Chemical and biological properties of supramolecular polymer systems based on oligocaprolactones

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Abstract

We show that materials with a diverse range of mechanical and biological properties can be obtained using a modular approach by simply mixing different ratios of oligocaprolactones that are either end-functionalized or chain-extended with quadruple hydrogen bonding ureido-pyrimidinone (UPy) moieties. The use of two UPy-synthons allows for easy synthesis of UPy-modified polymers resulting in high yields. Comparison of end-functionalized UPy-polymers with chain-extended UPy-polymers shows that these polymers behave distinctively different regarding their material and biological properties. The end-modified UPy-polymer is rather stiff and brittle due to its high crystallinity. Disks made of this material fractures after subcutaneous implantation. The material shows a low inflammatory response which is accompanied by the formation of a fibrous capsule, reflecting the inertness of the material. The chain-extended UPy-material on the contrary is practically free of crystalline domains and shows clear flexible properties. This material deforms after in-vivo implantation, accompanied with cellular infiltration. By mixing both polymers, materials with intermediate properties concerning their mechanical and biological behaviour can be obtained. Surprisingly, a 20:80 mixture of both polymers with the chain-extended UPy-polymer in excess shows flexible properties without visible deformation upon implantation for 42 days. This mixture, a blend formed by intimate mixing through UPy–UPy interaction, also shows a mild tissue response accompanied with the formation of a thin capsule. The material does not become more crystalline upon implantation. Hence, this mixture might be an ideal scaffold material for soft tissue engineering due to its flexibility and diminished fibrous tissue formation, and illustrates the strength of the modular approach.

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degradation in macrophages and giant cells takes place [7]. Many polymeric variations of PCL have been investigated ranging from blends [8] to co-polymers [9]. PCL was also used in shape-memory polymers, which can be used for minimally invasive surgery [10–12].

Recently, we have shown that a supramolecular and modular toolbox can be used for the assembly of bioactive polymers [13]. Using supramolecular building blocks, that assemble non-covalently via specific interactions, makes it possible to produce materials without tedious synthetic procedures but simply by assembly. In this way, it becomes easy to vary the amount and nature of the bioactive molecules and the nature of the polymers. This supramolecular approach bridges the gap between covalent modification and simple mixing of molecules and polymers. The first method leads to highly stable structures that lack dynamics. The latter, in contrast, results in very dynamic structures that lack stability. Using a supramolecular approach it is possible to control both stability and dynamics. The bioactive supramolecular system we have shown before, is based on oligocaprolactone end-functionalized with 2-ureido-4[1H]-pyrimidinone (UPy) groups [13]. These UPy-moieties strongly dimerize via quadruple hydrogen bonding and display high association constants ($K_a = 10^6–10^7 \text{L mol}^{-1}$) in organic solvents [14–17]. The UPy-functionalized oligocaprolactone [13] shows much better mechanical properties than its unfunctionalized variation. These supramolecular polymer systems find already many uses in polymer applications [18,19].

Here, we report that the addition of more than two UPy-units in the main chain can dramatically improve the material properties, while still supporting a modular approach (Fig. 1). A chain-extended supramolecular oligocaprolactone-based UPy-polymer was designed (PCL$_{1250}$UPy$_6$; Fig. 1B) that can be easily blended with the end-functionalized UPy-oligocaprolactone (PCL$_{2000}$ UPy$_2$; Fig. 1A). The material properties of the pure polymers were compared to mixtures of both polymers (Fig. 1C). Additionally, the in vivo behaviour was studied showing the tunability of the supramolecular system.

2. Materials and methods

2.1. General materials

The hydroxy-terminated PCLs ($M_a = 2.1$ kg/mol and $M_a = 1.25$ kg/mol) were purchased from Acros. 1,6-Diisocyanatohexane was obtained from Fluka. 2-Amino-4-hydroxy-6-methylpyrimidine, dibutyl tin dilaurate (DBTDL) and isophorone diisocyanate (IPDI) were purchased from Aldrich. Commercial products were used without further purification. All solvents purchased from Acros Chimica or Sigma-Aldrich were of...
p.a. quality. Deuterated chloroform was obtained from Cambridge Isotope Laboratories. Water was always demineralized prior to use. Phosphate-buffered saline (PBS) tablets were purchased from Sigma (dissolution of the tablets in water resulted in a 0.01 M phosphate buffer with 0.0027 M potassium chloride and 0.137 M sodium chloride, with pH = 7.4).

2.2. General methods

1H-NMR and 13C-NMR spectra were recorded on a Varian Mercury 400 MHz spectrometer at 298 K. Chemical shifts are given in ppm (δ) values relative to tetramethylsilane (TMS). Infrared (IR) spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer with a Universal ATR Sampling Accessory for solids. Elemental analysis was carried out on a Perkin Elmer 2400 apparatus. Matrix assisted laser desorption/ionization–time of flight (MALDI–TOF) mass spectroscopy was performed on a PerSeptive Biosystems Voyager-DE PRO spectrometer. 2D NMR spectra were collected on a Varian Mercury 400 spectrometer using 1D and 2D experiments. Gel permeation chromatography (GPC) was performed on a Shimadzu FCV-10 AL VP with a Shimadzu SCL-10AL VP system controller, equipped with a trilab refractive index detector and a Polymer Laboratories PL-ELS 1000 detector. Narrow polystyrene standards were used for calibration. Optical microscopy pictures of the explanted disks were taken on a Zeiss Axiosvert 25 microscope with a Sony (Cybershot, 3.3 Megapixels) digital still camera DSC-S75 (Carl Zeiss ACC Terminal). Normal photographs of these disks were taken with the same Sony digital still camera DSC-S75. Pictures of tissue slices were taken on a Leica DMLB microscope with Leica DC300 camera.

2.3. Synthesis UPy-hexyl-isocyanate synthon (1b)

The synthesis was performed as described before [15]. 2-Amino-4-hydroxy-6-methylpyrimidine (1a; 0.23 mol; 29.1 g) was dissolved in 1,6-diisooctanohexane (1.6 mol; 272.3 g) and heated at 100°C for 16 h. Pentane (ratio 10:1 = pentane:reaction mixture) was added to the reaction mixture after cooling. The resulting precipitate was filtered and thoroughly washed with pentane. The product was dried at 50°C under reduced pressure, yielding 1b as a white powder. Yield: 98%, 66.8 g; 0.23 mol. M.p.: 185°C. 1H-NMR (CDCl3): δ = 13.13 (s, 2H, CH2–NH–(C=O–O)–NH–(C=O–O)–OCH2, 1.76 (s, 2H, CH2–NH–(C=O–O)–NH–(C=O–O)–OCH2, 2.39 (m, 4H, CH2–CH2–C–N), 15.20 ppm. IR (ATR): 173.5, 173.1, 156.7, 156.5, 154.6, 148.2, 106.6, 69.0, 64.4, 64.1, 63.2, 40.6, 39.6, 34.0, 33.9, 29.7, 29.3, 28.7, 28.3, 26.2, 26.1, 25.5, 24.6, 24.5, 24.4, 18.9 ppm. IR (ATR): ν = 2943, 2865, 1721 (C=O stretch), 1698 (UPy), 1669 (UPy), 1584 (UPy), 1528 (UPy), 1471, 1419, 1397, 1366, 1294, 1240, 1185 (C–O stretch), 1107 cm⁻¹.

2.4. Synthesis PCL2000UPy2 (1)

PCL diol (Mn = 2.1 kg/mol; obtained via ring-opening polymerization initiated by diethylene glycol) was reacted with the UPy-hexyl-isocyanate synthon 1b, making use of a similar procedure as described before [15]. PCL diol (10 mmol; 20.0 g) was dissolved in dry chloroform (300 mL), after which 1b (38 mmol; 11.2 g) was added. After addition of two drops of DBTDL the solution was stirred at 75 °C for 16 h. The completeness of the reaction was checked with 1H-NMR and 13C-NMR for the presence of OH endgroups. Then 5 g silica kieselgel 60 and two drops DBTDL were added and the mixture was heated at 75 °C for 4 h. The reaction mixture was put overnight at room temperature. The silica was removed by filtration on a glassfilter over Hyflo after dilution of the mixture with chloroform (1:1 = chloroform:reaction mixture). With IR the absence of 1b in the solution was checked. Compound 1b was still present and the solution was heated again with 3 g silica and two drops DBTDL at 75 °C for 3 h. Again the silica was removed by filtration. The chloroform was removed under reduced pressure; the material was precipitated from chloroform in hexane and filtrated. The resulting white material was dried for 2 days under reduced pressure. With IR the absence of 1b in the material was checked again and it was found to be absent. Yield: 73%, 18.8 g, 7.3 mmol. 1H-NMR (CDCl3): δ = 13.13 (s, 2H, C–NH–C=N, UPy), 11.76 (s, 2H, CH2–NH–(C=O–O)–NH–(C=O–O)–OCH2, 5.85 (s, 2H, C=CH2, UPy), 4.90 (s, 2H, CH2–NH–(C=O–O)–OCH2, 4.23 (t, 4H, CH2–(C=O–O)–OCH2–CH2–O), 4.06 (t, 2H, CH2–(C=O–O)–OCH2–CH2–O), 3.69 (t, 4H, CH2–(C=O–O)–OCH2–CH2–O), 3.24 (m, 4H, CH2–(C=O–O)–OCH2–CH2–O), 3.16 (m, 4H, CH2–(C=O–O)–OCH2–CH2–O), 2.31 (m, 2H, CH2–(C=O–O)–OCH2–CH2–O), 2.23 (s, 6H, CH2–CH2–C–N), 1.64 (m, 4H, CH2–(C=O–O)–OCH2–CH2–O), 1.50 (m, 16H, NH–(C=O–O)–NH–CH2–CH2–CH2–CH2–NH–(C=O–O)–O), 1.39 (m, 3H, CH2–(C=O–O)–OCH2–CH2–CH2–NH–(C=O–O)–OCH2–CH2–CH2–NH–(C=O–O)–O), 0.14 ppm. IR (ATR): 1603, 1571, 1570, 1565, 1548, 1426, 1410, 1384, 1383, 1370, 1355, 1349, 1253, 1240, 1187, 1107 cm⁻¹.

2.5. Synthesis IPDI-UPy-IPDI synthon (2b)

5-(2-Hydroxyethyl)-6-methylisocyanate (2a) was synthesized from 2-acetylbutyrolactone according to the literature procedure [20]. 5-(2-Hydroxyethyl)-6-methylisocyanate (2a, 71 mmol; 12.0 g) was suspended in IPDI (715 mmol; 159 g) and was stirred for 16 h at 90 °C under an argon atmosphere. A clear solution developed. The solution was cooled and precipitated in hexane. The solid was filtered and stirred in another portion of hexane. The product was isolated by filtration and washed with hexane. The white residue was dried. Yield: 81%, 35.5 g; 58 mmol. M.p.: 204°C. 1H-NMR (CDCl3): δ = 12.93–12.83 (m, 1H, CH2–C–N), 12.01–11.92 (m, 1H, CH2–NH–(C=O–O)–NH), 10.18–10.13 (m, 1H, CH2–NH–(C=O–O)–NH), 5.15–4.54 (m, 1H, CH2–CH2–O–(C=O–O)–NH), 4.20 (t, 2H, CH2–CH2–O–(C=O–O)–NH), 3.98–3.26 (m, 3H, CH2–NH–(C=O–O)–O–CH2–CH2–O, 4.06 ppm. IR (ATR): ν = 2931, 2262 (NCO stretch), 1698 (UPy), 1667 (UPy), 1577 (UPy), 1519 (UPy), 1461, 1356, 1310, 1255 ppm. Anal. calcd. (%) for C13H19N5O3: C 53.2, H 6.5, N 23.9; Found (%): C 53.2, H 6.2, N 24.0.

2.6. Synthesis PCL1250UPy2 (2)

Telechelic hydroxy-terminated PCL (Mn = 1.25 kg/mol; obtained via ring-opening polymerization initiated by diethylene glycol) was dried in vacuo. The PCL diol (21 mmol; 25.9 g) was reacted with the IPDI-UPy-IPDI synthon 2b (18 mmol; 10.9 g) in dry ethylacetaete (130 mL) in the presence of two drops of DBTDL for 16 h at 70°C. After that, ethylacetaete and ethanol (70 mL:50 mL) were added to the reaction mixture, which was subsequently precipitated twice into ethanol. The polymer was isolated after drying, resulting in a white elastic material. Yield: 90%, 33.1 g, 2.7 mmol. 1H-NMR (CDCl3): δ = 12.84 ppm (s, 1H, CH3–C–N), 11.88 (s, 2H, CH2–NH–(C=O–O)–NH), 10.03 ppm (s, 1H, CH2–NH–(C=O–O)–O), 9.45–4.40 ppm (3s, broad, 2H, NH–(C=O–O)–O),
4.16 (t, 4H, \(\text{CH}_2\text{CH}_2\text{O})\), 1.30 (m, 2nH, \(\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\) at UPy), 1.12–0.86 (m, 26H, isophorone moieties). ppm. Note (1H-NMR): the integrals are based on 1 UPy-group.

2.7. Preparation of mixtures

Fibres were made of the pristine polymers PCL2000UPy2 and PCL1250UPy6 and of mixtures consisting of different ratios (in weight%) by dissolution of the polymers separately or simultaneously (in the case of mixtures) in THF while stirring the solution for 16 h at room temperature (concentration ≈ 0.1 g/mL). The polymer solutions were drop cast in teflon moulds. The generated fibres were dried in vacuo for 1–2 days at 37 °C. Mixtures consisting of 60:40 (m1), 50:50 (m2) and 20:80 (m3) PCL2000UPy2/PCL1250UPy6 were used. Mixtures m1 and m3 were used to perform the tensile testing experiments and in vivo studies. The materials that were used for in vivo experiments were sterilized with UV for at least 2 h on each side.

2.8. Tensile testing

Mechanical properties were performed in a tensile mode according to ASTM D1708-96 specifications in air at room temperature on a Zwick Universal Tensile Tester at an elongation rate of 20 mm/min with a grip separation of approximately 20 mm. The measurements were performed at least in quadruplicate. Yield stresses and yield strains were determined, due to the shape of the stress–strain curves, by taking the intersection point of the two tangents to the initial and final parts of the load elongation curves around the yield point [21]. An indicative Young’s modulus (\(E\)) was determined by calculating the slope at 0% strain.

2.9. Differential scanning calorimetry

Thermal properties were investigated with differential scanning calorimetry (DSC) on a Perkin Elmer Differential Scanning Calorimeter Pyris 1 with Pyris 1 DSC Autosampler and Perkin Elmer CCAF cooling element under a nitrogen atmosphere with heating and cooling rates of 20 °C/min (samples of 10–12 mg were measured). The temperature range was –100 °C to 120 °C.

2.10. In vivo implantations: PCL2000UPy2 versus PCL1250UPy6

Solution cast films of PCL2000UPy2 and PCL1250UPy6 were used to study these samples; mass loss (the dry mass was measured on a Sartorius microbalance), morphology, NMR (conc. 14 mg/mL in CDC13), IR and DSC measurements were performed. The second fraction was embedded in plastic (see above) and stained with toluidine blue. The third part was frozen in liquid nitrogen and cut into slices (7 μm) for immune staining with anti-collagen IV antibody (goat anti-type IV collagen-UNLB, Southern Biotechnology Associates), the ED1 marker (mouse anti-rat CD68 MCA341 R, Serotec) and the R73 antibody. The second antibodies that were used, were RAMPO (polyclonal rabbit anti-mouse immunoglobulins HRP, DakoCytomation) for ED1 and R73 and RAGPO (polyclonal rabbit anti-goat immunoglobulins HRP, DakoCytomation) for collagen IV. The final colouring reaction was performed with hydrogen peroxide and 3-amino-9-ethylcarbazole (AEC) resulting in a red colour if the staining was positive. Finally, the tissue slices were coloured with hematoxilin (blue). As positive control tissue rat spleen was used.

2.12. In vitro incubation

The pristine polymers 1 and 2 and the mixtures m2 and m3 (90–140 mg per sample) were incubated in PBS buffer (20 mL per sample) in duplicate for 126 days at 37 °C. Mass measurements (the wet mass (water absorption) and dry mass (mass loss) of the samples were measured on a Sartorius microbalance) and DSC experiments were performed, after rinsing the samples three times with water and drying them at 30 °C for 1.5 h.

3. Results and discussion

3.1. Synthesis of UPy-synthons and UPy-polymers

Both materials, the bifunctional and chain-extended UPy-polymers, were synthesized using two different UPy-isocyanate synthons, to facilitate the synthetic method. The bifunctional UPy-terminated PCL (PCL2000UPy2; Scheme 1A) consists of a PCL part with a molecular weight of 2.1 kg/mol. Methyl-isocytosine (1a) was reacted for 16 h at 100 °C in an excess of hexyldiisocyanate resulting in the formation of the UPy-hexyl-isocyanate synthon, 2(6-isocyanatohexylamino)-6-methyl-[4(1H)-pyrimidinone (1b) [15]. The UPy-hexyl-isocyanate synthon 1b was precipitated in pentane and was obtained as a white powder with
a yield of 98%. Hydroxy-terminated PCL was reacted with an excess of 1b in chloroform with DBTDL as catalyst at 75 °C for 16 h resulting in PCL2000UPy2 (1) [13,15]. The mixture became more viscous in the course of the reaction, indicating the formation of virtual high molecular weight polymers due to dimerization of the UPy-moieties (Fig. 1A). The excess of 1b was removed via stirring the reaction mixture with silica at 75 °C in the presence of DBTDL for 4 h. The silica was removed by filtration and 1 was precipitated in hexane [13,15] and obtained as a white material with a yield of 73%.

The molecular weight of the PCL prepolymer used for the chain-extended PCL (PCL1250UPy6; Scheme 1B) is 1250 g/mol. On average, six UPy-units are part of the main chain. The starting compound 5-(2-hydroxyethyl)-6-methyl-isocytosine (2a) was synthesized from 2-acetylbutyrolactone [20]. Compound 2a was reacted in an excess of IPDI for 16 h at 90 °C to yield IPDI–UPy–IPDI synthon 2b. The product was precipitated in hexane, which resulted in 81% 2b as a white powder. The hydroxy end-groups of the PCL (1.25 kg/mol) were reacted with 2b in a ratio of 7:6 in ethylacetate in the presence of DBTDL as catalyst for 16 h at 70 °C. Also, during this reaction the mixture became more viscous, indicating the formation of longer polymers via chain extension and the generation of physical aggregation between chains by UPy–UPy dimers (Fig. 1B). Polymer 2 was precipitated in ethanol and obtained as a white elastic material with a yield of 90%.

3.2. Mechanical properties

Mixtures consisting of different ratios (in weight%) of polymer 1 and 2 (60:40 (m1), 50:50 (m2) and 20:80 (m3) PCL2000UPy2:PCL1250UPy6) were used to correlate different material properties to their in vitro and in vivo behaviour. Tensile test experiments were performed on
the pristine polymers (1 and 2) and on the different mixtures (60:40 and 20:80 PCL-2000UPy2:PCL1250UPy6, m1 and m3, respectively) to investigate their mechanical properties.

The differences in mechanical properties between polymer 1 and 2 are clear (Fig. 2; Table 1). In the case of polymer 1, the Young’s modulus (E) and yield stress (σyield) are relatively high, whereas the elongation at break (εbreak) is relatively low. Polymer 1 is rather stiff, more brittle and less tough as compared to polymer 2, which is more flexible and ductile. Polymer 1 is able to form relatively high virtual molecular weight chains via hydrogen bonding between the end-capped UPy-groups (Fig. 1A). This dimerization gives the material its properties; the unfunctionalized hydroxy-terminated oligocaprolactone is a waxy, brittle solid. On the contrary, polymer 2 forms physical cross-links between the already longer chains that are formed by covalent chain-extension during synthesis resulting in a huge physical network (Fig. 1B). Mixtures of polymer 1 and 2 are visualized in Fig. 1C.

The tensile tests on the mixtures show that after addition of 40% 2 to 1 (m1), the E and σyield decrease, that εbreak increases and that the yield point (σyield) takes place at a larger elongation. This indicates that material m1 is less stiff, and more tough and flexible than 1. Noteworthy is also that the graph of m1 slowly moves in time to the position of 1, which might be caused by aging and crystallization. This is also reflected in the relatively higher margins of error for m1 (Table 1). Surprisingly, when more 2 is added to 1, a material (m3) is obtained that has a lower yield stress (σyield) and stress at break (σbreak) when compared to 2. Evidently, the relative low amount of 1 is not enough to increase the σyield and σbreak of 2, and the opposite is observed. This might be caused by the fact that the presence of 1 hampers the strain-induced crystallization that is present to some extent in pristine 2. This makes the material m3 weaker (by reduction of the stress at break). It is evident that the material properties can be tuned by mixing 1 and 2.

### 3.3. Thermal properties

The thermal properties of the polymers 1 and 2 and of the mixtures m1 and m3 were studied with DSC. Clear differences are visible between the second heating runs of the different polymers and polymer mixtures, reflecting the intrinsic polymer properties (Fig. 3A and B; supplementary information). The unfunctionalized PCL prepolymer do not show a glass transition temperature (Tg; data not shown). However, owing to UPy-functionalization a Tg appears for all polymers studied. The Tg shifts to higher temperatures upon increasing the relative amount of 2 in the mixtures (Fig. 3B). This reflects the intimate mixing between 1 and 2, showing that the polymers do not phase separate. The shifting of the Tg is possibly caused by the presence of a larger amount of longer chains, which leads to a decrease of the free volume. A decrease in the heat capacity, ΔCp, was measured upon addition of 2 (Fig. 3B).

Only the films consisting of 1 and m1 showed a melting peak (Tm) in the second heating run around 42 °C with a melting enthalpy, ΔHm, of 31 and 1.1 J/g, respectively. This lowering of ΔHm for m1 might be caused by suppression of the crystallization of 1 by 2. This might be due to the shorter PCL chains of 2. Another possibility is that the IPDI–UPy–IPDI units in 2 are less prone to crystallization than the UPy-hexyl moieties in 1, because of steric reasons and/or the presence of different ways of IPDI incorporation.

The films of 2 and m3 did not show a melting peak. The tendency of the PCL part to crystallize is higher for the PCL2000UPy2 polymer 1 than for the PCL1250UPy6 polymer 2, which is mainly caused by the chain length of the PCL part. The absence of a Tm for 2 and m3 might also be caused by the presence of the IPDI–UPy–IPDI moieties. The thermogram of 1 showed a second melting peak at 58 °C. This peak is probably originating from the melting of the UPy-hexyl-urethane dimer hard-blocks (the UPy–UPy stacks in the lateral direction). This peak might also be due to a second, more thermodynamically stable, crystalline form of PCL. This peak was not found for mixture m1, which is probably because of the presence of the long PCL1250UPy6 chains that create less ordered structures or caused by the IPDI–UPy–IPDI moieties. Only polymer 1 shows a crystallization (Tg) signal in the second heating run.

The DSC results discussed here show that as expected polymer 1 and 2 do not phase separate, which is possibly enhanced by the UPy–UPy interaction. However, phase separation between the UPy-units and PCL-chains might be present. To search for a material with both excellent material properties and good biological properties, these mixtures were studied in vivo.

| Table 1 | Tensile test data of the polymer films that were used for subcutaneous implantation in rats. The Young’s modulus (E in MPa), yield stress (σyield in MPa), yield strain (εyield in %), stress at break (σbreak in MPa), elongation at break (εbreak in %) and the maximum stress (σmax in MPa) are shown. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| E (MPa) | σyield (MPa) | εyield (%) | σbreak (MPa) | εbreak (%) | σmax (MPa) |
| 1 | 129 ± 7 | 6.3 ± 0.25 | 5.4 ± 0.1 | 6.0 ± 0.4 | 14 ± 4 | 6.5 ± 0.4 |
| m1 | 28 ± 10 | 2.2 ± 0.5 | 9.1 ± 0.9 | 2.0 ± 0.2 | 33 ± 18 | 2.4 ± 0.7 |
| m3 | 4.1 ± 0.7 | 0.72 ± 0.08 | 35.2 ± 6.5 | 0.9 ± 0.1 | 511 ± 143 | 1.0 ± 0.1 |
| 2 | 3.1 ± 0.6 | 0.83 ± 0.17 | 45.8 ± 4.6 | 2.4 ± 0.6 | 576 ± 172 | 2.5 ± 0.6 |

**References**

3.4. Tissue response of end-modified and chain-extended UPy-polymers

The pristine polymers 1 and 2 were subcutaneously implanted as disks in rats to study their biocompatibility and the onset, progression and resolution of the Foreign Body Reaction (FBR) in vivo. Despite the fact that the FBR is very low for both materials, differences between the two polymers were clearly visible on the level of cellular infiltration and immune response (Fig. 4). PCL2000UPy2 1 seemed chemically inert and stayed intact during the whole period. Fibrin was observed at the interface up to 5 days (Fig. 4A—1 and A—2). Remarkably, after 10 days PCL 1250UPy6 2 seemed to be slightly deformed. This deformation became more intense after 21 and 42 days. This phenomenon was attended by increased cellular infiltration of macrophages (Mφint: Fig. 4B—1) and giant cells (Gcin: Fig. 4B—2) in time. It is not inconceivable that this distortion of the shape of material 2 might be partially due to the ingrowth of these cells (supplementary information). This infiltration is probably caused by its low crystallinity (see below). In contrast, for 1, macrophage infiltration accompanied by some exudate formation was observed at day 2. The presence of macrophages at the interface became relatively low after 21 days (Mφint: Fig. 4B—1). Giant cells were sporadically present at the interface (Gcin: Fig. 4B—1). A clear fibrous capsule (c; Fig. 4A—1) was developed for 1 from day 10 up to 42, with an increased amount of fibroblasts (F; Fig. 4B—1). On the contrary, for 2 the capsule (c; Fig. 4A—2) was rather thin and the amount of fibroblasts (F; Fig. 4B—2) was low. Also, another distinction could be made concerning the vascularization in the surrounding tissue (Vas; Fig. 4B—1 and 4B—2). In general, vascularization was low for 2, while increased for 1 after 10 days. However, vascularization seems to be increased at the interface of 2. For both polymers 1 and 2 holds that polymorph nuclear cells and lymphocytes were...
scarcely present in the surrounding tissue at all time points, which again shows that the FBR is very mild for both materials.

The mild FBR in the case of material 1 is accompanied with fibrous capsule formation showing the inertness of the material. On the contrary, for material 2 major cellular infiltration, accompanied by a very thin fibrous capsule and an increased amount of vascularization at the interface, is observed. It was already demonstrated for other polymer scaffolds that an increased cellular invasion can be accompanied by higher vascularization [22]. Besides this low FBR, major deformation could be detected for 2. So this material might find its application as self-adapting wound filler.
3.5. Mass loss of implanted mixtures

A modular approach was applied to tune deformation and cellular infiltration. Consequently, both pristine polymers 1 and 2 and mixtures with different ratios, 60:40 (m1) and 20:80 (m3) PCL_{2000}UPy_2:PCL_{1250}UPy_6 were implanted subcutaneously for 42 days to study the resolution of the FBR. As a control experiment, the pristine polymers 1 and 2 and mixtures 50:50 (m2) and 20:80 (m3) were incubated in vitro in PBS buffer at 37 °C for 126 days.

The degradation of the implanted disks was investigated by measuring mass loss. The implanted disks were not degraded during the period of 42 days according to mass loss experiments (0.8 ± 0.2% for 1, 1.1 ± 0.3% for m1, 0.6 ± 0.1% for m3 and 0.90 ± 0.04% for 2). This was also confirmed with 1H-NMR. The spectra were exactly the same before and after implantation for 42 days; no structural differences or hydrolysis could be detected. Also, GPC did not show any hydrolysis. This in vivo experiment was compared to films made of the pure polymers 1 and 2 and of similar mixtures of PCL_{2000}UPy_2:PCL_{1250}UPy_6 (50:50 (m2) and 20:80 (m3)) that were incubated for 126 days in PBS buffer (pH 7.4) at 37 °C.

Upon addition of 2 the water absorption might become higher when compared to the pure 1 after 126 days (3 ± 3% for 1, 7 ± 2% for m2, 4 ± 1% for m3 and 6 ± 3% for 2). Unfortunately, the error in these measurements is large which prohibits real quantification. Furthermore, the mass loss in vitro is lower for mixtures with more 2 as compared to the pure 1 (5 ± 4% for 1, 1 ± 1% for m2, 0.2 ± 0.2% for m3 and 0.3 ± 0.1% for 2). This might be caused by the fact that 1 becomes more crystalline during incubation than 2 (see below) which leads to partial fragmentation of the film caused by increased brittleness of 1. However, even after 126 days in buffer the mass loss is negligible small for all films. In conclusion, the end-modified and chain-extended UPy-PCL polymers do not degrade during 42 days of subcutaneous implantation, nor in buffer at 37 °C for 126 days.

3.6. Morphology and crystallinity of implanted mixtures

The morphology and crystallinity of the mixtures after implantation was studied using optical microscopy, IR spectroscopy and DSC. The morphology of the disks changes during implantation (Fig. 5A). The disks of 1 and m1 are intact but break upon explantation, reflecting the brittle nature of the materials due to their higher crystallinity. Both materials can be removed easily from the capsule, indicating hardly any interaction with the cells. The surface of these materials is very smooth, which is also visible using optical microscopy (Fig. 5A). Material m3 stays intact and is not deformed. Disk 2 is much more difficult to remove from its capsule and shows on the contrary a massive deformation (Fig. 5A). The less smooth surface of 2 is confirmed with optical microscopy (Fig. 5A).

To study the surface morphology, the IR spectra of polymers 1 and 2 and mixtures m1 and m3 that were kept for 42 days at room temperature were compared to the spectra of the samples that were subcutaneously implanted in rats for 42 days. The IR spectra for all polymer films were similar (supplementary information). The spectra in general became broader owing to implantation, especially for disks 2, m1 and m3, indicating that the system shows less organization at the surface [23]. This more random state can be attributed to the presence of water, which is shown at 3300 cm^{-1}. This characteristic OH band became higher after implantation. Water might be incorporated or disturb the hydrogen bonding between the UPy-groups. However, no evidence in this study or earlier investigations is found for a significant effect of water on the quadruple hydrogen bonding units. The IR measurements also show that the disks do not degrade during the investigated time.

To investigate the nature of the fracturing and deformation of the disks after implantation, the thermal properties of the implanted disks were measured. From the second heating runs (Fig. 3A) it is clear that the polymers 1 and 2 and mixtures m1 and m3 display similar phase behaviour after subcutaneous implantation for 42 days in rats when compared to the samples that were kept at room temperature for 42 days. Very similar results were found for the samples (1, 2, m2 and m3) that were incubated in buffer for 126 days at 37 °C (supplementary information).

Whereas the intrinsic polymer properties do not change, clear differences are found concerning the thermal history of the disks retrieved from the first heating runs (Fig. 3C and D). The glass transition temperatures (T_g) stay more or less the same and follow the same trend as was observed for the second heating runs. Unfortunately, the heat capacities cannot always be determined. The onset of the melting temperature changes after incubation from approximately 30 °C to temperatures around 50 °C. This implies the formation of more stable PCL crystals upon annealing at 37–38 °C in the rat in all four films. The same results were found for the polymers 1, 2, m2 and m3 that were incubated in vitro (supplementary information) indicating that these phenomena are not due to subcutaneous implantation but probably caused by annealing at 37 °C and the presence of an aqueous environment.

The materials 1, 2 and m1 become more crystalline upon incubation; an increase in ΔH_m can be seen. The increase in ΔH_m for 1 is much larger than for the other mixtures. Striking is that the melting enthalpies, ΔH_m, for the polymers that were kept at room temperature, m1 and m3 are higher than for 1, indicating that the crystallization process at room temperature is slower for 1. After annealing in rats or in buffer these differences change. Hardly any change in ΔH_m for mixture m3 is found after annealing in rats or in buffer as compared with the samples that were left at room temperature (Fig. 3D). This indicates that m3 is already in its most thermodynamically stable state before annealing. So, disks made of 1 and m1 became more crystalline owing to subcutaneous implantation.
Fig. 5. In vivo behaviour of the disks of the pristine polymers PCL_{2000}{UPy}_2 (1) and PCL_{1250}{UPy}_6 (2) and of mixtures consisting of 60:40 (m1) or 20:80 (m3) PCL_{2000}{UPy}_2:PCL_{1250}{UPy}_6 after 42 days of subcutaneous implantation in rats. (A) The explanted disks. The first row consists of photographs. The second row shows optical micrographs with enlargements at 100 times magnification. All scale bars represent 1 mm. (B) Histological examination after toluidine blue staining; m indicates the material. The scale bars represent 100 μm. (C) Histological examination after toluidine blue staining. The following abbreviations are used: m = material, v = blood vessel, c = fibrous capsule, # = macrophage, * = giant cell. The scale bars represent 100 μm. (D) Immune-stained histology. The first row shows tissue sections that were stained using the anti-collagen IV antibody to detect vascularization. The second row consists of micrographs of tissue sections stained with the ED1 marker against macrophages. The third row shows tissue sections were stained with the R73 antibody against T-cell receptors on the lymphocytes. The scale bars represent 100 μm. m = material, v = blood vessel, c = fibrous capsule, # = macrophage, * = giant cell.
(displaying similar $\Delta H_m$, Fig. 3D) reflecting their brittleness and fracture upon explantation. The change in crystallinity of disk 2, however, was relatively low. This phenomenon was accompanied by the very small $\Delta H_m$, which resulted in deformation of disk 2. On the contrary, no change in crystallinity could be detected for m3, but its $\Delta H_m$ was high enough to resist deformation.

3.7. Tissue response of mixtures

The histology of the tissue sections of the mixtures was investigated to determine the cellular response. The toluidine blue-stained tissue sections reveal that 1 is an inert material and no cellular infiltration into the material was observed, as already shown above (Fig. 5B and C). This is in accordance with the ease of explantation. For polymer 2 the cellular infiltration is clearly visible by showing macrophages and giant cells at the interface (Fig. 5C and D). The amount of different cell types and vascularization were determined in the corresponding tissue sections of the mixtures m1 and m3 by immunohistochemistry and were compared to the pristine polymers 1 and 2 (Figs. 5D and 6). Mixture m3 is more comparable to polymer 2. In the case of polymer 2 the amount of macrophages ($M_{\phi_{in}}$, Figs. 5D and 6) at the interface is much higher and also giant cells ($G_{cin}$, Figs. 5D and 6) are visible. This surface reaction is also present for m3 but to a lesser extent. Mixture m1 shows a similar FBR as polymer 1 (Fig. 6). The fibrous capsule comprising of many fibroblasts is thicker for 1, m1 and m3 (F; Fig. 6). Hardly any macrophages are present in these capsules ($M_{\phi_{out}}$, Figs. 5D and 6). Also the amount of macrophages located in the surrounding tissue is very low ($M_{\phi_{out}}$, Figs. 5D and 6). Furthermore, lymphocytes are not present in all material explants (Fig. 5D). The vascularization in the surroundings is much higher for polymer 1 and m1 ($Vas$, Figs. 5D and 6). For material 2, vascularization is particularly higher at the interface. In the case of disks 1, m1 and m3 a more granular anti-collagen IV staining near the interface is observed (Fig. 5D). This might indicate desintegration of the extracellular matrix of blood vessels. It is clear that the inflammatory response on the different polymeric disks can be tuned via mixing polymer 1 and 2. Upon addition of more 2 in the mixture the response becomes more located to the surface. This reaction is accompanied with higher vascularization near the interface and a thinner fibrous capsule.

4. Conclusions

Intimate mixing through quadruple hydrogen bonding allows the formation of systems based on two oligocaprolactone-based building blocks: end-functionalized UPy-oligocaprolactone (PCL$_{2000}$UPy$_2$) and chain-extended UPy-oligocaprolactone (PCL$_{1250}$UPy$_6$). The two polymer building blocks show different material properties and biological behaviour in vivo. Making use of all the benefits of these reversible supramolecular polymer systems, a highly interesting biomaterial was formed, comprising of 20:80 PCL$_{2000}$UPy$_2$:PCL$_{1250}$UPy$_6$. This mixture not only displays good mechanical properties as it does not deform or become more crystalline after subcutaneous implantation, but also the foreign body reaction displayed is very mild.

In this way it is shown that with a modular approach the material properties and biological in vivo behaviour can be tuned. This approach opens the way to develop scaffolds with material properties tuned to the specific needs of the application and to use the UPy–UPy interactions to incorporate UPy-modified bioactive factors without the need for covalent modification.

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References


