Biomimetic Polyurethanes for Regenerative Medicine

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ABSTRACT

Degradable linear polyurethanes from non toxic building blocks were synthesized and characterized. An enzymatically degradable peptide was incorporated in polyurethane chains, in order to create a synthetic equivalent of the Extracellular Matrix. Furthermore amino-derived diamines and cyclic diols were used as alternative chain extenders. Mechanical tests showed the influence of the macrodiol and chain extender in mechanical properties. Cell adhesion test with myoblast showed the effect of mechanical properties on cell adhesion. For instance myoblast cells adhere preferently to polymer with lower Young Modulus and marked elastomeric behaviour, properties similar to those of musculoskeletal tissue.

1 INTRODUCTION

The main challenge in the design of a scaffold for Tissue Engineering is the realisation of biodegradable constructs with suitable properties that promote cell adhesion, proliferation and differentiation, and the Extracellular Matrix (ECM) production [1]. An ideal scaffold material should mimic the properties and behaviour of host tissue, have suitable surface and mechanical properties (including wettability, elastic modulus, mechanical strength) and, additionaly, include bioactive molecules (e.g peptides) of the ECM. High molecular-weight linear aliphatic polyester have been effectively used for clinically applicable products, but they possess mechanical properties (high modulus and low elongation at break) best suited for orthopedic tissue engineering. In order to meet the mechanical demands of force-generating contractile tissues, biodegradable elastomeric materials may serve as constituents of more appropriate scaffolds. In this work polyurethanes (PURs) with different composition were synthesized and characterized, in order to show the influence of the chain extender in bulk and surface properties and in biological response. For instance, both commercial aliphatic diols and diamines were used, as custom made peptides. In a previous work [2], we reported the functionalisation of polyurethanes with RGD peptides; here we describe the functionalisation with enzymatically degradable peptides (Ala-Ala sequences, degraded by elastase). Both surface and bulk characterizations were performed. Biological test conducted with myoblast (C2C12 cell line) showed that polymer stiffness is a driving force on cell viability and adhesion.

Keywords: polyurethanes, mechanical properties, peptides

2 MATERIALS AND METHODS

2.1 Synthesis of H-Ala-Ala-Put-H

The diamine putrescine (Put) (11 mmol) was dissolved in 75 ml $CHCl_3$. Boc_2O (2.2 mmol, 1 eq. relative to the amino Put groups) dissolved in 25 ml of $CHCl_3$ was added and the mixture was stirred overnight. The solvent was evaporated and the crude material was dissolved in water and filtered. The aqueous solution was extracted with CH_2Cl_2. The organic layers were dried over Na_2SO_4. The yield of N-Boc monoprotected Put was 53%. The latter (176.0 mg, 1.0 mmol, 1 eq.) was then dissolved in 25 ml CH_3CN and Z-Ala-OH (267.6 mg, 1.2 mmol, 1.2 eq.), 1-hydroxy-benzotriazole (162.0 mg, 1.2 mmol, 1.2 eq.), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (453.6 mg, 1.2 mmol, 1.2 eq.) and N,N-diisopropylethylamine (418.0 µl, 2.4 mmol, 2.4 eq.) were added to the solution. The reaction mixture was stirred overnight. The solvent was removed, the solid was dissolved in ethyl acetate and the organic layers were extracted with water, 5% $NaHCO_3$ and brine. The organic layers were finally dried over Na_2SO_4. The crude material was purified via flash chromatography (CH_2Cl_2/MeOH 95:5). The yield of Z-Ala-Put-Boc was 95%. The cleavage of the Z protecting group was carried out in methanol with 10% Pd/C overnight. The coupling of the second Z-Ala-OH was carried out with the same procedure and the total yield after chromatography was 80%. ^{1}H-NMR (Bruker AC200, 250 MHz, $CDCl_3$) for Z-Ala-Ala-Put-Boc (ppm): 7.4-7.3 (5H, Z); 6.6 (2mb, 2H NH(amide)); 5.3 (mb, 1H NH(Z)); 5.1 (s, 2H CH_2(Z)); 4.7 (mb, 1H NH(Z)); 4.4 (m, 1H Hα-Ala); 4.2 (m, 1H Hα-Ala); 3.1 (2qb, 4H, NCH_2); 2.8 (s, 6H, CH_3-Ala); 1.6-1.4 (m; s, 13H NCH_2CH_2; Boc). Then Z

protecting group was removed in methanol with Pd/C and the Boc protecting group was cleaved in CH_2Cl_2/TFA 9:1.

2.2 Synthesis of PURs

Biodegradable PURs were prepared as previously described [3] by using polycaprolactone (PCL) diol as soft segment in combination with diisocyanate (1,4-budandiisocyanate, BDI or 1,6- hexametilendiisocyante, HDI) and the following chain extenders: 1,4-cyclohexane dimethanol, L-Lysine ethyl ester dihydrochloride, N-Boc-serinol, (Sigma Aldrich) and custom made peptides. PUR nomenclature is based on the nature of the constituent segments, as follows: the first letter indicates the chain extender: C corresponds to CDM, K corresponds to lysine ethyl ester, NS to N-Boc-serinol and A to H-Ala-Ala-Put-H. The letter H corresponds to HDI and B to BDI; C1250 corresponds to PCL, M_n = 1250 and C2000 to PCL, M_n = 2000.

Polymer characterization was performed by differential scanning calorimetry (DSC) (TA INSTRUMENT DSC Q20), dynamic mechanical analysis (DMA) (TA ISTRUMENTS DMA Q800), attenuated total reflectance Fourier Transform Infrared Spectroscopy (ATR-FT-IR) (Perkin–Elmer Spectrum One spectrometer, film samples), Size Exclusion Cromatography (SEC) (Agilent Technologies 1200 Series), stress strain test (MTS QTest/10 Elite Controller), Contac Angle (KSV Instrument CAM200) and Atomic Force Microscopy (AFM) (NanoWizard II AFM, JPK Instruments, Berlin, Germany). DSC analysis were carried out heating the samples from -60 to 200°C at 20°C·min^{-1}. DMA analysis were conducted from -80 to 60°C, heating rate of 3°C·min^{-1}, and frequency of 1Hz. Stress strain test were carried out with a cell load of 10 N at speed rate of 10mm· min^{-1}. Intermittent-contact mode AFM was used to obtain height and phase data. Microfabricated Silicon cantilevers with force constants around 40N/m and fundamental frequencies around 300 KHz were used. The frequency of vibration of the cantilever was fixed near the resonance on the low-side. The ratio of the set-point amplitude to the free air amplitude of oscillation of the cantilever was maintained at 0.5. All samples were prepared by spin coating in order to have thin films. The thicknesses were approximately 20-40 μm. Mechanical test and contact angle measurements were taken respectively over 3 and 4 replicate samples and then averaged.

2.3 Biological tests

The adhesion at short time and the morphology of C2C12 myoblast cells seeded on cell culture Petri dishes (controls) and on different material were observed with an optical microscope. Images were collected at different times by a camera connected with the microscope.

Citotoxicity: MTT (3-Dimethylthiazol-2,5-diphenyltetrazolium Bromide) Colorimetric Assay and immunofluorescence for cellular morphology. After culturing cells for 24 h and 3 days, the medium was removed; 10% MTT solution (5 mg·mL^{-1} in DMEM; Sigma, Italy) was added to the cell monolayers and incubated at 37 °C for an additional 3h. After discarding the supernatants, the dark blue formazan crystals were dissolved by adding 100 μL of dimethylsulfoxide (DMSO, Sigma, Italy) and quantified spectrophotometrically (Secomam, Anthelie light, version 3.8, Contardi, Italy) at 570 nm. The results are reported as optical density units. Cells seeded on a cell culture plate, adequately treated for cell adhesion, were used as controls. The mean and the standard deviations were obtained from three different experiments. Samples were also analyzed under a fluorescence microscope (Leica DM2500) for revealing cell morphology (rhodamin-phalloidin staining actin filaments) after 3 days.

3 RESULTS AND DISCUSSION

3.1 Peptide characterization

The solution-phase of H-Ala-Ala-Put-H ran in good yield and the final product was confirmed by NMR.

3.2 Polyurethane characterization

The ATR-FT-IR spectra (data not shown) confirmed the structure of PURs. The ester groups showed the pronounced carbonyl peak near 1730 cm^{-1}. The urethane and amide groups also showed absorption at 3350 cm^{-1}. The absorptions of amide I and II were at 1670 and 1560 cm^{-1}, respectively. The absorbance at 1110 cm^{-1} was attributed to C-O stretching in PCL. Polyurethane average molecular weights obtained by Size Exclusion Cromatography are in the range of 15000-38000 Da, as reported in Table 1.

Sample	M_n [Da]	M_w/M_n
K-BC2000	18000	2.8
NS-BC2000	38000	1.6
A-BC2000	22000	1.4
C-HC1250	15000	1.3
C-HC2000	22000	1.7
C-BC2000	15000	1.5

Table 1: Polyurethane average molecular weight data

Thermal characterization:

PURs were characterized by DSC and DMA analysis and results are reported in table 2. Concerning melting point and glass transition temperature, the synthesized PURs showed thermal properties suitable for tissue engineering application. K-BC2000 and C-CH1250 showed two different crystalline phases and, consequently, two melting

points. However DMA analysis showed that these PURs have good mechanical properties at 37°C. The percentage of crystallinity, assessed by DSC analysis, is reduced for PURs with a pendant side chain (K-BC2000) and further decreases for the PURs synthesized with the lower molecular weight macrodiol. The higher crystallinity of A-BC2000 with respect to K-BC2000 and NS-BC2000 can be ascribed to an increase in hydrogen bonding, due to the presence of the peptide sequence.

Mechanical test:

PURs were characterized by stress strain tests and results are reported in table 3. Young Moduli are in the range of 9 to roughly 152 MPa. From stress-strain curves, it was observed that the use of a cyclic diol improved the mechanical performances (increase in Young Modulus).

Sample	T_m [°C]	T_g [°C]	Cristallinity [%]
K-BC2000	25.8 / 42.5	-38.2	22.3
NS-BC2000	53.3	-29.9	43.8
A-BC2000	70	-48	46.9
C-HC1250	35.3 / 54.2	-30.8	33.7
C-HC2000	62.3	-45.2	50.3
C-BC2000	56.4	-36.5	36.6

Table 2: Thermal analysis results

PURs synthesized with lysine ethyl ester and N-Boc-serinol as chain extender, showed an elastomeric behavior. The higher mechanical properties, with a particular reference to Young Modulus, for NS-BC2000 can be related to the higher molecular weight and crystalline content. The PUR functionalised with Ala-Ala sequence, showed an higher Young Modulus and lower strain at break compared to the K-BC2000 and NS-BC2000, according to the highest crystallinity of A-BC2000. PURs synthesized with cyclic diols as chain extender showed lower mechanical properties concerning the stress and strain at break. Moreover Young Modulus and stress at break increased with the molecular weight of the macrodiol. Comparing the polyurethanes C-BC2000 and C-HC2000, synthesized using two different diisocyanates, concerning the Young Modulus values and the strain at break, a similar mechanical behaviour was observed. For instance the low strain at break percentage indicated a brittle behaviour for these PURs. Elastic modulus were also evaluated with DMA at 30°C and 37°C, in order to simulate the physiological temperature (data not shown). These analyses showed a low decrease in Young modulus at 37° C compared to the value at 30°C, for the PURs synthesized using the PCL 2000 as macrodiol, but more evident decrease for the PUR C-HC1250. In conclusion, mechanical test showed that K-BC2000, NS-BC2000 and A-BC2000 are good candidate as scaffold materials for the regeneration of contractile tissues, because of their elastomeric behaviour. A-BC2000 showed no high strain at break, but adequate to the requirement of soft tissues, such as the cardiac tissue.

Sample	Young Modulus [MPa]	Stress at Break [MPa]	Strain at Break [%]
K-BC2000	8.8 ± 0.6	10.8 ± 2.3	691.3 ± 38.0
NS-BC2000	48.7 ± 6.9	4.9 ± 1.0	725.4 ± 20.0
A-BC2000	129.2 ± 15.6	9.7 ± 2.3	22.3 ± 3.1
C-HC1250	41.2 ± 0.1	1.4 ± 0.4	5.9 ± 1.8
C-HC2000	148.7 ± 3.9	4.8 ± 0.6	3.9 ± 0.4
C-BC2000	151.7 ± 0.1	3.6 ± 0.4	3.3 ± 0.8

Table 3: Stress strain test results

Contact angle measurement:

Hydrophilicity of the substrate is one of the thermodynamic parameters claimed to play an important role in cell adhesion. The importance of polymer surface hydrophilicity on cell adhesion was reported by Weiss [4], and confirmed by other studies. For instance hydrophilic surfaces promoted the highest levels of cell adhesion (maximun adhesion and growth at around water contact angles of 55°) [5]. All the synthesized PURs showed a moderate hydrophobic surface in the range of 77-90° (table 4).

Sample	Contact Angle [°]
K-BC2000	89±0.4
NS-BC2000	77±1.0
A-BC2000	90±0.8
C-HC1250	87±0.8
C-HC2000	84±0.9
C-BC2000	88±3.2

Table 4: Contact Angle Analysis

Atomic Force Microscopy:

The good biocompatibility of PURs is sometimes ascribed to a high degree of microphase separation, typical of this family of polymer. AFM phase images for some rapresentative PURs are reported in figure 1.

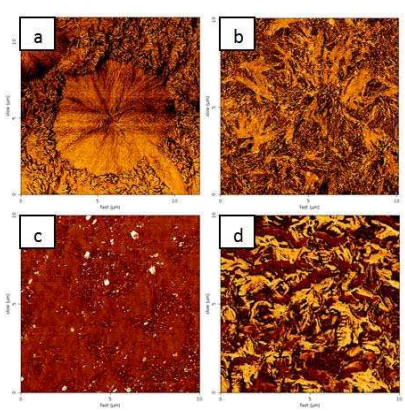

Figure 1: AFM phase images for: a) NS-BC2000; b) C-CH2000 c) K-BC2000, d) C-HC1250

Signs of the presence of the hard and soft domains are noted in all the samples, but the surface morphologies appear significantly different. The samples with higher crystallinity (C-HC2000 and NS-BC2000) showed not only a microphase but also a nanophase separation; furthermore spherulites can be observed. For instance, NS-BC2000 shows large spherulites (about 7 μm of diameter), probably due to the higher molecular weight of this PUR, which causes an increased growth of crystals, at a slower rate [6].

Biological Tests:

Cell morphology – Short time adhesion: Cell adhesion and spreading, observed at short time (from 1h to 24h), for K-BC2000 were similar to the control (figure 1). In these samples a stable adhesion started after 30 min. (data not shown) and a significant cell spreading was appreciable already after 3 hours incubation. C-HC1250 showed low adhesion and spreading at 24 h, when compared to K-BC2000. Cells seeded on the NS-BC2000 and C-BC2000 showed still a round shape even after 24 hours, indicating that these materials are not adequate substrates for cell adhesion. These results can be correlated to the mechanical properties of PURs. Some cell types appear to be sensitive to substrate stiffness [4].

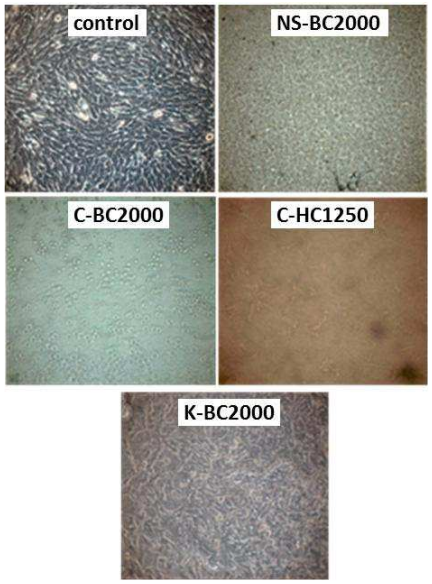

Figure 2: myoblast C2C12 Cell adhesion, 24 hours

For example, K-BC2000 has a Young modulus that is clearly lower than that of NS-BC2000 and C-BC2000, whereas it is similar to that of soft tissues; C-HC1250 had an intermediate Young modulus at 37°C. Differences in the biological response can not be ascribed to changes in wettability since the best performance in terms of cell adherence to the subtrate is given by K-BC2000, which is the more hydrophobic PURs of the family examined in this study.

Citotoxicity – MTT test: K-BC2000 showed a very low citotoxicity with values that were very close to the controls at 24 hours (figure 3) as well as at 3 days culture (data not shown). These data have been confirmed by cell morphology at day 3, where an uniform cellular monolayer was shown with fluorescence microscopy. C-HC1250 showed an higher toxicity.

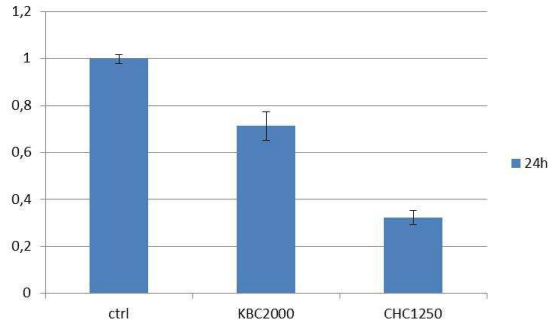

Figure 3: MTT test at 24 hours

CONCLUSION

In this work a series of polyurethanes were synthesized and characterized, showing the influence of the chain extender on the surface and mechanical stiffness and the effect of the latter on biological responses. Furthermore, the functionalization of PURs with bioactive peptides has been proposed.

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