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2014 Biofabrication 6 035009

(http://iopscience.iop.org/1758-5090/6/3/035009)

OPEN ACCESS
IOP Publishing
Biofabrication

Biofabrication **6** (2014) 035009 (10pp)
doi:10.1088/1758-5082/6/3/035009

Robust fabrication of electrospun-like polymer mats to direct cell behaviour

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Received 18 February 2014, revised 11 April 2014
Accepted for publication 28 April 2014
Published 28 May 2014

Abstract

Currently, cell culture systems that include nanoscale topography are widely used in order to provide cells additional cues closer to the *in vivo* environment, seeking to mimic the natural extracellular matrix. Electrospinning is one of the most common techniques to produce nanofiber mats. However, since many sensitive parameters play an important role in the process, a lack of reproducibility is a major drawback. Here we present a simple and robust methodology to prepare reproducible electrospun-like samples. It consists of a polydimethylsiloxane mold reproducing the fiber pattern to solvent-cast a polymer solution and obtain the final sample. To validate this methodology, poly(L-lactic) acid (PLLA) samples were obtained and, after characterisation, bioactivity and ability to direct cell response were assessed. C2C12 myoblasts developed focal adhesions on the electrospun-like fibers and, when cultured under myogenic differentiation conditions, similar differentiation levels to electrospun PLLA fibers were obtained.

S Online supplementary data available from stacks.iop.org/BF/6/035009/mmedia

Keywords: biophysical cues, cell adhesion, microfabrication, cell alignment, electrospinning, myogenic differentiation, poly(lactic acid)

(Some figures may appear in colour only in the online journal)

1. Introduction

Different applications of nanomedicine, tissue engineering and drug development use cell culture systems to mimic the *in vivo* extracellular matrix (ECM) environment with which cells interact. Nanoscale topography therefore becomes a key parameter since *in vivo* ECM possesses a complex mixture of fibers with different nanometric sizes [1, 2]. Moreover cells sense and respond to this nanotopography in terms of adhesion, proliferation, differentiation, migration and gene expression [3, 4]. Different biological systems have been used for this purpose, such as decellularised tissues, but the limitation of donors as well as the complexity and differences of each tissue have encouraged bioengineers to develop simple and more reproducible systems [5, 6]. As the ECM consists of fibrillar proteins with diameters ranging from tens to hundreds of nanometers, fibrous scaffolds have been engineered as ECM substitutes. Different techniques such as melt-blown multicomponent processes and mechanical fiber spinning have been used, but electrospinning has emerged as the most promising technique owing to its ability to generate fibers

similar to the fibrous structures of native ECM and to its flexibility in controlling fiber morphology [7, 8].

Electrospinning is based on the principle that at a certain voltage, a charged polymer jet is ejected from a polymer solution moving in the direction of the external electric field. This jet is then randomly deposited on a substrate as a non-woven mat of nanofibers with diameters ranging from a few nanometers to several microns [9]. Over 200 synthetic and natural polymers have been successfully processed into nanofibers by electrospinning, as well as mixtures of polymers with chromophores, nanoparticles, metals, ceramics and even living microorganisms [10–17]. The dimensions and morphology of the electrospun fibers depend on a large set of parameters including the properties of the polymer itself (such as molecular weight and distribution, glass-transition temperature, solubility etc), the properties of the polymer solution (such as viscosity, surface tension, viscoeleasticity, concentration, diffusion coefficient, vapor pressure, and dielectric and electrical properties) and other external parameters (such as feed rate, diameter of the needle, collector distance, temperature and humidity). Thus, electrospinning becomes a complex and poorly reproducible process that is usually largely controlled empirically [18–20]. Beading, branching and undulation are the most common undesired phenomena observed during electrospinning but other artefacts such as ribbon-like (instead of circular) fibers have been also observed [21]. This variability during electrospinning often hampers comparisons in biological studies since it is difficult to obtain similar fibers from different (or even the same) polymer solutions. In addition, the adequate and reproducible patterning of electrospun nanofiber scaffolds is a relevant process towards engineering functional tissues and organs, where ordered cellular organization is essential. New methods to obtain reproducible electrospun-like samples are therefore needed [17].

Here, we introduce a robust and simple method to engineer reproducible aligned and random electrospun-like polymer fibers. In brief, a single electrospun mat was used as a master template to fabricate silicone (polydimethylsiloxane; PDMS) molds. Afterwards, samples were obtained by solvent casting a polymer solution in the mold. This novel methodology was validated with poly(L-lactic) acid (PLLA). Replication of molded PLLA samples was studied by atomic force microscopy (AFM) and scanning electron microscopy (SEM). Cell adhesion and differentiation were assessed using immunofluorescence and SEM.

2. Materials and methods

2.1. Materials

Polymer sheets were obtained by bulk radical polymerization of ethyl acrylate (EA) (99% pure, Sigma-Aldrich, Steinheim, Germany) in anaerobic conditions by using 1 wt% benzoin (98% pure, Scharlau, Barcelona, Spain) as the photoinitiator. The polymerization was carried out up to limiting conversion by UV exposure over night. After polymerization, low molecular-mass substances were extracted from the material by drying in vacuum at 60 °C to constant weight. PLLA was obtained from NatureWorks (>99% pure, 144 g mol^{-1}, Netherlands).

2.2. Electrospinning

Poly(ethyl acrylate) (PEA) and PLLA solution were electrospun to produce master templates to manufacture the molds and obtain control electrospun samples, respectively. Polymer solutions were dissolved in hexafluoroisopropanol (HFIP; >99% pure, Sigma) and electrospun as described elsewhere [3, 4, 22]. Briefly, electrospinning was carried out using a needle of 0.15 mm of internal diameter (EFD International) and a constant feed rate of 900 μl h^{-1} with a programmable syringe pump (New Era Pump Systems, Wantagh, NY, USA) with a specific voltage (Glassman High Voltage, High Bride, NJ, USA) and collector distance (table 1). Randomly electrospun fibers were collected on glass coverslips placed on aluminum foil, while aligned fibers were collected by electrospinning the solution onto a rotating drum on which glass coverslips were attached.

2.3. Mold making

The pre-polymer Neukasil RTV-20 and the cross-linker Neukasil A2 (Altropol Kunststoff GmbH, Germany) were mixed according to the manufacturer's datasheets in a 10:1 proportion and introduced in a vacuum chamber for 20 min at the beginning of the polymerization for adequate degasification. Afterwards, the mixture was cast into a plastic cage were the PEA electrospun samples had been adhered previously, facing upward. Polymerization at room temperature (25 °C) took approximately 24 h. Then, the PDMS mold was carefully peeled off the cage and maintained at room temperature for another hour to assure complete polymerization of the surface in contact with the original PEA master templates. Finally, the mold surface was cleaned using an air gun to eliminate fibers that might have remained attached.

2.4. Solvent casting

Polymer solution was obtained by dissolving PLLA in chloroform (stabilised with ethanol, >99% pure, Scharlau) to produce a solution with a concentration of 20 mg ml^{-1}. 200 μL of PLLA solution was deposited in each silicone (PDMS) mold and allowed to evaporate at room temperature for 10 min. Drops of the same polymer solution were placed on glass coverslips and allowed to evaporate under the same conditions. The mold and coverslips were then heated at 200 °C for 5 min. Then, PLLA-coated coverslips were placed on top of the mold and pressed gently to adhere both parts. Subsequently, samples were further heated for 5 min at 200 °C and then the mold was quenched in cold water for a few seconds. Finally, the samples were peeled off the mold and dried in a vacuum (see figure 1).

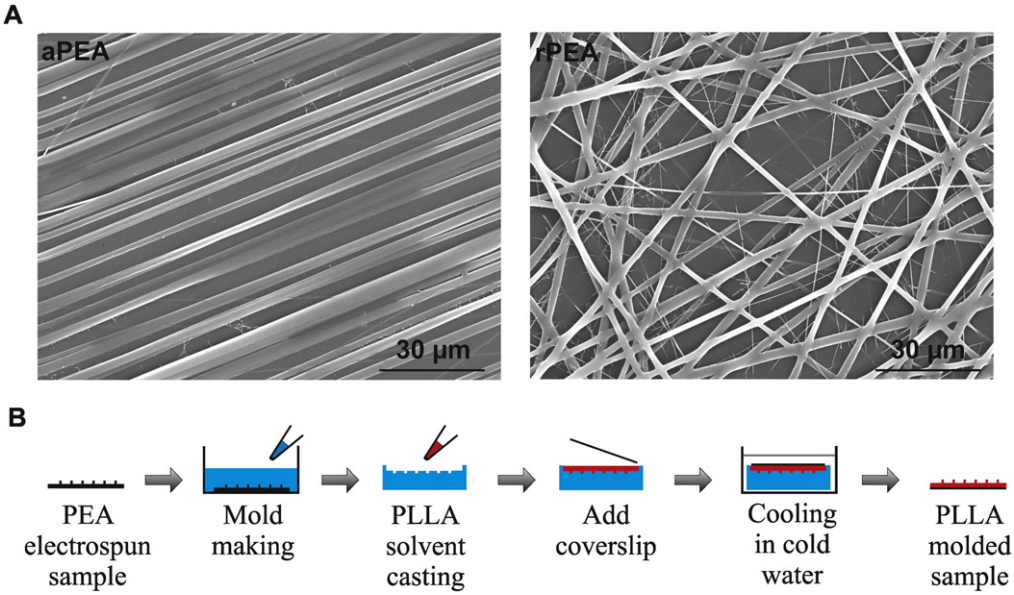

Figure 1. Molding process. (A) SEM images of electrospun PEA samples that were used as a template to fabricate the PDMS mold. (B) Diagram showing the main steps of the molding process.

Table 1. Electrospinning conditions for each polymer.

Polymer	Concentration (mg ml^{-1})	Voltage (kV)	Collector Distance (cm)	Linear speed (cm s^{-1})
PEA	20	12.5	20	337.5
PLLA	80	30	12	1125

2.5. Scanning electron microscopy

Samples were characterised using SEM with a JEOL JSM 6300 system (JEOL Ltd, Tokyo, Japan) operating at 10 kV. The electrospun and molded samples were sputtered with gold and visualised to obtain qualitative data regarding the fiber topography. When used for cell culture, the samples were fixed in 2.5% glutaraldehyde (Panreac, Spain) for 1 h at 4 °C and after several washes in DPBS, the samples were postfixed with 1% osmium tetroxide (>99% pure, Electron Microscopy Science) for 1 h, dehydrated in graded ethanol solutions (once in 30, 50, 70, 80, 90, 96% ethanol and three times in 100% ethanol), chemically dried with hexamethyldisilazane (>97% pure, Fluka, Switzerland) and finally coated with gold prior to SEM observations.

2.6. Image analysis

Cell morphology was quantified by calculating the aspect ratio (major axis/minor axis) and roundness (4 x area/π x [major axis]^2), which corresponds to a value of 1 for a perfect circle, using ImageJ software. In order to obtain quantitative data regarding cell anisotropy, a Fourier fast transform (FFT) of the squared SEM pictures was performed, and after a counter-clockwise rotation of 90° (in order to recover the original cell orientation modified by the Fourier transform) a radial projection of pixel intensity was determined.

2.7. Atomic force microscopy

AFM was performed in a JPK Nanowizard 3 BioScience AFM (JPK, Germany) operating in ac mode; the SPM and DP 4.2 software versions were used for image processing and analysis. Si-cantilevers from Nanoworld AG (Switzerland) were used with a force constant of 2.8 N m^{-1} and resonance frequency of 75 kHz. The phase signal was set to zero at a frequency 5–10% lower than the resonance frequency. Drive amplitude was 700 mV and the amplitude setpoint was 650 mV.

2.8. Fibronectin adsorption

Fibronectin (FN) from human plasma (Sigma-Aldrich) was adsorbed from a solution with a concentration of 20 μg ml^{-1} in Dulbecco's Phosphate Saline Buffer (DPBS) on the samples for 1 h. Afterwards, samples were rinsed with DPBS to eliminate the non-adsorbed protein excess.

2.9. Cell culture

Murine C2C12 myoblasts obtained from ATCC were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS; Fisher) and 1% penicillin–streptomycin (Lonza). Prior to seeding, samples were sterilized by UV exposure for 30 min and coated with FN as described previously. Then, 17 000 cells/cm^2 were seeded onto the different samples in serum-free conditions

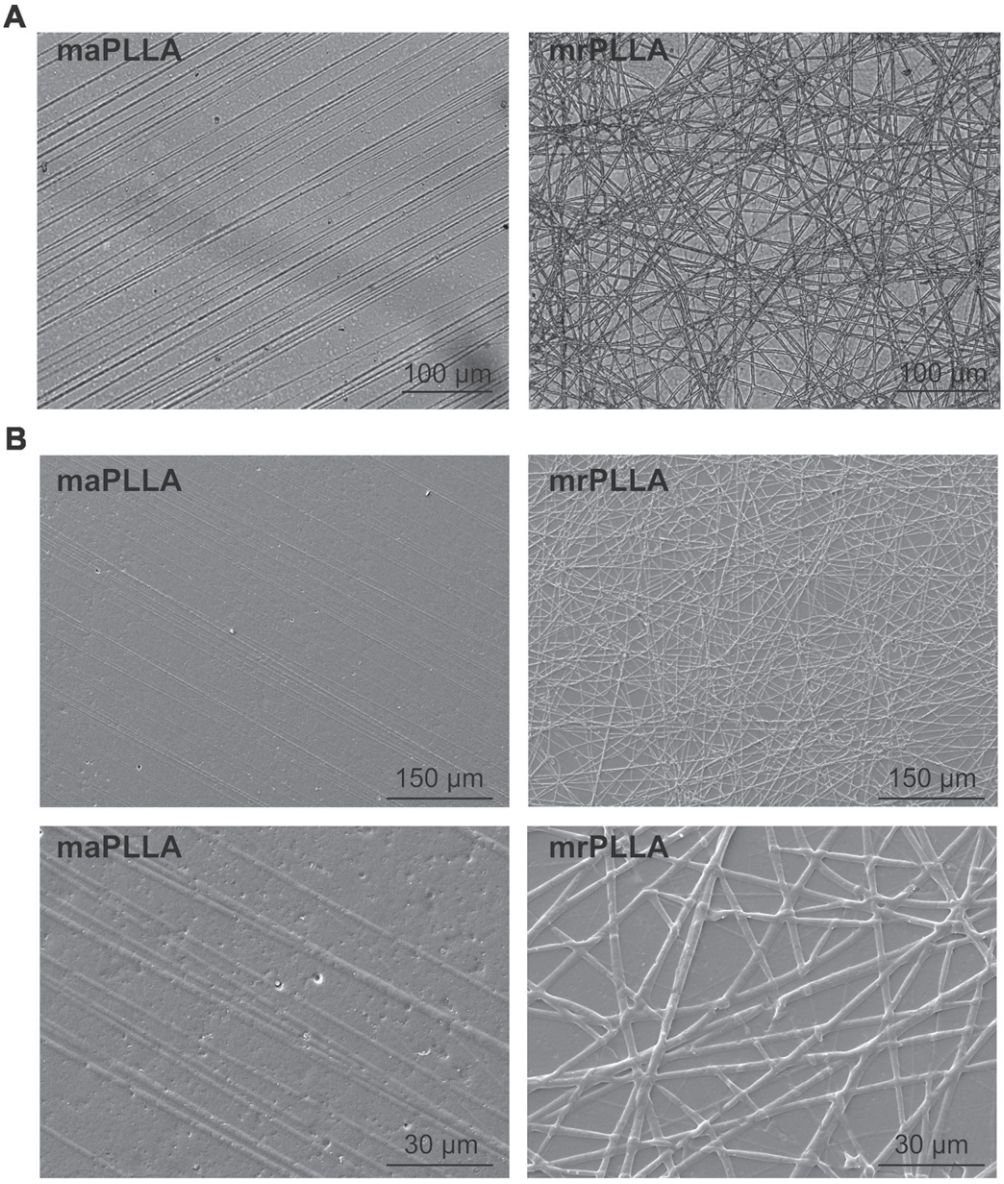

Figure 2. (A) Optical and (B) scanning electron microscopy of molded PLLA samples with aligned and random patterning (maPLLA and mrPLLA respectively).

Table 2. Fiber geometrical characterisation using AFM. Original template, mold and molded PLLA samples. No statistically significant differences between the mold and molded PLLA samples were observed. Statistically significant differences with the original template are indicated by *, $P < 0.001$.

	Random			Aligned		
	PEAr	Mold (random)	mrPLLA	PEAa	Mold (aligned)	maPLLA
Width (μm)	2.3 ± 0.6	3.4 ± 0.3	2.7 ± 0.5	2.7 ± 0.3	2.6 ± 0.4	2 ± 0.2
Height (nm)	1592 ± 337	534 ± 195*	673 ± 68*	675 ± 137	145 ± 51*	144 ± 20*

seeking to direct specific cell adhesion to the pre-adsorbed FN.

For cell adhesion experiments, the medium was changed to DMEM with 10% fetal bovine serum (FBS) after 3 h of culture. Once the culture was complete, the cells were fixed with 10% formalin (Sigma) for 30 min at 4 °C. After fixation, cells were permeabilised for 5 min at room temperature using 0.5% Triton X-100 (Sigma) in HEPES buffer 20 mM (Sigma) supplemented with 0.3 M saccharose, 50 mM NaCl (Sigma) and 3 mM $MgCl_2$ hexahydrate (Scharlab), and then incubated in blocking buffer (1% BSA in DPBS) for 30 min. Samples were then incubated with an anti-vinculin antibody (Sigma) diluted 1 : 400 for 1 h and then with anti-mouse Cy3-conjugated secondary antibody (Jackson Immunoresearch, UK) and BODIPY FL phallacidin (Molecular probes). Finally, the samples were washed before being mounted in Vectashield

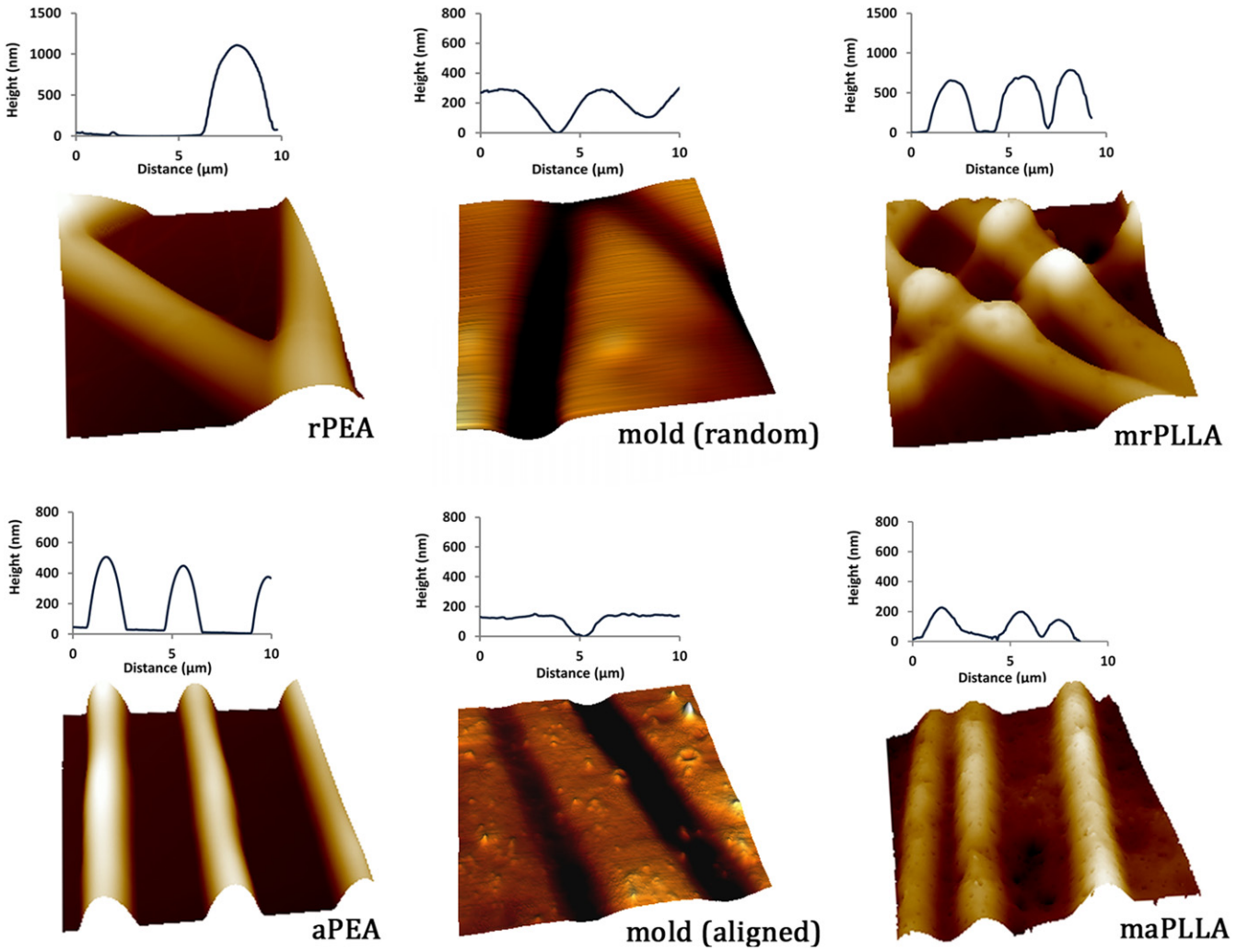

Figure 3. Surface characterization. Transversal sections and 3D images of a 10 × 10 μm surface area of each sample are shown.

containing DAPI (Vector Laboratories, UK). A Nikon microscope (Nikon Eclipse 80 i) was used.

For cell differentiation experiments, C2C12 cells were cultured for 4 d under differentiation conditions (DMEM supplemented with 1% penicillin–streptomycin and 1% insulin-transferrin-selenium-X (Invitrogen)) and immunostained for sarcomeric myosin (Developmental Studies Hybridoma Bank). In brief, cells were fixed with 70% ethanol, 37% formaldehyde glacial and acid acetic solution (20:2:1) at 4 °C for 10 min. After fixation, samples were rinsed with DPBS and blocked with 5% goat serum in DPBS for 1 h at room temperature. Blocking buffer excess was removed, and sequential incubations with MF-20 mouse antibody (Developmental Studies Hybridoma Bank, University of Iowa, USA) and anti-mouse Cy3-conjugated secondary antibody (Jackson Immunoresearch) were carried out. Finally, samples were washed before being mounted in Vectashield containing DAPI (Vector Laboratories, UK). Cultures were scored by the percentage of positive cells for myosin using CellC image analysis software [23].

2.10. Statistical analysis

The results are shown as average ± standard deviation and were analyzed by one-way ANOVA. If treatment level differences were determined to be significant, pair-wise comparisons were performed using the Tukey post hoc test.

3. Results and discussion

3.1. Molding and characterization

We have developed a robust and simple technique to obtain highly reproducible aligned and random polymer nanofibers similar to those obtained using conventional electrospinning. Following this methodology, a battery of topologically identical samples can be fabricated for biological studies. As different polymer solutions can be used, samples with different physico–chemical properties (e.g. chemistry and mechanics) can be fabricated while maintaining identical fiber topography. That is to say, we show a method capable of preparing sets of topologically identical samples using

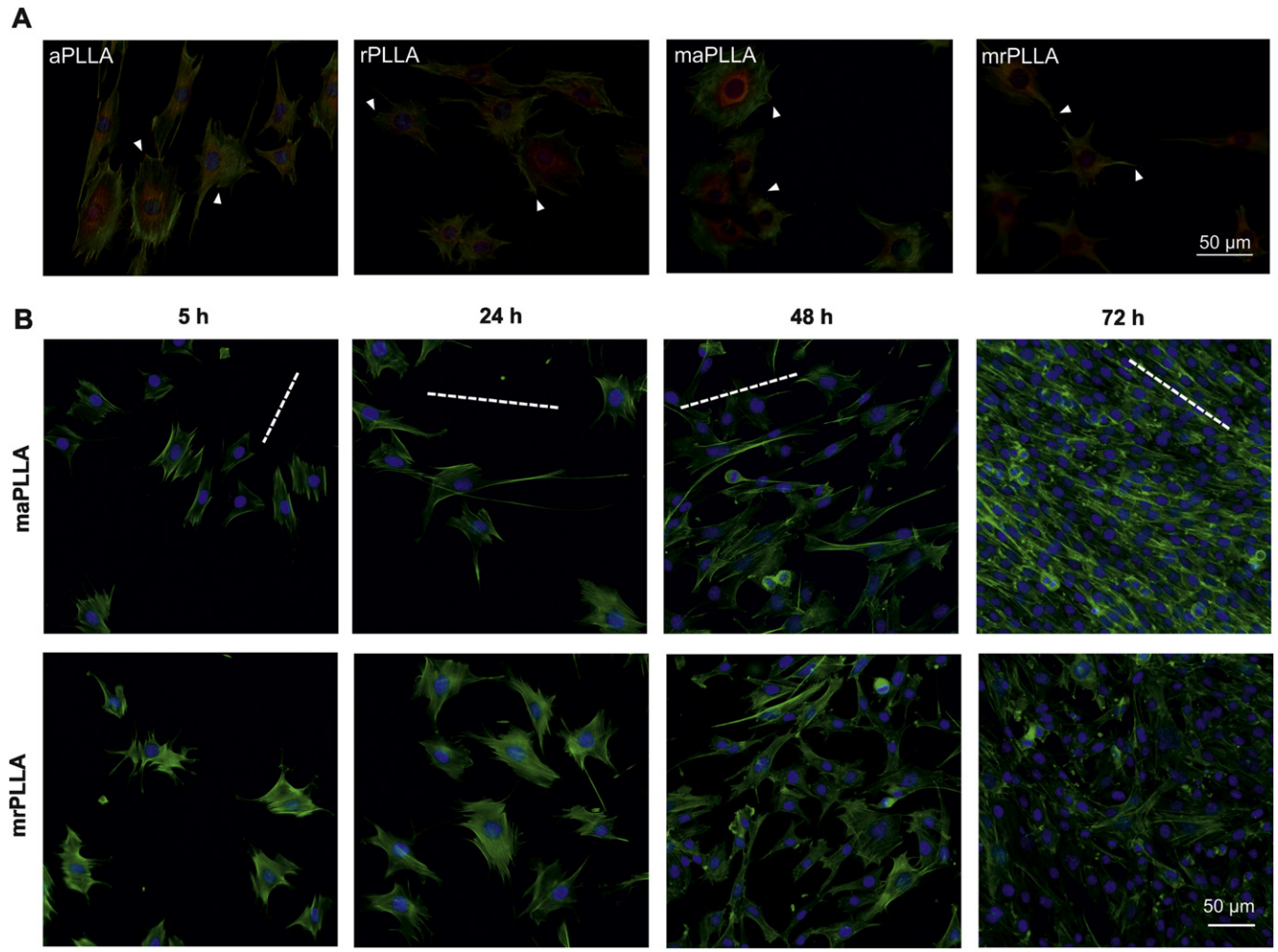

Figure 4. Cell orientation on elecrospun-like fibres. (A) Cell adhesion after 5 h of culture on the different FN-coated samples. Vinculin is stained red and actin is seen in green. Nuclei were counterstained with DAPI and appear blue. (B) Evolution of cell morphology and orientation on molded samples as observed by cytoskeleton (actin) detection. Dotted lines represent fibre orientation where necessary.

different polymers. We started by electrospinning PEA to fabricate the PDMS mold. Figure 1 shows SEM images for the original PEA template samples (aligned and random PEA fibers; PEAa and PEAr, respectively) and illustrates the entire fabrication process, from mold making to sample production.

As a result of electrospinning, smooth, circular PEA fibers without beading were obtained. In the case of random samples, there were some very thin fibers mixed up with the main fiber population of greater diameter (~2 μm, see table 2 for a summary of all fiber dimensions). Aligned fibers show a similar diameter but reduced height (see table 2). A high degree of alignment was obtained as a result of the manufacturing process.

Fiber topography from the original PEA electrospun sample was replicated in the PDMS mold with minimal defects (AFM in figure 3), which were formed in a few samples most likely due to the difficulty in the PDMS getting into the topography of the PEA fibers due to surface tension effects. According to this, topographical features in the mold reproduced the original template for both the random and the aligned patterns, and could therefore be used to obtain electrospun-like polymer fibers. As a proof-of-concept, we selected PLLA as a gold standard biodegradable polymer with an excellent track-record in tissue engineering [4, 24]. After solvent casting the PLLA solution and cooling down in cold water, molded samples were easily obtained. Fibers were observed by optical microscopy and at higher magnifications by SEM on both aligned and random molded samples (maPLLA and mrPLLA respectively) (figure 2). The molding process transfers the original fiber patterning from the electrospun PEA samples to the final molded PLLA samples using the mold as an intermediate step in the process.

AFM was performed on the molded fibers to quantify topographical cues in three dimensions (height, width and real shape of the fiber). As shown in figure 3 and table 2, solvent casted samples reproduced the mold topography accurately. In fact, the stretching of the original electrospun PEA fibers during alignment (figure 1 and table 2) was also observed in the molded samples, which supports excellent replication.

3.2. Cell adhesion

After assessing the quality of replication of the molded samples, cell adhesion and cytoskeleton development were

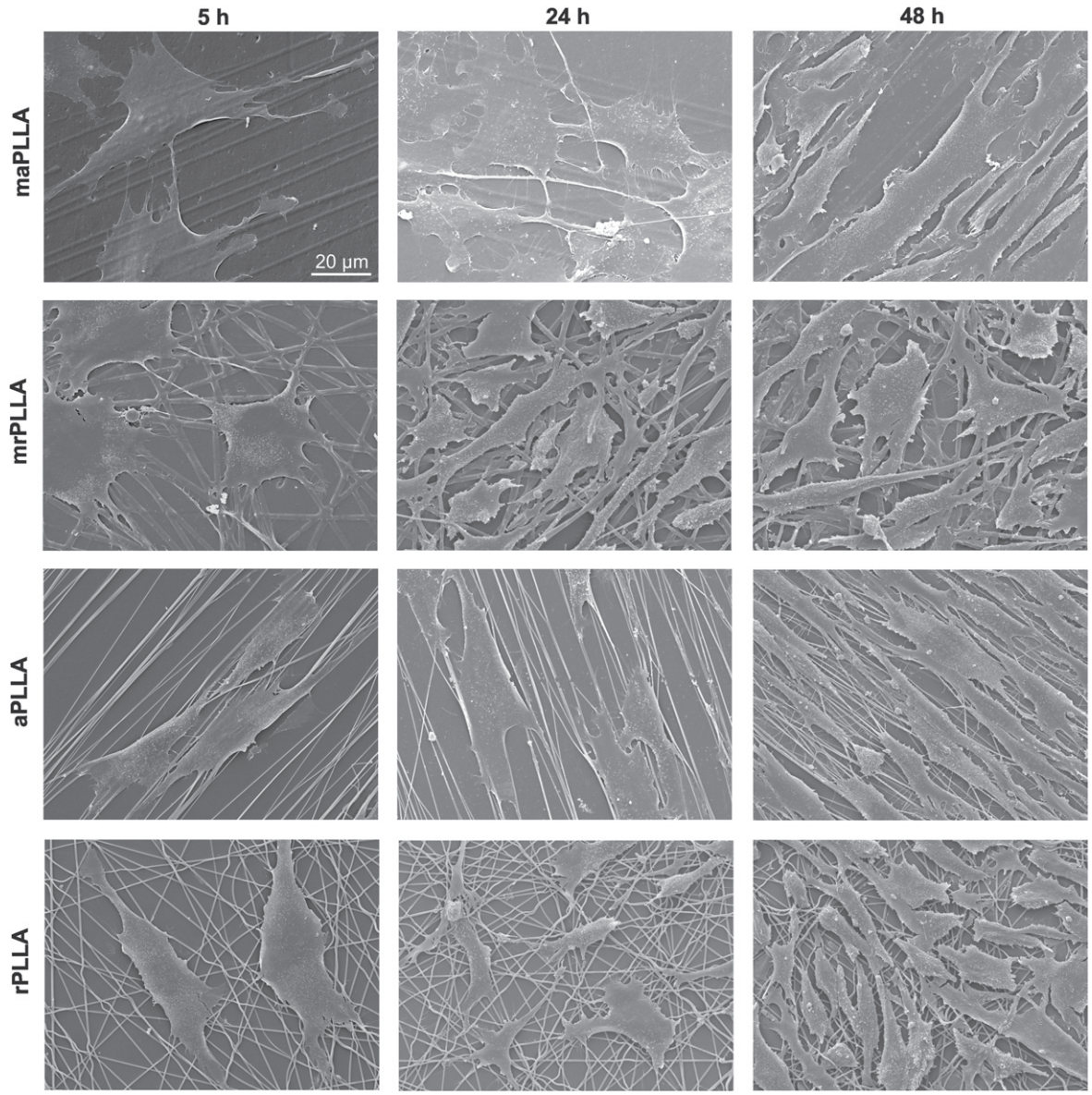

Figure 5. Cell adhesion on electrospun-like fibres at different time points as observed by SEM.

used as biological proof-of-concept for the novel methodology introduced in this work. As the molded samples are made of PLLA, PLLA fibers electrospun on glass were used as positive controls (aPLLA and rPLLA will be used to identify aligned and random electrospun PLLA fibers, respectively). Note that the size of these electrospun PLLA fibers differs from the molded fibers (supplementary figure 1, available at stacks.iop.org/BF/6/035009/mmedia). Figure 4 shows cells after 5 h of culture on the different substrates. Prominent actin fibers inserted into well-developed focal adhesion complexes containing vinculin were observed (white arrows) on both molded and electrospun PLLA fibers. As expected, alignment of filopodia and/or part of the actin cytoskeleton in the direction of the underlying fibers was seen on the electrospun aligned PLLA samples. Nevertheless, neither cell nor filopodia alignment was seen on the molded aligned samples after 5 h; which is likely due to the lower height of the molded fibers, which could delay cell alignment along the fibers [25]. We disregard any role of FN in the different cell responses between the electrospun and molded samples since the microfibers consist of PLLA on all samples and FN is consequently expected to adsorb similarly on the different PLLA fibers (electrospun or molded) in terms of quantity and conformation. We have previously characterised FN adsorption on PLLA at $\sim$1800 μg m^{-2} and as adopting a globular conformation [26, 27]. To confirm this hypothesis and refute that the molded samples are unable to direct cell alignment, cell orientation was tracked over 3 d of culture. As shown in figure 4(*B*), cells projected long pseudopodia in the direction of the fibers after 24 h and aligned after 48 h of culture (most of them) showing stressed actin fibers with the same orientation as the fiber patterning. The same trend was observed by SEM (figure 5) corroborating that, as the topology of the molded samples was lower than on the electrospun samples,

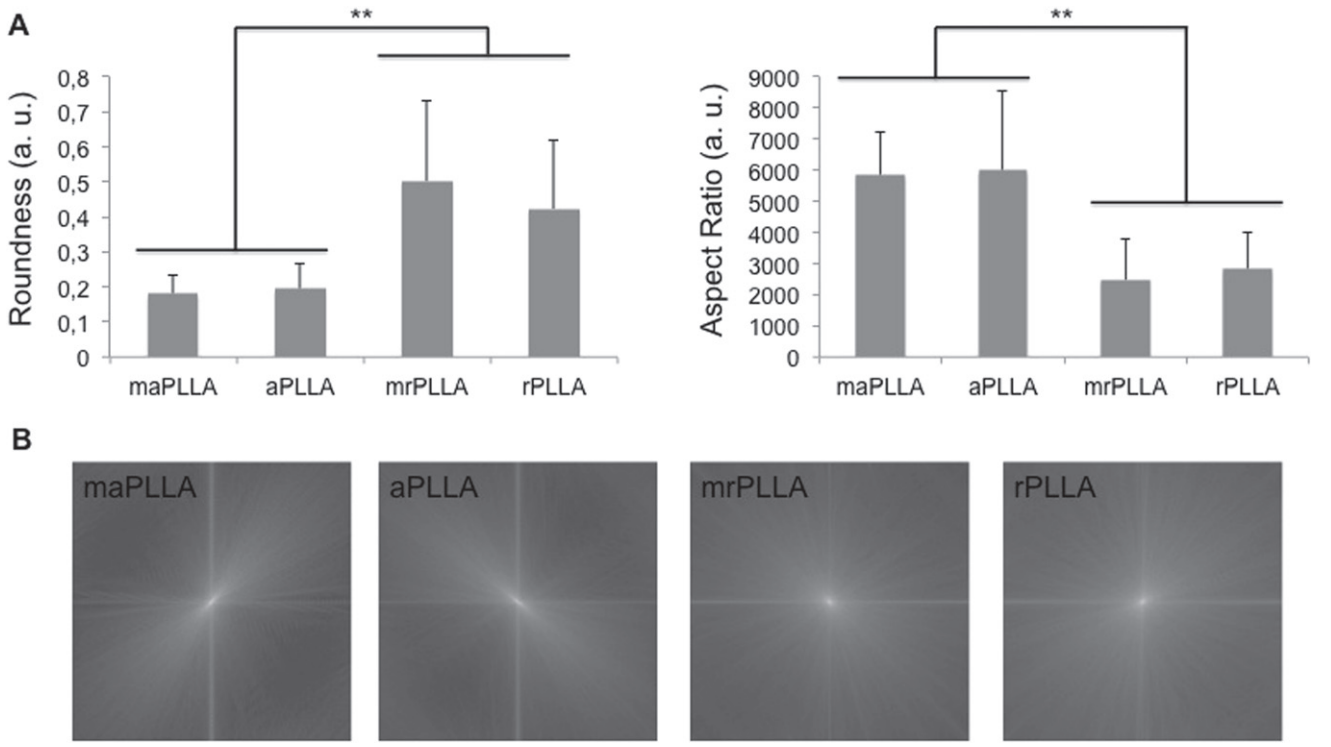

Figure 6. Cell morphology and alignment. (A) Roundness and aspect ratio of cells cultured on the different samples. (B) Cell alignment as calculated by image analysis (FFT of the normalized pixel intensity).

cells needed more time to align on molded samples due to the weaker topological stimulus. First, roundness and aspect ratio were evaluated, corroborating the elongated morphology of cells seeded on aligned patterned samples (aPLLA and maPLLA in figure 6). Then, in order to assess cell alignment in the direction of the fibers, the Fourier transform of each image was calculated showing preferential alignment on aligned electrospun and molded samples but not on random ones.

As fiber topography has a key role in cell fate [25] and electrospinning is an intrinsically unstable technique that makes the manufacturing of reproducible fibers difficult, new routes able to produce reliable samples are needed. The technique shown here not only produces reliable samples but may also be used to prepare samples made of different polymers with the same topography, which is not possible using electrospinning. This technique is therefore a robust tool to investigate cell behaviour under well-controlled stimuli.

3.3. Myogenic differentiation

Next, we examined the role of topological cues from the electrospun and molded fibers on myogenic differentiation by immunolabeling and quantifying positive sarcomeric myosin cells. As shown in figure 7, similar differentiation levels were found on the four substrates, with differences neither between the aligned and random samples nor between the electrospun and molded fibers [3, 4]. That is to say, molded samples trigger cell differentiation to the same extent as the original electrospun fibers.

4. Discussion

Microfibers have been extensively studied as a means to mimic the fibrillar structure of the natural *in vivo* environment in cell culture experiments. Electrospinning stands out among the large variety of techniques used, but it presents major drawbacks in terms of reproducibility of different batches of samples. New techniques for easy production of reproducible samples are therefore required. We have developed a PDMS mold-based technology as a robust and rapid method to obtain these micropatterned samples and overcome the limits of electrospinning. Moreover, we have validated the technology using a myoblast culture, showing that replicated samples triggered a similar response as electrospun samples in terms of focal adhesion formation, cell alignment and cell differentiation. We suggest that this method could profitably replace electrospinning for some specific experiments because it avoids using electric fields (a hazard for the research staff) and it is remarkably simple in terms of the technique, procedure and equipment required while allowing fast production of reproducible samples. We have checked that the PDMS molds can be reused approximately 15 times before they begin to degrade. Moreover, reproducible topological fibers can be obtained from a huge variety of polymer solutions, even aqueous or conductive solutions which are normally very difficult to electrospin. As no high electric fields are

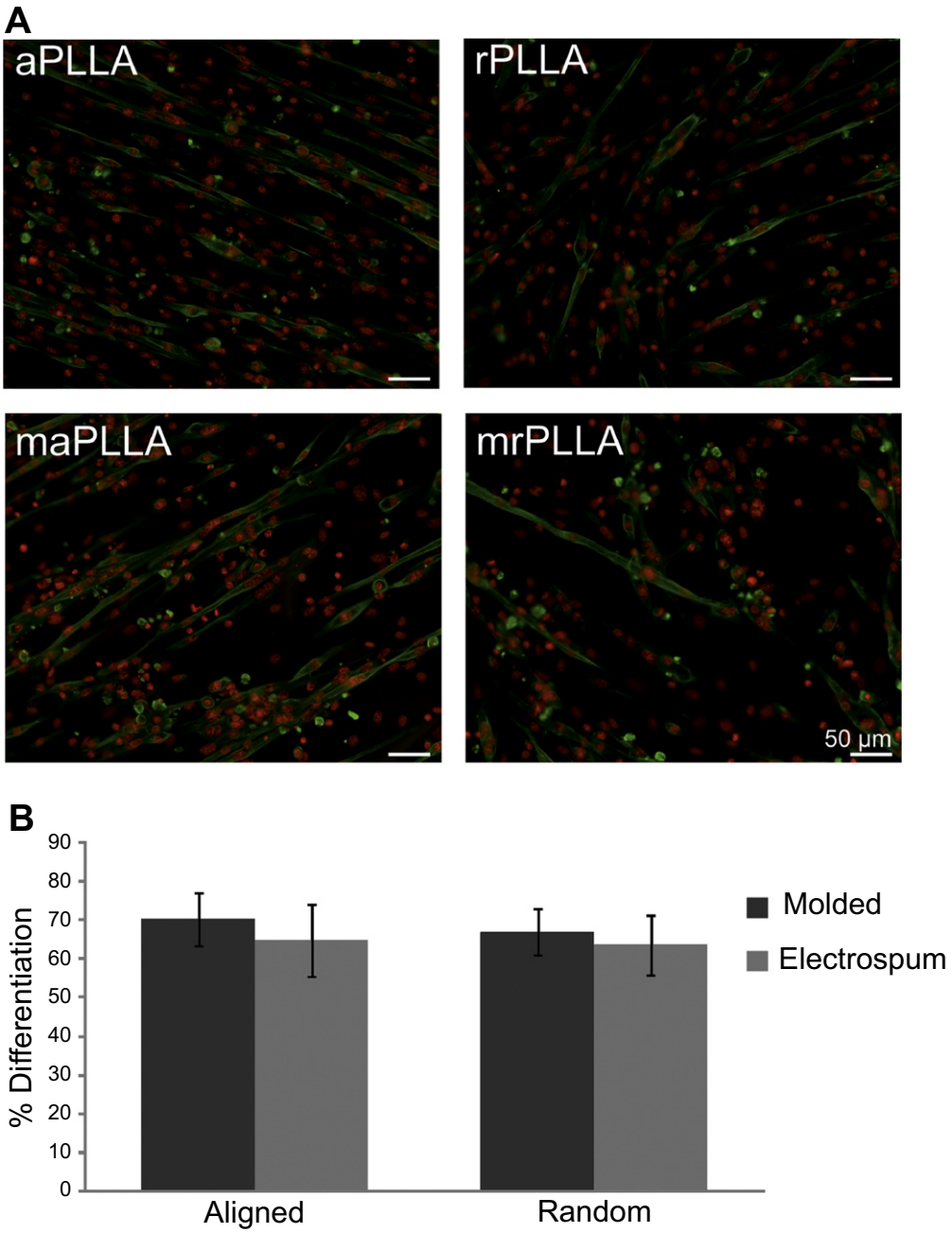

Figure 7. Myogenic differentiation of C2C12 myoblasts on PLLA samples. (A) Fluorescence staining showing sarcomeric myosin positive cells (green) and cell nuclei (red). (B) Myogenic differentiation as determined by the percentage of sarcomeric myosin-positive cells. No statistical differences were observed.

used, biologically active molecules (such as drugs for controlled release, growth factors) and even microorganisms (such as bacteria and cells) might be encapsulated without compromising their biological properties. Finally, polymeric fibers obtained using this technique are laid on a plain surface of the same polymer, whereas polymeric fibers obtained by electrospinning lie on a support—commonly glass—which makes comparison of results obtained from the fibers and the plain substrate difficult (fibers cannot be electrospun on the same polymer substrate as the residual solvent dissolves the underlying polymer, burying the electrospun fibers in the bulk). Solvent casting samples in a mold is therefore an easy and reliable method to produce micropatterned substrates for cell culture procedures in an easy way, therefore providing an interesting alternative to electrospinning for some specific studies.

5. Conclusions

Here, we have proposed a novel procedure that yields electrospinning-like polymer fiber samples with high reproducibility by solvent casting a polymer solution on a PDMS mold. A solution of 2% PLLA in chloroform was used and the

obtained samples replicated the master template in a highly reproducible process. These electrospun-like molded samples supported cell adhesion as shown by the developed vinculin focal adhesions and provided topological cues to direct cell orientation similarly to the control PLLA electrospun fibers. In addition, similar levels of myogenic differentiation were obtained for the molded samples and the PLLA electrospun fiber samples (which were used as controls) supporting its potential for cell behavior studies.

Acknowledgements

The support of ERC through HealInSynergy (306990) and FPU program AP2009-3626 is acknowledged.

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