



## **Biopolymer Systems in Soft Tissue Engineering: Cell Compatibility and Effect Studies Including Material**

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**Biopolymer Systems in Soft Tissue Engineering – Cell Compatibility & Effect Studies  
Including Material-, Catalyst- and Surface Properties**

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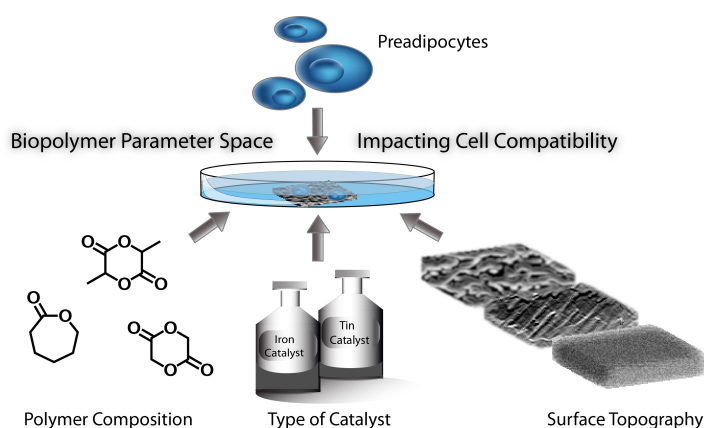
## ABSTRACT

Cell compatibility of biomaterials is one of the most important issues in Tissue Engineering. It is affected by the catalyst, the chemical composition and the surface topography of the material. Our aim was to study the influence of these factors for three standard FDA-approved biopolymers on the proliferation of preadipocytes. The analyzed materials were polyesters, which were synthesized by ring-opening polymerization. Tin(II)octanoate is the standard catalysts for the ring-opening polymerization (ROP); however, considered to be cytotoxic. Therefore, we compare its compatibility with iron catalysts. Our study suggests that neither polymers nor catalysts or mechanical properties have a significant impact on cell compatibility in short term 2D cell cultures *in vitro*. In contrast, nanoroughness can influence how cells attach and spread in a material, showing an influence on preadipocyte proliferation. Higher nanoroughness leads to higher cell compatibility, which becomes more pronounced with time.

## Introduction

Cell compatibility is one of the most important preconditions for the use of biopolymers in tissue engineering. Biopolymers can be divided into natural biopolymers like collagen and synthetic biopolymers. Among the synthetic biopolymers, poly( $\epsilon$ -caprolactone), poly(lactide) and poly(glycolide) are the most commonly used in tissue engineering since they are FDA-approved while poly(lactide) has also found wide application in daily products. In addition, they span a wide range of degradation times and elastic properties. Moreover, their different degradation times allow to adapt their use in many different applications: from fast degrading sutures made of poly(glycolide) to slow degrading contraceptive devices made of poly( $\epsilon$ -caprolactone).<sup>[1,2]</sup>

Even though these biopolymers found widespread applications in the biomedical field, their diversity in terms of their chemical nature (homo- or copolymers), chemicals used during synthesis (e.g. catalysts), as well as material properties resulting from polymer processing (e.g. surface topography/roughness) create a vast parameter range impacting cells, which has not yet been analyzed in concert to verify if critical differences occur and which limitations or freedom for material choices may exist (Scheme 1).



**Scheme 1:** Biopolymers parameter space impacting cell compatibility: composition, catalyst used in ring-opening polymerization and surface topography can affect cells – in this study

preadipocytes were used. On the lower left the three biopolymer constituting monomeric building blocks used for biopolymer synthesis are depicted:  $\epsilon$ -caprolactone, D,L-lactic acid and glycolide. The latter were used to prepare poly(DL-lactide) and poly( $\epsilon$ -caprolactone) homopolymers, as well as all copolymers combinations of the three monomers to binary copolymers and the ternary copolymer comprising all three monomers (see Table 1 & 2).

Synthetic biopolymers are usually polymerized with tin(II)octanoate as a catalyst. However, this catalyst is considered to be cytotoxic.<sup>[3]</sup> As a consequence, other catalysts like rare earth metals, iron, zinc or organo-catalytic systems or enzymes were examined.<sup>[4-8]</sup>

Many studies compare the cell compatibility of different polymers<sup>[9-12]</sup> but to our knowledge there are only a few studies which examine the cell compatibility of the catalysts, too.<sup>[3,13]</sup> Especially there are nearly no studies with a combined scope of the effect of surface topography and polymer identity/composition on cell proliferation. The choice for preadipocytes as cellular test system scan derives from their large potential in soft tissue engineering because of their potential to differentiate into different tissues. However, to the best of our knowledge there is no study analyzing cell compatibility of a combined approach including polymer identity/composition, catalyst and surface topography towards these cells.

Our main objective is to develop favourable scaffolds and growth chambers for soft tissue engineering composed of synthetic biopolymers. To achieve this goal, it is important to analyze the impact of polymers and catalysts towards cell compatibility. As it is hardly possible to completely remove tin(II)octanoate from the polymer, a potential influence towards cell compatibility has to be assumed.<sup>[14]</sup>

Schappacher et al. analysed the cell compatibility of several catalysts, by examining samples made with different catalysts with the finding that none of them were toxic.<sup>[13]</sup> However Tanzi et al. analyzed four different catalyst including tin(II)octanoate and found that this is the second most toxic catalyst among them.<sup>[3]</sup> Until now there is no study, which compares tin- and iron catalysts.

Concerning the cell compatibility of polymers, the current studies can be divided into *in vitro* and *in vivo* analyses or both simultaneously. *In vitro* studies do not show great differences between the cytocompatibility of different biopolymers.<sup>[10-12]</sup> Brackett et al. analyzed endodontic materials for 6 weeks including poly( $\epsilon$ -caprolactone), indicating that poly( $\epsilon$ -caprolactone) is non-cytotoxic, and was one of the best materials tested.<sup>[16]</sup> In the case of clinical application of poly(lactide), swellings could be observed three years after operation, induced by the degrading polymer.<sup>[17]</sup> Kobayashi et al. performed toxicity tests using implanted biodegradable polymers in rabbit cornea and found that biodegradable cyanoacrylates cause severe inflammation.<sup>[18]</sup> Other *in vivo* tests were conducted by implantation of biopolymers into animals: the implants were analyzed using different methods, most importantly histologically. Investigated biopolymers such as PLA mainly showed good biocompatibility.<sup>[19, 20]</sup> Until now there is no study, which analyzes the cell compatibility of different types of polymers with preadipocytes.

The effect of the substrate topography was investigated in cell-culture experiments. For example, it was shown that osteoblasts prefer surfaces with micro-roughness in contrast to fibroblasts.<sup>[21, 22]</sup> However, if surfaces with different nanoroughnesses are compared, the fibroblasts prefer rougher surfaces.<sup>[22]</sup> For osteoblasts a combination of both nanoroughness and microroughness enhances differentiation.<sup>[23]</sup> Neither the effects of nanoroughness nor the effects of microroughness on preadipocytes are described in the literature yet.

Due to their importance in soft tissue engineering, preadipocytes were chosen for our study. In this paper, we analyzed the influences of the following factors on preadipocyte

compatibility: biopolymer type, catalyst, and (nano)roughness. Although several previous studies reported in the literature, examine one of the mentioned issues, as far as we know the present study is the first one that addresses all three aspects at once. We examined different polymers and copolymers made of  $\epsilon$ -caprolactone, DL-lactide and glycolide. While their influence was already analyzed towards other cells, it was not yet analyzed towards preadipocytes.

## **1. Experimental Section**

### **1.1. Materials**

Iron(II)bromide anhydrous +98% was purchased from Alfa Aesar and used as received. DL-lactide and benzyl alcohol were purchased from Sigma-Aldrich and used as received. Iron(III) perchlorate from Aldrich was dried in vacuum prior to use and stored in a baked-out Schlenk-flask under nitrogen.  $\epsilon$ -Caprolactone was dried over  $\text{CaH}_2$ , distilled two-times and stored over molecular sieve and under nitrogen atmosphere in a baked-out Schlenk-flask. Chloroform from VWR was used as received. Methanol (VWR) was used as technical grade.

### **2.2 Preparation of Polymers**

The polymers were synthesized by Ring-Opening-Polymerization (ROP).

A 25-50 ml flask was baked out three times and flushed with nitrogen. The solid chemicals were weighed on a weighing boat and added to the flask. The liquid monomers were continuously kept under nitrogen atmosphere. They were added with Eppendorf microliter pipettes. Last the initiator was added. Usually the ratio (2000:1:2, M:C: I) was used; however, if the molecular weight was not high enough with this ratio, a ratio of (2000:1:5) was used instead.

For example, to a solution of 6.6 ml (6.85 g, 0.06 mol)  $\epsilon$ -caprolactone, 9.7  $\mu\text{l}$  (12.15 mg, 30  $\mu\text{mol}$ )  $\text{Sn}(\text{octanoate})_2$  and 6.2  $\mu\text{l}$  (6.5 mg, 60  $\mu\text{mol}$ ) benzyl alcohol were added. For other

polymers or copolymers, the same polymerization scheme was used. The flask was sealed, evaporated and flushed with nitrogen three times. The reaction was stirred at 140°C in an oil bath.

The raw product was dissolved in chloroform and poured into cold methanol in 50 ml falcon tubes. (3 ml of polymer solution to 25 ml of cold methanol). The falcon tube was centrifuged for 15 min at 12,000 rpm. Methanol was decanted and the polymer dissolved in chloroform. The chloroform was evaporated and the polymer dried until a constant weight was reached. Polymer chain lengths were characterized with PSS GPC SeCcurity and polystyrol standards. NMR spectra of yielded polymers were analyzed using a Bruker AVII-300 MHz.

## **2.3 2D-Spin-Coats**

### **2.3.1 Plasma-cleaned glass-slides**

The glass slides (VWR, 3 cm diameter, ECN 631-1585) were cleaned with acetone and a lint-free paper (VWR, Kimberly Clark, Kimcare). Then the cleaning process was continued by exposure to oxygen plasma (Femto 100 W, Diener electronic, Germany) for 300 s.

A 2% polymer solution in chloroform was prepared. The glass slides were spin-coated (Spincoater Model P6700, Speciality Coating System Inc.) at 200 rpm for 5 s and then 12000 rpm for 20 s. A volume of 120 µl to 150 µl of polymer solution was used, adjusted to the viscosity of the polymer solution.

### **2.3.2 Functionalized glass slides**

To verify, if the adhesion of the polymers to the glass slides can be enhanced, the glass slides were functionalized with silanes. The glass slides were cleaned with acetone and a lint-free paper (VWR, Kimberly Clark, Kimcare) and afterwards cleaned with Caro's acid (sulfuric acid with hydrogen peroxide) for 30 minutes. The glass slides were rinsed four times with distilled water and dried with compressed air followed by silanization for 2 hours with toluene:hexamethyldisilane (4:1). After this step, they were rinsed 4 times with distilled water



and dried with compressed air. The spin-coating process was the same as described for plasma-cleaned glass-slides. The films were spincoated using the spin coater SCC 200 KLM with a volume of 120  $\mu\text{l}$  to 150  $\mu\text{l}$  of polymer solution, adjusted to the viscosity of the polymer solution.

## **2.4 Mechanical Measurements**

Topography and film thickness were investigated by Atomic Force Microscopy (AFM) in intermittent contact mode in air with an AFM5500 (Agilent). Image processing and film thickness measurements were performed with Gwyddion software. All AFM force curves were taken in contact mode with a Z-closed loop system. Spring constants of silicon rectangular cantilevers ( $k = 2.1 \text{ N/m}$ ) used in this study were calibrated using thermal-oscillation.<sup>[24]</sup> The tip radii ( $R = 15\text{-}20 \text{ nm}$ ) were measured by blind reconstruction of the tip geometry.<sup>[25]</sup>

The characterization of hardness  $H$  and Young's modulus  $E$  were performed using an ultra nanoindenter from CSM-Instruments (Switzerland). The standard instrumented indentation tests were driven at constant load rate/load of  $0.05 \text{ s}^{-1}$  during both loading and unloading phases. Five different maximum loads were chosen: 0.1, 0.25, 0.5, 1.5 and 25 mN corresponding to a maximum penetration depth of about  $2.5 \mu\text{m}$  while a principle limitation in our samples is based on the thin polymer thickness. A constant load step of 200 s was achieved before unloading. Each test was performed three times to ensure reproducibility. Results reported here are averages and were calculated following the procedure described in the literature.<sup>[26]</sup>

## **2.5 Cell culture**

### **2.5.1 Human preadipocytes**

Fat tissue was obtained during surgery of the abdomen and used with the informed consent of the patient. Subcutaneous human preadipocytes were isolated as described in the literature.<sup>[27]</sup>

The cells were immediately stored in 200 ml  $\alpha$ -Minimal Essential Media ( $\alpha$ -MEM, Lonza). To the media was added 200 ml Hams F12 Nutrient Mixture (Lonza), 100 ml Fetal bovine serum (FBS, Biochrom), 5 ml chicken embryo extract (Seralab), Glutamine (Biochrom), 5 ml Penicillin Streptomycin (Pen-Strep, Biochrom) and 5 ml Amphotericin B (Ampho B, Biochrom). After 70-80% confluence the cells were frozen in liquid nitrogen. The cells were seeded at a concentration of  $5 \times 10^4$  cell per well. They were seeded on the spin-coated 3 cm glass plates, placed in 6-well dishes (TPP).

#### 2.5.2 Rat preadipocytes

Rat preadipocytes were isolated from Sprague-Dawley-rats, which were killed before. For isolation of the cells the same procedure as for human preadipocytes were used.<sup>[27]</sup> The cells were stored in medium 199 (Lonza) supplemented with 1%-Pen-Strep (Biochrom) and 10%-FBS. Media were changed the next day. After 70-80% confluency the cells were frozen in liquid nitrogen. The cells were seeded at a concentration of  $5 \times 10^4$  cell per well. They were seeded on the spin-coated 3 cm glass plates, in 6-well dishes (TPP).

#### 2.5.3 Cell counting

Dead cells swimming in the medium were extracted by suction. The living cells were trypsinized and suspended in the cell media mentioned above. The cell compatibility was analyzed by cell counting using a hemocytometer.

#### 2.5.4 Differentiation

Human preadipocytes were seeded on spin-coated glass slides at concentration of  $5 \times 10^4$  cell per well. The cells were differentiated in media consisting of 50 ml Dulbeccos Modified Eagle Medium (DMEM) (Lonza) and 50 ml Hams F12 Nutrient Mixture (Lonza), supplemented with 1ml glutamine (Biochrom), 1 ml Pen-Strep (Biochrom), 200  $\mu$ l Ampho B (Biochrom), 100  $\mu$ l calcium D-pantothenate (Sigma-Aldrich), 1  $\mu$ l Biotin (Serva), 200  $\mu$ l human apotransferrin (Sigma-Aldrich) and 400 $\mu$ l Insulin Rapid (Sanofi). In the first week of

differentiation the cells were cultured in a solution containing the media, 1.5 ml FBS (5%, Biochrom), 600  $\mu$ l isobutyl-1-methylxanthine (IBMX, Serva), 30  $\mu$ l cortisol (Sigma-Aldrich), 30  $\mu$ l Troglitazon (trogli, Sigma-Aldrich) and 150  $\mu$ l indomethacin (Sigma-Aldrich). In the second week only 1.5 ml FBS (5%, Biochrom) and 30  $\mu$ l trogli (Sigma-Aldrich) were added to the media.

The cells were stained with oilred. The surfaces with differentiated adipocytes were photographed in 4x and 10x magnification. The pictures were analyzed using ImageJ and the cells counted with the cell counting plugin.

#### 2.5.5 Statistic section

The results were analyzed with one-way ANOVA. The calculations were performed with Rstudio.<sup>[28]</sup> The p-value for significance was defined as  $p = 0.05$ . To be able to speak of a tendency for one possibility, an additional p-value of  $p = 0.3$  was defined. Additionally, when statistical significance or tendency is obtained, a Tukey HSD is performed.

### 3. Results and Discussion

The cell compatibility experiments, were composed of two sets of experiments, one with human preadipocytes (with the plasma-cleaned glass slides), the other with rat preadipocytes (with silanized glass slides). The second set of experiments was performed with rat preadipocytes due to limited access to human preadipocytes. The cells were isolated from five genetically identically Sprague-Dawley rats and not pooled. To isolate rat preadipocytes the rats were killed in accordance with the animal welfare officer of the University Tübingen and approval of the regional administrative authority Tübingen.

To verify if mechanical or topological qualities have an impact mechanical and topological measurements were made with AFM and AFM indentation. AFM indentation was used to measure the hardness and Young modulus (elasticity). In case of some polymer samples it

was hard to measure the mechanical properties of the films, because of not sufficient height of the film or inhomogeneity.

Poly(DL-lactide) samples gave improper results probably because of the morphology of the film: it is highly inhomogeneous so that the mechanical property assessment could not be correctly achieved. As reported in the materials and methods section, the tests were performed up to approximately 2.5  $\mu\text{m}$  and show for both investigated parameters (Young's modulus  $E$  and Hardness  $H$ ) a unique asymptotic behavior for all the samples when the penetration depth is higher than 600 nm, i.e. when higher than the film thickness. At this penetration depth the mechanical property of the substrate assembly (mineral glass + cyanoacrylate glue layer), impact the measured film properties: the characteristics of the films seem to be far lower than the one of the substrate assembly. Nevertheless, the representative values of the films, i.e. for small indentation depth, do not show a fundamental difference between the samples: the elasticity of most of the measured polymers was around 4-6 GPa. The poly(DL-lactide) samples polymerized with iron(III)bromide were inhomogeneous and showed deviations. The polymers exhibit hardness around 0.15-0.25 GPa. The elasticity and hardness characterization by nano-indentation induce interpretation cautions. The results presented here do not represent quantitative values but only trends of comparison since the substrate greatly influences the results. Therefore, the results are not intrinsic mechanical parameters of the considered films only but represent accessible values for the possible thicknesses reached.

As shown in **table 1**, poly( $\epsilon$ -caprolactone), poly(DL-lactide) and a copolymer poly( $\epsilon$ -caprolactone-DL-lactide) (50/50) were analyzed by AFM to characterize topographical properties of the systems. The nanoroughness is in the range of 2-11 nm; only poly( $\epsilon$ -caprolactone) samples polymerized with iron(III)bromide had a higher nanoroughness of 35 nm.

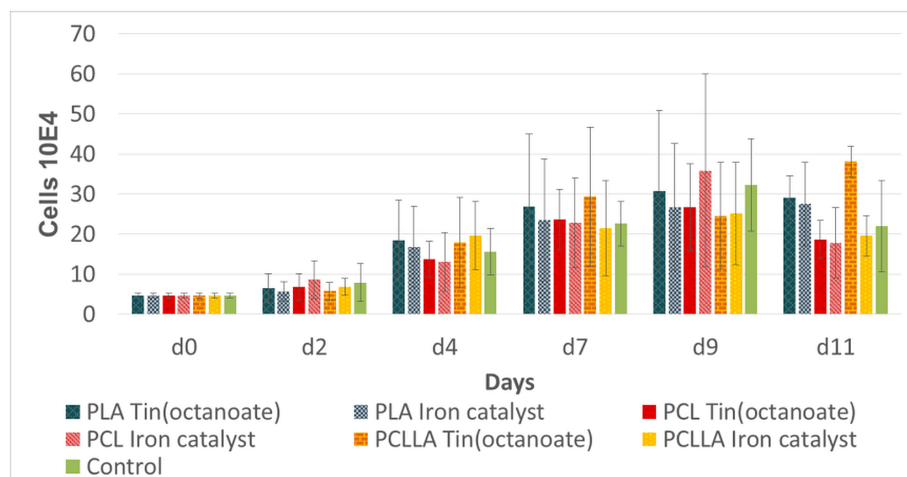
Polymer/copolymer	Catalyst	Average molecular weight Mw [kg/mol]	Thickness [nm]	Roughness [nm]	Tack trend (adhesion)
PLA	Sn(Oct) <sub>2</sub>	70	Not measurable	Not measurable	
PLA	FeBr <sub>2</sub>	48	500	3	-
PCL	Sn(Oct) <sub>2</sub>	80	650-700	11	+
PCL	FeBr <sub>2</sub>	34	470-600	35	--
PCLLA (50/50)	Sn(Oct) <sub>2</sub>	90	470	2	+
PCLLA (50/50)	FeBr <sub>2</sub>	22	180	3	++
Control	Pure glass				

**Table 1** Polymers used for cell studies with human preadipocytes. PCL=Poly( $\epsilon$ -caprolactone), PLA=Poly(DL-lactide), PCLLA=Poly( $\epsilon$ -caprolactone-co-DL-lactide)

### 3.1 First set of experiments: human preadipocytes

In the first set of experiments the focus was on the influence of the catalysts. Each biopolymer sample was synthesized two times, one using tin(II)octanoate as a catalyst and the other with iron(II)bromide or iron(III)perchlorate according to reported literature. <sup>[5]</sup>

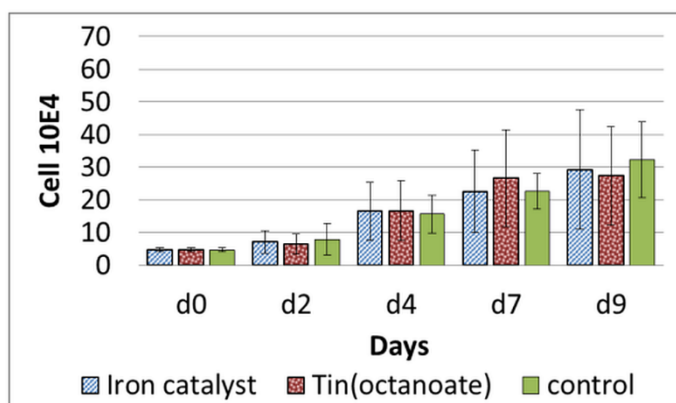
Human primary cells from four different donors were used, four experiments were performed for each polymer type, and the cells were counted three times each time a measurement was made. Cells were counted on day 1 and after 2, 4, 7, 9 and 11 days. The results are plotted in figure 1.



**Figure 1** Average cell growth of human preadipocyte cells on the polymer films and the glass control as function of time. No preference for one polymer or catalyst is generalizable.

In two of four cases, the number of cells was not enough to perform the analysis on the last day, day 11. As the results differ substantially from cell to cell, only results up to day 9 were analyzed.

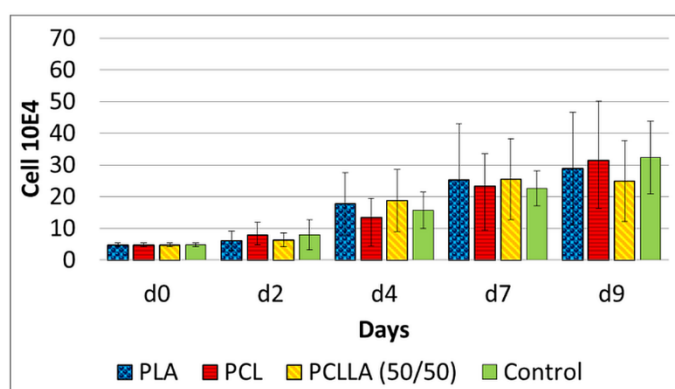
The cell compatibility of different polymers was examined regarding the influence of composition, catalyst, mechanical and topological properties. The tests were conducted with primary cells of rats and humans. The sample consisted in all cases of spin-coated glass slides. The cell growth on different polymer films as well as on the control is similar. In order to validate the impact of the catalyst, the cell growth of human preadipocytes was analyzed disregarding the influence of the polymers, shown in **figure 2**.



**Figure 2** Average cell growth (human preadipocytes) as function of time, disregarding the influence of the polymers. Cells growth on all surfaces is similar, no preference for a specific catalyst is found for short term 2D experiments.

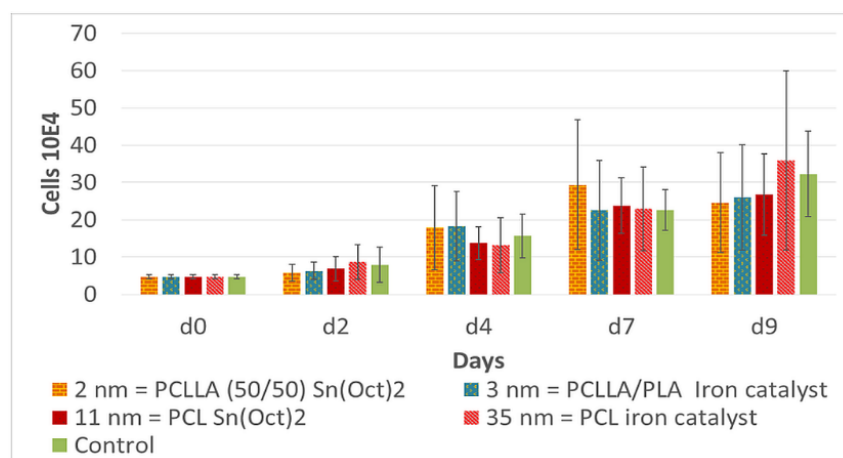
The preadipocytes seem to grow slightly better on the iron-catalyzed samples, but the difference is not significant. The noticeable small effect of the catalysts towards cell compatibility is supported by a study of Schappacher et al. They analyzed the cytotoxicity of poly(caprolactone) samples, polymerized with different catalysts, and the effects of the catalysts were found to be quite similar.<sup>[13]</sup> It is important to state, that these short time experiments only show acute toxicity and surface effects. Deviations result from cell-based diversity, as there is a cell to cell difference for catalyst and polymer preference if differences are not critical/toxic.

The next step was to analyze the influence of the polymers while disregarding the influence of the different catalysts, see **figure 3**. We find no clear preference of the cells for one surface.



**Figure 3** Average cell growth (human preadipocytes) as function of time, disregarding the influence of the catalysts.

The last aspect examined, was the surface roughness, measured by AFM and reported in table 1 and table 2. In **figure 4** the cell growth is presented in ascending order depending on the roughness of the polymer surfaces. In the case of the roughest surface with roughness 35 nm, the cell growth increases continuously. At day 9 one finds a preference for the roughest surface and a tendency of higher cell growth on higher roughness. The difference was not significant, but there was a tendency (ANOVA(Roughness): 0.3). The Tukey HSD test also showed that the p-value was smaller the bigger the roughness difference was. So nanoroughness seems to be a positive property human preadipocytes. The effect becomes more pronounced with time.



**Figure 4** Average cell growth (human preadipocytes) listed as a function of time with respect to surface roughness in ascending order of roughness. At day 9 there is a preference for the roughest surface. This matches the findings of a study of Chung et al. who found that fibroblast prefer nanorough surfaces in contrast to smooth surfaces. <sup>[22]</sup>



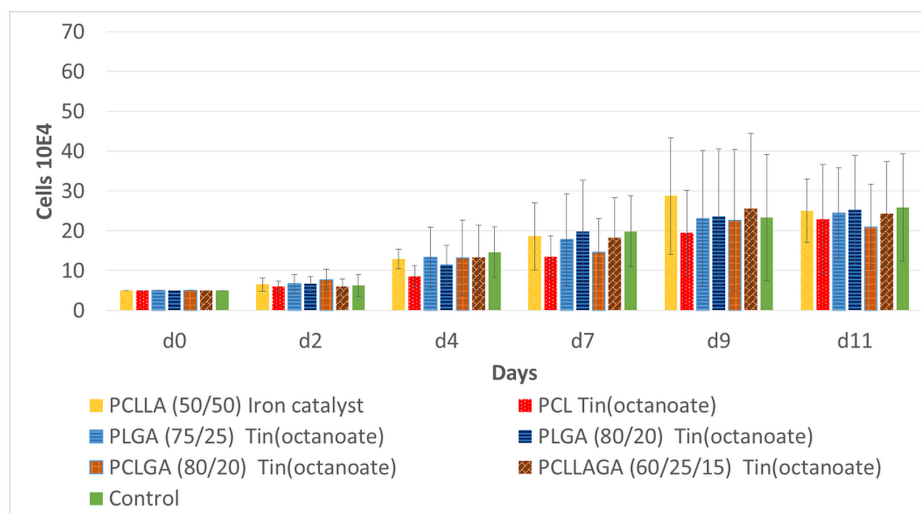
### 3.2 Second set of experiments: rat preadipocytes

The main focus of the second set of experiments was the influence of the chemical composition of the polymers. Here only one variation of each polymer was used, in order to be able to analyze a large diversity polymers. Five polymers were analyzed: poly( $\epsilon$ -caprolactone), poly( $\epsilon$ -caprolactone-co-DL-lactide) (PCLLA, 50:50), poly(DL-lactide-co-glycolide) (PLGA, 75/25), poly(DL-lactide-co-glycolide) (PLGA, 80/20) and poly( $\epsilon$ -caprolactone-co-DL-lactide-co-glycolide) (PCLLAGA, 60/25/15). The molecular weight and roughness of these polymers are given in **table 2**.

Polymer/copolymer	Catalyst	Average molecular weight Mw [kg/mol]	Thickness [nm]	Roughness [nm]	Tack trend (adhesion)
PLGA (75/25)	Sn(Oct) <sub>2</sub>	50	240	1	++
PLGA (80/20)	Sn(Oct) <sub>2</sub>	39	190	3	--
PCLLA (50/50)	Fe(ClO <sub>4</sub> ) <sub>3</sub>	22	180	3	++
PCL	Sn(Oct) <sub>2</sub>	80	650-700	11	+
PCLGA (80/20)	Sn(Oct) <sub>2</sub>	66	360	14	--
PCLLAGA (65/25/15)	Sn(Oct) <sub>2</sub>	53		Could not be measured	Could not be measured
Control	Pure glass				

**Table 2** Polymers used for rat preadipocyte cell cultures. PCL = Poly( $\epsilon$ -caprolactone), PCLLA = Poly( $\epsilon$ -caprolactone-co-DL-lactide), PLGA: Poly(DL-lactide-co-glycolide), PCLGA = Poly( $\epsilon$ -caprolactone-co-glycolide), PCLLAGA = Poly( $\epsilon$ -caprolactone-co-DL-lactide-co-glycolide).

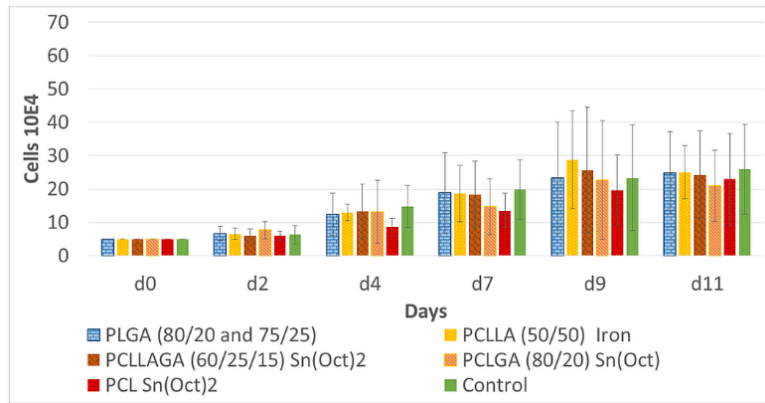
For the second set of experiments, polymer samples were prepared with a difference in nanoroughness of only 10 nm. Nearly all polymers except one were synthesized with tin(II)octanoate. Primary cells from five rats were used. The cells were counted at day 0, d2, d4, d7, d9 and d11. The results are shown in **figure 5**.



**Figure 5** Average cell growth of rat preadipocytes on polymer films and glass control as function of time.

The iron catalyzed PCLLA (50/50) sample is among the best surfaces at nearly all days investigated, see figure 2. At day 9, it is slightly better than the other surfaces. Poly( $\epsilon$ -caprolactone-DL-lactide) also showed good performance in the first part of the study, see figure 1.

Poly( $\epsilon$ -caprolactone) is part of nearly all polymer compositions in the second part of the study. The polymer compositions contain poly( $\epsilon$ -caprolactone) in different ratios. To investigate if there is a monomer ratio of the copolymer, which is preferred by rat preadipocytes, the average cell growth was ordered in respect to an increasing  $\epsilon$ -caprolactone ratio (see figure 6). In case of the PLGA samples, the average value was used.



**Figure 6** Average cell growth of rat preadipocytes shown as a function of time in ascending order of  $\epsilon$ -caprolactone ratio. Until day 11 the cell density on poly( $\epsilon$ -caprolactone) films is significantly lower than on the other polymer films.

Until day 11 the cell density is lowest on the pure poly( $\epsilon$ -caprolactone) sample. At day 7 and 9 the cell density decreases with increasing  $\epsilon$ -caprolactone-ratio. An explanation for this could be that with increasing  $\epsilon$ -caprolactone ratio the hydrophobicity also increases. The hydrophobicity seems to slow down cell proliferation. This matches the study of Altankov et al. who compared the biocompatibility of hydrophobic and hydrophilic surfaces with the result that the hydrophilic surfaces are better for the cells than the hydrophobic. <sup>[29]</sup> At day 11 the cell density is on nearly the same level on all surfaces. On the PCLLA-surfaces the cell density is significantly higher than on poly( $\epsilon$ -caprolactone)-surfaces (ANOVA(PCLLA/PCL: 0.02). Another reason why the PCLLA-surface could be preferred, could be the better adhesion on PCLLA compared with the PCL-surface. Comparing the best adhesive surfaces (PCLLA and PLGA (80/2) with PCL leads to significant results (ANOVA(PCLLA-PLGA80/PCL: 0.024). However, no significant influence of the adhesion could be shown in the first set of experiments.

In case the of the types of polymers, the study showed that the cell compatibility of the polymers does not differ significantly. This is in line with the study of Pariente et al. who

analyzed different natural and synthetic biomaterials concluding that all tested materials are biocompatible.<sup>[10]</sup>

### 3.2 Comparisons

The studied biopolymer systems provide important insights for material design in tissue engineering and cell culture application. The use of biopolymers with distinct compositions and surface properties are exemplified and validated for 2D cell cultures.

Concerning the cell compatibility of the catalysts no statistical influence could be seen in time frame typical for 2D cultures. In the second set of experiments, iron-catalyzed poly( $\epsilon$ -caprolactone-co-lactide) was significantly better than the tin-catalyzed poly( $\epsilon$ -caprolactone). However, in the first set of experiments the tin-catalyzed poly( $\epsilon$ -caprolactone-co-lactide) showed better cell compatibility than the iron-catalyzed. Since the absolute differences are minor, one cannot infer a strict preference for one catalyst over another despite statistical significance.

In contrast nanoroughness does seem to have a pronounced influence on cell compatibility of the surfaces. The iron-catalyzed poly( $\epsilon$ -caprolactone) sample showed substantial growth by day 9, see figure 1. The nanoroughness of this polymer sample is with 35 nm the largest surface roughness of all polymer systems investigated, see figure 5. In the first set of experiments, one can also see that the cell density increases with increasing nanoroughness. This matches the findings of a study of Chung et al. who found that fibroblasts prefer nanorough surfaces in contrast to smooth surfaces.<sup>[22]</sup> If the difference of the nanoroughness is only around 10 nm, as found in set 2, no influence of the roughness is found. This matches the study of Xie et al. which also showed no influence if the difference in nanoroughness was smaller than 12 nm.<sup>[30]</sup>

### 3.3 Differentiation experiments

Differentiation experiments were performed to examine the impact of different surfaces on the differentiation of preadipocytes. In order to investigate this question, cells were differentiated according to the method mentioned in section 2.5.4.

Regarding differentiation there is no obvious difference between different polymer systems or catalysts investigated; the adipocyte density resides in the same range. However, there is a big disparity between the polymer surfaces and the glass control (see supporting information). The adipocyte density is nearly 9 times lower than on the polymer surfaces. This is a clear indicator that polymer surfaces are better for adipocyte differentiation than glass.

#### **4. Conclusions**

The biopolymer systems studied represent a vast range of chemical entities and conditions covering important applicable combinations of the three major FDA-approved biopolymers in use. Major factors for materials used in tissue engineering such as the type of biopolymer, combinations thereof, effect of catalysts used in biopolymer synthesis as well as mechanical properties and surface topological effects (roughness) could be validated and give important guidelines for the use of biopolymers and their effects exemplified and validated for 2D cell cultures.

Neither the polymer, nor the catalyst nor the mechanical properties have a critical influence on cell behavior. One exception concerning the polymer compatibility was found for rat preadipocytes which significantly preferred the PCLLA-surface over the PCL-surface. Probably this is because of PCL's higher hydrophobicity compared to PCLLA. This is in line with a study of Altankov et al. who showed that cells prefer hydrophilic surfaces.<sup>[29]</sup> Also the better adhesion of cells on the PCLLA surface compared to the PCL surface can be important. The role of the catalyst was shown to have no critical effect in the context of these studies if cell proliferation and differentiation is concerned. This may be attributed to a limited liberation of catalyst from the material: the catalyst able to diffuse out of the material will be

quickly eluted and distributed within the comparably large volume of the culture media, in addition biopolymer degradation is low within the time frames investigated.

Increased nanoroughness seems to be advantageous for preadipocytes, and should be subject of further studies. If the nanoroughness is comparable for all slides, for example in the second set of experiments, the material composition becomes more important.

Concerning the differentiation, no polymer-specific effect could be identified, which significantly stimulates differentiation. But the comparison of the polymer films and the pure glass slides showed big differences. On the polymer films many preadipocytes differentiated into adipocytes (see Supporting Information). In contrast to this finding, nearly no preadipocytes differentiated on pure glass samples despite the applied differentiation cocktail. So as a major finding we can postulate that differentiation of preadipocytes favors polymer surfaces in contrast to glass surfaces.

In sum we can conclude that the type/chemical composition and catalyst as well as mechanical properties have limited influence on preadipocyte cell cultures. It could be shown, that a nanoroughness of 35 nm in contrast to 10 nm has a positive effect of preadipocytes. Therefore the type of biopolymer to be chosen for certain biomedical applications may be attributed to specific needs of the application such as mechanical stiffness, time of degradation and processability. This is an important and promising finding fostering a multitude of applications such as current efforts towards porous scaffolds for 3D tissue replacement explored by us.

## **Abbreviations**

PCL = Poly( $\epsilon$ -caprolactone)

PLA = Poly(DL-lactide)

PCLLA = Poly( $\epsilon$ -caprolactone-co-DL-lactide)

PLGA: Poly(DL-lactide-co-glycolide)

PCLGA = Poly( $\epsilon$ -caprolactone-co-glycolide)

PCLLAGA = Poly( $\epsilon$ -caprolactone-co-DL-lactide-co-glycolide)

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