Electrospun PCL in Vitro: a Microstructural Basis for Mechanical Property Changes

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Abstract
Polymeric tissue-engineering scaffolds must provide mechanical support while host-appropriate cells populate the structure and deposit extracellular matrix (ECM) components specific to the organ targeted for replacement. Even though this concept is widely shared, changes in polymer modulus and other mechanical properties versus biological exposure are largely unknown. This work shows that specific interactions of biological milieu with electrospun scaffolds can exert control over scaffold modulus. The net effects of biological and non-biological environments on electrospun structures following 7 and 28 days of in vitro exposure are established. Reduction of modulus, ultimate tensile strength and elongation occurs without the apparent involvement of classic hydrolysis mechanisms. We describe this phenomenon as deposition-induced inhibition of nanofiber rearrangement. This phenomenon shows that both mechanical and morphological characterization of electrospun structure under load in biological environments is required to tailor scaffold design to pursue specific tissue-engineering goals.

Keywords
Biomaterials, polycaprolactone, electrospinning, polymer scaffold, in vitro test, in vivo test

1. Introduction
Within the field of tissue engineering, the degradation of specific scaffold forms is not well understood. An improved understanding of breakdown is particularly important in specific applications, e.g., as vascular constructs in which the need to avoid ischemic stroke is paramount. Biodegradation in vitro is also important because of the widespread application of stress needed to precondition the architec-
ture of the developing cells. A key requirement of this vision is that the scaffold has no strongly negative effects on the adherent/developing biological tissues during these mechanical excursions. As an important subtext we need to understand how these scaffolds break down in these environments. Beyond the obvious potential for systemic consequences the scaffold must maintain the optimal (and usually poorly described) mechanical properties versus time. Very few scaffolds have been characterized in this way. Electrospun scaffolds appear to be gaining in acceptance and applications [1–15] and, thus, provide an excellent starting point.

Tissue engineering currently utilizes cells and a range of engineering materials to pursue the promise of functional replacement of diseased or failing human organs [16, 17]. However, a better match of the properties of these engineered scaffolds with the mechanical requirements of the target organ is needed to promote or preserve appropriate levels of biological activity. Scaffolds must also realistically consider multiple hierarchical scales, from the microscopic to the macroscopic, satisfying both long- and short-term biological considerations.

Even though this concept is widely shared, the extent of changes in polymer behavior, especially at the nanometer scale, versus these biological exposures is largely unknown. Any in vitro or in vivo exposure has the potential to physically (as well as chemically) alter the ability of the scaffold microstructure to respond to stress [18]. Such stresses may be passed onto the scaffold either as an implant in vivo or as architectural conditioning by deliberate, cyclically applied forces in vitro.

To begin to address this issue, we have carried out exposures of electrospun poly(ε-caprolactone) (PCL), a relatively slowly degrading biopolymer, to show that a nearly 60% degradation in tensile strength occurs following 28 days of exposure to simple biological fluids such as plasma, urine or milk. Equally long exposures to saline, de-ionized (DI) water and sodium bicarbonate solution produced strength degradations of as much as 40%. Plasma and saline represent important general components of both in vivo and in vitro environments. The use of urine and milk as exposures targets applications as tubular replacements [19] in the urinary tract and in breast reconstruction, respectively. Deionized water (DI) provides a clean, purely hydrolytic exposure.

Parallel measurements of weight versus exposure time showed that plasma and milk exposures caused a net gain in the mass of these fiber networks while all other solutions resulted in as much as a 33% loss over the same time period. The gain is attributed to adsorbed proteins in the electrospun fiber present even after rinsing. Based on our understanding of key aspects of fiber behavior that control extension [20], we conducted additional in vitro experiments to prove that biological deposition, rather than material degradation alone, is responsible for at least some of the observed changes in mechanical properties. Short-term but relatively efficient deposition of plasma proteins was achieved by utilizing a flow-through configuration. The result of deposition-induced inhibition appeared to effectively prevent the rearrangement of electrospun fiber central to its ability to achieve relatively higher levels of stress or strain.
A complete understanding of the potentially competitive effects of fiber degradation versus biological deposition during the growth of adherent mammalian cells on scaffold properties is a worthwhile long-term goal.

2. Materials and Methods

2.1. Electrospinning

A 12 wt% solution of poly(ε-caprolactone) (PCL, Sigma–Aldrich, 65 kDa) in anhydrous acetone (Mallinckrodt Chemicals) was prepared by heating acetone to 50°C followed by continuous stirring to dissolve pelletized PCL. After cooling to room temperature, the solution was placed in a 60 cc syringe (Becton–Dickinson) with a 20 gauge blunt tip needle (EFD Precision Tips) and electrospun using a high voltage DC power supply (Glassman) set to 24 kV, an 18 cm tip-to-substrate distance and a 24 ml/h flow rate. A 3 × 3″ (7.6 × 7.6 cm) sheet approx. 200 µm in thickness was deposited onto aluminum foil. The PCL sheets were then placed in a vacuum for 12 h to ensure removal of residual acetone. High-resolution ESI analysis (Esquire) was used to establish that the residual acetone content was beneath our ability to detect it (less than 10 ppm).

2.2. Tensile Testing

Tensile dog bones with a gauge length of 20 mm and a gauge width of 2.4 mm were cut from the sheets by placing the PCL electrospun sheet between two 3 mm-thick aluminum templates. A #15 rib-back carbon steel surgical blade (Bard–Parker) was used to cut the straight edges while a 3-mm dermal punch was used to cut the radii. Great care was taken in the preparation of the gauge length to minimize tearing or smearing. The tensile properties were determined utilizing a 1 kg load cell (model 31, Sensotec) with a strain rate of 5 mm/min on an Instron load frame (model 1322) and lightweight carbon fiber grips (model A2-166 Fibre Clamp Assembly, Instron).

Tensile sample thickness was measured using a digital micrometer; the gauge length of each specimen was confined between two glass microscope slides and the total thickness determined. Subtraction of the thicknesses of the individual glass slides provided the gauge length thickness. A control group of 6 samples was tested without any exposure to establish the as-fabricated baseline ultimate tensile strength (UTS), elongation to failure and modulus. The modulus was determined by taking the slope of a best fit line through the initial linear regime or within the range of 5–15% elongation.

2.3. In Vitro Exposures

Twelve samples were then placed into each of six solutions; sodium bicarbonate (5 wt% NaHCO₃), sterile saline, pasteurized whole cow’s milk (protein content 4.5 g/dl, fat content 3.5 vol%), bovine urine, bovine plasma (protein content 7.0 g/dl) and de-ionized water. The solutions containing the samples were kept in
an incubator at 37°C and the solutions were replaced every three days. The pH of each solution was measured initially and then again after every 3 days of exposure. After 7 days of exposure, 6 samples were removed from each solution and immediately (without drying) tested in tension. Following 28 total days of exposure the remaining samples were removed from solution and were also tested immediately.

Additional electrospun sheets (approx. 3 × 1.5″ (7.6 × 3.8 cm)) were made using identical electrospinning parameters, held under vacuum for 12 h, weighed, exposed to these six solutions for 28 days, rinsed in 500 ml de-ionized water, allowed to air dry, returned to vacuum for an additional 12 h, and then re-weighed to establish net changes in mass.

An exposure to bovine plasma in vitro complementary to those described above occurred as follows: a 3 × 3″ (7.6 × 7.6 cm) sheet of electrospun PCL was mounted to a 5-cm-diameter Buchner funnel connected to a recirculation pump. Approximately 250 ml fetal bovine serum at 37°C was recirculated at 600 ml/h through the sheet for 24 h after which tensile dog bones were cut as described above followed immediately by tensile testing.

2.4. In Vivo Exposure

Six additional tensile dog bone samples were prepared as described above and implanted into a subcutaneous pocket in a bovine model. The samples were mounted inside a poly(propylene) mesh cage that protected the samples from in vivo mechanical forces. After 28 days, the samples were removed, cleaned and exposed to a 5 wt% papain solution at 37°C for 10 min. The papain solution released the samples from the tissue encapsulation. They were then tested in tension as described above. The Institutional Animal Care and Use Committee of Ohio State University approved all in vivo experimental protocols.

2.5. Scanning Electron Microscopy

To examine microstructural changes, all of these samples were coated with 8 nm of osmium (model OPC-80T, SPI Supplies) prior to viewing in a scanning electron microscope (XL-30 ESEM). The use of osmium plasma deposition instead of gold or gold–palladium sputtering eliminated concerns regarding PCL melting and allowed for higher resolution imaging of the fiber surface.

To establish microstructural changes in situ, additional tensile samples were strained to 80% elongation and immediately adhered to double-sided carbon tape to maintain the specific state of strain. At no point during the mounting process did the samples de-adhere or cause bowing of the tape. The samples were then coated with 8 nm of osmium for viewing in the SEM as before.

2.6. X-Ray Diffraction

The XRD spectra were acquired with a Rigaku Ultima-III diffractometer operated in parallel beam mode. The X-ray source was Cu Kα and both incident and diffracted beam monochromators were used. Percent crystallinity was determined by taking the ratio of the crystalline area to the amorphous area in the XRD spectrum [21].
Areas were determined by profile fitting of the crystalline peaks and amorphous ‘humps’ between 10 and 60° 2θ. The stepsize in 2θ was selected to assure a minimum of 10 points above half-maximum intensity for each peak. The integration time at each peak was selected so that all PCL peak intensities were greater than three times the estimated noise level [22]. A constant background was used in the profile fitting to eliminate potential variations in area determination caused by subjective selection of shape and level.

3. Results

3.1. Mechanical Behavior

Figure 1 provides stress–strain curves for the as-fabricated electrospun PCL. The spread in the data is fairly typical reflecting variations in the randomly deposited electrospun material. Figure 2 displays the average ultimate tensile strengths (UTS), Fig. 3 the average elongations to failure and Fig. 4 the average moduli of the various exposures. The plots show the mean value with the error bars representing the standard error. The final properties are summarized in Table 1 as mean value ± standard error.

3.2. Weight Changes

The percentage weight change after 28 days of exposure is summarized in Fig. 5. The saline, sodium bicarbonate and DI water exposures consistently show 15–20% weight loss following 28 days of exposure. The urine data show a larger spread and a greater total weight loss (33%) consistent with the relatively large decrease in strength. On the other hand, the plasma-exposed samples showed a consistent weight gain; the milk-exposed samples showed both weight gains and losses and the widest spread in the observed data. Both the milk and the urine exposure resulted in signs of macroscopic physical degradation such as crumbling of the material and discoloration.

![Figure 1. Stress–strain behavior of as-electrospun PCL.](image-url)
3.3. Microstructural Changes

In Fig. 6a–c, SEM micrographs show that adsorption to the nanofiber surfaces occurs. In contrast, the saline-exposed sample in Fig. 6d appears relatively clean and is quite similar in appearance to the original nanofiber matrix.
Figure 4. Variation in modulus versus exposure.

Table 1.
Mechanical properties versus exposure (mean ± SE)

<table>
<thead>
<tr>
<th></th>
<th>UTS (MPa)</th>
<th>Elongation (%)</th>
<th>Modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.29 ± 0.04</td>
<td>102 ± 6</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>28 days in vivo</td>
<td>0.70 ± 0.16</td>
<td>59 ± 5</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>24 h plasma flow</td>
<td>1.0 ± 0.1</td>
<td>93 ± 5</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>7 days bicarbonate</td>
<td>0.94 ± 0.03</td>
<td>100 ± 6</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>7 days milk</td>
<td>0.54 ± 0.05</td>
<td>56 ± 7</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>7 days saline</td>
<td>1.10 ± 0.02</td>
<td>114 ± 5</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>7 days urine</td>
<td>0.68 ± 0.07</td>
<td>109 ± 8</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>7 days plasma</td>
<td>0.98 ± 0.08</td>
<td>108 ± 5</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>7 days DI water</td>
<td>1.24 ± 0.09</td>
<td>103 ± 9</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>28 days bicarbonate</td>
<td>0.74 ± 0.03</td>
<td>124 ± 8</td>
<td>1.56 ± 0.06</td>
</tr>
<tr>
<td>28 days milk</td>
<td>0.43 ± 0.07</td>
<td>51 ± 4</td>
<td>1.36 ± 0.08</td>
</tr>
<tr>
<td>28 days saline</td>
<td>0.85 ± 0.06</td>
<td>110 ± 6</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>28 days urine</td>
<td>0.50 ± 0.02</td>
<td>103 ± 10</td>
<td>1.10 ± 0.05</td>
</tr>
<tr>
<td>28 days plasma</td>
<td>0.92 ± 0.05</td>
<td>77 ± 7</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>28 days DI water</td>
<td>1.06 ± 0.03</td>
<td>111 ± 2</td>
<td>2.0 ± 0.1</td>
</tr>
</tbody>
</table>

In Fig. 6c, the SEM micrograph shows globular, calcium-rich (by EDS) deposits coating nearly all of the exposed nanofibers even after rinsing. Those nanofibers that are visible appear to have a smaller diameter following exposure to urine, resulting in the largest weight loss of all the in vitro solutions, even though these calcium-rich deposits are present.
Figure 5. Percentage changes in the weight of electrospun PCL samples following 28 days of the indicated exposures.

Figure 6. (a) Matrix adsorption to the milk-exposed PCL following 28 days of exposure. (b) Matrix adsorption to the bovine plasma-exposed PCL following 28 days of exposure. (c) PCL nanofibers exposed to bovine urine for 28 days. An extensive globular coating of calcium-rich deposits is present. (d) PCL nanofibers exposed to saline for 28 days. In the absence of obvious deposition, small segmental defects are visible (circled) that at least partially account for the observed decreases in strength.
3.4. Crystallinity Changes

The percent crystallinity for the control sample was determined to be 52%. After 28 days in vitro exposure, the amount of crystallinity detected versus exposure was: urine 31%, saline 27%, milk 34%, plasma 40%, bicarbonate 56% and de-ionized water 53%.
Figure 9. SEM micrograph of the “back side” of electrospun PCL after 24 h of serum flow-through followed by the application of 80% strain.

Figure 10. SEM micrograph of explanted electrospun PCL showing the extensive matrix deposition characteristic of in vivo exposures.

Table 2.
Average pH values from in vitro solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>After 3 days</th>
<th>Initial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bicarbonate</td>
<td>10</td>
<td>7.9</td>
</tr>
<tr>
<td>Milk</td>
<td>6.4</td>
<td>6.6</td>
</tr>
<tr>
<td>Saline</td>
<td>7.9</td>
<td>4.7</td>
</tr>
<tr>
<td>Urine</td>
<td>9.6</td>
<td>8</td>
</tr>
<tr>
<td>Plasma</td>
<td>8.8</td>
<td>7.5</td>
</tr>
<tr>
<td>Water</td>
<td>7.7</td>
<td>6.6</td>
</tr>
</tbody>
</table>

4. Discussion

The concepts behind the hydrolysis and degradation of bulk PCL and other biodegradable polymers are widely agreed upon. However, the effects of biodegra-
dation on electrospun fibers are relatively novel. PCL is relatively slow to degrade versus other absorbable polymers; this has undoubtedly been a factor in its broad use [1–15], as it provides a relatively topographically and chemical stable platform upon which to culture mammalian cells, but the behavior of this polymer in vivo in a nanofiber form is not well understood. Being able to predict this behavior and the resulting effects on mechanical properties would constitute a significant step toward the use of PCL nanofiber tissue scaffolds in vivo.

The varying degrees of degradation in the tensile properties of the electrospun PCL observed in this study appear to follow classic biomaterials trends expected based on the environment. The rich array of hydrolase enzymes present in milk, lipoprotein lipase, plasmin and alkaline phosphatase [23] has the expected effects on the tensile properties, decreasing the strength and elongation to approximately half that of the control. The most basic environment, urine exposure (pH 8.0), produced the second-lowest values of tensile strength. The standard interpretation is that sufficient hydrolysis occurred during exposure to have affected the tensile properties. Any basic environment should catalyze a greater degree of hydrolysis (relative to a neutral environment) by cleaving the ether/ester groups along the PCL main chain to produce various oligomers and the corresponding hydroxy acid, caproic acid [24].

The large PCL sheets demonstrated weight loss (Fig. 5) that corresponds to the trends in tensile property degradation (Fig. 2). The greatest weight loss was seen following exposure to urine (33%), followed by bicarbonate (20%), saline (18%) and DI water (15%). The corresponding UTS reductions were urine (61%), bicarbonate (43%), saline (34%) and DI water (18%). The exceptions to this trend are the milk and plasma exposures. This apparent discrepancy between loss of strength and weight gain in the milk and plasma solutions can be explained by adsorption of specific components of the in vitro environment to the surface of the nanofibers. The SEM micrograph in Fig. 6a shows milk proteins adhered to the nanofiber surface, while Fig. 6b shows the strong adherence of plasma proteins. The large variance in the weight data for the milk samples could be due to loosely adhered proteins that were washed away either during the exchange of solutions every three days or during post-test rinsing. In the case of exposure to plasma, the proteins appear to be better adhered to the nanofiber surface and, thus, result in a more consistent weight gain.

The large weight loss and decline in tensile properties in the urine samples suggests that a large degree of biodegradation occurred in the highest pH environment. Accordingly, the bicarbonate exposure also resulted in significant reductions in both weight and tensile strength. Biodegradation of electrospun PCL can be driven by not only enzymatic activity (present during the milk exposure), but also by pH that plays its usual role in driving hydrolysis forward.

Supporting the trends in UTS degradation, the in vitro exposures also resulted in the same trends in modulus reduction. After 28 days in vitro, urine exposure lead
to a modulus decrease of 67%, milk 59%, bicarbonate 53%, saline 51%, \textit{in vivo} 48%, DI water 38% and plasma 29%. Since tissue cells are now thought to sense matrix modulus \cite{25–27}, changes in modulus after exposure could affect the behavior of adherent cells. One reason why the milk and plasma exposures do not result in exactly the same trends of modulus reductions seen for UTS is the adsorption of proteins into/onto the nanofiber matrix. These adsorbed proteins create a deposition-induced inhibition of nanofiber rearrangement and essentially make the scaffold ‘stiffer’. This effect is most notable in the plasma samples as the proteins appear to be more strongly adhered to the scaffold (Fig. 6b).

The effect of protein adsorption is also manifest in elongation to failure (Fig. 3). After 28 days \textit{in vitro}, systems in which deposition occurs show clear decreases in elongation: milk 50%, \textit{in vivo} 42% and plasma 24%. In contrast, elongation to failure increases in systems that do not experience deposition: saline 8%, DI water 9%, bicarbonate 21%. The exception to this trend is urine, where the elongation essentially remains unchanged possibly due to a combined effect of deposition (Fig. 6c) and degradation.

Previous extension data \cite{20} established a clear baseline for the behavior of electrospun nanofiber sheets. These nanofibers undergo substantial alignment that varies in accordance with the amount of applied strain. Clearly, the process of elongation at least partially relies on extensive reorganization \cite{20} of initially randomly oriented nanofiber. This also provides some insight into how the deposition observed in Fig. 6a–c might influence elongation. Furthermore, such extensive rearrangement clearly has the potential to compromise the integrity of adherent mammalian cells.

To establish that biological deposition alone can truly affect the mechanical behavior of electrospun scaffolds, we recirculated fetal bovine serum at a rate of 600 ml/h at 37°C through the electrospun sheet for a period of 24 h. This resulted in substantial physical deposition, but was a short enough exposure to expect little, if any, degradation of the polymer itself. The UTS decreased by 22%, the elongation to failure 9% (not significant) and the modulus 55%. These results show that biological deposition alone can substantially decrease mechanical properties.

To provide further insight into how biological deposition affects tensile response, we looked at the failure surface of the tensile samples in the SEM. Large sections of adhered proteins envelop the fibers and prohibit generalized rearrangement (Figs 7 and 8). Fiber extension/alignment is localized between regions of extensive deposition, suggesting that failure likely occurs in these areas rather than more generally. As a result, fewer fibers are able to align and bear the load, lowering the UTS and elongation to failure. Figures 7 and 8 were taken from the “front side” of the serum flow-through configuration that experiences relatively extensive deposition as the electrospun mesh acts as a filter. Conversely, Fig. 9 shows the “back side” of the serum flow-through sheet that sees considerably less deposition. Fixed in place at 80% elongation, the fibers show alignment but not to the extent demonstrated pre-
viously [20]. Quantification of the fiber alignment from this work was not possible due to the significant amount of biological deposition.

This suggests that not only can PCL biodegradation affect the mechanical properties, but that protein deposition, extracellular matrix production and cell adhesion can, as well by inhibiting fiber rearrangement and alignment. This effect will likely become more dominant as the fibers themselves become progressively weaker through biodegradation. During the relatively short time-scale of this in vitro study, only decreases in UTS and modulus are observed. However, looking at potentially longer time scales relevant to tissue engineering, it is possible to imagine that as the cellular network and native tissue formation is developed, mechanical property degradation will be reversed and begin to overcome the effects of polymer degradation.

Prior literature [24] makes it clear that, as these nanofibers break down, their size dictates that they will be phagocytosed internally by macrophages due to their relatively small initial diameter. In the in vivo SEM image (Fig. 10), however, we see no clear evidence of PCL particle formation; in fact, soft tissue invasion partially obscures the nanofibers themselves making any observation regarding integrity difficult. However, given that the amount of mechanical property degradation is similar to that observed in both urine and milk, it is reasonable to assume that the underlying structure undergoes similar levels of physicochemical degradation partially responsible for the observed property decreases. This suggests that these in vitro solutions are a good prediction of in vivo mechanical behavior for electrospun PCL scaffolds during this relatively short time period.

XRD was used to determine the crystallinity of the meshes after exposure. Zeng et al. [4] demonstrated that crystallinity can increase with biodegradation. This was believed to be possible due to degradation occurring primarily in the amorphous domains and because PCL has a relatively modest cold crystallization temperature. The latter could allow PCL crystals to grow at the expense of amorphous PCL during in vitro exposure at 37°C. The results of the DI water and sodium bicarbonate exposures appear to be consistent with this observation. After sodium bicarbonate exposure, the crystallinity of electrospun PCL appeared to increase while the macroscopic UTS decreased by nearly half. However, for bulk polymers, as percent crystallinity increases, strength increases. This suggests that the macroscopic properties of these scaffolds are not directly related to traditional molecular-scale interactions, but are instead dominated by microstructural interactions involving fiber-based structures.

In contrast, decreases in crystallinity are observed after exposure to urine, saline, milk and plasma. This suggests that other mechanisms of degradation can operate within these high surface area materials. Random chain scission could attack chains within crystals, decreasing the $T_m$ and resulting in losses of crystallinity at 37°C. Such material changes could conceivably play a role in the macroscopic softening, decreased strength, modulus and increased elongation, observed in these scaffolds after exposure.
5. Conclusions

The net effects of exposure to various biological and non-biological environments on electrospun structures following 7 and 28 days of in vitro exposures are readily apparent. All the exposures employed lead to a net decrease in the UTS and modulus of electrospun fiber. Trends in strength reduction were somewhat as anticipated: de-ionized water had the least effect, while exposures to milk and urine produced the greatest decreases in strength. The ‘classic’ biomaterials view, i.e., that polymer hydrolysis is the sole determinant of property degradation, does not fully account for the observed behaviors; a deposition-induced inhibition of nanofiber rearrangement exists and is significant. Some exposures lead to a gain in mass resulting from this biological deposition. Such processes clearly have the potential to compete with each other to control the mechanical properties of electrospun structures. By providing a baseline morphological characterization of the response to chemical and mechanical stresses, the effects of both biological infiltration and biochemical exposures can be further understood and tailored for specific tissue engineering applications. This work establishes that non-hydrolytic, non-enzymatic mechanisms of biological activity can have negative effects on the properties of electrospun fiber matrices. The opposite issue, how the fibers influence the viability and activity of adherent cells, is of even greater importance and will be addressed in future studies.

Acknowledgements

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