J Mater Sci: Mater Med (2008) 19:257–267
DOI 10.1007/s10856-006-0048-3

Three-dimensional nonwoven scaffolds from a novel biodegradable poly(ester amide) for tissue engineering applications

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Received: 5 February 2006 / Accepted: 20 November 2006 / Published online: 28 June 2007
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Abstract Biodegradable polyesters are established biomaterials in medicine due to their chemical characteristics and options for material processing. A main problem, however, is the release of acid degradation products during biodegradation with severe local pH-drops and inflammatory reactions. Polyesteramides, in contrast, show a less prominent pH-drop during degradation. In this study, we developed a simple, reproducible synthesis of the poly (ester amide) (PEA) type C starting from ε-caprolactame, 1,4-butanediol, and adipic acid in a one-batch two-step reaction and conducted the manufacturing of PEA-derived 3D textile scaffolds applicable for tissue engineering purposes. The thermal and mechanical properties of PEA-type C were analysed and the structural conformity of different batches was confirmed by NMR spectroscopy and size exclusion chromatography. The polymer was formed into nonwovens by textile manufacturing. Cytotoxicity tests and X-ray photoelectron spectroscopy (XPS) were used to analyze the effect of scaffold extraction before cell seeding. The manufactured carriers were seeded with human preadipocytes and examined for cellular proliferation and differentiation. The production of PEA type C successfully occurred via simultaneous ring-opening polymerization of ε-caprolactame and polycondensation with 1,4-butanediol and adipic acid at 250 °C under high-vacuum. Soxhlet extraction allowed optimal cleaning of nonwoven scaffolds. Extracted PEA-derived matrices were capable of allowing good adherence, proliferation, and differentiation of preadipocytes. These results are encouraging and guidance towards an optimally prepared nonwoven carrier applicable for clinical use.

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Introduction

Polymers are established biomaterials in several clinical applications due to their chemical characteristics and options for material processing, combinations and modifications. Besides the classification into homo- or copolymers, synthetic or natural polymers, the division into absorbable and nonabsorbable polymers is essential. The option for absorption opens new fields for biomaterials since these carriers only remain within the biological system for a certain period of time. Biodegradable polymers need to have chemical bonds which can be cleaved by hydrolytical or enzymatical mechanisms. Today, poly(glycolic acid) (PGA) and poly(lactic acid) (PLA) are by far the most commonly used synthetic polymers in tissue engineering. These polymers are also extensively utilised in other biomedical applications such as drug delivery. They have a long history of use in humans since their introduction as suture materials in the 1970s and particularly in regard to

their FDA approval for a variety of applications [1]. However, low thermal, mechanical, and processing performances greatly restrict the practical use of these materials [2]. An additional disadvantage of PLA and PGA in tissue engineering applications is their release of acidic degradation products by a bulk degradation mechanism and the subsequent loss of mechanical properties very early during in vivo degradation [3]. Such acidic degradation metabolites could lead to undesirable pH changes in cellular microenvironment in long-term organotypic cultures [4, 5]. Polyesteramides, in contrast to many other synthetic, biodegradable polyester-based biomaterials, show a less prominent pH-drop during degradation. Furthermore, the addition of amino acids as monomer component allows the introduction of functional groups in the polymer which create specific surface modifications. Research on poly (ester amides) (PEA) has been conducted with the objective of incorporating an amide linkage into the backbone of hydrolytically labile polyesters. The amide moiety enables interchain linkage via hydrogen bonding for producing filaments with improved fiber strength and durability. PEAs are not yet available as commercial biomaterial, however, they are the subject of ongoing development [6–8] and have shown promising results so far. Because of their low immunogenity, good biocompatibility, and degradability, they may soon be widely used in pharmaceutical and other medical applications, such as sutures, implants, and temporary matrices or scaffolds in tissue engineering.

In the last years, research efforts allowed the development of a new class of bioabsorbable co-poly(ester amides) with adjustable profiles concerning thermal, mechanical and hydrolytical properties of the scaffold [9, 10]. The objectives of this present study were (i) to establish a simple and reproducible synthesis of the novel poly(ester amide) PEA type C starting from ε-caprolactame, 1,4-butanediol, and adipic acid; (ii) to process the raw polymer material into filaments, staple fibers and subsequently into nontoxic three-dimensional nonwoven scaffolds as long-term biodegradable matrices; (iii) to analyze cell adherence, proliferation, and cellular differentiation capacity of preadipocytes on nonwovens with defined voids for optimal spatial and nutritional conditions for cell maintenance. Preadipocytes, adipose-tissue-derived progenitor cells, were used for seeding of scaffolds since these cells are capable of differentiating into a variety of cell types, including osteoblasts, chondrocytes, endothelial cells, myoblasts, neuron-like cells, and adipocytes [11–13]. Therefore, they are a promising material for tissue engineering of bones, cartilage, muscle, fat and other mesenchymal tissue types. Our experimental design focused on the application of preadipocytes for soft-tissue defect reconstruction since successful long-term treatment of these defects remains an unresolved problem in plastic and reconstructive surgery.

Materials and methods

Reagents

ε-Caprolactame, 1,4-butanediol, and adipic acid were purchased from Merck (Germany). The catalyst titanium(IV)isopropoxide was a product of Lancaster Synthesis (UK) and LiCl was purchased from Sigma-Aldrich Chemie GmbH (Germany). Isopropanol was obtained from Fluka Chemie GmbH (Germany) in a bottle with crown cap over a 4-Å molecular sieve. Resomer® R203 S (Poly(D,L-lactide)) was purchased in medical grade quality by Boehringer Ingelheim Pharma GmbH & Co.KG (Germany). Collagenase solution type I, M199, Dulbecco's modified Eagle medium (DMEM), RPMI-1640 medium, Ham's F12 (F12), and fetal calf serum (FCS) were from Biochrom AG (Germany). Trypsin/EDTA was purchased from PAA Laboratories GmbH (Germany). Basic fibroblast growth factor (bFGF) was from Tebu GmbH (Germany). All other reagents, chemicals, and solvents were of best quality and purchased from diverse conventional suppliers.

Synthesis of PEA-type C

The PEA type C was synthesised from ε-caprolactame, 1,4-butanediol, and adipic acid (compare Fig. 1) in a custom-built batch reactor equipped with a condensor, destillation bridge, and a stirrer, designed for mixing of media of higher viscosity and therefore combined with a viscosity-controller. ε-Caprolactame (2 mol) and adipic acid (0.8 mol) were filled into the reaction chamber and dried over night under high vacuum (1×10^{-3} mbar). The reaction mixture of this one-batch two-step reaction was accomplished by adding 1,4-butanediol (1 mol) and 1% titanium(IV)isopropoxide solution in isopropanol. The reaction mixture was stirred and heated up to 250 °C immediately. When the temperature at the head of the column had decreased to 80 °C the first time, the reaction was proceeded under high vacuum conditions. From that moment on, the viscosity of the reaction mixture was controlled continuously. The reaction was completed after approximately 4 h. The hot and low viscous polymer mixture was filled into aluminium cups and cooled down to room temperature which created a colorless and hard solid polymer. The polymer was milled to powder, dried under high vacuum, and stored under nitrogen at 4 °C.

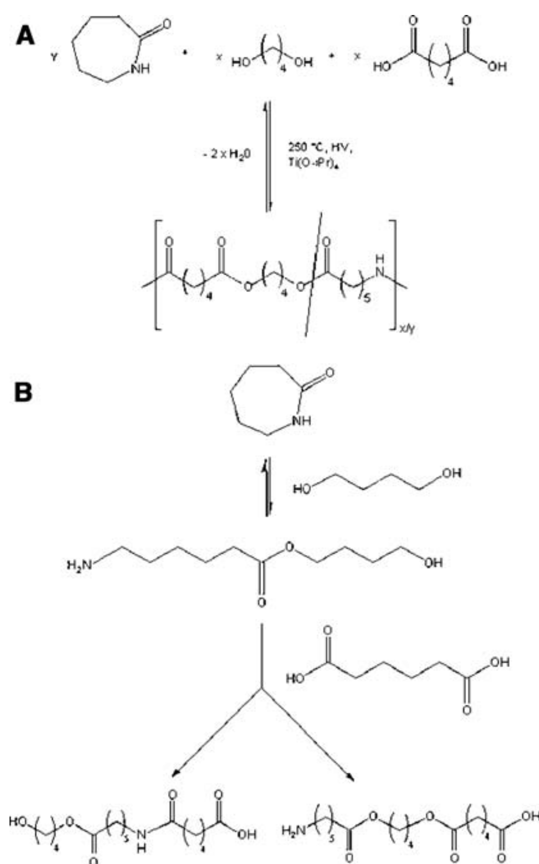

Fig. 1 Scheme of the PEA type C synthesis from ε-caprolactam, adipic acid, and 1,4-butanediol. (**A**) The synthesis of the novel PEA type C was carried out in a solvent-free continuously running two-step process. (**B**) The reaction includes simultaneous ring-opening polymerisation of ε-caprolactam and the polycondensation of the resulting 6-amino-hexanoic acid esters with 1,4-butandiol and adipic acid, respectively. The reaction was achieved under high vacuum conditions and high temperature to remove water and alcohol respectively

Preparation of poly(D,L-lactide) films

Poly(D,L-lactide) (PDLLA) films were prepared by a casting method. PDLLA powder (10 g) was dissolved in 60 ml chloroform and 68 g of the 10 wt% polymer solution were casted into a PTFE mold. The mold was placed on a table with integrated water-level. PDLLA films with a size of 18 cm × 18 cm (l × w) and a thickness of 270 µm were produced. The mold was covered and connected to a vacuum line to slowly remove chloroform by a controlled evaporation process over 48 h. Quality control of PDLLA films concerning chloroform residual content was carried out by solid phase micro-extraction (SPME) and subsequent gas chromatography mass spectroscopy analysis on a GC 3800 + MS Saturn 2000 from Varian GmbH (Germany). Specimens of 20 mm × 20 mm (l × w) and a thickness of 270 µm were prepared as reference by cutting.

Characterization of the poly(ester amide) PEA type C

Gel permeation chromatography (GPC) analyses were conducted for polymer solutions in *N,N*-dimethylacetamide (DMAc) containing 0.2% (w/v) LiCl (0.8 mL/min flow rate) with a Bischoff 2200 HPLC system (pump and autosampler) equipped with a refractive-index detector (Waters, Germany) and Jordi columns (MZ-Analysentechnik, Germany). Polystyrene standards from PSS (Polymer-Standard-Service, Germany) were used for calibration. NMR spectra were recorded with an INOVA-400 NMR spectrometer operating at 399.97 MHz and 100.57 MHz for ^{1}H and ^{13}C NMR investigations, respectively. Dimethyl sulfoxide (DMSO)-d_6 was used as solvent while tetramethylsilane was applied as an internal standard. The thermal behavior of PEA type C was investigated with a PerkinElmer DSC-7 (USA) at a heating rate of 20 °C/min in a nitrogen atmosphere. The intrinsic viscosity η of PEA type C was measured in *m*-cresol at 25 °C and set into the ratio with η_0 of the solvent. The measured parameters are the flow rates of defined volumes of the polyesteramide and of the solvent itself. X-ray photoelectron spectroscopy (XPS) was recorded on a KRATOS ANALYTICAL AXIS HSi 165 Ultra (UK). Scanning electron micrographs were carried out with a Hitachi S-3000N. The mechanical properties were analysed on a Zwick Z2.5/TN1S WN:144029 (Germany).

Analysis of the degradation behavior of PEA type C

The degradation behavior of the polymer PEA type C was determined under physiological conditions in PBS (pH 7.4) at 37 °C. Therefore, films were melt pressed and the specimens (20 mm × 20 mm (l × w), thickness: 80 µm) were placed in the PBS buffer containing sodium acid (0.03 wt%) as a microbial growth inhibition. Films were placed in small Duran™ bottles (Schott, Germany) filled with buffer and incubated at a temperature of 37 ± 1 °C and shaking speed of 150 rpm. Each sample was periodically taken out from the solution as indicated and carefully analysed after drying under reduced pressure.

Manufacturing of nonwoven scaffolds

Nonwovens made of the novel bioabsorbable PEA type C were produced at the *Institut für Textiltechnik* (ITA) of the RWTH Aachen University. Before the nonwovens were formed, the polymer was extruded by melt-spinning and subsequent stretching to fabricate filaments. To minimise adhesion between the filaments and the mechanical units of the spinning machine and between the filaments themselves, silicon oligomers were applied. The filament production was carried out by Membrana GmbH

(Obernburg, Germany), part of Polypore International Inc. (USA). Next, the filaments were cut into fibers. After dissolving the crimped staple polymer fibers, the material was formed into nonwovens by textile manufacturing techniques. In order to achieve webs without a main fiber orientation, the aerodynamic web formation process was used. To achieve a thickness of approximately 4.5 mm and an open porous structure, eight unbonded webs (each 2.5 g and 80×210 mm^2) were piled up. Since no thermal, chemical or humid bonding techniques could be employed to avoid contamination or accelerated degradation of the polymers, the needle felting technique was applied. To needle the thin and brittle fibers, fine needles were applied. The stitching density amounted to 350 stitches/cm. For cell seeding experiments, nonwovens were provided as cylinders (diameter = 20 mm, height = 4.6 mm; area mass = 540 g/m^2) with an open porous structure (compare Fig. 5).

Cleaning and sterilization of nonwoven scaffolds

The nonwoven scaffolds were Soxhlet-extracted continuously in hexane/isopropanol (9:1; v/v) for 6 h and finally dried at 50 °C in vacuum. Scanning electron microscopy (SEM) (compare Fig. 6) and X-ray photoelectron spectroscopy (XPS) (compare Table 2) were used to analyze the scaffolds before and after extraction to examine whether the cleaning process could successfully remove contaminations. Measured in XPS was the element composition of carbon (C1s 285.0 eV), oxygen (O1s 532.5 eV), nitrogen (N1s 102.5 eV), and silicon (Si2p 102.5 eV).

Vacuum-dried nonwovens were stored in tissue-culture polystyrene (TCPS) dishes, which were explicitly designed to avoid contaminations by polymer additives. Furthermore, they were intended to prevent a significant build-up of hydrocarbon and other contaminations during storage. The cleaned and packed substrates were stored at 4 °C until use. Before application in in vitro cell culture experiments, the samples were sterilised by gamma-irradiation (BGS, Germany). The energy dose on the sample surface amounted to 23.9×10^3 Gray (Gy).

Cytotoxicity tests with L929

In vitro cell culture experiments concerning the indirect cytotoxicity of PEA type C were carried out with L929 murine fibroblasts. Cells were routinely cultured in RPMI-1640 medium supplemented with L-glutamine and 10% FCS at 37 °C and 5% CO_2, and used for experiments between the 3rd and 8th passage. Cytotoxicity tests to screen the degradation products of PEA type C for indirect cytotoxicity were performed during storage of the scaffolds in medium over 52 days. Cell viability was analysed with the two fluorescent dyes fluorescein diacetate (FDA), a permeable substrate of cell membrane esterases, and impermeable propidium iodide (PI), which binds to DNA by intercalating between the bases.

Isolation, culturing, and seeding of preadipocytes on nonwovens

Preadipocytes were isolated out of fragments freshly obtained from human subcutaneous adipose tissue of healthy donors who had undergone elective operations (e.g. abdominoplasties) at the Department of Plastic Surgery and Hand Surgery—Burn Center. Fibrous tissue and visible blood vessels were removed; adipose tissue was minced and digested by collagenase CLS type I 0.2% at 37 °C for 45 min under constant shaking. The ratio of tissue to enzyme was 1:1. Digestion was stopped by adding two volumes of DMEM/F12 medium (1:1) supplemented with 10% FCS. After filtration through a sieve (pore diameter 250 μm, Verseidag Techfab GmbH, Germany), the fat layer was removed and the cell suspension was centrifuged again. Finally, the cell pellet was seeded on tissue culture dishes with DMEM/F12 (1:1) plus 10% FCS with a seeding density of 3×10^4 cells/cm^2. Preadipocytes of the second passage were trypsinised at confluence and used for seeding of scaffolds.

Preparation of nonwovens for in vitro analyses

Scaffolds were either precoated with fibronectin or directly used for seeding. Inoculation with different amounts of cells was performed as indicated. Cellular growth, expansion, and differentiation of preadipocytes on the nonwovens were carried out as following: Human preadipocytes were seeded on scaffolds in DMEM/F12 (1:1) medium supplemented with 10% FCS, and 10 ng ml^{-1} bFGF [14], 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. Medium was changed every third day. For differentiation analyses, adipogenic conversion was promoted for 21 days by changing medium to DMEM/F12 (1:1) without serum addition, supplemented with 66 nM insulin, 100 nM dexamethasone, 0.5 mM IBMX, 0.1 μg ml^{-1} pioglitazone, 1 nM triiodo-L-thyronine, and 10 μg ml^{-1} human transferrin. After 5 days of incubation, medium was used as before but without IBMX and pioglitazone until histological evaluation after 21 days.

Determination of three-dimensional growth of preadipocytes on nonwovens

To monitor preadipocyte proliferation and viability on nonwovens, the MTT assay was chosen. Nonwovens were

inoculated with preadipocytes, and formazan formation by the composites was analysed 6 h and 9 days after seeding. As control one scaffold for each time point was cultured without cells. To measure formazan formation biohybrids were transferred to a 24-well plate and washed twice with PBS^{2-}. Each composite was then treated with 5 mg/ml MTT in a cell incubator for 45 min at 37 °C. Thereafter all probes were washed again prior to extraction with isopropanol/0.04 M HCl for 3 h. Extraction was completed by vortexing and centrifugation for 5 min at 13,500 rpm. Supernatant was used in a 1:4 dilution with isopropanol/0.04 M HCl and extinction was analysed in a microtiter plate (570 nm).

Histological evaluation

For histological examination, the cell-loaded and control scaffolds were fixed in 4% buffered formaldehyde solution, embedded, vertically sectioned, and stained. The formalin fixed pieces were dehydrated in ascending series of alcohols and embedded in paraffin. Tissue slices of 45 µm were prepared, stained with hematoxylin–eosin and DAPI, and analysed under the microscope at a magnification of 200 and 400, respectively.

Statistical evaluation

Data of overall cellularity in the scaffolds (MTT-test) and biodegradation (weight residues) were expressed as mean value ± SD. The significance of differences was evaluated by the *t*-test for in vitro analyses of scaffolds. For biodegradation, differences at $p < 0.05$ were considered significant (*), $p < 0.01$ highly significant (†).

Results

Characterization of the polymer

The poly(ester amide) (PEA) type C was synthesised via a continuously running two step synthesis in a custom-built batch-reactor by simultaneous ring-opening polymerization of ε-caprolactame and polycondensation with 1,4-butanediol and adipic acid at 250 °C under high-vacuum in the presence of titanium(IV)isopropoxide as a catalyst (Fig. 1). The synthesis was confirmed by ^{1}H and ^{13}C NMR spectra shown in Fig. 2. The relative viscosity η_{rel} of PEA type C and all of its thermal and mechanical properties are summarised in Table 1. η_{rel} values of different PEA type C batches were in the range of 2.2 and 2.4. The number-average molecular weight M_n was estimated to be 61.000. The calorimetric analysis demonstrated during the first heating run a broad melting transition with two predominant melting points at 57.5 °C and 122.6 °C. During the cooling run, a crystallization point of 57.5 °C was measured. Stress–strain measurements at 22 °C delivered a breaking elongation ε_R of 703%, a maximum of tensile strength σ_{max} of 31 MPa and an E modulus of 84 MPa (compare Table 1).

Degradation behavior of PEA type C

The biodegradability of film specimens, monofilaments and staple fibers of PEA type C was estimated from the weight loss caused by hydrolytic cleavage of the polymer chains. Poly(D,L-lactide) (PDLLA), a well-known and often applicated degradable biomaterial, was selected as control. Figure 3A shows the weight loss of film specimens of PEA type C in comparison to PDLLA samples of the same size over a period of 168 days. After 168 days, the PEA probes showed only a weight loss of around 5%. In the case of PDLLA, a significant mean weight loss of around 10% was measured. Analyzing the biodegradation of PEA type C melt pressed film versus monofilament versus staple fibers (Fig. 3B), we found that after 168 days of incubation, the industrially manufactured monofilaments as well as the staple fibers showed little, but significant weight loss of around 10% in contrast to the melt pressed specimens of PEA type C which revealed a weight residue of 98%. A change in pH level was also investigated. After 150 days of degradation, a strong and significant decrease of the pH values of the solutions with PDLLA probes was observed whereas PEA type C showed no significant change in pH level (data not shown). Comparing the breaking elongation between PEA type C and PDLLA, we found the breaking elongation ε_R of PEA type C to be 703% at the beginning,

Table 1 Thermal and mechanical properties of PEA type C

Polymer	η_{rel}	T_g (°C)	T_{m1} (°C)	T_{m2} (°C)	T_c (°C)	ε_R (%)	σ_{max} (Mpa)	E (Mpa)
PEA type C	2.3	–25.3	57.5	122.6	57.5	703	31	84
PDLLA	0.3	55	[a]	[a]	[a]	3–10	4–48	1.500–3.200

The PEA type C was analysed as described in ''Methods'' to determine thermal and mechanical properties. Analysed were relative viscosity η_{rel}, glass transition temperature T_g, melting points (T_{m1} and T_{m2}), crystallization temperature T_c, breaking elongation ε_R, maximum of tensile strength σ_{max}, and E modulus

[a] PDLLA is amorphous. The values of the mechanical properties of PDLLA are from literature [15, 16]

decreasing continuously and reaching an ε_R value of 240% after 70 days (compare Table 1; PDLLA: ε_R = 5–10% [17].

Manufacturing of three-dimensional nonwoven structures of PEA type C

In order to fabricate three-dimensional nonwoven structures, the novel bioabsorbable PEA type C polymer was extruded by melt-spinning and subsequently stretched to fabricate filaments. The filaments were then cut into fibers, dissolved, and formed into nonwoven structures by an aerodynamic web formation (Fig. 4A) and the needle felting technique (Fig. 4B, C). To achieve a thickness of approximately 4.5 mm and an open porous structure, several webs were piled up and connected to each other by needles punching into the web. The obtained block structure (Fig. 5A) was cut into cylinders (Fig. 5B) for cell culture experiments.

Nonwoven scaffolds before and after extraction

To extract degradation products and to remove both lipophilic and hydrophilic biological as well as synthetic substances from the nonwoven cylinders, a cleaning and decontamination protocol was developed. Samples were analysed before and after extraction by means of scanning electron microscopy (SEM) and X-ray photoelectron spectroscopy (XPS). SEM images of the PEA type C nonwoven samples before (Fig. 6A) and after (Fig. 6B) the extraction process demonstrate significant differences when inspecting the fiber surface. The fibers have a very smooth and clean surface after the extraction process. This was confirmed by chemical analyses when measuring the molecular amounts of oxygen, nitrogen, carbon, and silicon atoms in the samples (Table 2). Unextracted samples contain high concentrations of silicone revealed by high amounts of silicon in XPS. Soxhlet-extracted carriers, in contrast, are completely free of silicon residues.

Cytotoxicity test with L929 fibroblasts on PEA type C

Cytotoxicity analyses were performed according to ISO 10993-5/EN 30993-5 with untreated nonwovens as well as with degradation products from scaffolds that had been released to culture medium after incubation for 16 h to 52 days. L929 fibroblasts were stained with FDA/PI for viability analysis. No cytotoxic reaction of the L929 cells in contact with the PEA type C materials was observed, neither with extracted nor with unextracted nonwovens. Furthermore, the degradation products of the different specimens of PEA type C showed no cytotoxic effects (data not shown).

Table 2 Elemental composition of the PEA type C scaffold surfaces before and after extraction

	PEA type C unextracted [atom-%]	PEA type C extracted [atom-%]
Carbon	55.00	74.3
Oxygen	21.98	18.4
Nitrogen	–	7.3
Silicon	23.02	–

Unextracted and extracted PEA type C were characterised by means of XPS for elemental composition and ratios of carbon (C1s 285.0 eV), oxygen (O1s 532.5 eV), nitrogen (N1s 102.5 eV), and silicon (Si2p 102.5 eV)

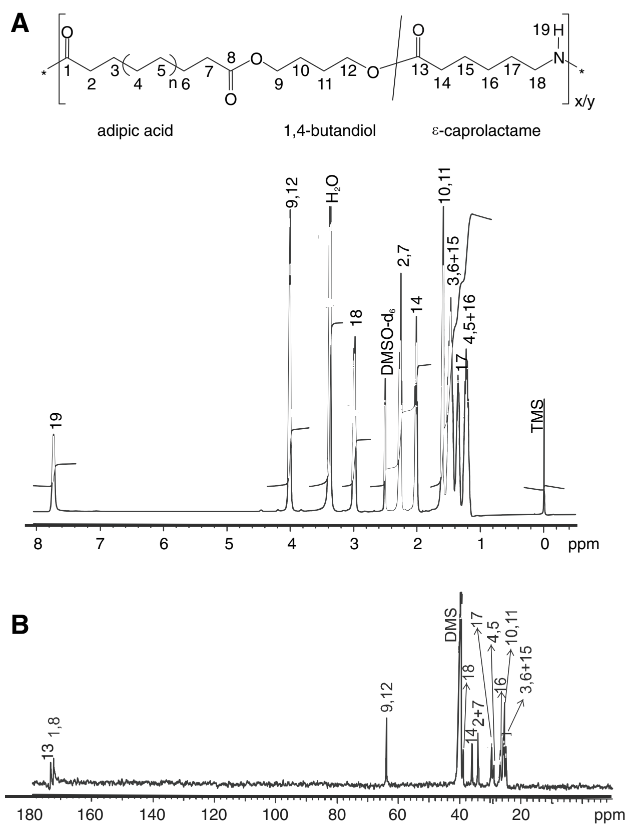

Fig. 2 NMR spectra of PEA type C. Shown here are the ^{1}H NMR and ^{13}C NMR spectra of PEA type C in DMSO-d6. (**A**) ^{1}H NMR: δ in ppm: 7.74 (s, 1H, 19); 4.02 (m, 4H, 9,12); 3.00 (m, 1H, 18); 2.27 (m, 4H, 2, 7); 2.03 (m, 2H, 14); 1.64 (m, 4H, 10, 11); 1.49 (m, 6H, 3, 6, 15); 1.35 (m, 2H, 17); 1.19 (m, 2H, 16). (**B**) ^{13}C NMR: δ in ppm: 173 (1C, 1); 172 (2C, 13, 8); 64 (2C, 9, 12); 39 (1C, 18); 36 (1C, 14); 34 (2C, 2, 7); 30 (1C, 17); 27 (1C, 16); 26 (2C, 10, 11); 26–25 (3C, 3, 6, 15)

In vitro examination of preadipocyte-loaded nonwovens

Preadipocytes of the second passage were trypsinised at confluence and used for seeding of nonwovens. Scaffolds were either precoated with fibronectin and then loaded with

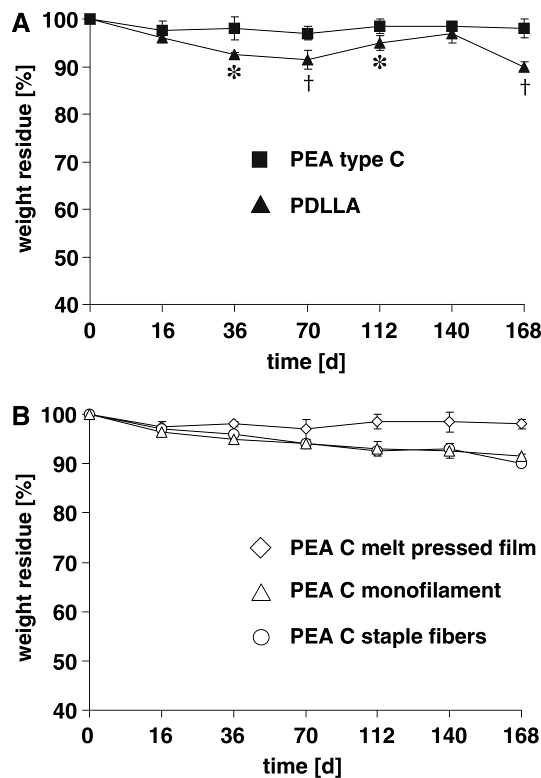

Fig. 3 Biodegradation behavior of PEA type C. The biodegradability of film specimens, monofilaments, and staple fibers of PEA type C was estimated from the weight loss caused by hydrolytic cleavage of the polymer chains. Poly(D,L-lactide) (PDLLA), a well known and often applicated degradable biomaterial, was selected as control. (**A**) Shown is the change in weight (weight residue given as mean ± SD, $n = 6$) of film specimens of PEA type C (■) versus PDLLA (▲) during hydrolysis under physiological conditions over a period of 168 days. Data refer to three individual experiments. Differences at $p < 0.05$ were considered significant (*), $p < 0.01$ highly significant (†). (**B**) Given is the weight residue (mean ± SD, $n = 6$) of different specimens of PEA type C during hydrolysis in PBS (pH 7.4) at 37 °C over a period of 168 days. (◇) melt pressed film, (△) monofilament, (○) staple fibers. Data are from three individual experiments

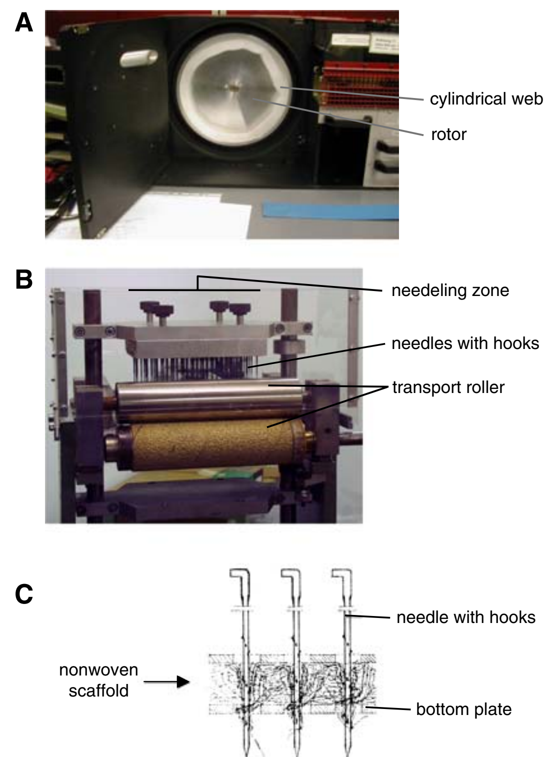

Fig. 4 Technical manufacturing of nonwoven three-dimensional structures**.** Shown here are two important steps of the manufacturing procedure of three-dimensional nonwoven scaffolds. In order to achieve webs without a main fiber orientation, the aerodynamic web formation process was used. Shown in (**A**) is the plant to produce webs. Staple fibers are fed to the combing roll. The isolated fibers are transported in the rotor and form a cylindrical web. Since no thermal, chemical, or humid bonding techniques can be employed to avoid contamination or accelerated degradation of the polymers, the needle felting technique was applied (**B**, **C**). The needle loom punches into the web. The needles pull down single fibers with their hooks (**C**). The friction locks the fibers in this position and thus increases the density and stiffness of the nonwoven

cells or directly used for seeding without fibronectin addition. After 1 week, scaffolds were microscopically analysed for cell adherence and proliferation on carriers. In addition, three-dimensional proliferation on the nonwovens was evaluated enzymatically with MTT assay. We find preadipocytes evenly distributed on the nonwovens. The tight attachment of the cells is especially obvious in the fluorescence images (compare arrows in Fig. 7A, B). Comparing attachment of cells with versus without fibronectin 6 h after seeding, we find that fibronectin binds approximately 150% cells more to the nonwoven matrix than observed with standard treatment ($p < 0.001$, compare Table 3). However, preadipocyte proliferation on the scaffolds (analysed after 9 days) is quicker in the absence of fibronectin than with fibronectin ($p < 0.001$).

For preadipocyte differentiation, all matrices were coated with fibronectin. Scaffolds were analysed 3 weeks after initiation of differentiation. As can be seen from bright field and fluorescence images (Fig. 7C–F), fibronectin-coated nonwovens allow good differentiation of the adipogenic precursor cells. Especially in areas where cells appear as clusters, complete morphological transformation from a thin, needle-like structure to a round, spheroidal form can be seen.

Discussion

This study aimed at developing a simple and reproducible synthesis of the poly(ester amide) (PEA) type C starting from ε-caprolactame, 1,4-butanediol, and adipic acid and to conduct the manufacturing of PEA-derived three-dimensional

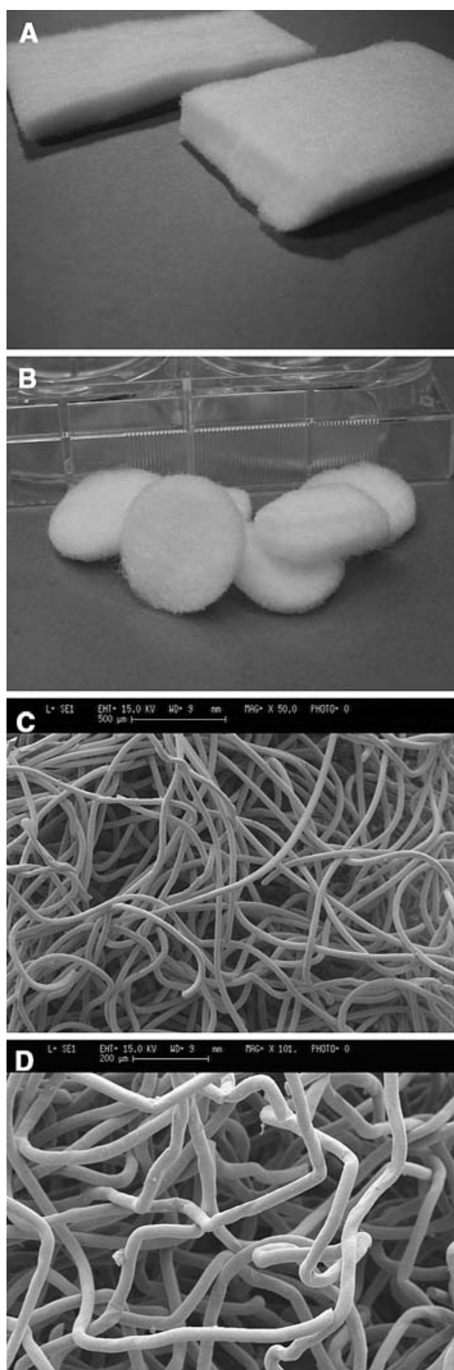

Fig. 5 PEA type C nonwoven scaffolds at various magnifications. (**A**, **B**) Macroscopical view of PEA type C nonwoven structures before (**A**) and after (**B**) preparation of discs forms (diameter: 20 mm, heigth: 4.6 mm, area mass: 540 g/m^2). (**C**, **D**) Electron micrographs of nonwoven carriers (**B**: magnification ×50,000, **C**: magnification ×101,000) demonstrate the open porous structure of the scaffold without a main fiber orientation

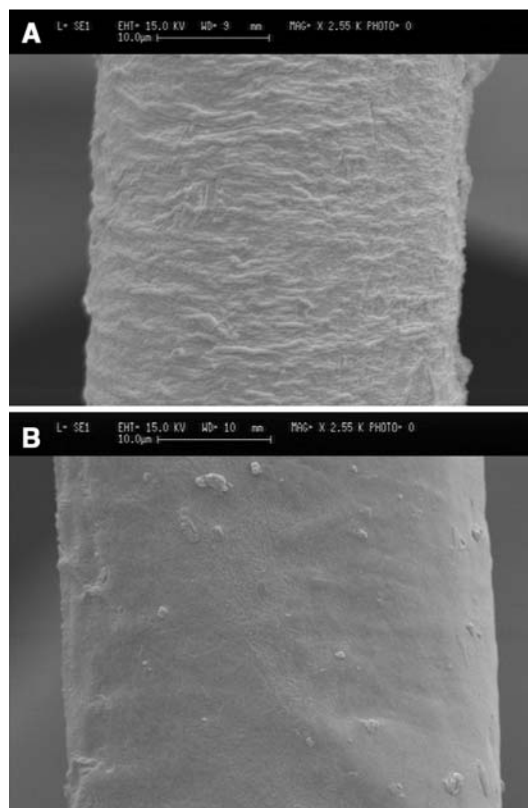

Fig. 6 SEM images of PEA type C nonwoven scaffolds before and after soxhlet extraction. To remove contaminating particles and substances from the nonwovens, extraction of the scaffolds was performed with a mixture of organic solvents. Nonwoven samples were placed in a custom-built Soxhlet extractor and were continuously extracted with hexane/propan-2-ol (9:1; v/v) for several hours. Samples were analysed before and after extraction by means of SEM. Shown in (**A**) is an electron micrograph of an unextracted nonwoven structure. Obvious is the inhomogenous surface of the fiber evidencing various contaminations. (**B**) Electron micrograph after solvent extraction of the contaminated PEA type C nonwovens scaffold. All contaminations on the fiber surface have been removed

textile scaffolds applicable for tissue engineering purposes. Our results demonstrate the successful synthesis of PEA type C in a one-batch two-step reaction using ε-caprolactame, 1,4-butanediol, and adipic acid in a molar ratio of 2:0.8:1. The outline of PEA type C synthesis is illustrated in Fig. 1. The procedure was performed under high temperature and high vacuum to remove reaction water thereby shifting the equilibrium to PEA of higher molecular weight. In the past, it was a main problem to synthesise the polymer in steady quality from batch-to-batch. Large variations in molecular weight, molecular weight distribution, and undesirable side products like oligomers caused problems during the subsequent fiber production. We here minimised the inhomogeneities by stepwise optimising the reactor setup and the synthesis parameters. The overlays of NMR spectra indicated no inhomogeneities. The peaks in the ^{1}H NMR spectrum marked with numbers 1–19 are caused by the corresponding hydrogen atoms of PEA type C (Fig. 2A). PEA type C was synthesised by using adipic acid as dicarboxylic component. Its methylene protons are marked with the

numbers 2,7. Hydrogen atoms 9,12 correspond to the methylene protons of 1,4-butanediol, and the methylene protons 14 and 18 to ε-caprolactame. At the beginning of the reaction, the N–H protons of ε-caprolactame appeared as a signal at 7.4 ppm. A few minutes afterwards, a new distinct peak was detected at 7.8 ppm (19, singlet, N–H). This peak shift to a lower field from 7.4 ppm to 7.8 ppm caused by three electronical effects (hybridisation, anisotropy, and electronical inductivity) is an expected result of introducing a C=O double bond to form carbonamide bonds. According to the stoichiometry of the synthesis, the theoretical ester content of PEA type C should have been 40 wt%. From calculation by ^{1}H NMR, it was 56 wt%. Additionally, the ^{13}C NMR spectrum (Fig. 2B) demonstrates signals characteristic for polyesteramides, i.e. an amide-carbonyl-carbon structure (13) at 173 ppm and ester-carbonyl-carbon structures (1, 8) at 172 ppm [2].

To characterise the thermal properties of PEA type C, differential scanning calorimetry (DSC) was applied. The calorimetric analysis of the polymer comprised three DSC runs. During the first heating two predominant melting points at 57.5 °C and 122.6 °C were observed (compare Table 1). Likely, the first melting point characterises the polyester part of the PEA while the melting point at 122.6 °C corresponds to its polyamide portion. Polyamides allow the formation of intermolecular hydrogen bonds which greatly enhance molecular stability. The increased stability of the polyamide part in contrast to the polyester part is reflected in the high second peak of 122.6 °C.

A major aim of this study was also to manufacture PEA-derived three-dimensional textile scaffolds with (i) slower degradation than poly(D,L-lactide) (PDLLA), (ii) less acidic degradation products than PDLLA and poly(L-lactide) (PLLA), and (iii) with higher mechanical stability during degradation than both lactide acid based polymers. Comparing the breaking elongation between PEA type C, PDLLA, and PLLA, we found the breaking elongation ε_R of PEA type C to be 703% at the beginning of the degradation experiments, decreasing continuously over degradation time and reaching an ε_R value of 240% after 70 days at 37 °C in PBS. PDLLA, in contrast, has an ε_R of 5–10% and PLLA of 2–6% [17]. The continuous decline of ε_R of PEA type C indicates a hydrolysis which starts from the surface of the PEA material and not from the bulk as it has been observed for the materials based on lactide and glycolide acid. Bulk degradation leads to an internal increase in acidic degradation products with autocatalytic and accelerated degradation of the material. Therefore, the high ε_R value of PEA type C in comparison to PDLLA and PLLA after 70 days of degradation demonstrates a surface degradation mechanism. Furthermore, the modulus E of PEA type C was measured to be 84 MPa which is 20 times smaller than the modulus E of PDLLA and PLLA [17]. This makes PEA type C a much more flexible but still highly stable material, compared to PDLLA and PLLA.

It is a well-known problem, that polymer materials, e.g. degradable materials, are sensitive concerning further technical processing. Additionally, the manufacturing of monofilaments, their subsequent processing to staple fibers and to nonwoven scaffolds can lead to degradation and numerous contaminations on the surface of the fibers. Therefore, degradation of PEA type C and cytotoxicity of the nonwoven material due to contaminations were investigated. To analyse degradation of PEA type C the weight loss of film specimens of PEA type C in comparison to PDLLA samples of the same size was measured over 168 days (Fig. 3A). After 168 days under physiological conditions, the poly(ester amide) probes showed only a weight loss of around 2%. In the case of PDLLA, a mean weight loss of around 10% was measured, verifying the promising material properties of PEA type C. Analyzing the biodegradation of different shapes of PEA type C, i.e. melt pressed films, monofilaments, and staple fibers, we found a weight loss of 10% at maximum in PBS (pH 7.4) at 37 °C after 168 days (Fig. 3B). This degradation stability of PEA type C is caused by H-bond formation between ester- and amide-segments. For tissue engineering purposes, especially for the generation of adipose tissue, it is important that the supporting matrix does not degrade too rapidly since it has to function as a spacer and allow tissue formation and organization over several months. Our findings of the biodegradability of PEA type C therefore demonstrate the suitability of this novel synthetic polymer for tissue engineering purposes. During degradation of a biomaterial, another very important parameter is the decrease of the pH value. Polyesteramides show a less prominent pH-drop during degradation than other synthetic polymers. This positive property of PEAs could also be demonstrated for the novel PEA type C.

In order to fabricate three-dimensional nonwovens from PEA type C, the polymer was extruded by melt-spinning and subsequently stretched to fabricate filaments. The filaments were cut into fibers, dissolved, and formed into nonwovens by an aerodynamic web formation and the needle felting technique (Fig. 4). The obtained block structure was cut into cylinders for cell culture experiments (Fig. 5). Cytotoxicity analyses were performed with untreated nonwovens as well as with degradation products from scaffolds that had been released to culture medium after incubation for 16 h to 52 days. No cytotoxic reaction of L929 cells in contact with the PEA type C materials was observed, neither with the nonwovens nor with the degradation products of the different specimens of PEA type C. Although no toxicity was observed, it had to be excluded that cell adhesion to the biomaterial was influenced by residues from the manufacturing process. Therefore,

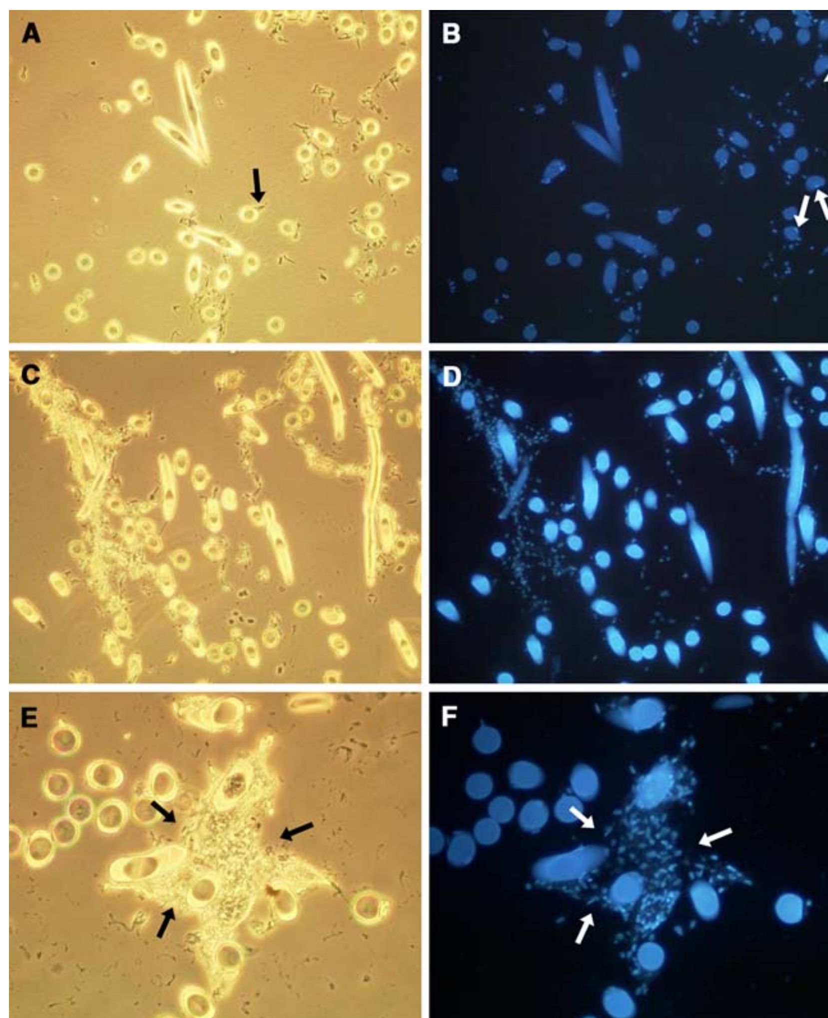

Fig. 7 In vitro examination of preadipocyte-loaded nonwovens. Preadipocytes were isolated out of human subcutaneous adipose tissue and seeded on nonwoven carriers. Scaffolds were either precoated with fibronectin or directly used for seeding. Inoculation with cells was performed in 300 µl culture medium. After 8 h, 2 ml culture medium were added. After 1 week or 3 weeks, respectively, scaffolds were histologically analysed for cell proliferation on carriers. (**A**, **B**) Bright field micrograph (**A**) and fluorescence image (**B**) of uncoated nonwoven scaffolds seeded with 3×10^6 preadipocytes after proliferation for 1 week. Arrows indicate cells which are tightly attached to carrier material. Magnification ×20. (**C**–**F**) For preadipocyte differentiation, inoculation was performed with 8×10^6 cells/ in 130 µl culture medium per scaffold quarter. After 8 h, 2 ml culture medium were added. After 3 weeks, scaffolds were histologically analysed for cell differentiation. Shown are bright field micrograph (**C**, **E**) and fluorescence image (**D**, **F**) of preadipocyte-loaded nonwovens at magnification ×20 (**C**, **D**) and magnification ×40 (**E**, **F**). Indicated by arrows in (**E**) and (**F**) is an aggregation of differentiating preadipocytes

Table 3 Determination of three-dimensional growth of preadipocytes on fibronectin-coated versus uncoated nonwovens

		Number of cells	
		6 h post seeding (%)	9 days post seeding (%)
Number of cells	No fibronectin	100	252.66 ± 31.67
	With fibronectin	242.9 ± 97.76	328.91 ± 46.23

nonwovens were screened for contaminating particles on the surface of the scaffold by Soxhlet extraction. The effectiveness of the extraction was confirmed by means of SEM and XPS. SEM images of the nonwoven samples before (Fig. 6A) and after (Fig. 6B) extraction demonstrate significant differences on the fiber surface. The very smooth and clean surface of the fibers after the extraction process was also confirmed by chemical analyses

measuring the molecular amounts of oxygen, nitrogen, carbon, and silicon atoms in the samples (Table 2). Unextracted samples contain high concentrations of silicone revealed by high amounts of silicon in X-ray photoelectron spectroscopy (XPS). Soxhlet-extracted carriers, in contrast, are completely free of silicon residues. The silicon residues are most likely a product of the manufacturing process since silicon was used to minimise adhesion between the polymer filaments and the mechanical units of the spinning machine and between the filaments themselves. Our results thus demonstrate that the extraction allows a sufficient and reliable cleaning of the fiber surfaces in the nonwoven scaffolds.

Preadipocytes, adipose-tissue-derived progenitor cells, were used for seeding of the nonwoven carriers since these cells are capable of differentiating into a variety of cell types, including osteoblasts, chondrocytes, endothelial cells, myoblasts, neuron-like cells, and adipocytes [11–13]. Therefore, they are a promising material for tissue engineering of bones, cartilage, muscle, fat and other mesenchymal tissue types. This present study focused on the branch of adipogenic differentiation of preadipocytes, since the generation of adipose tissue for successful long-term reconstruction remains a challenging aim.

Our examinations of preadipocyte-loaded nonwovens demonstrate the suitability of PEA type C-based biomaterials for tissue engineering applications since preadipocytes were evenly distributed on the nonwovens with most cells tightly attached to the carrier material (Fig. 7A, B). Fibronectin demonstrates a beneficial effect raising the amount of attached cells by approximately 150% compared to seeding in the absence of this glycoprotein. This is in accordance with the literature which states a higher yield of preadipocytes in the presence of fibronectin, for instance after isolation from adipose tissue [18–20]. Interestingly, when comparing proliferation of preadipocytes over 9 days on fibronectin-coated versus uncoated nonwovens, we find proliferation on the scaffolds to be quicker if cells were seeded without fibronectin. This might be due to a glue-like effect of fibronectin which on the one hand fixes cells but on the other hand limits proliferation activities as well. A solution for this problem could be a lower concentration of fibronectin still allowing to attract the cells to the fibers of the nonwoven without limiting the proliferation activities of the cells. For differentiation of adipogenic precursors, fibronectin was used constantly since previous experiments had demonstrated a clearly beneficial effect on the extend of differentiation [21].

In summary, extracted PEA-derived matrices were capable of allowing good adherence, sufficient proliferation, and differentiation of preadipocytes on the nonwoven structures. These findings are encouraging for further investigations and help to define an optimally prepared PEA-based nonwoven applicable for clinical use.

Acknowledgements This research work is part of a project funded by the ‘‘HOLSTE-Stiftung’’ of the RWTH Aachen University. Additionally, the Federal Ministry of Economics and Labour (BMWA = Bundesministerium für Wirtschaft und Arbeit) and its partner organisation the German Federation of Industrial Research Associations ‘‘Otto von Guericke’’ (Arbeitsgemeinschaft industrieller Forschungsvereinigungen ‘‘Otto von Guericke’’ e.V.) are gratefully thanked for financial support of the research project (AiF-No. 107 ZN/1). The authors are also grateful to the DECHEMA (Society for Chemical Engineering and Biotechnology) for cooperation.

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