Resorbable Polyesters in Cartilage Engineering: Affinity and Biocompatibility of Polymer Fiber Structures to Chondrocytes

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The resorbable polymers polyglycolic acid (PGA) and polylactic acid (PLA) are gaining increasing importance in tissue engineering and cell transplantation. The present investigation was focused on the biocompatibility and cell retaining behavior of PGA/poly-L-lactide (PLLA) (90/10) and PLLA nonwoven structures for the *in vitro* development of chondrocyte–polymer constructs. The effect of the relevant monomers to chondrocytes was analyzed. Type II collagen and poly-L-lysine were compared to improve loading of PGA/PLLA and PLLA polymer nonwovens with chondrocytes. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) test was applied for quantifications. At concentrations above 2 mg/mL, glycolic acid was more cytotoxic than lactic acid. As shown by pH equilibration, the cytotoxic effect is not due merely to the acidity of the α-hydroxy acids. Regarding the degradation products, glycolic acid, and L(+) lactic acid, nonwovens of PLLA are more biocompatible with chondrocytes than nonwovens of polyglycolide. Collagen type II and poly-L-lysine generally improved cell seeding on resorbable polymers in tissue engineering; however, their efficiency varies depending on the type of fiber structure. © 1996 John Wiley & Sons, Inc.

INTRODUCTION

The development and design of structures and delivery substances for 3-dimensional cell cultures and tissue engineering are of increasing importance.[1–4] Conventional organ and tissue transplantation are severely limited by graft versus host reactions, often necessitating permanent immunosuppressive treatments. Autologous cell and tissue transplantation offer promising approaches to overcome these immunological problems. The source of implant material is primarily obtained from the patient. Cells from biopsies can proliferate in efficient culture systems using appropriate growth factors.[5,6] This also opens possibilities for genetic manipulations on isolated cells from the patient before preparation of autologous implants by tissue engineering.[7] Artificial autologous tissues therefore may become important for the repair of severe tissue lesions such as cartilage defects or for the repair of biological functions such as enzyme deficiencies carried out by gene therapy in cell culture. Tissue engineering requires a 3-dimensional arrangement of cells.[3] Conventional culture dishes are not suitable to form tissues like cell aggregates *in vitro*. Suspension of cells in agarose gel was found to improve the expression of the appropriate phenotype.[8] Recently, the use of resorbable polymer structures has been the focus of increasing attention by investigators.[3,9] The resorbable polymers polyglycolic acid (PGA) and polylactic acid (PLA) can be formed to nonwovens with high internal surface tension and broad variations in their mechanical and degradation behavior. Such polymer nonwovens were found to be a promising tool to guide tissue formation *in vivo*[10] and *in vitro*.[3]

This study was designed to evaluate the application of resorbable fiber structures for cartilage engineering. The effect of α-hydroxy acids and the efficiency of suitable adhesion factors, as used in cell culture for preparation of chondrocyte–polymer tissues to engineer autologous cartilage transplants, were analyzed.

MATERIAL AND METHODS

Cell Preparation

Hyaline cartilage was obtained from macroscopically normal femoral heads of humans between 30 and 65 years of age within 12 h of death or from patients undergoing reconstructive surgery. Chondrocytes were isolated using an enzymatic solution as described previously.[11] Briefly, the extracellular matrix of cartilage slices was digested for

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12 to 18 h at 37 °C in the presence of 2 mg/mL type II collagenase (Seromed, Berlin, Germany), 0.1 mg/mL hyaluronidase (Serva, Berlin, Germany), and 0.15 mg/mL DNase II (Paesel, Frankfurt, Germany) in Ham's F12 medium. The resulting cell suspension was filtered through a nylon sieve with a pore size of 88 μm. Chondrocytes were suspended in Ham's F12 with 10% fetal calf serum (FCS) (BRL, Eggenstein, Germany) in a spinner flask for 15 h. The resulting cell suspension was centrifuged at 1500 rpm for 7 min and resuspended in Ham's F12 containing 20% FCS.

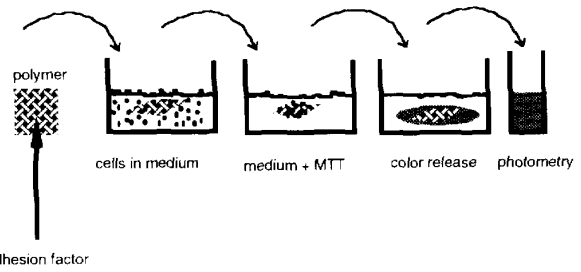

Figure 1. Modified MTT assay to investigate the amount of cells bound in polymer fiber structures.

Cell Lines

The cell lines utilized in the study were HTB 139, TE671 rhabdomyosarcoma (ATCC, USA) and HTB 94 chondrosarcoma (ATCC).

Polymers

In-house polymerized poly-L-lactide (PLLA) was processed at the Institute of Textile and Processing Engineering to nonwovens (V7-2) by the melt spinning process. The strength of the nonwoven was enhanced by needle punching.

The mechanical properties of the nonwovens were determined by tensile test in the wet and dry stage with reference to ISO 5081. The sample size was reduced to 150 × 60 mm to limit the amount of testing material for the degradation study. It was ensured by comparative measurements that this reduction of the size did not change the strength values significantly. For testing in the wet condition the sample was immersed for 1 h in distilled H_2O.

The strength of the nonwoven was calculated maximum force by width and thickness of the probe (cN/mm^2). This did not consider porosity of the nonwoven. The influence of the porosity can be taken into account by dividing the maximum force by weight per unit area ($cN*m^2/g$); this formula contains the specific weight of the material to consider comparing different materials.

The thickness of the samples was measured by a digital micrometer at a pressure of 5.31 cN/cm^2 (531 Pa). The porosity was calculated from the specific weight of the nonwoven related to the specific weight of the polymer.

The degradation of the nonwovens was performed in accordance with ISO-DIS 13781-1995 at 37 °C in phosphate buffered solution of pH 7.4. It was evaluated regarding the loss of strength (tensile test according to ISO 5081) and the decrease of molecular weight by inherent viscosity (Ubbelohde 0c; 0.1% in chloroform, 25 °C). The samples were washed 5 times in distilled H_2O to extract the salts from the nonwovens, which would affect both the strength and the viscosity measurements.

The PLLA nonwoven was sterilized by 250 Gray cesium γ irradiation (Amersham Buchler GmbH, Germany) and stored at −20 °C. Sterile PGA/PLLA (90:10) (Ethisorb 210) nonwovens were provided by Ethicon (Norderstedt, Germany) and stored at −20 °C. Sterile PGA net was provided by B. Braun-Dexon GmbH (Spangenberg, Germany) and was also stored at −20 °C.

Analysis of Monomer, Doses, and Effects

Chondrocytes after the first passage and cell lines were seeded into microtiter plates and cultured with Ham's F12 supplemented with 10% FCS and glycolic acid or L(+) lactic acid with concentrations between 0.001 and 10 mg/mL for 24 h; 10 mg/mL is considered at least as an imaginable local concentration *in vivo* by other investigators.[12] Subsequently the mitochondrial activity was analyzed by the assay.[13]

Evaluation of Degradation Time

Polymer nonwovens, 10 mg/mL, were incubated in RPMI medium/15 m*M* HEPES without serum at 37 °C. The pH of the culture medium was measured after 1, 3, 5, 7, 14, and 28 weeks of incubation.

Cell Attachment Assays

Analysis of attachment onto polymer fiber structures was carried out using a modified MTT assay (Fig. 1).[14] Polymer fabrics (3 × 3 × 2 mm) were dipped into solutions of adhesion factors for 30 min and subsequently lyophilized or air dried overnight. The meshes were placed in chondrocyte suspensions of the second or third passage (6×10^5 cells/mL) in 24-well culture plates, and cells were allowed to attach for 60 or 20 min. Subsequently, the structures were dipped twice in plain culture medium and placed into microtiter wells with medium containing 5 mg/mL thiazolyl blue (Sigma, Munich, Germany) and incubated for 4 h. Then the cells were treated with 20 μL 3% SDS and color crystals were dissolved with 100 μL isopropanol. Absorption at 560 nm was measured using an ELISA-Reader (Titerteck-Multiskan-Plus MK II, Labsystems, Finland).

TABLE I. Properties of Poly-L-Lactide Nonwoven V7-2

	Dry	Wet
Strength (cN/mm^2)	133 ± 16	51 ± 10
Max. force by wt/unit area (cN*m^2/g)	85 ± 7	32 ± 6
Elongation (%)	58 ± 4	55 ± 9
Thickness (mm)	1.97 ± 0.05	
Porosity (%)	91 ± 1	

Statistical Analysis

The Tukey–Kramer multiple comparison test was applied.

RESULTS

The properties of the PLLA-nonwoven V7-2 are found in Table I. The strength of nonwovens is mainly determined by the entangling of the fibers and the fiber strength itself. It is drastically reduced by water compared to the dry material. This is due to reduced fiber/fiber friction by water contact. Elongation in the wet stage is not affected.

The degradation behavior is shown in Figure 2 in comparison to filament materials of the same polymers. The respective original values are set to 100%.

The degradation of the nonwoven and suture braid are similar. Compared with the strength, the molecular weight (measured by inherent viscosity) of the nonwoven decreases faster in the beginning but reaches a plateau in the intermediate stage. Data on the final degradation are still lacking at the moment, but the degradation needs more than 1 year for both structures.

Chondrocytes were exposed to different concentrations of glycolic acid and L(+) lactic acid in monolayer culture for 24 h (Fig. 3). Concentrations up to 1 mg/mL did not significantly affect the mitochondral activity of the chondrocytes. When the chondrocytes were incubated with 10 mg/mL glycolic acid, the activity dropped to almost zero after 24 h. In contrast, 10 mg/mL lactic acid were tolerated by the chondrocytes within this time.

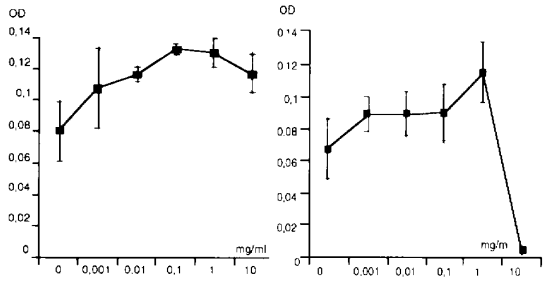

Figure 3. Mitochondral cell activity of chondrocytes after short time exposure (24 h) to L(+) lactic acid (left) and glycolic acid (right).

As a control, the same experiments were done with two cell lines. Compared to chondrocytes, the optical densities were higher indicating a more active metabolism of these cells. Glycolic acid concentrations up to 0.1 mg/mL had no effect on the cells; 1 mg/mL significantly decreased the cell activity whereas 10 mg/mL completely inhibited the activity (Fig. 4, left). With 1 mg/mL lactic acid the cell metabolism is reduced only slightly, but 10 mg/mL also stopped the activity in both cell lines (Fig. 5).

For the analysis of long-term incubation with monomers, chondrocytes were incubated 12 days with three different concentrations in the expected range of interest: 0.5, 1, and 2 mg/mL.

The lower concentrations (0.5 and 1 mg/mL) did not significantly decrease the mitochondral activity of the chondrocytes during 12-day incubation (data not shown). However, 2 mg/mL α-hydroxy acids in the culture medium affected the cell metabolism after 10 days (Fig. 6, left). The decrease was more predominant with glycolic acid than with L(+) lactic acid. These effects are partly due to the acidity of the monomers as shown in Figure 6 (right). When the pH of the medium was adjusted, only glycolic acid lowered the metabolism significantly after 12 days of incubation.

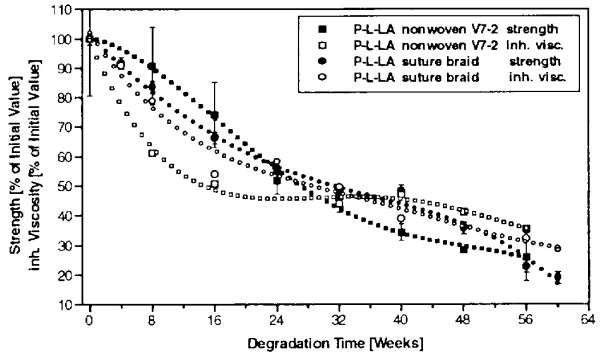

Figure 2. Degradation of PLLA nonwoven compared with suturelike material.

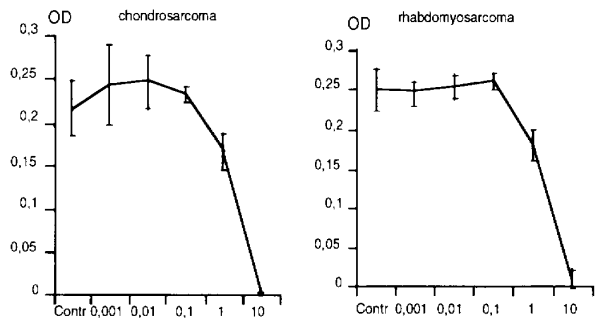

Figure 4. Mitochondral cell activity of two cell lines (rhabdomyosarcoma and chondrosarcoma) after 24-h exposure to glycolic acid. n = 6, mean ± SD.

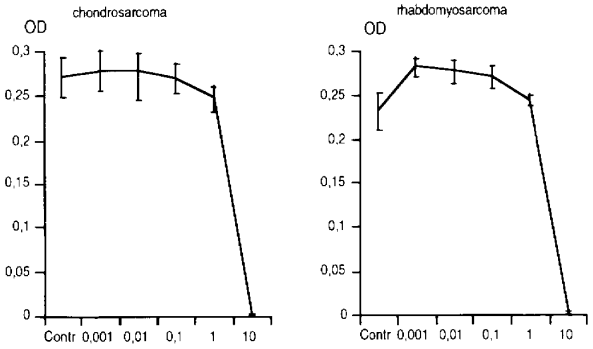

Figure 5. Mitochondral cell activity of two cell lines (rhabdomyosarcoma and chondrosarcoma) after 24-h exposure to L(+) lactic acid. $n = 6$, mean ± SD.

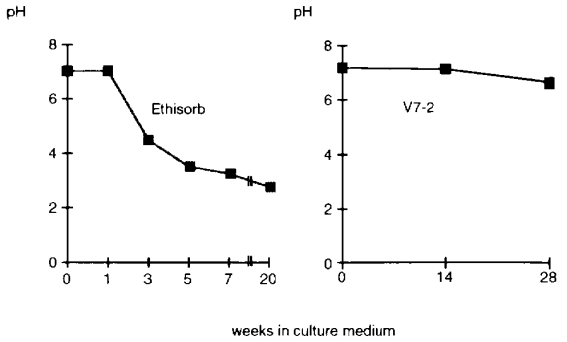

Figure 7. Resorbable polymer nonwovens, 10 mg/mL, in RPMI cell culture medium. The pH was reduced below 5 after 3-week incubation of PGLA (Ethisorb 210). Instead, there was almost no change of pH when the PLLA nonwoven (V7-2) was incubated for 14 weeks.

Polymer Degradation in Culture Medium

To roughly evaluate the degradation time of both nonwovens, they were simply placed in culture medium with 15 m*M* HEPES at 37 °C. The degrading Ethisorb nonwoven strongly reduced the pH of the medium after 3 weeks. In contrast, with PLLA nonwovens, even after 28 weeks, only a slightly lower pH was measured (Fig. 7). Consequently, the PLLA nonwoven is favorable for *in vitro* culture times beyond 3 weeks.

Loading of Cells into Polymer Fiber Structures

To evaluate the amount of cells retained in a 3-dimensional structure, the MTT colorimetric assay was applied. A preliminary experiment was done to verify a proportional correlation between serially diluted cells seeded in multiwell dishes and the measured optical densities after lysis and color release. The results showed a rather linear correlation between cell number and optical density.

Preliminary experiments (Fig. 8) were done with PGA net structures (Dexon). A striking improved cell attachment was seen with poly-L-lysine, which was also clearly visible in the microscope. However, the thin woven structures were not found suitable for cartilage engineering. Therefore, further experiments were carried out with the nonwoven structures of interest.

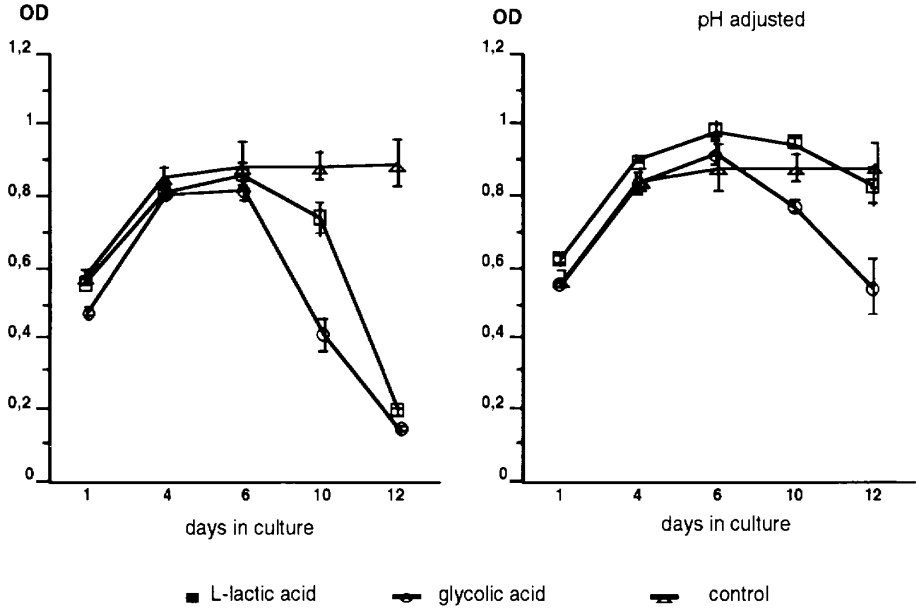

Figure 6. Chondrocytes in culture were incubated with 2 mg/mL L(+) lactic acid and glycolic acid for 12 days. The influence on the mitochondral activity is plotted: left, no pH adjustment; right, with pH adjustment. $n = 6$, mean + SD.

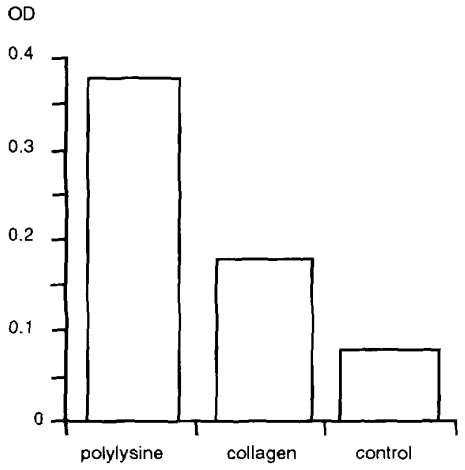

Figure 8. Chondrocytes on woven PGA meshes coated with collagen type II or poly-L-lysine. Measured mitochondral activity in the cell–polymer construct.

PLLA and PGLA nonwoven structures were coated with adhesion factors promoting the chondrocyte phenotype and placed in a suspension of chondrocytes for 60 min (Fig. 9). In both cases, a maximum amount of cells was retained in nonwovens coated with poly-L-lysine ($p < 0.001$). Collagen type II was less effective (Ethisorb: $p < 0.05$; V7-2: $p < 0.001$), even though it significantly increased the recovered amount of cells. The differences were more apparent with the PLLA nonwoven. The PGLA nonwoven (Ethisorb 210) control held more cells than the PLLA control. The PLLA nonwoven was more hydrophobic and soaked more slowly.

One can assume that the measured optical densities (ODs) correlate with the number of cells in the nonwoven, although the average metabolic activity per cell during the assay procedure is not known. For cells in the monolayer, an OD of 0.432 was measured for 5000 chondrocytes ($n = 11$). The presented experimental setup, however, did not give any information on whether the cells were actually attached to fibers or merely entrapped as aggregates in areas where the structure was more dense.

DISCUSSION

Recent progress in new designs, structures, and compositions of resorbable polymers such as nonwovens offer novel biomedical applications. The development of suitable polymer nonwovens could be the key to construction and design of vital tissues *in vitro*. This raises questions on the interactions of the polymer structure or its degradation products to the tissue forming cells. The experiments described focused on *in vitro* cartilage synthesis with nonwoven structures of PGLA and PLLA. Both of these polymers had been described and analyzed for their biocompatibility by other investigators.[15,16] The behavior of polymer degradation products after hydrolysation of the polymer is known to be crucial for surrounding cells and tissues.[17] Even though there are some highly biocompatible biomaterials, the general goal in tissue engineering should be to minimize the amount of biomaterial to also minimize negative interactions with cells and tissues.[18] So far polymer nonwovens manufactured of extremely thin fibers of PGLA and PLLA with a diameter of about 10 μm appear to fulfill this requirement to a great degree and are superior to porous structures in that respect. Having such nonwovens available, information about optimized cell affinity and the effect of degradation products on the applied cell type is needed.

Chondrocytes and α-Hydroxy Acids

In the present experiments, concentrations of the monomers up to 1 mg/mL seem to be tolerated by human cells. The results also imply that lactic acid compared to glycolic acid is more biocompatible as a degradation product to chondrocytes (Figs. 3, 6). Cartilage cells tolerated 2 mg/mL of lactic acid during an incubation time of 12 days as long the pH was adjusted. In contrast, the same treatment with glycolic acid severely affected cell metabolism.

Both α-hydroxy acids are found naturally in the human organism.[19,20] Lactic acid is produced during glycolysis in working muscles and metabolized in the liver for gluconeogenesis.[21] Glycolic acid is found as a metabolite of glycine synthesis through glyoxyl acid.[22] However, degradation products are not released gradually, because autocat-

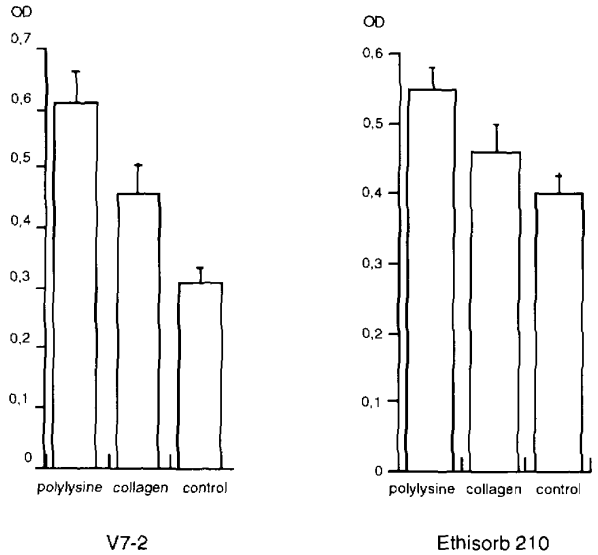

Figure 9. Chondrocytes in polymer nonwovens V7-2 (PLLA) and Ethisorb 210 (PGLA) coated with the adhesion factors collagen type II and poly-L-lysine. The optical density gives the mitochondral activity measured in the structure after 60 min in a cell suspension. $n = 6$, mean + SD.

alytic degradation processes lead to bulky releases of degradation products.[23] It is a question of which *in vivo* concentrations are realistic. Concentrations of lactic acid up to 10 mg/mL are at least imaginable.[24] In our experiments, chondrocytes tolerated more lactic acid than cell lines. It is known that the concentration of lactate is increased in articular cartilage because of a normally slightly anaerobic metabolism[25] supported by an elevated glucose supply from the synovia (Crabtree effect).[25] This could be an explanation for the toleration of lactic acid by chondrocytes in culture.

The effects of glycolic acid in our cell culture experiments are possibly based on a release of H_2O_2 by the enzyme glycolic acid oxidase combined with an accumulation of oxalic acid.[26] Figure 6 shows that glycolic acid does not affect chondrocytes merely by reducing the pH. Considering the monomers of resorbable polyesters, the results of the experiments suggest the design of polymers mainly leading to a release of L(+) lactic acid instead of glycolic acid for cartilage engineering, because the degradation time depends only in part on the α-hydroxy acids. However, further experiments need to clarify the effect of fragments or oligomers as relevant degradation products enclosed in developing artificial cartilage tissue. It is also not yet clear how the cartilage matrix formation is influenced by resorbable polymers or its degradation products.

Fiber Structure and Cell Adhesion

Optimized microstructures of resorbable cell carriers certainly provide a major tool for tissue engineering. Many preliminary experiments have been carried out with gels, porous structures, and fibers. The results propose nonwovens for *in vitro* tissue formation.[27] However, cells of a single cell suspension need to be evenly seeded 3-dimensionally and must rather quickly attach to their scaffold. A rapid attachment to fibers can be improved with adhesion factors. Although 3-dimensional culture can induce redifferentiation of chondrocytes,[8] it is still difficult to differentiate chondrocytes that have been cultured in a monolayer for many passages. To promote cartilage development in nonwoven polymer structures, type II collagen and poly-L-lysine were of special interest, because they are known to support the cartilage phenotype.[28] In contrast, fibronectin is known to induce cell spreading and to inhibit type II collagen synthesis. One has to consider that a chondrocyte on the polymer fiber surface could behave like on a 2-dimensional substrate. The presented experiments prove that poly-L-lysine and collagen type II improve the loading of cells into fiber structures. The applied factors were more effective in the PLLA nonwovens. This may be due to the average distance between fibers that appeared to be slightly higher in the PLLA nonwoven or to a difference of hydrophobicity. In the PGLA nonwoven a larger proportion of cells is possibly trapped as aggregates in dense fiber rather than actually attached to fibers. It also might be soaked more efficiently, because it is more hydrophilic. This problem indicates the current difficulty with polymer nonwovens: the homogenous cell distribution. Although it is not specifically addressed in other important literature,[29,30] we believe cell distribution is a crucial step in such tissue engineering approaches. Open structures have to be very sticky to collect enough cells, whereas the more dense structures tend to clot and the cells are mainly found in the surface layer as large aggregates. Despite these limitations, resorbable fiber structures could be the most promising approach for tissue engineering, especially when preshaping and temporary mechanical stability is required. The results presented here suggest that further improvement of the nonwoven structures to a more homogenous cubic mesh of thin fibers is needed.

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