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Biocompatible α-aminoacids based aliphatic polyamides

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Summary

Biodegradable aliphatic polyamides have been prepared from C10 and C14 dicarboxylic acids and amide-diamines containing one or two preformed amide linkages susceptible to enzymatic cleavage in their molecules. L-phenylalanine, either by itself or together with L-valine, was used to generate amide bonds which are cleavable by chimotrypsine. Improved chain flexibility and hydrophilicity of the polyamides was obtained using a triethyleneoxy based amide-diamine. Characterization of the novel monomers and polymers by FTIR and ^{1}H-NMR spectroscopy confirms the expected structures. Thermal data and solubility tests indicate that both T_m and solubility depend on the length and nature of the aliphatic segment present in the amide-diamine as well as on the "density" of interchain hydrogen bonds. Finally the polymers tested are capable of supporting fibroblast adherence and proliferation, and proved to be non cytotoxic.

Introduction

Aliphatic polyamides (PAs) are interesting materials for application in medicine due to their physico-mechanical properties and biocompatibility. In recent years, efforts have been directed towards the preparation of biodegradable, resorbable condensation copolymers containing amide bonds (polyesteramides and polyurethaneamides)[1-4] with controlled lifetime which can be used as temporary implants or as controlled drug release matrixes. Synthetic high molecular weight aliphatic polyamides are generally reported as being resistant to microbial and enzymatic attacks, although examples of degradation to some extent has been reported in literature[5-7]. PAs present amide linkages as in natural proteins and their low biodegradation rate has been attributed to a packing into highly ordered structures due to the short and regular repeat units and to strong interchain interactions caused by hydrogen bonding. Loose packing, improved conformational chain flexibility, hydrophilicity and the introduction of substituents such as benzyl, methyl, hydroxyl groups into the chain may enhance the susceptibility of the amide linkages of synthetic polyamides to enzyme catalyzed hydrolysis[6,8]. A further approach to improve PA biodegradability involves inserting hydrolyzable ester linkages along the PA chains, as in multiblock poly(esteramide)s with short poly(L-lactide) sequences such as polyester blocks[9]. In a previous paper we reported the synthesis and the characterization of both alternating or block poly(esteramide)s obtained from diamines containing enzymatically cleavable amide bonds[10]. The present work describes a series of aliphatic polyamides of different chain flexibility and hydrophilicity containing one or more peptide bonds susceptible to enzymatic attack by chimotrypsine in the chain repeat unit.

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Experimental

Materials and techniques

The solvents were purified according to standard procedures. N-Boc-1,6-diaminohexane hydrochloride, Boc-L-valine (Boc-Val), Boc-L-phenylalanine (Boc-Phe), Z-L-phenylalanine (Z-Phe), tetrabuthylammonium hydrogen sulfate (TBHS) and 1,12-diaminododecane were supplied by Fluka and used as received. Sebacoyl chloride, 4,7,10-trioxa-1,13-tridecanediamine (Fluka) and tetradecandioyl chloride were purified by fractional distillation under vacuum. Infrared (IR) analysis was performed using a Bruker IFS 66 FTIR spectrophotometer. The ^{1}H-NMR spectra were recorded at 25°C using a Bruker DRX 400 instrument and chloroform-*d* ($CDCl_3$), dimethylsulfoxide-d_6 (DMSO-d_6), methanol-d_4 (CD_3OD) and $CDCl_3$/trifluoroacetic acid-*d* mixtures as solvents. The thermal data were obtained using a Mettler TA-3000 differential scanning calorimeter (DSC). Approximately 6-7 mg of the sample were heated under nitrogen from 50°C to 250/300 °C at a rate of 10°C/min. The inherent viscosities were measured on a Cannon-Ubbelhode viscosimeter at 25°C in *m*-cresol (c= 0.5 g/dL). Dynamic mechanical thermal analysis (DMTA) was performed using a DMTA analyzer (Polymer Laboratories) on 0.4-0.5 mm thick films obtained by compression molding.

Synthesis of diamines

1,12-di(L-phenylalaninamido)dodecane **(1)**

Isobuthylchloroformate (12.3 mL, 94.2 mmol) was added dropwise under stirring at -15 °C to 300 mL of a chloroform solution containing Boc-Phe (25.0 g, 94.2 mmol) and triethylamine (9.51 g, 94.2 mmol) under nitrogen. After stirring for an additional 20 min, 1,12-diaminododecane (9.44 g, 47.1 mmol) dissolved in 100 ml of chloroform was added dropwise and the resulting solution was stirred overnight at room temperature. The chloroform solution was first washed with sodium carbonate and citric acid solutions, and then with water before being finally dried over Na_2SO_4. 26.5 g of crude 1,12-di-(Boc-phenylalaninamido)dodecane (I) were recovered by removing the solvent. Crystallization from ethyl acetate/ethanol (3:1) gave 16.2 g of white crystals (61% yield, m.p.139 °C). Trifluoracetic acid (TFA) (35.7 mL) was added to 16.2 g of (I) under stirring at 0 °C and after two hours anhydrous ethyl ether (300 mL) was added dropwise to the viscous solution. The precipitated white trifluoracetate salt (II) was recovered by filtration, repeatedly washed with ether and dried. Crude **1** was obtained by adding a 0.8 M Na_2CO_3 solution to II dissolved in the minimum amount of water. The suspension obtained was repeatedly extracted with chloroform and the organic layer was dried over Na_2SO_4. Purification by column cromathography (silica gel 60, propanol/CH_2Cl_2 3:10 as eluent) afforded **1** as a white solid (yield 80%, m.p. 123 °C). ^{1}H-NMR ($CDCl_3$): δ = 3.21 (α, 2H),1.50 (β, 2H),1.31 (γ, 2H), 2.68-3.23 (ε, 2H), 3.57 (CH, 1H), 7.32 (NH, 1H), 7.32 (Ph, 5H) (see Figure 1).

1-amino-6-(L-phenylalaninamido)hexane **(2)**

2 was prepared from Boc-L-Phe and N-Boc-1,6-diaminohexane hydrochloride as reported for **1**. The 1-Boc-amine-6-(Boc-L-phenylalaninamido)hexane (III) intermadiate was crystallized from ethanol/diethyl ether 5:1 (59% yield, m.p., 121°C). Crude **2**, obtained as a yellow oil, was purified by column chromatography (silica gel 60, chloroform/methanol-1% NH_4Cl/methanol 10:2:3.5 mixture as eluent). Crystallization from toluene/chloroform 3:1 and diethyl ether as non solvent gave a white solid (46% yield, m.p. 137°C). ^{1}H-NMR (CD_3OD): δ = 3.27 (α, 2H), 1.65 (β, 2H), 1.45 (γ, 2H), 1.30 (δ, 2H); 2.9 0-3.00 (ε, 2H), 1.45 (η, 2H), 2.90 (ϖ, 2H), 3.55 (CH, 1H), 7.32 (NH, 1H) 7.25 (Ph, 5H) (see Figure 1).

1,12-di(L-phenylalanyl-L-valinamido)dodecane **(3)** *and 1,12-di(L-valyl-L-phenylalaninamido)dodecane* **(4)**

3 and **4** were prepared from **1** and 1,12-di(L-valinamido)dodecane by reacting them with Boc-L-Val and Boc-L-Phe, respectively, as reported for **1.** Purification on a silica gel column (eluent propanol/CH_2Cl_2, 3:10) gave pure **3** (m.p. 161 °C) and **4** (m.p. 144 °C). ^{1}H-NMR (DMSO-d_6). **3** : δ = 2.95 (α, 2H), 1.30 (β, 2H), 1.20 (γ, 2H), 2.85-3.00 (ε, 2H), 4.50 (χ, 1H), 3.00 (ψ, 1H), 1.85 (ξ, 1H), 7.20 (Ph, 5H), 0.6-0.80 (CH_3, 6H); **4**: δ = 3.00 (α, 2H), 1.40 (β, 2H), 1.25 (γ, 2H), 2.65-3.10 (ε, 2H), 4.10 (χ, 1H), 3.45 (ψ, 1H), 1.90 (ξ, 1H), 7.25 (Ph, 5H), 0.85 (CH_3, 6H) (see Figure 1).

1,13-di(L-phenylalaninamido)4,7,10-trioxatridecane **(5)**

Isobuthylchloroformate (9.65 mL, 74.4 mmol) was added dropwise under stirring at -15 °C to 215 mL of a chloroform solution of Z-L-Phe (22.3 g, 74.4 mmol) and triethylamine (11.1 mL; 74.4 mmol). The solution was stirred for 20 min and 4,7,10-trioxa-1,13-tridecanediamine (8.2 g, 37.2 mmol) dissolved in 80 mL of chloroform was added dropwise. After stirring overnight at room temperature, the solution was first washed with citric acid and Na_2CO_3 solutions and then with water before being dried over Na_2SO_4. 1,13-di(Z-L-phenylalaninamido)-4,7,10-trioxatridecane (IV) was obtained as a white solid by removing the solvent under reduced pressure (25.8 g, 89% yield, m.p. 116 °C). 7.6 g of IV were hydrogenated at room temperature for 5 h in 320 mL of methanol containing 1.9 g of 10% palladium on charcoal. After removal of the catalyst, the yellow oil obtained by solvent evaporation was purified by column chromatography (silica gel, chloroform/methanol 6:1 as eluents) giving pure **5** as a clear viscous oil.(3.6 g, 73% yield). ^{1}H-NMR ($CDCl_3$) : δ = 3.34 (α, 2H), 1.75 (β, 2H), 3.47 (γ, 2H), 3.55-3.58 (δ, 4H), 2.75-3.25 (ε, 2H), 7.60 (NH, 1H), 7.28 (Ph, 5H) (see Figure 1).

Polymerization

a) Interfacial polycondensation

Sebacoyl chloride (0.949 g, 3.97 mmol) dissolved in 20 mL of a chloroform/hexane 1:1 mixture was quickly added under vigorous stirring to 100 mL of a water solution containing **5** (2.04 g, 3.97 mmol) and 0.64 g of NaOH, at 0-5°C. After 10 min the white precipitate was collected on a glass filter, repeatedly washed with water and dried *in vacuo* at 60°C giving 1.63 g of **5-C10** (60% yield), η_{inh} = 0.92 dL/g (*m*-cresol, T = 25 °C, 0.5 g/dL). This procedure was also used to prepare the polyamide **2-C10**.

b) Modified procedure of interfacial polymerization

1 (0.756 g, 1.53 mmol) dissolved in 5 mL of $CHCl_3$ was stirred in a blender with 60 mL of a water solution containing 0.24 g of NaOH and 0.061 g of TBHS. A solution of sebacoyl chloride (0.366 g, 1.53 mmol) in 8 mL of chloroform was added under vigorous stirring, at 0-5°C. After 10 min the precipitate was collected by filtration, repeatedly washed with water and dried *in vacuo* at 60°C giving 0.706 g of **1-C10** (70% yield), η_{inh} = 0.82 dL/g (*m*-cresol, T = 25 °C, 0.5 g/dL). This procedure was also followed to prepare the polyamide **1-C14.**

c) Dispersion polycondensation

0.25 g of NaOH and 0.063 g of TBHS were added to a solution of **1** (0.771 g, 1.56 mmol) in 23 mL of tetrahydrofuran (THF) and 32 mL of water. Sebacoyl chloride (0.337 g, 1.56 mmol) dissolved in 8 mL of anhydrous THF was quickly added to this solution under vigorous stirring, at 0-5°C. After 10 min, the reaction mixture was poured into icy water. The precipitate was collected on a glass filter, repeatedly washed with water and dried *in vacuo* at 60 °C giving 0.958 g of polymer **1-C10** (93% yield), η_{inh} = 1.00 dL/g (*m*-cresol, T = 25 °C, 0.5 g/dL). Polyamides **3-C10** and **4-C10** were obtained by this procedure.

Cytocompatibility assay

In order to determine cell adhesion and cell growth, sterilized polymers were placed in the bottom of each well of a 24-well tissue culture plate. BALB/3T3 ambryonic mouse fibroblasts were cultured in complete DMEM with 10% Fetal Calf Serum (FCS) and antibiotics. Flasks were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. At confluency, cells were trypsinized using 0.2% Trypsine/0.2% EDTA centrifuged at 200 g for 10
min and resuspended in the same culture medium. Single cell suspensions were seeded on the testing materials at a density of $2x10^5$ cells/well and allowed to attach at 37 °C. By way of positive control, cells were plated onto tissue-culture plates. Three independent growth experiments were undertaken for each material tested. Cell cultures were followed at 3, 6, 12 and 24 h after plating by optical microscope.

Cytotoxic assay

Cytotoxicity was evaluated not only by microscopic examination but also by LDH concentration in the cell conditioned medium. Lactate dehydrogenase (LDH) is an enzyme that is released from the cell when the cell membrane is ruptured. Thus, the amount of LDH present in the media when the test is completed correlates to the amount of cell death in the culture. Four control cultures included in the experiments were also used as controls for LDH analysis. After 24 h incubation, two of the four control cultures were given 100 µl of Triton X100 and held at 37°C for 2 h. These cultures served as positive controls for LDH testing given that Triton causes complete cell death. The other two control cultures served as negative controls for LDH analysis.

Results and discussion

Monomers

Various amide-diamine monomers, different in the number or sequence of aminoacids present, the number of preformed enzymatically degradable amide bonds, chain flexibility and hydrophilicity, were prepared from C6 and C12 aliphatic α,ω-diamines or 4,7,10-trioxa-1,13-tridecanediamine. L-phenylalanine or L-phenylalanine-L-valine and L-valine-L-phenylalanine sequences were used together with the above diamines to build up peptide linkages susceptible to cleavage by chymotripsine in the amide-diamines. The general procedure followed for their synthesis was the well known "mixed anhydride method". The amide-diamines prepared are listed in Figure 1. Amide-diamines **1-4** are crystalline solids which melt in the range 123-161 °C; attempts to crystallize the highly flexible monomer **5** were unsuccessful. All diamines are soluble in chloroform, ethanol and THF; **2** and **5** are highly soluble also in water, while **1**, **3** and **4** are only slightly soluble at room temperature because of the hydrophobic character of the $-CH_2-$ sequence. The structures of the amide-diamines were checked by FTIR and ^{1}H-NMR spectroscopy. The FTIR spectra show characteristic absorptions of the amide group at 3355-3279, 1653-1628 and 1557-1531 cm^{-1}, and of the aromatic C-H at 3065-3026 cm^{-1}; the spectrum of diamine **5** shows additional absorption due to the C-O-C group at 1108 cm^{-1}. The ^{1}H-NMR spectra (see Experimental) are in agreement with the structures shown in Figure 1.

Polymers

Enzymatic degradation of polymers, as widely reported[1,6,8], requires polymer chain flexibility, hydrophilicity and a steric environment around the cleavable bond, all of which favour the interaction between the polymer and the active site of the enzyme. All the PAs prepared and investigated are characterized by the presence of selected chymotripsine-

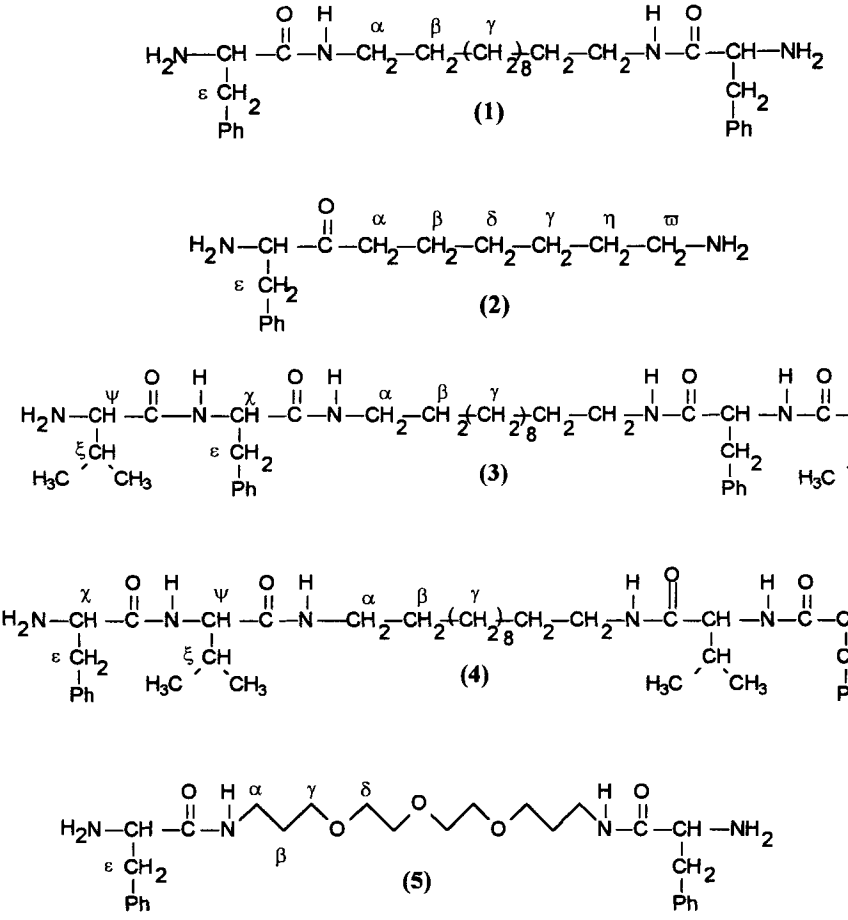

Figure 1. Structure of the amide-diamines used in the synthesis of polyamides. The Greek letters refer to the ^{1}H-NMR spectra (see Experimental).

susceptible amide bonds incorporated into the polymer chain. Such linkages, generated by the amidation reaction of the L-Phe carboxylic group, were preformed in the amide-diamines **1-5**. Sebacic acid (C10) and, in one case, tetradecanedioic acid (C14) were used to impart conformational flexibility to the polymer chain without any great increase to the hydrophobicity. Similarly, as far as the amide-diamine moiety is concerned, the overall chain flexibility and hydrophilicity of PAs were enhanced using monomer **5**, which is derived from the 4,7,10-trioxa-1,13-tridecanediamine. The polyamides were obtained by stirred, low temperature polycondensation of **1-5** with the acyl chlorides of 1,10-decanedioic acid and 1,14-tetradecanedioic acid. The standard interfacial technique was followed using the water soluble diamines **2** and **5** whereas modifications were devised for diamines **1**, **3** and **4**, which are slightly soluble in water. In this case the diamines were dissolved in the minimum amount of chloroform and the solution was mixed, under stirring, with the appropriate volume of water containing an acid acceptor and TBHS as emulsifying agent. The acyl chloride, dissolved in chloroform, was added under stirring to this emulsion. Following a different procedure, known as dispersion polymerization, a THF

solution of the acyl chloride was added under stirring to a THF/water 1:1.5 solution containing the diamine, the emulsifying agent and the acid acceptor. The modified interfacial technique and the dispersion procedure both afforded polymers with comparable polymerization degrees, as indicated by the inherent viscosities obtained for the PA derived from **1** and sebacoyl chloride synthesized according to the two different procedures. The polyamides prepared, together with some of their properties are listed in Table 1.

Table 1. Synthesis and thermal data of aliphatic polyamides prepared from diamines **1-5** and the acyl chlorides of C10 and C14 dicarboxylic acids.

Code	η_{inh} dL/g	yield %	T_m (°C)	ΔH_m J/g	T_g[(a)] (°C)
1-C10[(b,c)]	0.82[(b)], 1.00[(c)]	70	197	58	104
2-C10[(d)]	0.78	57	147[(e)]	39[(e)]	n.d
3-C10[(c)]	0.46	46	272	60	118
4-C10[(c)]	0.30	54	248	41	125
5-C10[(d)]	0.92	60	181[(e)]	25[(e)]	46*
1-C14[(b)]	0.86	80	187	29	104 ; 100*

(a) Glass transition temperature measured by DSC or DMTA (*). (b) Modified interfacial polymerization. (c) Dispersion polycondensation. (d) Interfacial polycondensation. (e) After annealing at 145°C (**5-C10**) and 135 °C (**2-C10**) for 60 min.

All the PAs prepared are white powders soluble in typical polyamide solvents such as formic acid and *m*-cresol; the polyamide **5-C10** is also soluble in chloroform. The inherent viscosity values are indicative of moderate polymerization degrees. Tough, flexible films can be prepared by slowly evaporating a polymer solution in formic acid. The molecular structure of the polyamides was confirmed by FTIR and ^{1}H-NMR spectroscopy measurements. The IR spectra show characteristic amide bands at 3285, 1639 and 1588-1549 cm^{-1}, aromatic C-H stretching at 3080-3030 cm^{-1} and aliphatic C-H bands at 2927-2857 cm^{-1}. In addition, the spectrum of **5-C10** shows absorption at 1122 cm^{-1} due to C-O-C stretching. The ^{1}H-NMR spectrum of the polyamide **5-C10**, shown in Figure 2, is representative of this class of polymers. The resonances were attributed on the basis of literature data and of the spectra of the corresponding amide-diamines.

The relevant thermal data are reported in Table 1. The polyamides are semicrystalline, as shown by the presence of melting endotherms in their DSC traces. In the case of the **5-C10** and **2-C10** polymers, however, the thermograms of the DSC analysis performed on the "as polymerized" PAs display two broad endotherms that coalesce to a single peak by annealing at $T_m1 > T > T_m2$. This result can be explained considering the irregular chain packing caused by the constitutional disorder induced by the unsymmetrical diamine **2** or by the presence of the flexible triethyleneoxy group of the diamine **5** which both lead to the formation of crystallites of different order degree. After an initial heating-cooling cycle, melting endotherms can still be detected in the second run DSC traces of all PAs; however, with the exception of **1-C10**, a marked lowering of both T_m and ΔH_m values is observed. This behaviour can be reasonably ascribed to a difficult crystallization from the melt. Furthermore, partial degradation which occurs during the melting is also to be accounted in

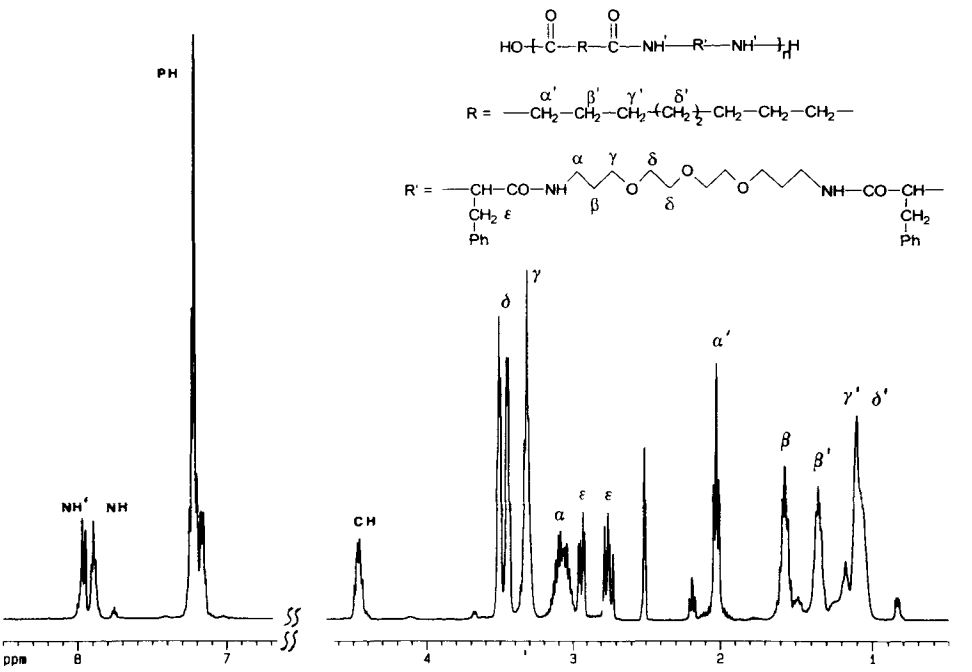

Figure 2. 400 MHz ^{1}H-NMR spectrum of the **2-C10** polyamide at 25 °C in DMSO-d_6 .

the case of **3-C10** and **4-C10** PAs, due to their high T_m values. Infact the melting temperatures, T_m , are spread over a wide range, 147-248 °C, and are strictly related to the diamine structure. Thus, comparing the T_m values of **3-C10** (272 °C) and **4-C10** (248 °C) with that of **1-C10** (197 °C), an increase in the melting temperature by increasing the number of α-aminoacid units in the diamine is observed as expected on the basis of the stronger interchain interactions due to the higher hydrogen bond density. The very low T_m (147 °C) of the **2-C10** PA is caused by the constitutional disorder introduced by diamine **2**. Finally, a comparison of the T_m values of **1-C10** (197 °C) and **1-C14** (187 °C) indicates that the influence of the diacid length on T_m is not particularly relevant (ΔT_m = 10 °C).
The glass transition temperatures, T_g , could not be easily detected by the DSC technique for all PAs therefore, they were measured by DMTA when suitable specimens could be obtained by melt casting (see Table 1). The T_g values were generally higher than 100 °C. A markedly low T_g was observed only when highly flexible triethyleneoxy groups were present in the amide-diamine moiety (**5-C10**).

Cytocompatibility

In this work, it was thought particularly interesting to evaluate the cytocompatibility of **1-C10**, **5-C10** and **1-C14** polymers, which were chosen on the basis of the different hydrophobicity/hydrophilicity surface characteristics. Cell viability, as determined by cytotoxic assays, was neither affected by incubation with the materials nor with material conditioned media (see Table 2). Optical microscopic observation of cultured cells showed that the polymers were capable of supporting fibroblast adherence and proliferation.

Table 2. Cytotoxicity test of the polyamides investigated

Polymer	1-C10	5-C10	1-C14	control
% of LDH/cm^2	2.36±0.39	1.41±0,17	4.22±0.51	0.00

Conclusion

Incorporation of L-phenylalanine and L-valine residues along the chain of aliphatic polyamides yields biocompatible, potentially resorbable polymers which are useful for biomedical applications. The crystallinity, melting temperature and solubility of the polymers, as well as their hydrophobic/hydrophilic character, can be properly regulated by varying the chemical structure of the amide-diamine monomers.

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