PROTEIN ADSORPTION ON BIODEGRADABLE MIKRO/NANOFIBRES MATERIALS FOR TISSUE ENGINEERING

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Abstract

Due to their specific properties, nanofibrous materials are increasingly used in regenerative medicine and tissue engineering. Nanofibrous materials can be used as tissue scaffolds for injured (damaged) tissue. The main factor for tissue scaffolds is their biocompatibility. One of the main factors influencing the organism's physiological response is the interaction of the material with proteins. Proteins adsorbed on the material's surface give the tissue scaffolds a "biological identity" [1]. Cells in the organism subsequently interact with proteins adsorbed on the material's surface and determine the entire organism's response to the implanted material. This work deals with the influence of the morphology and chemical composition of polyester nanofibrous materials on the adsorption of proteins. The materials produced by electrospinning (DC spinning) were characterised from the point of view of morphology and wettability. Then, the adsorption of weakly and strongly bound proteins on the fibre surface was evaluated. Cell adhesion and proliferation on the tested materials were also observed. The results of protein adsorption were compared with the results of cell adhesion and proliferation to determine the effect of the amount of adsorbed proteins on the interaction of cells with the tested materials.

Keywords: Nanofibers, protein adsorption, cell adhesion

1. INTRODUCTION

Tissue engineering is a constantly developing multidisciplinary field of science combining material science, engineering, molecular biology and medicine. Methods and production of tissue carriers (scaffold) and drug carriers are constantly evolving. Polymeric materials widely used for constructing carriers intended for the regeneration of soft tissues or the delivery of drugs have enormous variability in their chemical composition, which can be controlled to a certain extent according to the desired properties of the given carrier and in the production possibilities [3].Material biocompatibility is the most important parameter for implanting a material into the human body. The property of the material determines biocompatibility to integrate into the organism and fulfil a supporting and regenerative function to repair damaged tissue effectively. At the same time, biocompatibility determines whether the material triggers an unwanted inflammatory reaction in the body or not. The main factor influencing the resulting biocompatibility of the material is the adsorption of proteins on its surface [2]. Adsorption of proteins is the first interaction with the tissue scaffold after implantation into the organism. Adsorption of proteins occurs in the first seconds after contact with body fluids (blood and its derivatives). Adsorbed proteins give the material a so-called biological identity [1].

In addition to monitoring the overall composition of protein layers, which can consist of a whole range of proteins, attention is paid to the resulting structure of proteins after adsorption to the surface of materials. The interaction of proteins with materials is most widespread for nanoparticles [2].

This work focuses on analysing nanofibrous materials with different morphologies and chemical compositions. The weakly and strongly bound proteins were analysed using bovine serum albumin (BSA) as a model protein. [4].

2. MATERIALS AND METHODS

2.1. Materials

For preparation of nanofiber layer were used biodegradable polyesters, Poly-ε-caprolactone (PCL, Mw 80000, Sigma-Aldrich), Poly-ε-caprolactone (PCL, Mw 45000, Sigma-Aldrich), Poly(lactide-co-ε-caprolactone) (PLCL, PLC 7030, Purasorb) and Polylactic acid (PLA, Mw 45000-55000, Polyscitech). The solvent system for the polymer solution was chloroform with ethanol (ratio 8:2). Materials were prepared by DC electrospinning by Nanospider™ NS 1WS500U (Elmarco).

2.2. Morphological and physical analysis

The morphology of the materials was evaluated using scanning electron microscopy (Vega 3, Tescan). Fiber diameters were measured from these images. In addition, the materials were evaluated for their weight per square meter and specific surface area. Area weight was measured by weighting square samples 5x5 cm. Each material was measured in four times. The specific surface area was measured by Brunauer-Emmett-Teller (BET, Autosorb iQ-KR/MP, Quantachrome) analysis by nitrogen absorption and desorption from the surface of the samples.

A microtensiometer (Krüss) was used to measure the wettability of the materials, which was used to determine the sorption of water into the material samples for 10 minutes. Every sample was prepared in a rectangle 3x4cm. The sample was clamped in the jaws of the device, which are clamped on very sensitive scales. Then, the sample is brought close to the liquid level. Sorption of the liquid was determined as a weight gain as a function of time.

2.3. Protein interaction

Materials for testing protein interaction were prepared in square 2x2 cm. Four samples were incubated from each material with a model protein solution and one with PBS as negative control. Every sample was weighted. After weighing, the sample was placed in a 1.5 ml microtube. A solution of the model protein was BSA (50 mg/ml) dissolved in PBS (pH 7.4). The sample with solutions (proteins and pure PBS) was incubated at 37 °C for 1 hour. During incubation, proteins from the solution could adsorb on the surface of tested materials. After incubation, every sample was washed in 1 ml PBS at room temperature (RT) for 5 minutes. This solution contains capillarity and weakly bound proteins from materials. Then, materials were put in 1ml desorption solution, 1% SDS in PBS, and incubated at RT for 1 hour. This solution has strongly bound proteins. The solution with weakly or strong proteins was analysed by spectrophotometry and SDS-PAGE. The scheme of the experiment is shown in **Figure 1**.

Quantification of weakly proteins was analysed by Bradford solution by spectrophotometry. Strongly bounded proteins were quantified by Quant-it protein assay kit. A different method was used to quantify strongly bound proteins due to the presence of SDS from the desorption solution. SDS interacts adversely with Bradford's reagent. To verify the measured data, a separation method using SDS-PAGE was performed. A 10% polyacrylamide gel separated the molecules according to size. The protein molecules bound in the separation gel were subsequently stained in a Coomassie blue solution.

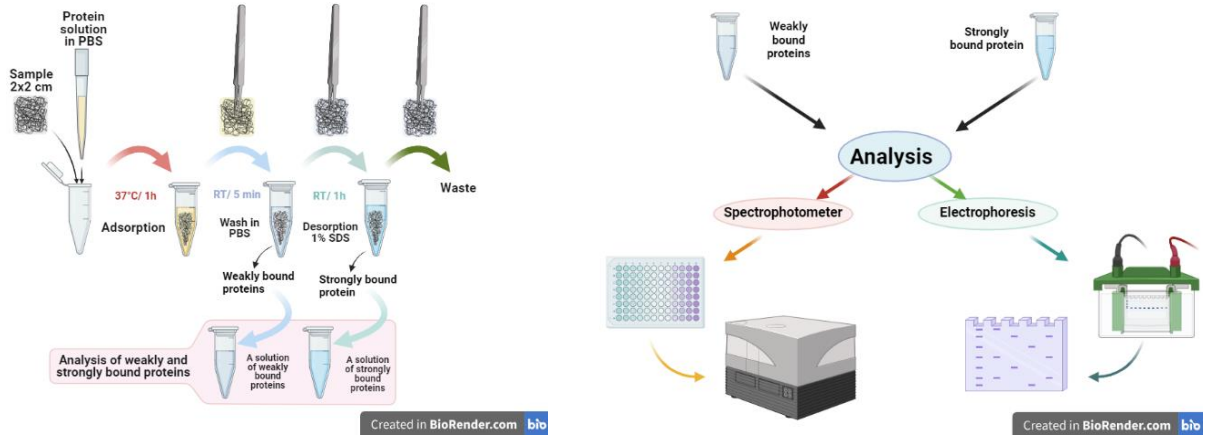


Figure 1 Schema testing protein interaction with biodegradable mikro/nanofibrous materials. Online program BioRender created schema.

2.4. Cell adhesion

The cell line for testing used 3T3 mouse fibroblasts. Medium for incubation was DMEM High Glucose (Dulbecco's Modified Engles Medium, Merck) with 10% Fetal Bovine Serum (Biosera), 1% glutamine (Biosera, Czech Republic) and 1% antibiotic - Pen / Strep Amphotericin B (Lonza). From each material, 6 sample circles with a diameter of 1.5 cm (24-well plates) were prepared. Five samples were seeded by cells, and 1 sample was incubated in pure DMEM as a negative control for each test material. The concentration of cells seeded on the materials was $1*10^4$ cells/ml. Samples were incubated with cells for 1 and 3 days in a CO_2 incubator at 37 °C (Heracell Vios 160i, Thermo Fisher Scientific). For the evaluation of cell adhesion and proliferation on materials, the CCK metabolic test (spectrophotometry analysis). Cells adhered to the surface of the tested samples were fixed using 2.5% glutaraldehyde. For fluorescence microscopy (Axio Observer, Zeiss), cells were stained with phalloidin-FITC (actin filaments in the cytoplasm) and DAPI (DNA in the nucleus). On the materials, the morphology of the cells was monitored.

The software Graph-Pad Prims 8 was used for statistic evaluation.

3. RESULTS AND DISCUSSIONS

In this work, different types of biodegradable polyester nanofibrous material were prepared by DC electrospinning (Nanospider). The materials produced were similar in terms of areal weight. The PCL45 material showed the lowest fibre diameters (**Figure 2C**); the highest fibre diameters were measured for the PLCL material. PCL80 and PLCL materials had nearly comparable fibre diameters. The morphology of the nanofibrous layers is depicted in **Figure 2**. The specific surface area of the materials agreed with the measured fibre diameters. As the fibre diameters in the material decreased, the specific surface area measured by BET analysis increased. The PLA material had the highest specific surface. This is due to the surface structure of the fibres. In the case of PLA material, the produced fibres are not smooth but have tiny pores on the surface of the fibres, which increases the overall specific surface of the material (**Figure 2B**). PCL45, PCL80 and PLCL materials had smooth fibres. The measured values of individual morphological analysis are summarised in the table in **Figure 3**.

Figure 4A describes the liquid sorption experiment in the test samples. The table in **Figure 4B** shows the average values of the mass of water absorbed in the individual materials, converted to the mass of 1g of the sample. The average wettability of the individual tested materials is shown in the graph in **Figure 4C**. There are statistically no differences in liquid sorption for PCL80, PLCL and PLA materials. There was a statistically significant difference only in the case of the PCL45 material with significantly lower wettability. The PCL45

material showed significantly the lowest wettability compared to the other materials after 10 minutes of liquid sorption testing. The low sorption of the liquid could be related to the polymer's hydrophobic nature and the fibres' thickness, where the PCL45 material has lower fibre diameters. The PLA material is relatively hydrophilic; therefore, the measured liquid sorption is also the highest. The difference in sorption between PCL80 and PLCL materials can be related to the specific surface area of the materials. Both polymers show a hydrophobic character. Therefore, different behaviours during wetting will be related to the morphology of the materials.

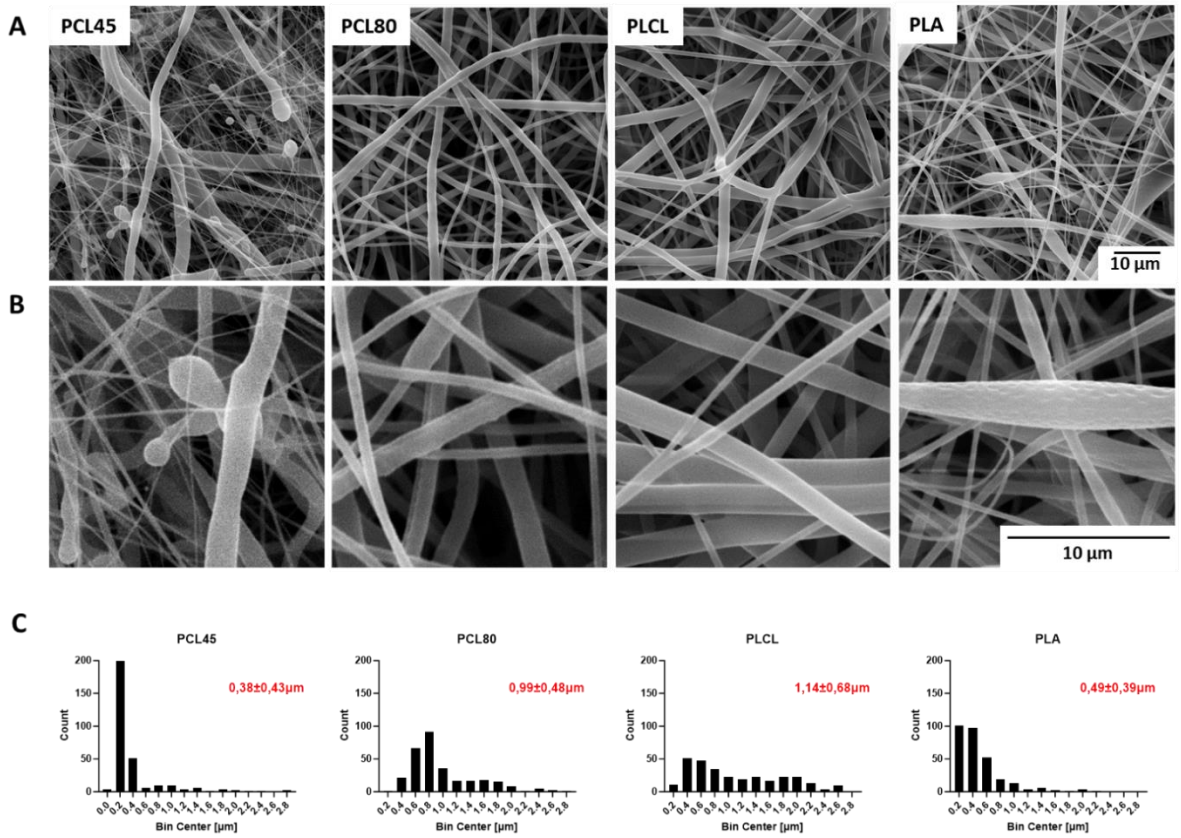
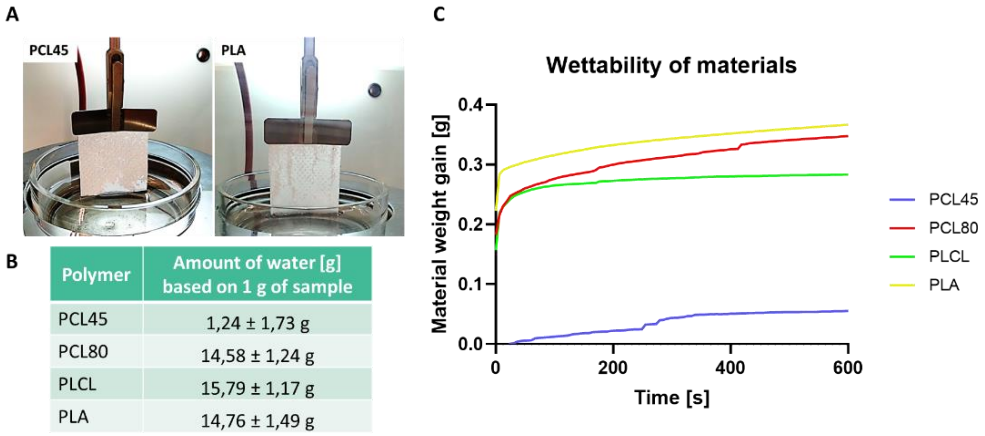


Figure 2 Scanning electron microscope images of fibres prepared DC spinning from biodegradable polyesters: PCL45, PCL80, PLCL and PLA (A) and fibres detail of these materials (B). The scale bar is 10 µm. A histogram of the frequency of measured fibre diameters is shown for each material (C).

Marked	Polymer	Fibre diameter [µm]	Areal weight [g/m²]	Specific surface area [m²/g]
PCL45	Polycaprolacton (Mw 45 000)	0,38 ± 0,43	20	1,82
PCL80	Polycaprolacton (Mw 80 000)	0,99 ± 0,48	22	1,40
PLCL	Poly(lactide-co-ε-caprolactone)	1,14 ± 0,68	20	1,00
PLA	Polylactic acid	0,49 ± 0,39	21	2,20

Figure 3 The table containing the results of the morphological analysis.

The issue of wetting of electrospun layers is dealt with by Szewczyk at all. [5]. The authors proved that from the surface point of view, the materials retain their hydrophobic character even with a change in surface morphology, e.g. foil or fibre. The results of their work show that the wetting of electrospun materials is closely related to roughness and fibre fraction.



B

Polymer	Amount of water [g] based on 1 g of sample
PCL45	1,24 ± 1,73 g
PCL80	14,58 ± 1,24 g
PLCL	15,79 ± 1,17 g
PLA	14,76 ± 1,49 g

Figure 4 Pictures of the liquid sorption experiment in the PCL45 and PLA samples (A). The table with values of the mass of water absorbed in the individual materials converted to the mass of 1g of the sample (B). The course of the curve wettability for each material is shown in graph (C).

The amount of proteins adsorbed on the materials were analysed spectrophotometrically by the Bradford method (weakly bound proteins), Quant-it protein assay kit (strongly bound protein) and also using SDS-PAGE (**Figure 5**). The graph in Figure 5A shows the calculated values of the weakly and strongly bound BSA. The obtained solutions of protein molecules were also analysed using SDS-PAGE (**Figure 5B**). The images of the separation gels show the difference in the amount of weakly and strongly bound BSA protein (approx. 66 KDa [7]) on the individual materials. The results of the SDS-PAGE analysis correspond with the results of the spectrophotometric analysis.

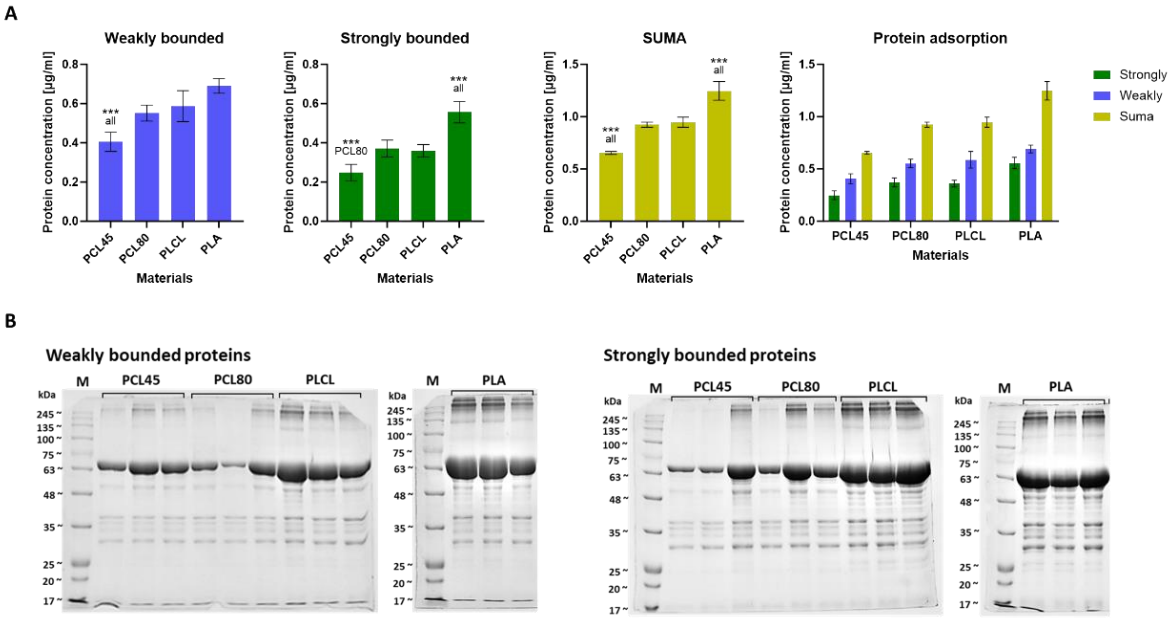


Figure 5 The graph describes weakly bound proteins, strongly bound proteins, their sum and their comparison with each other (A). Images of electrophoretic records of SDS-PAGE separation gels of weakly and strongly bound proteins (B).

The results show that the amount of weakly and strongly bound proteins is closely related to the chemical composition of the material and its morphological and physical properties. PLA material adsorbed the most weakly and strongly bound proteins, while PCL45 adsorbed the least proteins. The morphology of the materials

is closely related to the adsorption of proteins on the tested materials. The materials PCL80 and PLCL, morphologically similar but differ in chemical composition, show comparable amounts of adsorbed proteins. For the materials PCL45 and PCL80, which are chemically comparable, it is evident that lower fibre diameters (PLC45) negatively affect protein adsorption. The influence of fibre diameters for strongly bound proteins is more apparent, where the amount of strongly bound proteins significantly differed from PCL80. Depending on the internal conformation of the molecules, the BSA protein binds to the surface of materials using hydrophobic interactions or is attracted by electrostatic forces (especially for hydrophilic materials) [8]. When comparing the materials PCL45 (hydrophobic material) and PLA (hydrophilic material), which had a similar morphology, a significant difference is evident, especially for strongly bound proteins. The PLA material showed a significant difference in strongly bound protein with all materials.

The materials were incubated for 3 days with the 3T3 mouse fibroblast cell line. The samples were taken after 1 and 3 days from the seed of the cell suspension on the tested materials. The metabolic activity of the cells was monitored using a spectrophotometric CCK-8 assay kit. The graph shown in **Figure 6A** describes the metabolic activity of cells on individual materials. Samples of materials with adhered cells were subsequently fixed using glutaraldehyde solution and staining for fluorescence microscopy. Images from each material after 1st and 3rd day after seeded cells are shown in **Figure 6B**. From the individual images of the cells captured in the image, the morphological distribution of the cell line on the surface of the materials is evident. It can be seen from the pictures that after the 1st day of incubation, the cells adhered more willingly on the PLCL and PLA material. After the 3rd day of incubation, an increase is visible on all materials. The pictures also show the difference in the number of cells on the individual materials. Microscopic analysis corresponds to cell viability results measured by the CCK-8 assay.

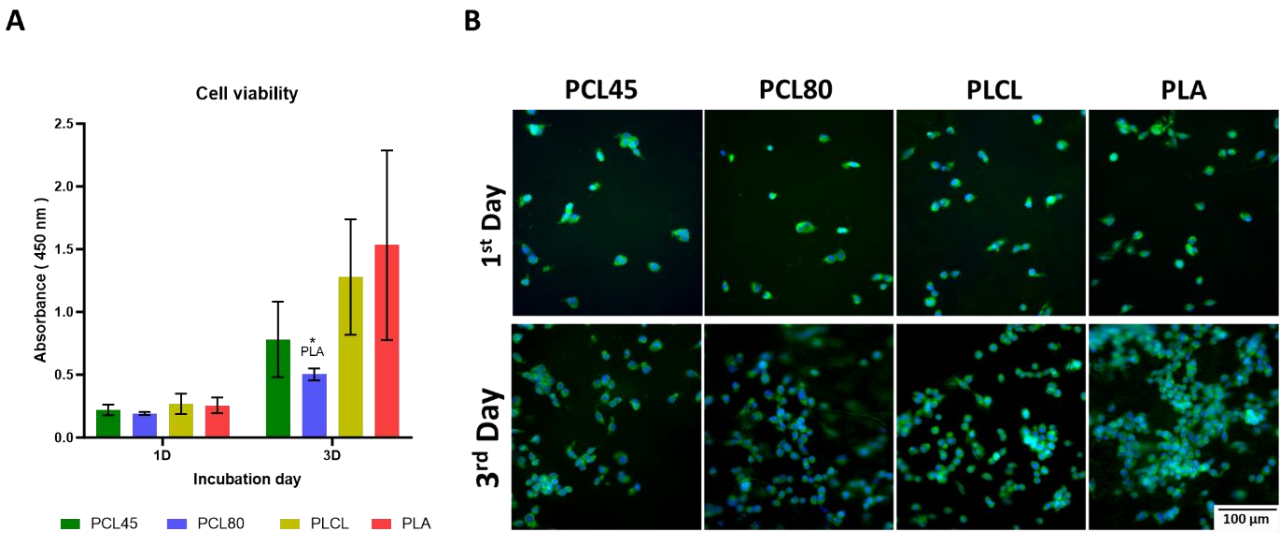


Figure 6 The graph describes the metabolic activity of cells adhered to the tested materials after 1st day and 3rd days from seeding cells on the materials (A). Images of Fluorescence microscopy analysis of cells on the materials after 1st day and 3rd days incubation (B).

The results of the experiments show that the amount of weakly and strongly bound proteins is related to the chemical and physical properties of the produced nanofibrous layers. With a higher specific surface, the wettability of the material increases and thus the amount of adsorbed proteins. However, it is evident from the results that both wettability and subsequent protein adsorption are closely related to the chemical composed and surface and morphological properties of the material. This is evident in PCL45 and PCL80 materials. It is the same polymer with a different molecular weight. Here, the morphological influence on the wettability and adsorption of proteins is most evident. PCL45 material with a lower fibre diameter is very reluctant to wet and

the protein interaction is the lowest. However, the results do not clearly link the amount of weakly and strongly bound proteins and cell adhesion. For PCL80, PLCL, and PLA materials the results show that cell adhesion increases with higher amounts of weakly and strongly bound proteins. However, this trend was not clearly demonstrated for the PLC45 material. More cells adhered to the PCL45 material than to the PCL80 material. It is therefore likely that cell adhesion is not only related to the amount of weakly and strongly bound proteins but to other parameters like morphology.

4. CONCLUSION

As part of this work, the influence of the morphology and chemical composition of polyester fibre materials on protein adsorption and cell adhesion was studied. Fiber materials from degradable polyesters were used for testing: PCL45, PCL80, PLCL and PLA. Areal weight, fibre diameters and specific surface area morphologically characterized the materials. The lowest fibre diameters and the highest specific fibre surface were measured for PCL45 and PLA. Materials were tested for wettability using liquid sorption into the sample structure. Only the PCL45 material, which sorbed the liquid the least, was statistically different. The other materials were not significantly different from each other.

As part of biological testing, protein adsorption and cell adhesion were studied. Protein adsorption was investigated using the model protein BSA. The amount of weakly and strongly bound proteins adsorbed on the materials was determined. Overall, the most significant amount of proteins adsorbed on the PLA material, where strongly bound proteins were most represented simultaneously. Weakly and strongly bound proteins adsorbed the worst on PCL45 materials. In in-vitro testing with 3T3 mouse fibroblasts, CCK testing focused on cell viability after 1st and 3rd days after seeding cells. Results showed the highest cell viability 1st and 3rd day for PLA material. The lowest cell viability was in PCL80. Differences between cells which adhered to the materials are apparent on 3rd day, with significant differences for PCL80.

For further characterization of the materials from the point of view of protein interaction and cell adhesion and proliferation, it would be appropriate to prepare the tested materials with different fibre morphology and repeat the experiment.

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