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Hydrolytic and oxidative degradation of electrospun supramolecular biomaterials: In vitro degradation pathways

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ABSTRACT

The emerging field of in situ tissue engineering (TE) of load bearing tissues places high demands on the implanted scaffolds, as these scaffolds should provide mechanical stability immediately upon implantation. The new class of synthetic supramolecular biomaterial polymers, which contain non-covalent interactions between the polymer chains, thereby forming complex 3D structures by self assembly. Here, we have aimed to map the degradation characteristics of promising (supramolecular) materials, by using a combination of in vitro tests. The selected biomaterials were all polycaprolactones (PCLs), either conventional and unmodified PCL, or PCL with supramolecular hydrogen bonding moieties (either 2-ureido-[1H]-pyrimidin-4-one or bis-urea units) incorporated into the backbone. As these materials are elastomeric, they are suitable candidates for cardiovascular TE applications. Electrospun scaffold strips of these materials were incubated with solutions containing enzymes that catalyze hydrolysis, or solutions containing oxidative species. At several time points, chemical, morphological, and mechanical properties were investigated. It was demonstrated that conventional and supramolecular PCL-based polymers respond differently to enzyme-accelerated hydrolytic or oxidative degradation, depending on the morphological and chemical composition of the material. Conventional PCL is more prone to hydrolytic enzymatic degradation as compared to the investigated supramolecular materials, while, in contrast, the latter materials are more susceptible to oxidative degradation. Given the observed degradation pathways of the examined materials, we are able to tailor degradation characteristics by combining selected PCL backbones with additional supramolecular moieties. The presented combination of in vitro test methods can be employed to screen, limit, and select biomaterials for pre-clinical in vivo studies targeted to different clinical applications.

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1. Introduction

Tissue engineering aims to restore tissue structure and function of diseased or damaged tissues by implantation of specifically designed biodegradable materials, with or without the addition of cells [1–3]. Conventional tissue engineering aims to collect autologous cells from patients, which are utilized for the in vitro generation of new tissues, and are often cultured in bioreactors for several weeks before implantation. A new and promising

approach is in situ tissue engineering, in which in vitro culture is omitted and the patient's body is used as a bioreactor [4–7]. New tissue will be regenerated directly in the body by host cells after implantation of, for example, a biodegradable electrospun polymeric scaffold. This makes the overall procedure less demanding in terms of costs, time, and regulatory challenges, and creates off-the-shelf availability.

In situ tissue engineering of load-bearing tissues places high demands on the biodegradable scaffolds, as these scaffolds should be able to provide mechanical stability immediately upon implantation, and for a prolonged period thereafter, until sufficient mature neo-tissue is formed by recruited cells to take over the mechanical function of the scaffold. Various synthetic biodegradable polymers are used for tissue engineering applications, and

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these polymers include aliphatic polyesters (e.g. polylactic acid (PLA), polyglycolic acid (PGA) and polycaprolactone (PCL)), as well as various polyurethanes [8–11]. A new set of synthetic materials are the supramolecular polymers, which are formed by arrays of directed, non-covalent interactions between the building blocks, and can form complex 3D-structures by self assembly [12]. Material properties such as mechanics and resorption rate, which are critical for the success of in situ tissue engineering can be modified by combining or changing ratios of the same building blocks. This potentially allows for a variety of polymers with varying properties to be synthesized in a relatively short time span, thereby accelerating the development process. Monomeric units of the supramolecular polymers possess a relatively low molecular weight, resulting in beneficial processing properties, e.g. easy dissolution in organic solvents. Furthermore, supramolecular polymers may show self-healing properties [13–15], can easily be made bioactive [16,17], and allow for a more controlled way of synthesis, which can result in complex molecular structures [12]. Because of these features, these materials pose excellent candidates for use in in situ tissue engineering. Particularly, we are interested in biomaterials that either have 2-ureido-[1H]-pyrimidin-4-one (UPy) [18–21] or bis-urea (BU) [17] hydrogen bonding supramolecular motifs incorporated into their molecular structure, as these contain elastomeric properties, which makes them suitable candidates for cardiovascular applications. In vitro tests of these materials resulted in satisfactory fatigue properties (unpublished results), which is mainly important for in situ tissue engineering of heart valves, which will be exposed to cyclic loading. In vitro toxicity tests performed by Dankers et al. [16] and Wisse et al. [17] indicated that the UPy- and BU-moieties are not toxic and thus biocompatible. Furthermore, it was shown that solution cast polymer films comprising UPy or BU moieties were shown to be non-toxic after subcutaneous implantations in rats [16,17].

To enable the formation of a completely autologous tissue, the scaffold should degrade at the right pace during neo-tissue formation, leaving behind a living implant that is able to remodel and grow. In vivo, degradation of implanted scaffold materials can be accomplished via different pathways that operate at the same time, and that even may affect each other [22–25]. A well-known pathway is hydrolytic degradation, where chemical bonds of the polymer chains are cleaved by reaction with water molecules, forming oligomers and ultimately generating small molecules that can be cleared from the body [22,23]. Previous studies have reported that several enzymes, like proteases and esterases, which are present in human serum or are expressed by macrophages and other activated cells that are in contact with the scaffold, are known to catalytically accelerate this process [22,26,25,27,28]. Another well-described pathway is oxidative degradation, which is mediated by reactive oxygen species (ROS) that are secreted by macrophages, neutrophils and giant cells that are in contact with the scaffold [29,22]. These ROS include hydrogen peroxide (H_2O_2), nitric oxide (NO), hydroxyl radical ($\cdot OH$) and the superoxide anion (O_2^-). Previous studies have investigated that oxidation of polymers is often initiated by abstraction of a hydrogen atom by radicals, resulting in chain scission and/or crosslinking of the polymer [30,31]. Mapping the degradation characteristics of promising (supramolecular) materials for use in in situ tissue engineering approaches, as well as their susceptibility for certain degradation pathways, paves the way for screening and selection of materials for various clinical implantation sites.

The degradation properties of widely used and well-known materials such as polyesters, polycarbonates and polyurethanes have been examined extensively, both in vitro and in vivo [32,33,28,34,35,11,36,37]. In general, results of these studies show that polymers containing ester or anhydride linkages react with water molecules and undergo hydrolysis [33,23,38,39]. The water

molecules can access those chemical species more easily, and thus increase the hydrolytic activity, when the polymer is amorphous or contains aliphatic structures [40,23]. Other polymers, including polyethers and polyurethanes, were found to be more susceptible to the oxidative pathway, as these materials contain α -methylene groups adjacent to ether or urethane groups, which are more prone to the formation of carbon centered radicals by abstraction of a hydrogen atom [41,42,23,24,31,43]. Just a few studies reported on the degradation characteristics of various polymers (PCL, polycarbonates, or polyurethane) modified with UPy or BU units. These were performed by incubating the materials in phosphate buffer saline (PBS) or solutions of various lipases at 37 °C [20,16,44,45]. These studies showed that the rates of enzymatic degradation can span a wide range, from less than 1% degradation after 1 month [45] to 90% after only 15 days [16], depending on the types of lipase and polymers used. No hydrolytic degradation, in terms of weight loss, of the UPy containing materials was observed for 126 days when samples were incubated with PBS [20], and a decrease in weight of only 2% after 120 days was observed for BU-containing materials [44].

Although these studies gave some insight into the degradation properties of biodegradable materials, the major part of these studies were performed on films or disks which are quite dense, while degradation rate of electrospun scaffolds, that are more porous and have higher surface to volume ratio, can be different. Studying the degradation properties of electrospun meshes is, from a clinical point of view, more relevant as these are more likely to be implanted as a tissue replacement, rather than a compact, solid construct. Furthermore, most research has focused on a single degradation pathway, while it is of importance to assess either the enzyme-accelerated hydrolytic and the oxidative degradation pathways, since in vivo both pathways may be operative and consequently, both may affect the implanted scaffold.

Here, an in vitro study was designed to investigate both degradation pathways in an accelerated fashion and was used to assess the degradation of several promising supramolecular biomaterials for in situ tissue engineering. We have chosen three previously reported supramolecular biomaterials, in which PCL backbones are combined with either UPy hydrogen bonding groups (materials PCL₂₀₀₀-UPy and PCL₈₀₀-UPy) [46] or BU hydrogen bonding groups (PCL₂₀₀₀-BU) [17]. High molecular weight PCL, a material frequently used for tissue engineering scaffolds, was added as a benchmark. All materials were electrospun and the resulting scaffold meshes were either exposed to enzymes that catalyze hydrolysis or to oxidative conditions. Degradation was monitored over time by examining the remaining scaffold with respect to weight, molecular weight, fiber diameter, and mechanical properties. Statistical analyses were performed to analyze changes in properties over time of all polymers with the various treatments, as well as to investigate their susceptibility to degradation and its mechanism (surface or bulk erosion). Finally, an explorative feasibility study was performed to show the effect of activated macrophages on the degradation of PCL scaffolds.

2. Materials and methods

2.1. Materials

All reagents, chemicals, materials, and solvents were obtained from commercial sources and were used without further purification, unless otherwise noted. The polycaprolactone based supramolecular biomaterials PCL₂₀₀₀-UPy, PCL₈₀₀-UPy and PCL₂₀₀₀-BU were synthesized as previously described from polycaprolactone diol building blocks of molecular weights 800 or 2000 [17,46]. These PCL₂₀₀₀-diol and PCL₈₀₀-diol building blocks are prepared by initiation from diethylene glycol, so they contain

one ether bond in their structure. Conventional PCL (Purasorb PC 12, IV = 1.24 dl/g) was purchased at Purac Biochem, Gorinchem, the Netherlands. Thermal characterization of these materials was performed by differential scanning calorimetry (DSC) on a Perkin Elmer Pyris 1 or on a TA Instruments Q2000. Reported data are from the melt, so after the sample has been in the isotropic state, and were determined in the second heating run at a heating rate of 10 °C/min. The glass transition temperature (T_g) is reported as the inflection point, while the melting transition (T_m) is reported as the peak of the transition.

2.2. Scaffold preparation

Scaffolds were fabricated in a climate-controlled electrospinning cabinet (IME Technologies, Geldrop, The Netherlands) using the conventional electrospinning method as described before [47]. Rectangular strips (25 (l) × 5 (w) × 0.44 (t) mm) were punched out of the electrospun scaffold meshes. Initial weight (W_0) and thickness of all individual strips were measured using a digital balance (Mettler Toledo, XS105, Greifensee, Switzerland) and a digital thickness gauge (Mitutoyo, SGM, Groningen, The Netherlands). Prior to incubation for degradation, the meshes were centrifuged at 4500 rpm in purified water for 5 min to remove air bubbles.

2.3. Accelerated in vitro degradation

Strips ($n = 60$ per material) were incubated at 37 °C in 1.5 ml enzyme solution, referred to as enzymatic degradation, or in a 4 ml oxidative degradation solution each. The enzyme solution consisted of 100 U/ml lipase from *Thermomyces lanuginosus* (L0777, Sigma–Aldrich) in PBS or 10 U/ml cholesterol esterase from bovine pancreas (C-3766, Sigma–Aldrich) in PBS. These enzymes, which are present in serum and are secreted by activated macrophages, are known to cleave ester and urethane bonds to a higher extent as compared to other secreted enzymes [48,49,36]. The oxidative solution comprised of 20% hydrogen peroxide (Sigma–Aldrich) and 0.1 M cobalt(II) chloride (Sigma–Aldrich) in purified water (pH of this solution is 4.5). Hydrogen peroxide and cobalt(II) chloride undergo a Haber–Weiss reaction, creating reactive hydroxyl radicals [31]. Incubation times of the scaffolds in lipase, cholesterol esterase, or oxidative solutions were up to 56, 96 and 400 h, respectively. Based on literature [42,50], solutions were changed every 3–4 days to maintain enzymatic activity and a constant concentration of radicals.

2.4. Scaffold characterization

Analyses of the (remaining) scaffolds were performed at 5 time points for the enzymatic groups and 7 time points for the oxidative group ($n = 4$ per group per time point). Mass loss, molecular weight, fiber diameter, and mechanical properties were determined.

2.4.1. Mass loss

Scaffold strips were removed from the degradation solution, washed three times with purified water, dried under vacuum at 37 °C for 16 h and weighed (Mettler Toledo, XS105, Greifensee, Switzerland), to assess weight loss due to scaffold degradation. Mass loss of the scaffolds ($n = 4$ per group per time point) was determined using the equation: $W_1/W_0 \times 100\%$, where W_0 is the initial scaffold weight and W_1 indicates remaining scaffold weight.

2.4.2. Scanning electron microscopy (SEM)

Scaffold fiber morphology and average fiber diameters were assessed and determined by scanning electron microscope (SEM),

(Phenomworld, Eindhoven, The Netherlands) of one sample per group per time point. Average fiber diameters were determined by 20 individual measurements performed on four SEM images per scaffold strip, using Phenomworld software (Fibermetric, Phenom pro suite version 2.0).

2.4.3. Mechanical properties

To study the effect of degradation on the mechanical properties of the scaffolds, uniaxial tensile tests in longitudinal direction of the strips ($n = 3$ per group per time point) were performed. Due to a loss of mechanical integrity over time, associated with degradation, it was not possible to perform tensile tests on all PCL-BU and PCL-UPy strips of the latest oxidation time points. Sample thickness and width were measured with an electronic caliper. Stress–strain curves were obtained (Mecmesin multiTest-i) at an elongation rate of 100% per minute and the mechanical test data was averaged per group per time point. The elasticity modulus (Young's modulus) was determined as the slope of the initial linear part of the curve, as a measure for stiffness. As a measure for strength, the ultimate tensile strength (UTS) was defined as the peak stress value, while strain at break is a measure for the maximal elongation of the samples until break.

2.4.4. Molecular weight (GPC)

After tensile testing, one strip per group per time point of each material was taken and dissolved in dimethylformamide ((DMF), Sigma) in order to determine the mass averaged molecular weight (M_w) of the samples by gel permeation chromatography (GPC) analysis. GPC was performed on a Varian/Polymer Laboratories PL-GPC 50, using DMF with 10 mmol/L lithium bromide as eluent and maintaining the temperature of the equipment at 50 °C. The relative or apparent molecular weights (M_w) were determined with respect to polyethylene glycol standards. Samples were measured in duplicate and the M_w was averaged from this duplicate measurement.

2.5. Feasibility study of cell-mediated scaffold degradation

2.5.1. Cell culture

The human monocytic cell line THP-1 was purchased from Cell Lines Service (CLS, Eppelheim, Germany) and cultured according the suppliers' recommendation in a humidified atmosphere containing 5% CO₂ at 37 °C.

2.5.2. Seeding of monocytes and transformation into macrophages

PCL scaffold strips ($n = 18$) were placed into 2 ml vials containing 1.5 ml culture medium, 3.0×10^6 THP-1 monocytes, and 50 ng/ml of phorbol 12-myristate 13-acetate ((PMA), Sigma Aldrich) to transform the monocytes into macrophages. PCL scaffold strips that were kept unseeded ($n = 5$) were also placed in 2 ml vials containing the same medium, but without cells. The vials were rotated for 16 h in a humidified atmosphere containing 5% CO₂ at 37 °C to allow cells adhere to the scaffold strips. After seeding, each scaffold strip was placed into a well of a 12-wells plates containing 1.5 ml culture medium and 50 ng/ml PMA for another 24 h.

2.5.3. Activation of macrophages

Activation medium consisted of 100 ng/ml Lipopolysaccharide S ((LPS), Sigma Aldrich) and 20 ng/ml Interferon gamma ((IFN- γ , Peprotech, London, UK) in culture medium. Medium change was performed 3 times per week. Seeded scaffold strips were cultured for 2 days, 1 week and 4 weeks, ($n = 6$ per time point). Unseeded scaffold strips were kept in culture for 2 days ($n = 1$), 1 week ($n = 2$) and 4 weeks ($n = 2$). After culture, strips were fixated in formalin for 24 h, which was replaced with sterile PBS afterwards and stored at 4 °C until use.

2.5.4. Morphology of scaffold fibers

Scaffold fibers were visualized by SEM to analyze the morphology of the fibers. Samples were dehydrated in a graded ethanol series, starting from 50% to 100% in 5% to 20% increments. The ethanol was then allowed to evaporate, and samples were visualized by SEM. After SEM analysis, samples were treated with 4.6% sodium hypochlorite for 15 min at room temperature, to remove all cells. Samples were washed twice in water and the same samples were analyzed by SEM again, in order to visualize parts of the scaffold fibers that were covered by the cells during the first SEM analysis.

2.6. Statistical analyses

All data are presented as mean $\pm$ standard deviation. Statistics were performed using GRAPHPAD Prism (version 5) and differences were considered significant for p -values <0.05 .

2.6.1. Changes over time

Regression analyses were performed to determine changes in weight, Mw, fiber diameter, Young's-modulus, UTS, and strain at break over time. Both a one-phase decay model (assuming the rate at which changes occur is proportional to the amount that is left) and a linear model (assuming a constant rate) were used to fit the data. In case of a significant increase or decrease with $p < 0.05$ or $p < 0.01$, the percentual in- or decrease was calculated from the predicted model equation and classified as non-relevant (0–10%), small (10–25%), moderate (25–100% for an increase and 25–50% for a decrease), and severe ($>100\%$ for an increase and 50–100% for a decrease).

2.6.2. Susceptibility to degradation and its mechanism

The susceptibility for both enzymatic and oxidative degradation was determined via correlation analyses of all measured parameters. Significant correlations were classified as weak ($p < 0.05$), average ($p < 0.01$), and strong ($p < 0.001$). Susceptibility for degradation was calculated as the number of significant correlations (with more weight to the average and strong correlations as compared to the weak correlations) divided by the maximum number

of possible correlations and expressed as a percentage. Susceptibility was classified as not susceptible ($<20\%$), susceptible (20–60%), or highly susceptible ($>60\%$). To obtain insight into the mechanism of degradation, correlations were either attributed to surface erosion or to bulk erosion. Correlations that were considered to attribute to surface erosion were correlations between mass loss and fiber diameter, between mechanical properties, between mechanical properties and fiber diameter, and between mass loss and mechanical properties. Correlations that were considered to attribute to bulk erosion were correlations between Mw and mass loss, mechanical properties, or fiber diameter and inverse correlations between parameters. The susceptibility to either enzymatic or oxidative degradation was subsequently determined as described above with similar classifications for susceptibility.

3. Results

3.1. Material properties

The studied supramolecular biomaterials PCL₂₀₀₀-UPy, PCL₈₀₀-UPy and PCL₂₀₀₀-BU are in fact thermoplastic elastomers with PCL soft blocks and hard blocks composed of interacting and phase separated hydrogen bonding units (Fig. 1A and B). PCL is a semi-crystalline polyester ($T_g = -64^\circ\text{C}$, $T_m = 52^\circ\text{C}$), while the PCL₂₀₀₀-BU thermoplastic elastomer shows a first melting transition (T_m1) of the semi-crystalline PCL soft block at a lower temperature and a second melting transition (T_m2) of the BU hard block at a higher temperature ($T_g = -54^\circ\text{C}$, $T_m1 = 27^\circ\text{C}$, $T_m2 = 98^\circ\text{C}$) [17]. Both PCL₈₀₀ and PCL₂₀₀₀-UPy are also thermoplastic elastomers (PCL₈₀₀-UPy: $T_g = -39^\circ\text{C}$, $T_m1 = 65^\circ\text{C}$, $T_m2 = 91^\circ\text{C}$; PCL₂₀₀₀-UPy: $T_g = -58^\circ\text{C}$, $T_m1 = 53^\circ\text{C}$, $T_m2 = 116^\circ\text{C}$).

3.2. In-vitro degradation as monitored by scaffold mass loss and Mw

Enzymatic degradation (Fig. 2A–D) of conventional PCL scaffolds resulted in moderate (44%, $p < 0.01$) to severe (92%, $p < 0.01$) mass loss by lipase and cholesterol esterase treatment, respectively, while

Fig. 1. Schematic overview of the materials examined in this degradation study, with bis-urea (BU) (A), and ureidopyrimidinone (UPy) (B) based supramolecular biomaterials.

Fig. 2. Influence of enzymatic (A–D) and oxidative degradation (E, F) on mass loss (A, C, E) and Mw (B, D, F) of conventional and supramolecular PCL-based scaffold strips. Significant and relevant changes over time are indicated by lines between datapoints. Conventional PCL was mainly affected by enzymatic degradation with moderate to severe mass loss, but with stable molecular weight. The supramolecular materials were mostly affected by oxidative degradation, with mass loss as well as decreases in molecular weight. (A, C, and E) Contain error bars.

Mw remained constant over time. For the supramolecular materials, only the PCL₂₀₀₀-BU was affected by enzymatic degradation with moderate weight loss by both lipase (30%, $p < 0.01$) and cholesterol esterase (22%, $p < 0.01$) treatment (Fig. 2A and C). Mw of PCL₂₀₀₀-BU did not change with lipase treatment, while a small decrease in Mw (14%, $p < 0.05$) was observed during cholesterol esterase treatment. The PCL-UPy materials did not show changes in weight and Mw over time due to enzymatic degradation.

Oxidative degradation (Fig. 2E and F) did not affect mass and Mw of conventional PCL scaffolds, while all supramolecular materials were affected. Both PCL-UPy materials showed moderate mass loss (42% and 27%, $p < 0.01$ for PCL₈₀₀-UPy and PCL₂₀₀₀-UPy, respectively) and a severe reduction in Mw (71% and 83%, $p < 0.01$ for PCL₈₀₀-UPy and PCL₂₀₀₀-UPy, respectively). The PCL₂₀₀₀-BU also demonstrated moderate mass loss (35%, $p < 0.01$) and a severe reduction in Mw (94%, $p < 0.01$) due to oxidative degradation.

3.3. In-vitro degradation as monitored by scaffold fiber diameter and morphology

Enzymatic degradation (Fig. 3A and B) of conventional PCL scaffolds resulted in small to severe fiber diameter reduction, depending on the enzyme used (18% and 62%, $p < 0.01$ for cholesterol

esterase and lipase treatment, respectively). Enzymatic degradation did not affect the fiber diameter of both PCL-UPy materials, but resulted in a moderate reduction in fiber diameter in PCL₂₀₀₀-BU scaffolds after both lipase (31%, $p < 0.05$) and cholesterol esterase (25%, $p < 0.01$) treatment.

Oxidative degradation (Fig. 3C) did not affect the fiber diameter of the conventional PCL scaffolds, but resulted in moderate reduction in fiber diameter for the PCL-UPy materials (45% and 49%, $p < 0.01$ for PCL₈₀₀-UPy and PCL₂₀₀₀-UPy, respectively). PCL₂₀₀₀-BU showed a small reduction in fiber diameter after oxidative treatment (10%, $p < 0.01$).

SEM images of scaffold strips before and after enzymatic and oxidative degradation treatment show these changes in fiber diameter (Fig. 4A–P). They further demonstrate that the surface of the conventional PCL fibers is clearly affected by degradation, while the fiber surface of the supramolecular materials seemed less affected as compared to the conventional PCL, though more fragmented fibers were observed in the supramolecular scaffold groups.

3.4. Changes in mechanical properties during in vitro degradation

Enzymatic degradation (Fig. 5A, C and E) resulted in overall weakening of conventional PCL scaffolds with severe reductions

Fig. 3. Influence of enzymatic (A, B) and oxidative degradation (C) on the fiber diameter of conventional and supramolecular PCL-based scaffold strips. Significant and relevant changes over time are indicated by lines between datapoints. The fiber diameter of conventional PCL scaffolds was affected only by enzymatic degradation, while the supramolecular materials showed mainly reduced fiber diameters with oxidative degradation.

Fig. 4. SEM images with PCL-based scaffold strips before (A–D) and after enzymatic (E–L) and oxidative (M–P) degradation. Conventional PCL is mainly affected by enzymatic degradation, resulting in thinner and clearly affected fibers, while the supramolecular materials were mainly affected by oxidative degradation with thinner fibers. The fiber surface of the supramolecular materials seemed less affected as compared to the conventional PCL, though more fragmented fibers were observed. The white dots on the conventional PCL scaffold after oxidative degradation are presumably cobalt chloride remnants. White scale bars represent 20 μm.

in Young's modulus (96%, $p < 0.01$ and 57%, $p < 0.05$ for lipase and cholesterol esterase, respectively), UTS (96%, $p < 0.05$ and 51%, $p < 0.01$ for lipase and cholesterol esterase, respectively), and strain at break (80% and 66%, $p < 0.05$ for lipase and cholesterol esterase, respectively). The PCL-UPy materials did not demonstrate weakening, but changed mechanical properties with small to moderate increases in modulus, depending on the PCL soft segment length, for both lipase (13% and 44%, $p < 0.01$ for PCL₂₀₀₀-UPy and PCL₈₀₀-UPy, respectively) and cholesterol esterase treatment (19% and 99%, $p < 0.05$ for PCL₂₀₀₀-UPy and PCL₈₀₀-UPy, respectively). PCL₂₀₀₀-UPy further showed a moderate reduction in strain at break with lipase treatment (27%, $p < 0.05$), indicating a change toward a more brittle material. PCL₂₀₀₀-BU showed a combination of weakening and a change toward a more brittle material with moderate reductions in UTS (40%, $p < 0.01$) and strain at break (39%, $p < 0.01$) by cholesterol esterase treatment, and a severe increase in modulus (56%, $p < 0.01$) after lipase treatment.

Oxidative treatment (Fig. 5B, D and F) only affected strain at break of conventional PCL scaffolds with a moderate decrease (25%, $p < 0.05$), while modulus and UTS remained unaffected. The PCL-UPy materials showed a combination of weakening and a change toward a more brittle material with severe reductions in

UTS (96%, $p < 0.05$ and 87%, $p < 0.01$ for PCL₈₀₀-UPy and PCL₂₀₀₀-UPy, respectively) and strain at break (100% and 96%, $p < 0.01$ for PCL₈₀₀-UPy and PCL₂₀₀₀-UPy, respectively), and a severe increase in Young's modulus ($>300\%$, $p < 0.01$) after oxidative treatment. Similar weakening and changes toward a more brittle material were observed for PCL₂₀₀₀-BU with severe reductions in UTS (80%, $p < 0.05$) and strain at break (99%, $p < 0.05$), accompanied by a severe increase in Young's modulus ($>1000\%$, $p < 0.01$) after oxidative treatment.

3.5. PCL scaffold degradation by activated macrophages

Fig. 6 show representative SEM images of PCL scaffold fibers after seeding (A–C), after removal of the cells (D–F) and without cell seeding (G–I). After 2 days, 1 week, and 4 weeks of culture, surface erosion of the fibers was visible at various spots in the scaffold strips in the samples that had been exposed to activated cells. Up to 1 week, no damage in terms of broken fibers, surface erosion or cracks in the fibers were observed in the scaffolds cultured without cells, while after 4 weeks minor surface erosion could be observed at some places in the scaffolds which is due to hydrolysis of the scaffold fibers (I).

Fig. 5. Influence of enzymatic (A, C, E) and oxidative (B, D, F) degradation on the Young's modulus (A, B), UTS (C, D), and strain at break (E, F) of PCL-based scaffold strips. The results for cholesterol esterase treatment are not shown, but are comparable to those of the lipase treatment. Significant and relevant changes over time are indicated by lines between data points. The mechanical properties of conventional PCL were mainly affected by enzymatic degradation and represented by overall weakening. The mechanical properties of the supramolecular materials were affected by enzymatic degradation, but to a larger extent by oxidative degradation. Here, a change to a more brittle material was evident, accompanied by an overall weakening of the material.

Fig. 6. Representative SEM images of PCL scaffolds incubated in medium with or without activated cells. Photos were taken after 2 days, 1 week, and 4 weeks of culture with activated macrophages (A), after removal of activated macrophages (B), and for reference scaffolds that were kept in culture without the presence of cells (C). It shows that addition of activated macrophages resulted in degradation of the scaffold fibers. When no cells were added to the scaffold, only minor damage was observed, which was due to hydrolytic degradation (white arrow). White scale bars represents 50 μm in pictures (A–C) and 30 μm in pictures (D–I).

3.6. Susceptibility to degradation and its mechanisms

Correlation analyses revealed that the conventional PCL scaffolds were susceptible to enzymatic degradation, with the degree of susceptibility depending on the enzyme used. Susceptibility was higher for lipase (62%) as compared to cholesterol esterase (36%) and surface erosion seemed the dominant degradation mechanism (77% and 33% for lipase and cholesterol esterase treatment, respectively). Conventional PCL scaffolds were not susceptible (13%) to oxidative degradation.

The PCL-UPy materials were not susceptible to enzymatic degradation, neither lipase (2% for both PCL₈₀₀-UPy and PCL₂₀₀₀-UPy) nor cholesterol esterase (7% for both PCL₈₀₀-UPy and PCL₂₀₀₀-UPy). The susceptibility for oxidative degradation was dependent on the PCL soft segment length, with no susceptibility for PCL₈₀₀-UPy (13%) to susceptible for PCL₂₀₀₀-UPy (40%). Both surface erosion (23%) and bulk erosion (33%) seemed involved. PCL₂₀₀₀-BU was susceptible to enzymatic degradation, though only to cholesterol esterase (16% and 31% for lipase and cholesterol esterase, respectively), and oxidative degradation (24%). Surface erosion seemed the dominant mechanism in degradation of PCL₂₀₀₀-BU (33% and 23% for enzymatic and oxidative degradation, respectively).

4. Discussion

Electrospun biodegradable scaffold meshes represent promising candidates for use in in situ tissue engineering to replace diseased or damaged tissue parts. While providing initial mechanical stability upon implantation, host cells are recruited over time for neo-tissue formation, taking over the mechanical function of the scaffold. Supramolecular polymers represent interesting candidates to replace soft and elastic dynamically-loaded tissues, such as heart valves. To allow for the development of a stable fully

autologous implant that can grow and remodel in the patient, the scaffold should degrade in pace with neo-tissue formation. Here, we aimed to map the degradation characteristics of promising (supramolecular) materials, as well as their susceptibility to certain degradation pathways, for use in in situ tissue engineering approaches. An in vitro test was designed to investigate the degradation of electrospun biomaterial scaffolds either by enzyme-accelerated hydrolysis or by oxidation. In addition to changes in fiber morphology of the meshes, changes in mass of the scaffold, and changes in molecular weight, this in vitro study also monitored and assessed changes in the mechanical properties of electrospun scaffolds over time. The investigated scaffolds were prepared from polycaprolactone-based supramolecular biomaterials and conventional PCL served as a benchmark. Fig. 7 provides a schematic summary of the results obtained in this study indicating the changes over time with both enzymatic and oxidative degradation as well as their susceptibility for each polymer group.

The conventional PCL scaffolds were rapidly degraded by enzymatic hydrolysis, using lipase or cholesterol esterase, as evidenced by mass loss, changes in fiber morphology, and overall weakening, while molecular weight remained unaffected. These results are consistent with findings by others, although different types and concentrations of the enzymes resulted in slower or faster degradation of the PCL [27,28,51–54]. Polymer degradation by enzymes can be either surface erosion or bulk degradation, depending on the accessibility of the interior of the polymer to the enzyme. Surface erosion was identified here as the dominant degradation mechanism with clear effects to the fiber surface, thus apparently, the ability of the enzymes to infiltrate the hydrophobic semi-crystalline PCL is limited (or the activity of the enzyme becomes compromised upon infiltration) [22,55].

In contrast to the conventional PCL meshes, the supramolecular UPy- and BU-based polycaprolactones demonstrated to be less

Fig. 7. Schematic summary of the obtained results indicating the changes over time in mass, Mw, fiber diameter, and mechanical properties by either enzymatic and oxidative degradation over time for conventional and supramolecular PCL-based scaffolds. Furthermore, the susceptibility of each polymer group to enzymatic as well as oxidative degradation is represented by a color scale, with red indicating a high susceptibility, green referring to the material being not susceptible to degradation, and yellow showing an intermediate susceptibility. Illustrations by Anthal Smits.

prone to hydrolyze enzymatically with no or minimal changes in mass, molecular weight and fiber diameter. The PCL-UPy materials were classified as not susceptible to enzymatic degradation, though they demonstrated an increased Young's modulus, accompanied by a reduction in strain at break, which indicates a change to a more brittle material. This change may be caused by annealing of the material at 37 °C, resulting in a material with an increased crystallinity of the PCL phase, and thus a more brittle material. The PCL-BU was classified as susceptible to cholesterol esterase and not to lipase, though also with a change to a more brittle material. Clearly, the introduction of the BU or UPy hard blocks in the polycaprolactone backbone has a marked stabilizing effect on the enzymatic degradation rate, despite increasing the overall polarity of the biomaterial by introduction of the polar BU or UPy groups. Presumably, the different morphology of the materials as compared to conventional PCL is causing the changes in hydrolytic enzymatic degradation behavior. For PCL-BU, it is known that phase separation of the PCL soft block and the BU hard block is on the nanometer scale (ca. 10 nm scale) [17], implying that the partly amorphous PCL soft block in PCL-BU may be less accessible as compared to the more sizable amorphous PCL phases in conventional semi-crystalline PCL. Moreover, the molecular dynamics of the segmented PCL chains may be compromised, first as these chains are relatively short, and second as they are kept into position at both ends by the immobile UPy or BU hard blocks. According to the above factors, we propose that the PCL chains in the supramolecular biomaterials are less accessible to enzymes, and therefore causing the lowered enzymatic degradation susceptibility. Among the supramolecular materials, the PCL-BU was more susceptible to enzymatic degradation as compared to PCL-UPy, though similar PCL soft segment length were used in the backbone of PCL₂₀₀₀-UPy and PCL₂₀₀₀-BU. Apparently, the ester bonds in the BU-based material are more accessible and/or prone to hydrolysis as compared to those in the UPy containing material. Both materi-

als have phase separated soft and hard blocks and the exact manner in which this phase separation takes place may influence and determine their degradation. However, the exact differences in morphology, e.g. level of phase separation, mobility of the PCL chains, and the level of crystallinity of the PCL soft phase, between PCL-BU and PCL-UPy are not known and beyond the scope of this study.

Oxidative degradation gave the opposite result as that observed for the enzyme-accelerated degradation. Conventional PCL scaffolds were not susceptible to oxidative conditions, with stable mass, molecular weight, fiber diameter, Young's modulus, UTS, and only a small decreased strain at break. The presence of merely an aqueous solution without enzymes was clearly not enough to hydrolyze conventional PCL. Conventional PCL only has ester groups in its structure, and apparently these ester groups are not significantly degraded by the offered oxidative cobalt (II) solution, despite the fact that the amorphous phase in semi-crystalline PCL must be accessible to the presented small oxidative cobalt (II) and H₂O₂ (derived) species. The supramolecular materials on the other hand did show susceptibility to oxidative degradation, with decreases in mass, molecular weight, fiber diameter and a combination of weakening of the materials with a change to a more brittle material, with some fragmentation of fibers. We primarily attribute the augmented oxidative degradation of the supramolecular PCL-based biomaterials to their chemical differences with PCL (and not to morphological features), whereby these differences are represented by the presence of ureidopyrimidinone for PCL-UPy, which contain urethane groups, and the presence of urea groups for PCL-BU. Moreover, all supramolecular polycaprolactones are based on PCL-diols initiated from diethylene glycol, hence they comprise a single ether group in every PCL soft block, which also might result in an increased sensitivity to oxidative degradation, while conventional PCL contain ester groups only. Remarkably, the PCL soft segment length influenced the susceptibility to oxidative degradation, with the PCL₈₀₀

soft block providing more resistance to oxidative degradation. Oxidative degradation of the supramolecular materials was classified as surface erosion, though for the PCL-UPy bulk erosion was also noted, indicating diffusion of the small oxidative cobalt and H₂O₂ (derived) species into these materials.

It has to be noted that degradation is a dynamic process, as the mechanical, morphological and chemical properties of the polymers change during degradation, and all can affect surface and bulk mobility, accessibility by enzymes, and the diffusion of small molecules such as water, oxidative species and degradation products. Here, we have investigated degradation by enzymatic hydrolysis and oxidation separately. Implant degradation, however, may involve simultaneous action of both degradation pathways. Macrophages play an essential role in the degradation of polymeric scaffold meshes in situ tissue engineering as an inflammatory response provides the basis for neo-tissue formation. These macrophages secrete both enzymes as well as oxidative species, therewith triggering both degradation pathways. Accordingly, and in a feasibility study, we have exposed conventional PCL scaffolds to activated macrophages, and have monitored these scaffolds with SEM for a period of 4 weeks. Activated cells are known to produce high levels of enzymes and reactive oxygen species that could contribute to scaffold degradation [56]. SEM images showed more local damage of the scaffold fibers after removal of the activated macrophages compared to the damage observed for scaffold samples that were unseeded and that were only exposed to medium. This result demonstrated cell-mediated degradation of the scaffold fibers. Further in vitro experiments need to provide more insight into the degradation mechanisms of these cells on various materials.

Depending on the application, either fast or slow degradation is desired. When scaffold degradation is too slow it can result in stress shielding of the growing tissue, thereby impeding the regeneration process [57] or leading to undesirable outcomes. When the degradation process is too fast, the mechanical integrity of the implant is not sufficient, as the neo-tissue is not sufficiently developed yet to bear the full mechanical force required [58], leading to failure of the implant. Furthermore, the site of implantation might influence the degradation rate of a biomaterial. Mechanical forces, like compression, fatigue and shear stress, or external factors like pH might affect the degradation rate of the implanted material [23]. This again demonstrates the need to tailor the properties of biodegradable polymers specifically to the intended application.

5. Conclusion

In this study, we have demonstrated that conventional and supramolecular polycaprolactone-based polymers respond differently to enzyme-accelerated hydrolytic or oxidative degradation, depending on the morphological and chemical composition of the material. Conventional PCL is more prone to hydrolytic enzymatic degradation as compared to the examined supramolecular materials, while in contrast the supramolecular materials degrade faster by an oxidative pathway. Given this knowledge on degradation characteristics of different (supramolecular) materials, we are able to tailor degradation characteristics by combining different PCL backbones with additional supramolecular moieties. The toolbox of techniques that has been used to study the degradation of biomaterials can be applied and employed to screen, limit and select biomaterials that are going to be used for pre-clinical in vivo studies with regard to a variety of (TE) clinical applications.

Disclosures

Marieke Brugmans and Anandkumar Nandakumar are employed by Xeltis. Martijn Cox is employed by Xeltis and has

shares of Xeltis. Frank Baaijens and Anita Driessen-Mol have shares in Xeltis.

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