospun PLLA-based membranes surfaces are at the origin of these behaviours. Although previous studies showed that shorter exposure times (lower than 1 min) can be used to generate superhydrophilic electrospun PLLA membranes, some alterations of the polymer fibrillar matrix were also observed[28b, 30, 33]. More importantly, the stability of the superhydrophilic surface oven time, which is a key parameter in the scope of the present work and applications, has not been demonstrated under the softer plasma treatment conditions used in these studies.

Figure 4a shows the contact angle measurement results of the commercial *Whatman no.1* and non-oriented and oriented electrospun PLLA-based membranes, whose values remain stable for at least 1 month.

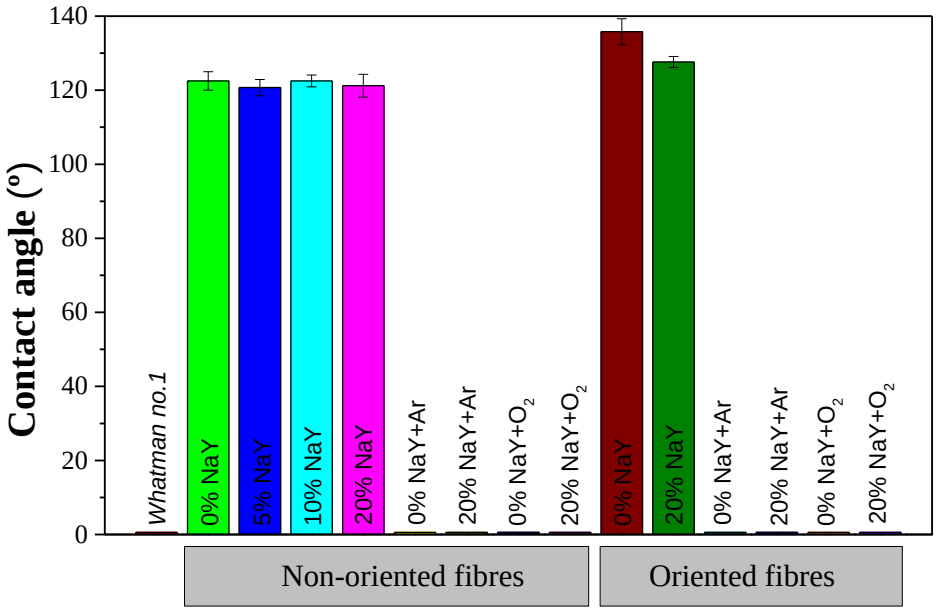


Figure 4: Contact angles of commercial *Whatman no.1* and electrospun PLLA-based membranes, whose values remain stable for at least 1 month.

As previously stated, the major drawback of PLLA membranes comparatively to cellulose membranes for microfluidic applications is associated to their strong hydrophobicity that must be overcome to allow the generation of capillary flows, which cause the fluid to move through the membrane without the need for external actuation systems. Thus, the proper characterization of this parameter is of major relevance in the scope of this work. Surface wettability is governed by surface chemistry, surface energy and morphology[38] as well as by the properties of the liquid[39].

A simple and effective way to express wetting properties of a surface is by the contact angle (CA) of a droplet resting on a surface. A surface is generally called hydrophobic when its contact angle is higher than 90º, whereas a hydrophilic surface features a contact angle lower than 90º. Superhydrophobic and superhydrophilic are terms also used to characterize surfaces that present contact angles higher than 150º and lower than 10º, respectively[38-39]. While in flat and dense superhydrophilic surface it is expected that the

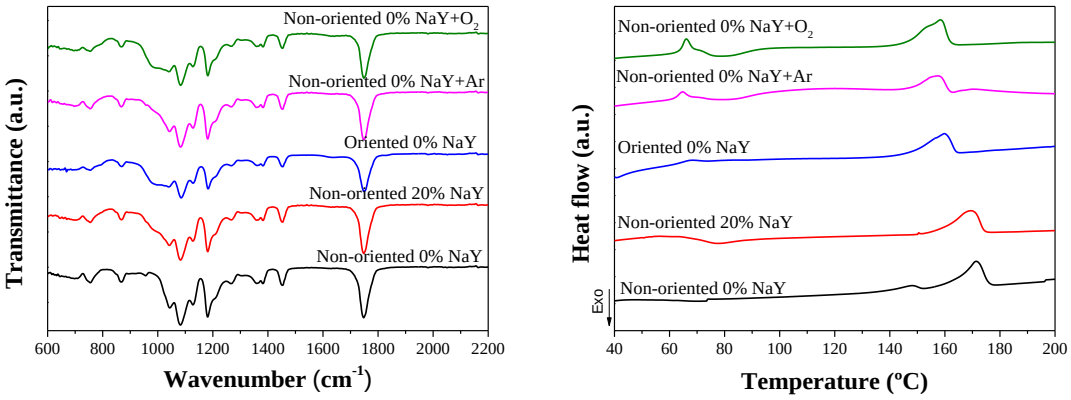
water spreads completely as a flat film, in the case of porous films, i.e. membrane, it is expected that the water is absorbed through the material.

Figure 4 shows that *Whatman no.1* membranes express a superhydrophilic behaviour with the water droplets being absorbed almost instantly, giving them suitable properties as substrate for microfluidic systems. In turn, the water surface contact angles of untreated electrospun PLLA-based membranes is 121.7±0.8º for non-oriented fibres and 131.7±4.1º for oriented fibres, demonstrating the strong hydrophobicity associated to PLLA[28b]. The addition of zeolites between 0 and 20 % has no significant effect on the surface wettability of these samples, remaining hydrophobic. In this study, the Y zeolite (faujasite structure) on the sodium form (NaY) has been used because of its hydrophilic properties, related with its low Si/Al ratio[40]. Accordingly, a decrease in the contact angle with the addition of zeolites would be expected, which was not found, leading to the conclusion that the filler is wrapped by a polymer thin layer that does not allow the interaction between zeolite and water.

On the other hand, the post-treatment of electrospun PLLA-based membranes with both Ar and O_2 plasma gases leads to a strong reduction of the contact angle (Figure 4), preventing its measurement due to the immediate and total absorption of water droplets. This is justified by the increase of the surface energy after plasma treatment, resulting in a reduction of the contact angle. Thus, plasma treated electrospun membranes exhibit a paper-like superhydrophilic behaviour, suitable for microfluidic applications, with proven stability over 1 month, independently of the fibre orientation and presence of zeolites. This stability can be explained by the fact that after O_2 and Ar plasma treatments, stable physico-chemical fiber modifications are induced, as previously shown, and indicated, among others, by the slight decrease in the amount of carbon atoms and increase in the oxygen content[33]. The increase in oxygen content is more noticeable for

PLLA fibers after O_2 exposure.Although the addition of zeolites has no significant effect on the wettability of the PLLA membranes, their presence will have a quantifiable effect on capillary flow tests, in the samples treated with plasma, as will be discussed in section 3.3.

FTIR-ATR measurements were carried out to study possible chemical variations in the PLLA polymer chain of the electropun membranes. In addition, DSC measurements were conducted to evaluate possible changes on the thermal behaviour of the PLLA samples, in particular regarding the glass transition temperature that will set the higher temperature at which wax-based hydrophobic microfluidic channels can be printed and processed without deterioration of the structural properties of PLLA substrate. FTIR-ATR spectra and DSC curves of representative PLLA-based membranes are illustrated in Figures 5a and 5b.



a) b)

Figure 5: a) FTIR-ATR spectra and b) DSC curves of representative electrospun PLLA-based membranes.

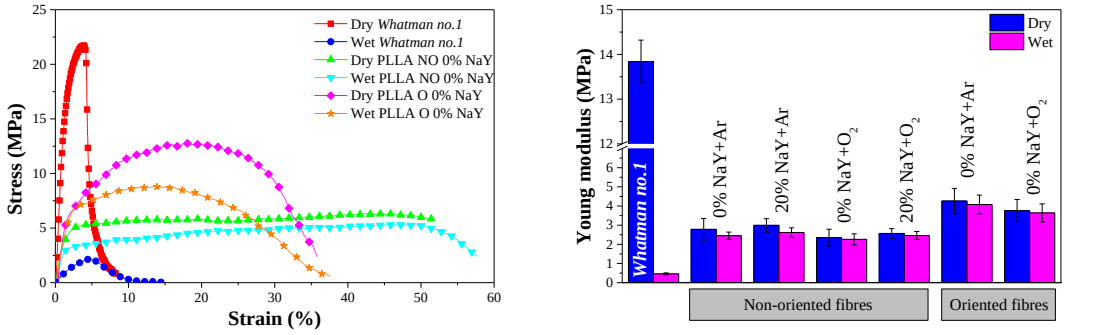
No noticeable variations are observed on the FTIR-ATR spectra of the electrospun PLLA-based membranes varying the processing conditions, namely in terms of orientation of the fibres, addition of zeolites, post-treatment with Ar or O_2 plasma, or combination of

them. All spectra feature characteristic absorption bands at 755 and 870 cm^{-1}, related to CH_3 stretching and rocking, respectively[41]. The band at 1044 cm^{-1} is assigned to the stretching vibration of the C-CH_3 bond, while 1130, 1357 and 1450 cm^{-1} are attributed to $r_s(CH_3)$, δ(CH) and δ_{as}(CH) stretch, respectively. In turn, the absorption bands at 1083, 1185, 1271 and 1747 cm^{-1} are ascribed to ν_s(C-O-C), ν_{as}(C-O-C)+r_{as}(CH3), ν(CH)+ν(C-O-C) and ν(C=0), which is characteristic of the presence of O_2.

Regarding the thermal characterization, it is also shown that the processing conditions do not significantly influence the thermograms of the samples. All DSC curves are characterized by two endothermic peaks at ~65 ºC that correspond to the glass transition and a melting peak between 140 and 160 ºC. A slight variation in the dynamic of the glass transition seems to occur in the PLLA samples treated with plasma, where the endothermic peak appears more evident. As previously stated, this temperature is relevant as it limits the temperature at which these substrates can be used without deterioration of their properties. This is not a concern for their use as microfluidic substrates for biomedical applications, where such high temperatures are hardly reached. However, the glass transition temperature should be considered during the fabrication of microfluidic systems when, for instance, wax printing is used. In this case, a post heating must be carried out to ensure a proper melting of the printed hydrophobic barriers to occupy the entire cross section of the microfluidic substrate, confining, therefore, the fluids. In addition, a broad exothermic cold crystallization peak occurs approximately above the glass transition, at ~85 ºC. This behaviour is related to the formation of a well-defined crystal structure above the glass transition as a result of the ordering of the PLLA polymer chains in a regular array[35]. The electrospinning process leads to numerous crystal nuclei present in the polymer as a result of non-equilibrium chain conformations that crystallize in the heating process above the glass transition.

The degree of crystallinity of the PLLA samples remains approximately constant regardless of processing conditions, with a value of ~30 %.

Membranes with proper mechanical properties are important for their use as microfluidic substrates, in particular when wetted, during functionalization and/or immersion in a solution containing the (bio)molecules to be quantified. Thus, strain-stress measurements were performed on dry and wet commercial *Whatman no.1* and representative electrospun PLLA-based membranes. The characteristic stress-strain curves are presented in Figure 6a. From the linear regime of the curves, the Young's modulus was obtained by applying Hooke's law and presented in Figure 6b.



(a) (b)

Figure 6: a) Characteristic stress-strain curves and b) Young's modulus of commercial *Whatman no.1* and representative electrospun PLLA-based membranes in dry and wet state.

Ductile materials are characterized by a linear elastic regime, followed by the yielding region, where the material enters in the plastic regime and suffers permanent deformation to any further increase in load or stress. By further stretching the samples, the rupture region is reached. In turn, brittle materials usually present little or no plastic deformation, fracturing near the end of the linear-elastic region of the curve. The mechanical response of the commercial *Whatman no.1* paper features almost no plastic regime in both dry and wet state, contrary to the electrospun PLLA-based membranes. In all tested substrates,

wet membranes show lower mechanical properties. In fact, less stress is required to deform wet membrane, being more significant for commercial *Whatman no.1* papers. By analysing in more detail the Young's modulus obtained from the linear regime, where materials are generally used, it is concluded that dry commercial *Whatman no.1* presents a more rigid behaviour with the highest Young's modulus of ~13.8±0.5 MPa, decreasing significantly to ~0.5±0.1 MPa when wet. In turn, electrospun PLLA-based membranes are more stable, nearly maintaining their elastic behaviour, with Young's modulus only undergoing a slight reduction when the membranes are wet. An average Young's modulus of ~2.6±0.3 MPa was obtained for the non-oriented electrospun PLLA-based membranes, increasing to ~3.9±0.5 MPa with the orientation of the PLLA fibres. No significant variation were observed with the addition of zeolites and plasma treatments.

3.2. Cytotoxicity essay

The absence of cytotoxicity is a key issue during the development and further use of materials for biomedical applications. Thus, cell viability of PLLA samples was assessed by indirect contact using MC3T3-E1 pre-osteoblast cell line. The results are presented in Figure 7.

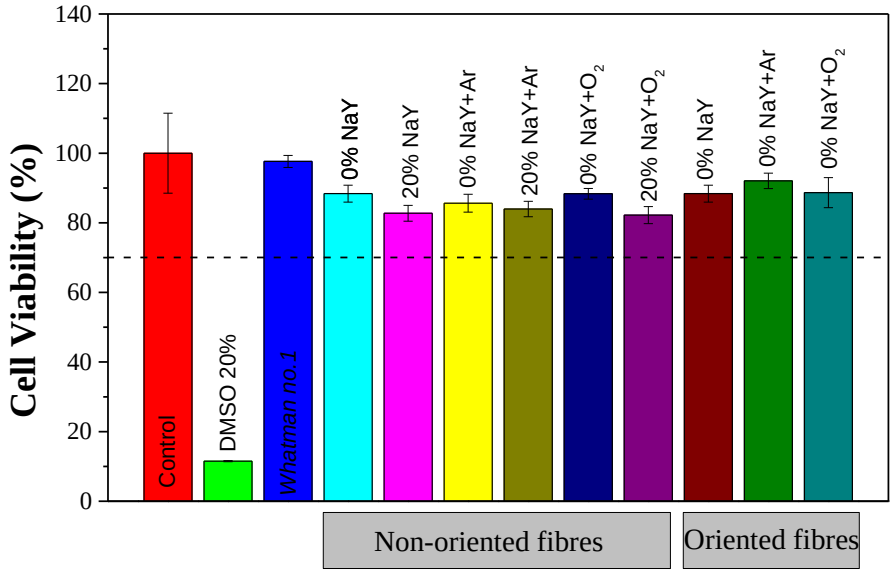


Figure 7: Cytotoxicity assay of MC3T3-E1 pre-osteoblast cells in contact with the as-prepared extraction media exposed to commercial *Whatman no.1* and representative electrospun PLLA-based membranes for 72 h (relative cell viability was presented as the percentage of the negative control (n=4±SD)).

It is demonstrated that the processed samples do not show cytotoxic effect, with cell viability values higher than 70 %, which is the threshold accepted according to the ISO standard 10993-5. The addition of zeolites and plasma treatments with Ar and O_2 has no significant effect on the toxicity of the material. The same behaviour is presented by the commercial *Whatman no.1*. Thus, it is proved that the processed samples can be used for biomedical applications.

3.3. Capillary flow rate tests

Capillary flow tests were performed in order to validate the possible use of the processed PLLA substrates in the development of microfluidic systems, where passive flows must be generated. As previously stated, thin strips of each sample were cut and dipped vertically and partially into a water solution containing food colouring. The results

presented in Figure 8 were obtained calculating the time required for the dye to travel through the strip with a length of 2 cm (plus 0.5 cm dipped in solution).

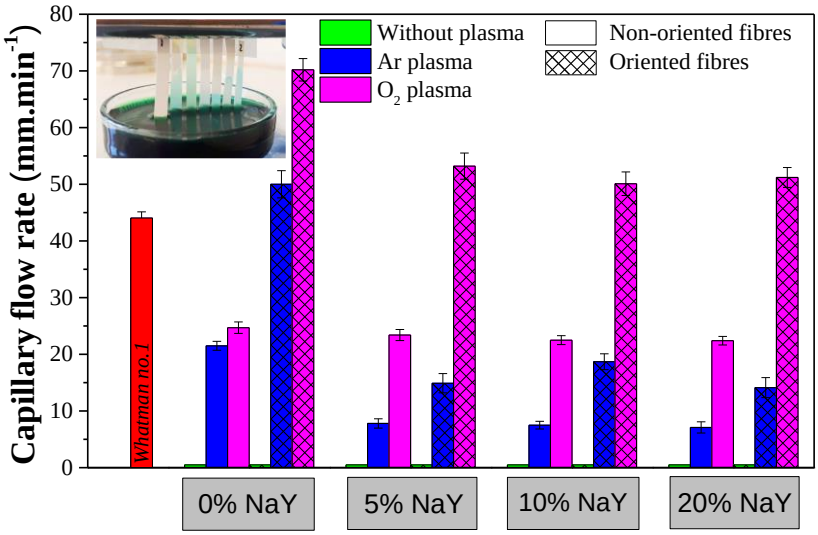


Figure 8: Capillary flow rates in the "antigravity" direction of dye solution in commercial *Whatman no.1* and electrospun PLLA-based membranes strips.

The results demonstrate that the hydrophobic nature of the PLLA membranes, without plasma treatments, has a negative effect on the capillary flow rate, presenting no flow, regardless of zeolite concentration. In turn, because of its superhydrophilic behaviour, the plasma treated PLLA samples show capillary flow rates varying from 7.1±0.9 to 70.2±1.9 $mm.min^{-1}$, being the effect superior in samples treated with O_2 plasma. An interesting observation comes from the treated samples with zeolites, with capillary flow rates that tend to decrease with the presence of this additive, as demonstrated by comparing the samples with 0% and 5% filler content. This result is explained by the high adsorption ability and the microporous structure of NaY zeolites that will retain H_2O molecules, leading to a consequent decrease in flow rate. Further, similar flow rates are observed for zeolite concentrations above 5%, indicating that a saturation is reached with respect to the effect of the zeolite content and corresponding water adsorption in the flow

rate.. Thus, although the presence of zeolites does not have the ability to change the surface hydrophobicity of the PLLA membranes by themselves, they demonstrate to have a relevant effect on the passively generated flow rate after plasma exposure. Another important conclusion is the capillary flow rate that increases significantly with the orientation of the fibres. Maximum values of 50±2.4 $mm.min^{-1}$ and 70.2±1.9 $mm.min^{-1}$ were obtained for the oriented PLLA fibres without zeolites and treated with Ar and O_2 plasma, respectively, comparatively to 21.5±0.8 $mm.min^{-1}$ and 24.7±1.0 $mm.min^{-1}$ obtained for the non-oriented PLLA fibres also without zeolites and treated with Ar and O_2 plasma, respectively. The higher fibre density and preferential orientation in the oriented PLLA samples compared to the non-oriented, as previously exposed in Figure 2, can justify this result by allowing the dye to flow more easily, and consequently faster along the fibres of the PLLA strip. The lowest capillary flow rates were observed in the PLLA membranes constituted by non-oriented fibres with 20 % of zeolites and treated with Ar and O_2 plasma, with values of 7.1±0.9 $mm.min^{-1}$ and 22.4±0.8 $mm.min^{-1}$, respectively. In comparison, commercial *Whatman no.1* features a capillary flow rate of 44±1.13 $mm.min^{-1}$. These results prove the ability of the processed PLLA substrates to generate passive flows. Controlling properly the processing parameters, namely in terms of PLLA fibres orientation, zeolites concentration and plasma treatment with Ar or O_2, it is possible to adjust the capillary flow rate according to a specific application that require controlled flow times and thus controllable reaction times, for example.

3.4. Proof-of-concept

Colorimetric essays based on the detection and quantification of glucose were evaluated on commercial *Whatman no.1* and non-oriented PLLA membranes with 10 % NaY (as representative example) to demonstrate the viability and potential of the processed

substrates for the fabrication of microfluidic systems in specific applications. Digital images of the glucose essays on the printed microfluidic systems are illustrated in Figure 9 and the corresponding calibration curves are presented in Figure 10. In this case, mean grey values are presented as a function of the logarithm of the glucose concentration ranging from 10 to 500 mg.dL^{-1}.

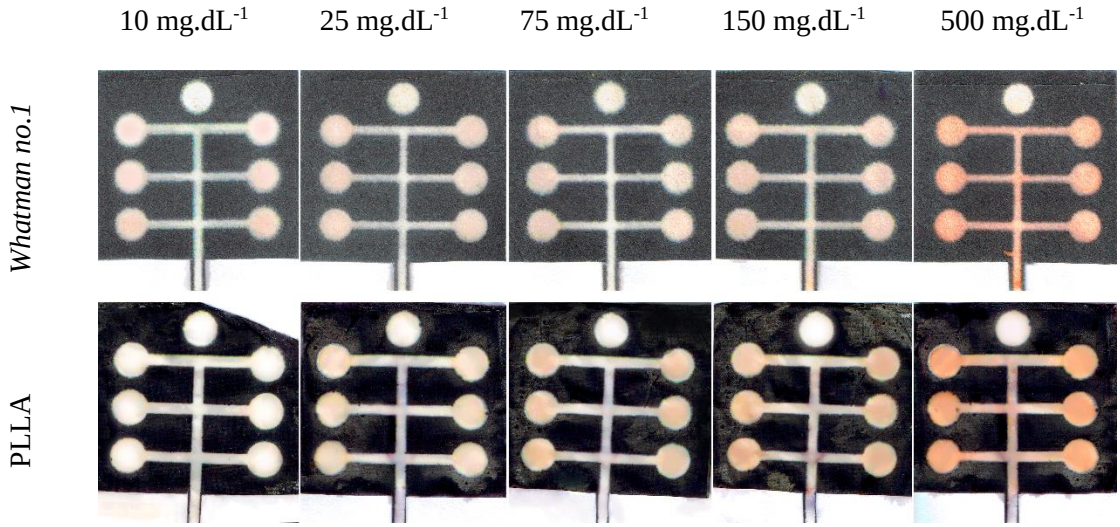
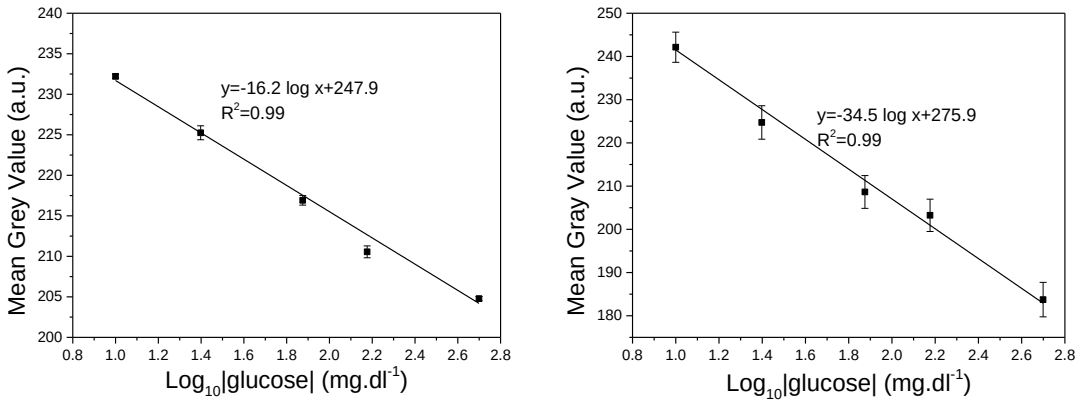


Figure 9: Digital images of microfluidic systems after glucose essays on commercial *Whatman no.1* paper and non-oriented electrospun PLLA membranes with 10 % NaY (as representative example). From left to the right: glucose concentration of 10, 25, 75, 150 and 500 mg.dL^{-1}.



a) b)

Figure 10: Calibration curves of glucose for a) commercial *Whatman no.1* and b) non-oriented electrospun PLLA membranes with 10 % NaY (as representative example). The results are presented as mean grey value and corresponding standard deviation measured on the reaction

chambers of the microfluidic substrates using *ImageJ* software according to the method described in [41].

Analysing first the print quality of the microfluidic systems on both substrates, it is concluded that the design loses some resolution in commercial *Whatman no.1* paper, where the wax expands approximately 2 mm during the post-thermal treatment. Accordingly, this unavoidable effect must be taken into account when designing the microfluidic system since the width of the channels and, therefore, the dimensions of the entire system, is changed after the printing process, affecting not only the passive capillary flow rate but also the viability of the microfluidic system, depending on the application requirements. In turn, electrospun PLLA-based membranes feature the advantage of maintaining the dimension and definition of the microfluidic design. Regarding the colour of the reaction chambers, it is observed a gradual increase of the red colour intensity with increasing glucose concentration. The calibration curves, which present the correlations between the colour intensity, denoted as mean grey values, and the logarithm of glucose concentrations, demonstrate a good linearity with correlation coefficients R^2 of 0.99, in both substrates. It should be noted that the grey values were only analysed on the reaction chambers and the calibration curves built accordingly. Thus, the reddish colour observed on the channels of the microfluidic systems, which comes from colorimetric reaction between the reagent that flows passively outside the reaction chambers during functionalization and glucose, does not interfere with the measurements. In addition, the electrospun PLLA-based membranes demonstrate an improved sensibility with a curve slope of -34.5 comparatively to -16.2 obtained with the commercial *Whatman no.1*. Thus, these results demonstrate the potential of the processed electrospun PLLA-based membranes as complementary or alternative substrates to the commonly used *Whatman* papers for the fabrication of microfluidic systems not just for colorimetric

detection, as proven in this section, but also for other relevant (bio)technological applications that can take advantages of the demonstrated beneficial physicochemical properties of the processed substrates.

4. Conclusions

The present work reports on the processing and evaluation of electrospun PLLA membranes as innovative substrates for the fabrication of microfluidic systems, complementary or alternatives to the µPADs based on paper substrates. The influence of fibre orientation, addition of hydrophilic additives (NaY zeolites) and plasma treatments (Ar and O_2) on the morphology, physicochemical properties, biocompatibility and capillary flow rate were properly studied and compared with the commonly used paper substrates based on commercial *Whatman no.1*. The results demonstrate that controlling properly the processing conditions, it is possible to tailor the morphology, surface hydrophilicity and capillary flow rate of the electrospun PLLA-based membranes, which is beneficial in order to reach specific applications requirements, which can be difficult to obtain with the current commercially available paper substrates. Moreover, the developed materials are biocompatible, show good wet strength and appropriate thermal properties to be used as substrates for the fabrication of microfluidic substrates using wax printing technology. A proof-of-concept based on the colorimetric detection of glucose in printed microfluidic systems demonstrated the potential of PLLA substrates for the fabrication of portable analytical devices that benefit from the demonstrated controllable physicochemical and capillary flow rate properties. It is to notice that the smart properties of PLLA based on its piezoelectricity can be further explored to give rise to active multifunctional microfluidic substrates.

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Notes

The authors declare no conflict of interest

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Table of contents (TOC)