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Synthesis and properties of novel biodegradable polyamides containing α-amino acids

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Dedicated to Professor Imanishi on the occasion of his retirement

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Abstract

Polyamides are useful biomaterials owing to their biodegradability and good mechanical strength. We have obtained novel polyamides from succinylsarcosine and ethylenediamine by polycondensation in water using 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (EDC·HCl) and 1-hydroxybenzotriazole. The molecular weight of the obtained polyamides depends on the concentration of the monomers or EDC·HCl, and reached a maximum of over 200,000. The optimal polycondensation temperature was between 10 and 20 °C. We also obtained copolymers of succinylsarcosine and succinylisoleucine by the same method. When the succinylisoleucine content increased, the obtained polyamides showed lower melting points, higher enzymatic degradability, and higher cell adhesion rates. Thermally responsive polyamides containing an elastin-derived pentapeptide, VPGVG, succinylsarcosine, and succinylisoleucine were obtained by the same method. The temperature-dependent precipitation of the polyamide occurred reversibly, and the temperature of precipitation varied from below room temperature to over 80 °C, depending on the ratios of succinylsarcosine and succinylisoleucine.

Keywords: Polyamide; Succinylamino acid; Cell adhesion

1. Introduction

Various bioresorbable and bioerodible materials have been investigated for medical applications such as sutures, drug delivery devices, and orthopedic fixation devices [1]. Polyamides may especially be very useful for medical purposes, because of their biodegradability, safety, and good mechanical strength [2]. Poly(α- or β-amino)acids are biodegradable, however they show high melting points, low solubility, and little variety of their properties [3]. Although Nylon 6,6 or poly(ε-caprolactam) has good mechanical strength, it shows low biodegradability, and has a high melting point.

Therefore, we have designed safe and highly functional polyamides that have proper biodegradability, from α-amino acids, succinic acid, and ethylenediamine. However, it is generally difficult to synthesize poly(α- and β-) amides. The polymerization of *N*-carboxyanhydride (NCA)-derivatives of α-amino acids is highly sensitive to contaminants, such as water and amines, and highly toxic reagents are required for the synthesis of the NCA monomers. Polymerization using acyl chloride derivatives is also highly sensitive to contaminants, and produces toxic substances. Polymerization using active esters of α-amino acids [4–7], and polycondensation of γ-glutamic acid using 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (EDC·HCl) and 1-hydroxybenzotriazole (HOBt) [8] have been reported.

Initially, we synthesized polyamides containing sarcosine, by polycondensation of succinylsarcosine and ethylenediamine in water using EDC·HCl and HOBt. Use of *N*-methyl derivatives of α-amino acids may increase the solubility of the monomer and the obtained polyamide. Succinylation of α-amino acids may suppress diketopiperazine formation in the direct condensation of the α-amino acids. As ethylenediamine is a stronger nucleophile than the α-amino group, it may raise the efficiency of the polymerization.

Secondly, we synthesized a copolymer of succinylsarcosine, succinylisoleucine, and ethylenediamine by the same method. Isoleucine should provide some plasticity and solubility in organic solvents for the obtained copolymer.

Finally, we synthesized polyamides containing an

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Table 1
Conditions of polymerization and the peak molecular weight of the obtained polyamides

Entry	Succinylsarcosine (M)	Ethylenediamine (M)	HOBt (M)	EDC·HCl	Temp, (°C)	Yield (%)	M_p[a] ($\times 10^3$)
1	0.410	0.410	0.930	2.050	20	41.4	173 (n = 5)
2	0.307	0.307	0.694	1.540	20	43.5	81 (n = 3)
3	0.205	0.205	0.463	1.030	20	41.8	68 (n = 3)
4	0.102	0.102	0.232	0.515	20	16.1	33 (n = 2)
5	0.410	0.410	0.930	1.640	20	48.4	105 (n = 3)
6	0.410	0.410	0.930	1.230	20	66.5	50 (n = 3)
7	0.410	0.410	0.930	0.820	20	–	<10 (n = 3)
8	0.410	0.410	0.930	2.050	10	38.1	200 (n = 3)
9	0.307	0.307	0.694	1.540	10	47.9	123 (n = 3)
10	0.205	0.205	0.463	1.030	10	38.5	85 (n = 3)
11	0.102	0.102	0.232	0.515	10	38.3	29 (n = 2)
12	0.410	0.410	0.930	1.640	10	49.4	110 (n = 3)
13	0.410	0.410	0.930	1.230	10	56.5	56 (n = 3)
14	0.410	0.410	0.930	0.820	10	–	< 10 (n = 3)

[a] Data represents mean peak molecular weight.

elastin-derived pentapeptide, VPGVG, succinylsarcosine, and succinylisoleucine by the same methods, and investigated a correlation between the temperature-dependent precipitation and the ratios of succinylsarcosine and succinylisoleucine.

Here, we report on and discuss the polymerization conditions of these novel polyamides, and discuss their chemical and biological properties.

2. Experimental

2.1. Materials

The 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide and the 1-hydroxybenzotriazole were purchased from the Peptide Institute, Inc. (Osaka, Japan). The amino acids and other reagents were supplied by Wako Pure Chemicals (Osaka, Japan). Ethylenediamine was purified by distillation before use, and the succinic anhydride was purified by recrystallization from hot ethanol before use.

An elastin-derived peptide, $(VPGVG)_4$, was synthesized on an automated peptide synthesizer (ABI 433A, Applied Biosystems Japan Ltd, Tokyo, Japan) using Fmoc synthesis chemistry at the 0.1 mM scale. The synthesized peptides were cleaved from the resin by TFA containing 5% water, and purified by reverse-phase HPLC on an ODS cartridge column, eluting with an acetonitrile–water gradient containing 0.05% TFA. Analytical HPLC on an ODS column eluting with a linear gradient of 5–50% acetonitrile–water containing 0.05% TFA for 30 min revealed a single peak at 18.8 min ascribed to $(VPGVG)_4$. The molecular weight of the peptide was 1656 as analyzed by Fab-MS (JMS-700, JEOL, Tokyo, Japan), and this aligns with the calculated value of 1656.0.

2.2. Succinylation

Succinylsarcosine was synthesized by mixing 3.56 g of sarcosine (40 mmol) and 4.0 g of succinic anhydride in 40 ml of water, and adjusting the pH to 7.0 using 4 N NaOH at room temperature for 30 min. The reaction mixture was used for polymerization without further purification. FT-IR spectrum (Spectrum GX, Perkin Elmer Japan Co., Ltd, Yokohama, Japan) of the obtained product with KBr tablet showed peaks at 1612 cm^{-1} (νCOO^-), 1490 cm^{-1} (δCH_3), and 1400 cm^{-1} (νCOO^-). ^1H NMR spectrum was recorded on a JNM-ECP 600 (JEOL) 600 MHz spectrometer. The ^1H NMR spectrum in D_2O with tetramethylsilane as an internal reference showed peaks at 2.44 ppm (2H, $OC(O)CH_2CH_2C(O)N$, dd), 2.54 ppm (2H × 0.49, $OC(O)CH_2CH_2C(O)N$, t), 2.69 ppm (2H × 0.51, $OC(O)CH_2CH_2C(O)N$, t), 2.88 ppm (3H × 0.44, NCH_3, s), 3.10 ppm (3H × 0.56, NCH_3, s), 3.93 ppm (2H × 0.55, $C^{\alpha}H_2$, s), and 4.02 ppm (2H × 0.45, $C^{\alpha}H_2$, s). The ^1H NMR spectrum suggested that succinylsarcosine was a mixture of *cis* and *trans* isomers (1:1.2). The molecular weight of succinylsarcosine was 234.0 as analyzed by Fab-MS, and this aligns with the calculated value of 233.0 for $C_7H_9NO_5Na_2$.

Succinylisoleucine was synthesized from 1.31 g of isoleucine (10 mmol) and 1.0 g of succinic anhydride (10 mmol) in 10 ml of water by the same method as described before. The FT-IR spectrum showed peaks at 3261 cm^{-1} (νN–H), 2967 cm^{-1} (νCH_3), 1583 cm^{-1} (νCOO^-), and 1403 cm^{-1} (νCOO^-). The ^1H NMR spectrum in D_2O with tetramethylsilane as an internal reference showed peaks at 0.90 ppm (6H, $C^{\beta}H(CH_3)C^{\gamma}H_2C^{\delta}H_3$, m), 1.17 ppm (1H, $C^{\gamma}H^1$, m), 1.42 ppm (1H, $C^{\gamma}H^2$, m), 1.85 ppm (1H, $C^{\beta}H$, m), 2.47 ppm (2H, $OC(O)CH_2CH_2C(O)N$, m), 2.50 ppm (1H, $OC(O)CH_2CH^1C(O)N$, m), 2.57 ppm (1H, $OC(O)CH_2CH^2C(O)N$, m), and 4.10 ppm (1H, $C^{\alpha}H$, d). The ^1H NMR spectrum showed no amide H. The molecular weight of succinylisoleucine was 276.1 as analyzed by Fab-MS, and this aligns with the calculated value of 275.1 for $C_{10}H_{15}NO_5Na_2$.

Succinyl$(VPGVG)_4$ was synthesized from 11.6 mg of

Table 2
Conditions of copolymerization and some properties of the obtained copolymer

Product	Ethylenediamine (mol%)	Succinylsarcosine (mol%)	Succinylisoleucine (mol%)	Yield	M_p[a] ($\times 10^3$)	T_m (°C)
1	50	50	0	40.4	212	172.5–211.0[b]
2a	50	40	10	35	17	120.7–127.2
2b	50	30	20	34	94	134.0–143.6
2c	50	20	30	21 + 29[c]	45	136.3–145.5
2d	50	10	40	14 + 43[c]	11	120.0–129.2
3	50	0	50	0 + 78[c]	–	204.9[b]

[a] Peak molecular weight was estimated by GPC.
[b] Decomposition.
[c] Precipitation.

$(VPGVG)_4$ (7.0 μmol) and 0.7 mg of succinic anhydride (7.0 μmol) in 3 ml of water, and the pH was adjusted to 7.0 using 0.1 N NaOH by the same method as above. Succinyl$(VPGVG)_4$ was eluted as a single peak at 20.3 min on the analytical HPLC. The molecular weight as analyzed using Fab-MS was 1800, which compared well with the calculated value of 1800.0 for succinyl-$(VPGVG)_4$·2Na.

2.3. Polymerization

Succinylsarcosine and ethylenediamine were dissolved in water, and the HOBt and EDC·HCl were added to the mixture under stirring under the conditions listed in Table 1. Twenty-four hours after the addition of the EDC·HCl, the reaction mixture was passed through a PD-10 column (Amersham Biosciences K. K., Tokyo, Japan) and a high molecular weight fraction was collected. GPC analysis on the obtained polyamides was carried out using an AKTA purifier system (Amersham Biosciences K. K., column = Superdex HR 75 10/30 or Superdex 200 HR 10/30, elution buffer = 10 mM phosphate buffer containing 150 mM NaCl (PBS, pH 7.4), flow rate = 0.5 ml/min, detection = 215 nm). The peak molecular weight was calculated from the standard curve obtained using a gel filtration calibration kit (Amersham Biosciences K. K.). The melting point was measured using a Yanaco MP-S3 (Yanaco LID Co. Ltd, Kyoto, Japan).

The copolymers of succinylsarcosine and succinylisoleucine were synthesized by the same method as described before, under the conditions listed in Table 2. The copolymers of succinyl$(VPGVG)_4$, succinylsarcosine, and succinylisoleucine were synthesized by the same method as described earlier.

2.4. Enzymatic degradation

Twenty microliters of the obtained polyamide solution in PBS (10 mg/ml) was mixed with either 80 μl of the elastase solution (20 U/ml PBS, porcine pancreas, EC 3.4.21.36, Sigma-Aldrich Japan K. K., Tokyo, Japan) or collagenase solution (*Clostridium histolyticum*, EC 3.4.24.3, Sigma-Aldrich Japan K. K., 1 U/ml PBS containing 0.9 mM $CaCl_2$ and 0.49 mM $MgCl_2$), and the mixture was incubated for 24 h at 37 °C. To precipitate the enzyme, 200 μl of ethanol was added to the reaction mixture, and the supernatant of the centrifuged solution (15,000 rpm, 15 min) was subjected to GPC analysis.

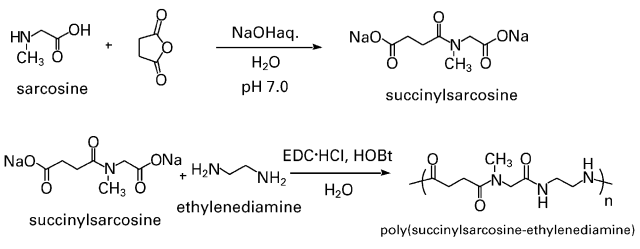
2.5. Cell adhesion assay

Eighty microliters of the obtained polyamide solution containing succinylsarcosine and/or succinylisoleucine in water (1 mg/ml) were coated onto the surface of a 96-well multiwell plate (Nalge Nunc International K. K., Tokyo, Japan) by air-drying. Twenty-five thousand NIH3T3 cells (ATCC CRL-1658, Dainippon Pharmaceutical Co., Ltd, Osaka, Japan) in Dulbecco's MEM were added to each well of the multiwell plate, and these were incubated under 5% CO_2-air at 37 °C for 1 h. The supernatant was removed, and the cells were washed twice with PBS. The nuclei of the adhered cells were stained using propidium iodide (Molecular Probes, Inc., Eugene, OR, 0.15 mg/ml) containing 1.2% Triton X-100 (Sigma-Aldrich Japan K. K.) and 5% EDTA (pH 7.0), and the fluorescence measured using a fluorescence plate reader (Ex = 485 nm, Em = 585 nm, TECAN SPECTRAFluor, Austria).

3. Results and discussion

3.1. Polymerization of succinylsarcosine and ethylenediamine

The polyamides containing succinylsarcosine were obtained by the method shown in Scheme 1. The



Scheme 1.

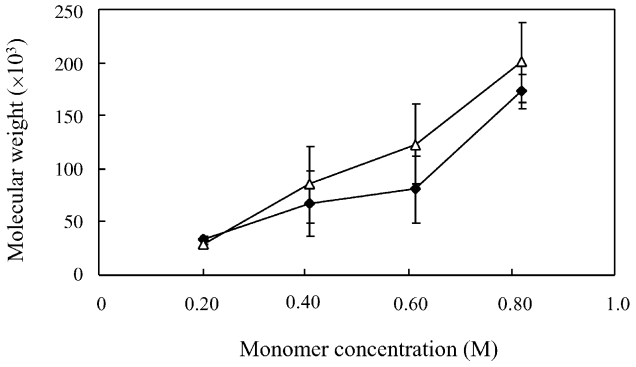


Fig. 1. Effect of the monomer concentration on the molecular weight of the obtained polyamides. The polymerization was carried out at 10 °C (–△–) and 20 °C (–◆–). The concentrations of EDC·HCl and HOBt were 2.5 equivalent and 1.13 equivalent to the monomer concentration, respectively.

polymerization conditions and the results are summarized in Table 1. The ^{1}H NMR spectrum in D_2O with tetramethylsilane as an internal reference showed peaks at 2.53 ppm (2H, NC(O)CH_2CH$_2$C(O)N(CH$_3$), broad d), 2.64 ppm (2H × 0.29, NC(O)CH$_2$CH_2C(O)N(CH$_3$), broad d), 2.78 ppm (2H × 0.71, NC(O)CH$_2$CH_2C(O)N(CH$_3$), broad d), 2.92 ppm (3H × 0.27, NCH$_3$, broad d), 3.12 ppm (3H × 0.73, NCH$_3$, broad d), 3.33 ppm (4H, NHCH_2CH_2NH, broad m), 4.05 ppm (2H × 0.71, C$^\alpha$H$_2$, broad d), and 4.18 ppm (2H × 0.29, C$^\alpha$H$_2$, broad d). The ^{1}H NMR spectrum showed no amide H, and suggested that poly(succinylsarcosine-ethylenediamine) was a mixture of *cis* and *trans* isomers (1:2.5). With increasing concentration of monomers and EDC·HCl, the molecular weight of the obtained polyamide increased up to a value of 200,000 (see Figs. 1 and 2). As the polymerization progressed from competition between the monomer and water molecules, the higher molecular weight products were obtained from the higher concentrations of monomer and EDC·HCl. The optimum reaction temperature was between 10 and 20 °C (see Fig. 3). It is generally difficult to obtain polyamide by direct condensation of α-amino acids. To obtain high molecular weight polyamide, highly active intermediate substance

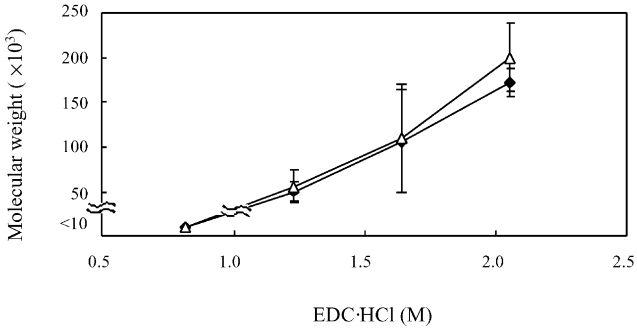


Fig. 2. Effect of EDC·HCl concentration on the molecular weight of the obtained polyamide. Polymerization was carried out at 10 °C (–△–) and 20 °C (–◆–). Each monomer concentration was 0.41 M, and concentration of HOBt was 0.93 M.

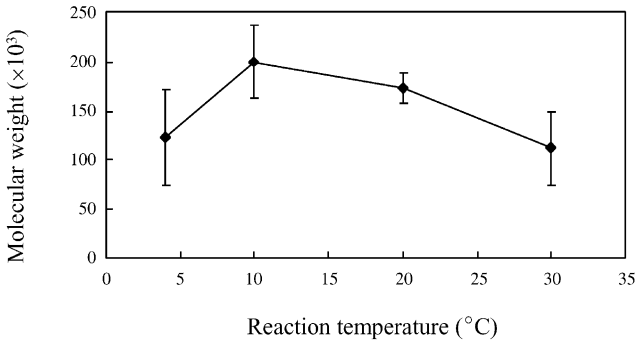


Fig. 3. Effect of reaction temperature on the molecular weight of the obtained polyamides. The polycondensation reaction was carried out in the presence of 0.82 M total monomer, 0.93 M HOBt, and 2.05 M EDC·HCl.

such as NCA and highly purified monomers and solvents are needed. Our method reported here gave a high molecular weight polyamide that included α-amino acids in water using usual grade reagents under a mild condition. It may be caused by changing low nucleophylic α-amino group into carboxyl group. Since the resultant dicarboxylic acid reacted with a highly active amino group in ethylenediamine, high molecular weight polyamide was obtained.

3.2. Copolymers of succinylsarcosine and succinylisoleucine

The conditions and the results for the copolymerization of succinylsarcosine and succinylisoleucine are summarized in Table 2. In the FT-IR spectra of the obtained products, peaks

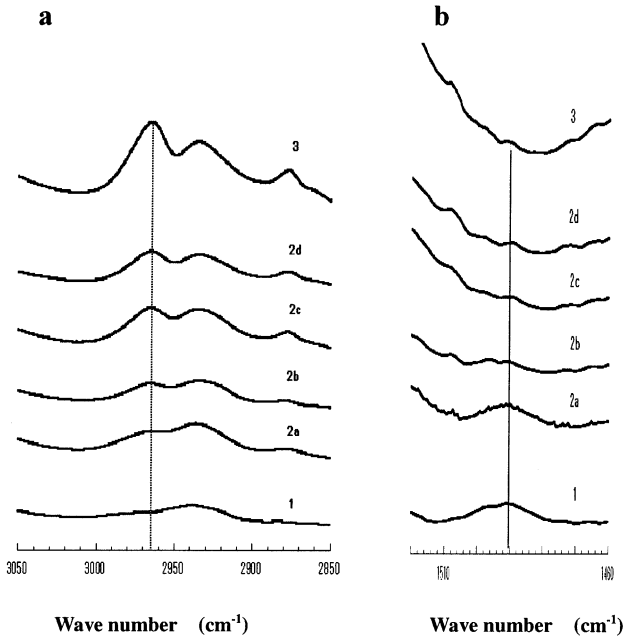


Fig. 4. FT-IR spectra of the (a) C–H region and (b) amide region of poly(succinylsarcosine-ethylenediamine) **1**, poly(succinylsarcosine-ethylenediamine-*co*-succinylisoleucine-ethylenediamine) **2a**–**2d**, and poly(succinylisoleucine-ethylenediamine) **3**.

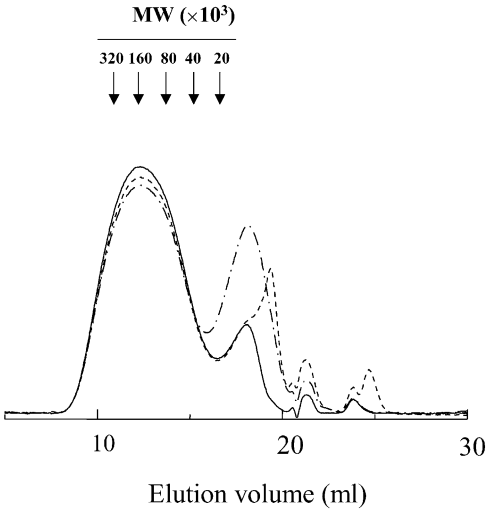


Fig. 5. GPC elution profiles of poly(succinylsarcosine-ethylenediamine) **1** incubated with buffer (—), elastase (---), and collagenase (—·—) at 37 °C for 24 h.

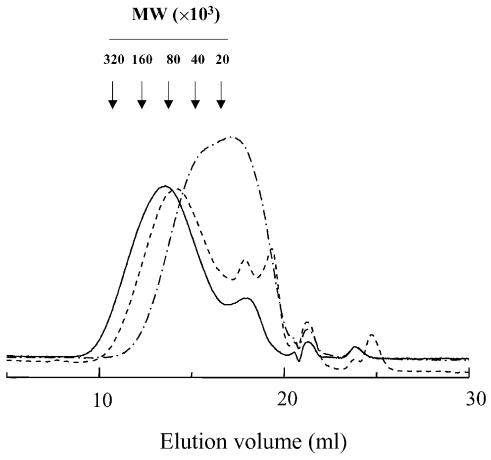


Fig. 6. GPC elution profiles of poly(succinylsarcosine-ethylenediamine-*co*-succinylisoleucine-ethylenediamine) **2b** incubated with buffer (—), elastase (---), and collagenase (—·—) at 37 °C for 24 h.

characteristic of polyamide were observed, which were at 3303–3311 cm^{-1} (νN–H), 1638–1644 cm^{-1} (amide I), and 1545–1550 cm^{-1} (amide II). As the succinylisoleucine content increased, the peak at 2965 cm^{-1} which was attributed to the isoleucine methyl group increased, and the peak at 1490 cm^{-1}, which was attributed to the tertiary amide group of succinylsarcosine, correspondingly decreased (Fig. 4). The ^{1}H NMR spectrum of the obtained copolymer (**2b**) in D_2O with tetramethylsilane as an internal reference showed the complex one. However, the peaks clearly attributed to each of sarcosine residue and isoleucine residue were observed, which were at 0.88 ppm (6H, isoleucyl-$C^{\beta}H(CH_3)C^{\gamma}H_2C^{\delta}H_3$); 1.18–1.44 ppm (2H, isoleucyl-$C^{\gamma}H_2$); 1.85–1.91 ppm (1H, isoleucyl-$C^{\beta}H$); 2.91 and 3.12 ppm (3H, sarcocyl-NCH_3); 4.05 and 4.18 ppm (2H, sarcocyl-$C^{\alpha}H_2$); 4.10 and 4.27 ppm (1H, isoleucyl-$C^{\alpha}H$). The ^{1}H NMR spectrum suggested that the copolymer (**2b**) was a mixture of some conformational isomers, and also suggested that the contents of sarcosyl residue and isoleucyl residue in copolymer (**2b**) were nearly equal in amount. These results show that the obtained copolymer included each monomer unit, almost in proportion to the initial composition. Although the polyamide that includes no succinylisoleucine decomposes at 208–212 °C, the copolymer, of which the initial succinylisoleucine content was 20 mol%, showed a melting point between 120 and 146 °C, and formed a film on heating. The copolymer, of which the initial succinylisoleucine content was 20 or 30 mol%, dissolved not only in water, but also in methanol and in dimethylformamide. The polyamide that included no succinylisoleucine dissolved in water only. Both the decrease in melting point and the increase in solubility may be caused by the decrease in crystallinity because of the bulky isoleucine side chains. Copolymerization of two different amino acid components conferred different properties on the copolymer.

The enzymatic degradation profiles of the polyamide and the copolymer are shown in Figs. 5 and 6, respectively. Although neither collagenase nor elastase digested the polyamide that included no succinylisoleucine, the copolymer, of which the initial succinylisoleucine content was 20 mol%, was digested by both enzymes. Polyamides such as Nylon 6,6 are not digested by the enzymes. However, Nylon 6,6, which includes a phenylalanine residue, is digested by α-chymotrypsin and Subtilisin [8]. Our results show that polyamides having the α- and β-amide groups may be digested by some enzymes, and that they could be degradable in vivo.

3.3. Cell adhesion to the copolymer

As the initial succinylisoleusine content increased, the adhesion of NIH3T3 cells to the copolymer clearly increased compared to the tissue culture plate, and to the polyamide that contained no succinylisoleucine (Fig. 7). The increase in cell adhesion may be caused by an increase in the hydrophobicity of the copolymer. As this copolymer

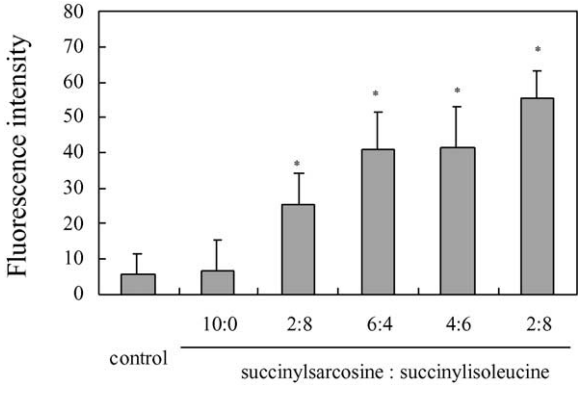


Fig. 7. Effect of the ratio of the succinylsarcosine and succinylisoleucine content on the adhesion of NIH3T3 cells. *These data are significantly high values compared with control ($p < 0.0001$) calculated by ANOVA.

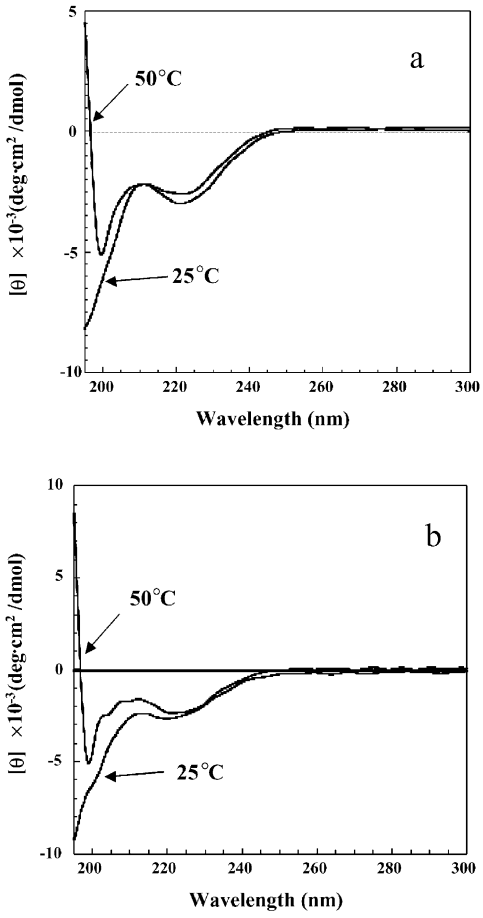


Fig. 8. Circular dichroism spectra of (a) $(VPGVG)_4$, and (b) copoly(succinyl$(VPGVG)_4$-ethylenediamine)$_1$-(succinylsarcosine-ethylenediamine)$_{1.5}$-(succinylisoleucine-ethylenediamine)$_{4.5}$.

showed biodegradability and enhanced cell adhesion, it may be useful as a scaffold for tissue engineering.

3.4. Polyamides, including $(VPGVG)_4$

It has been reported that poly(VPGVG) shows an inverse temperature transition behavior because of a conformational change triggered by the dehydration of the hydrophobic side chains of the peptide [9–11]. Copolymerization of succinyl$(VPGVG)_4$, succinylsarcosine, succinylisoleucine, and ethylenediamine, was performed by the same method as discussed, and a polyamide was obtained that had a molecular weight of 55,700. The polyamide that included succinyl$(VPGVG)_4$ could cause the same temperature-dependent conformational change as that of $(VPGVG)_4$, because both $(VPGVG)_4$ and the polyamide that included succinyl$(VPGVG)_4$ showed the same temperature-dependent change in their circular dichroism spectra (Fig. 8). The polyamide that included succinyl$(VPGVG)_4$ may take an extended form at 25 °C, and may form the β-spiral structure at 50 °C. This conformational change

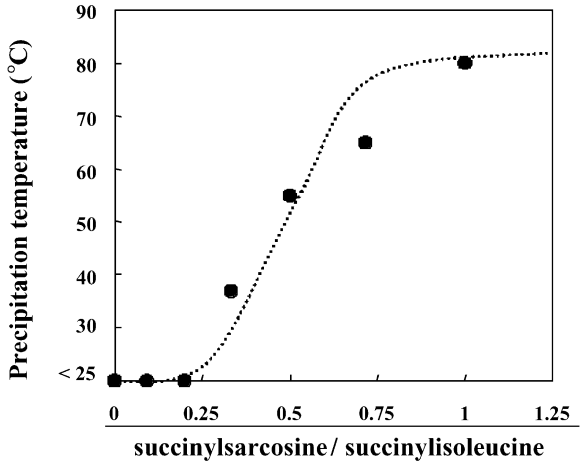


Fig. 9. Effect of the ratio of the succinylsarcosine content to succinylisoleucine content on the precipitation temperature. Polymer concentration was 20 mg/ml in PBS.

make both $(VPGVG)_4$ and the polyamide that included succinyl$(VPGVG)_4$ more hydrophobic. However, only the polyamide that included succinyl$(VPGVG)_4$ showed a reversible temperature-dependent precipitation. The precipitation may need longer hydrophobic chain than that of $(VPGVG)_4$. As the initial content of succinylsarcosine and succinylisoleucine varied, the precipitation temperature varied from below room temperature to about 80 °C (Fig. 9). This phenomenon was caused by a change in the hydrophobicity of the polyamide. Therefore, the transition temperature of the polyamide may be controlled by the initial content of the hydrophobic and hydrophilic amino acid components. This polyamide may be a useful intelligent material for drug delivery systems, wound dressings, and adhesion prevention materials.

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