Photoresponsive Peptide and Polypeptide Systems. 13. Photoinduced Cross-Linked Gel and Biodegradation Properties of Copoly(L-lysine) Containing ϵ-7-Coumaryloxyacetyl-L-lysine Residues

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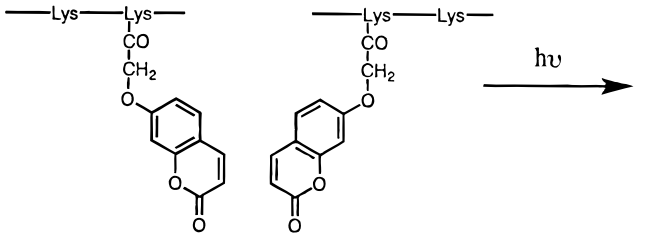
ABSTRACT: Copoly[LysLys(Cou)] containing ϵ-7-coumaryloxyacetyl-L-lysine [Lys(Cou)] residues were synthesized by the *N*-carboxyanhydride method. When the aqueous solutions of copoly[LysLys(Cou)] containing 5−10 mol % of Lys(Cou) are irradiated by light, the photo-cross-linking reaction proceeds slowly between coumarin moieties in the side chains to give a cis head-to-head cyclocoumarin, and after 6−24 h the solutions become transparent hydrogels. The gels exhibit solvent-induced reversible expansion and contraction behavior in water and in ethanol. The biodegradation of the hydrogels by some proteolytic enzymes and soil filamentous fungi has been investigated using photo-cross-linked copoly[LysLys(Cou)] gels. The photo-cross-linked copoly[$Lys^{90-95}Lys(Cou)^{10-5}$] gels are degradable by trypsin and protease type XXIII or two microorganisms, *Aspergillus oryzae* and *Penicillium citrinum*, suggesting potential for a controlled biodegradation by the different cross-linking densities in the gel matrixes upon the controlled irradiation.

Introduction

Our group has been studying photoresponsive peptide and polypeptide systems.[1] Photosensitive biopolymers are interesting systems because of their relevance to the molecular mechanism of photochemistry in biological materials and process and have received increased attention due to their broad applications as new formulation systems. Also, cross-linked biological polymers in watery systems have long been an important class of materials and are used in a diverse assortment of applications as hydrogels, including medical wound dressing; progress continues in developing approaches to describe the molecular structure of cross-linked polymers.[2,3] In particular, photo-cross-linked hydrogels have been reported to immobilize cell adhesive peptide and artificial fibrin glue.[4,5] In the course of our studies on bioadhesive material science,[6] we have reported some characterstics of polyamino acid hydrogels such as swelling properties, anion adsorption capability, adhesion, and biodegradation by proteolytic enzymes and microbial organisms.[7−12]

In this article, we describe first synthesis of utilized photochemical techniques for the development of an artificial three-dimensional matrix and preparation of the photo-cross-linked poly(L-lysine) hydrogels, **2**, from water-soluble copoly[LysLys(Cou)], **1**, containing ϵ-7-coumaryloxyacetyl-L-lysine [Lys(Cou)] residues. Into the cross-linked hydrogel matrixes many bioreactive materials can be incorporated.

In addition, during investigations of biodegradation of cationic biohydrogels, we reported the biodegradation characteristics of chemically cross-linked cationic polypeptides and polysaccharide gels by enzymes and microorganisms.[10−12] The present article describes secondarily the biodegradation characteristics and the possibility for its use as biodegradable materials of photo-cross-linked hydrogels, since polylysine and coumarin are nontoxic biomaterials.



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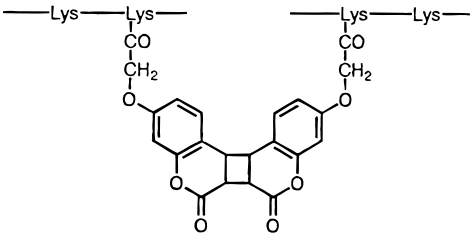

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Experimental Section

Materials. Ethyl bromoacetate, bromoethane, triethylamine (TEA), dicyclohexylcarbodiimide (DCCD), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), tetrahydrofuran (THF), dimethylformamide (DMF) and 25% hydrogen bromide in acetic acid (HBr/AcOH) were purchased from Wako Pure Chemical Industries, Ltd. 4M hydrogen chloride in dioxane was purchased from Kokusan Chemical Works, Ltd. 7-Hydroxycoumarin was purchased from Tokyo Chemical Industry Co., Ltd. α-*N*-*tert*-Butyloxycarbonyl ϵ-*N*-benzyloxycarbonyl-L-lysine dicyclohexylamine [Boc−Lys(Z)DCHA] was purchased from the Peptide Institute, Inc.

Enzymes. Trypsin (from porcine pancrease; EC 3.4.21.4; activity, 30−5200 USP units/mg) was purchased from Wako

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Pure Chemical Industries, Ltd. Protease type XXIII (from *Aspergillus oryzae*; activity, approximately 4 units/mg) was purchased from Sigma. Microorganisms employed for the biodegradation of copoly[LysLys(Cou)] were two pure-line soil filamentous fungi, *Penicillium citrinum* and *A. oryzae*.

Methods. The absorption spectra were measured with a Hitachi U-2000 spectrophotometer. The circular dichroism (CD) spectra was measured at 25 °C with a Jasco J-600 spectropolarimeter. The spectroscopic data were expressed in terms of molar extinction coefficient ϵ ($dm^3\ mol^{-1}\ cm^{-1}$) and mean residue ellipticity [θ] (deg $cm^2\ dmol^{-1}$). The specific rotation was measured with a Jasco DIP-4 spectropolarimeter at 589 nm and at 25 °C. Elementary analysis was carried out with a Yanagimoto CHN recorder MT-3 apparatus.

Irradiations of the sample solutions were carried out with a high-pressure mercury lamp (400 W) without a filter at room temperature. The light intensity was determined by chemical actinometry using potassium ferrioxalate to be 5.1×10^{18} photons $cm^{-2}\ s^{-1}$.[13]

The degree of swelling (q) was determined from the ratio w_s/w_o, where w_s is the weight of the swelled gels and w_o is the drying weight of gel materials or the ratio $(d_s/d_o)^3$, where d_s is the diameter of the cylindrical swelled gels after they reached equilibrium in the medium and d_o is the initial diameter of gels which were prepared in a capillary tube.[14]

The extent of cross-linking was calculated from Flory's theory using the equation $q_e = (vM_c)(1 - 2M_c/M)^{-1}(^1/_2 - \chi)/v_o$, where q_e is the equilibrium swelling ratio, v_o is the molar volume of the solvent, v is the specific volume of polymer, M_c is the molecular weight per cross-linking unit, M is the primary molecular weight of the polymer, and χ is the interaction parameter for solvent with polymer.[15]

Biodegradation of hydrogels was observed as a collapse of the hydrogel accompanying the growth of microorganisms. To evaluate the growth of microorganisms quantitatively, relative amounts of mature sporangia of the microorganisms were estimated by imaging analysis on each photograph of the culture, defining the area size of sporangia fraction measured on the calture with starting hydrogel as 100%, as described in detail in our earlier article.[11] The analysis was performed on a Kontron Electonik Imaging System KS300 (Carl Zeiss Vision K.K., Munich, Germany).

Synthesis. Cis Head-to-Head Coumarin Dimer. A suspension of coumarin (3.0 g) in 100 mL of water was irradiated by light without a filter for 2 weeks. The insoluble sticky product mixtures were collected by centrifugation, and the pale yellow residues (480 mg, 17.8%) obtained were washed with ethanol to give a white crystal. This was recrystallized from dichloromethane (30 mL) and ethanol (600 mL): yield, 90 mg (3.0%); mp 257−260 °C (lit.; mp 260 °C).[16]

Copoly[LysLys(Cou)]. General Procedure. All the synthetic reactions were monitored by tlc. Tlc was carried out on DC-Alufolien (Kieselgel 60 F_{254}, Merck). The following solvent systems were used: A, chloroform−methanol−acetic acid (95:5:3, v/v); B, *n*-butanol−acetic acid−water (4:1:5, v/v, upper layer); C, chloroform−methanol−water (90:10:1, v/v); D, 2-propanol−acetic acid−water (72:7:21, v/v). Spots were located on the sheets by spraying with a 1% solution of ninhydrin in water-saturated butanol and heating (amino acid derivatives), or inspected the fluorescent spots under light irradaition (Irie Seisakusho, model UV-LS-B1) at 254 and 365 nm (coumarin derivatives).

The molecular weight was estimated from an empirical equation in dichloroacetic acid (DCA) at 25 °C; $\log Dp = 1.47 \times \log[\eta] + 2.99$ for poly[Lys(Z)].[17]

Coumaryloxyacetic Acid. Coumaryloxyacetic acid ethyl ester was prepared from 7-hydroxycoumarin (24.3 g, 0.15 mol), ethyl bromoacetate (18.2 mL, 0.165 mol) and anhydrous potassium carbonate (22.8 g, 0.165 mol) in dry acetone as described in the earlier article by Chujo et al.[18] and recrystallized from ethanol: yield 83.9%; mp 112 °C; R_f 0.78 (A). This ester (29.8 g, 0.12 mol) in 400 mL of dioxane was saponified to coumaryloxyacetic acid using 2 N sodium hydroxide (400 mL) as described by Chujo et al.[18] The crude product was recrystallized from ethanol and *n*-hexane: yield 93.2%; mp 225 °C; R_f 0.17 (B). Anal. Calcd for $C_{11}H_8O_5$: C, 60.0; H, 3.7%. Found: C, 60.0; H, 3.6%.

Boc−Lys(Z) Ethyl Ester. Boc−Lys(Z) [isolated from 56.2 g, 0.1 mol, of Boc−Lys(Z) DCHA using 1N H_2SO_4], in 300 mL of DMF, sodium bicarbonate (16.8 g, 0.2 mol), and bromoethane (37 mL, 0.5 mol) in 300 mL of DMF were added together at room temperature with stirring. After 24 h, the reaction mixture was diluted with water and the product was extracted with ethyl acetate. The organic layer was dried over Na_2SO_4. After filtration, the solvent was removed under reduced pressure. The product was obtained as an oil: yield 87.0%; R_f 0.66 (A).

Boc−Lys Ethyl Ester Hydrochloride. To a Boc−Lys(Z) ethyl ester (16.2 g, 40 mmol) solution in 160 mL of ethanol−dioxane (1:1, v/v) was added 10% Pd on charcoal (1.6 g) under nitrogen. The mixture was subjected to catalytic hydrogenolysis for 3 h. After reduction was complete, 9.7 mL of 4 N HCl/dioxane was added and the mixture was filtered. The filtrate was evaporated to dryness under reduced pressure to give a pale yellow oil: yield 95.2%, R_f 0.05 (A).

Boc−ϵ-Coumaryloxyacetyl-L-lysine Ethyl Ester. To a solution of coumaryloxyacetic acid (10.3 g, 46.8 mmol) in 1000 mL of THF was added DCCD (10.7 g, 51.6 mmol) at 0 °C. After 2 h, Boc−Lys ethyl ester (isolated from 12.2 g, 39.3 mmol, of Boc−Lys ethyl ester hydrochloride using 6 mL of TEA) in 200 mL of THF was added dropwise to the cold solution. After 22 h, a few drops of acetic acid were added, and the reaction mixture was evaporated to dryness under reduced pressure. Ethyl acetate was added to the residues. The insoluble dicyclohexylurea was filtered off. The filtrate was washed successively three times with a 2% sodium bicarbonate solution, water, 5% citric acid, and water and dried over Na_2SO_4. The solvent was evaporated under reduced pressure. The product was recrystallized from ethyl acetate and *n*-hexane: yield 89.0%; mp 120−125 °C; R_f 0.49 (A); $[\alpha]^{25} = -9.1°$ ($c = 1$, methanol). Anal. Calcd for $C_{24}H_{32}N_2O_8$: C, 61.3; H, 7.1; N, 6.0. Found: C, 60.5; H, 6.8; N, 5.9.

Boc−ϵ-Coumaryloxyacetyl-L-lysine. Boc−ϵ-coumaryloxyacetyl-L-lysine ethyl ester (15.6 g, 32.7 mmol) in 250 mL of methanol was saponified to Boc−ϵ-coumaryloxyacetyl-L-lysine using 1 N sodium hydroxide (in total 113 mmol) at room temperature. After 11 h, methanol was removed under reduced pressure. The solution was acidified to Congo Red with 10% citric acid. The product was extracted with ethyl acetate. After the organic layer was washed with water and dried over Na_2SO_4, the solvent was evaporated under reduced pressure. The crude product obtained as a white crystalline was recrystallized from ethanol and *n*-hexane: yield 70.7%; mp 153 °C; R_f 0.09 (C); $[\alpha]^{25} = -2.0°$ ($c = 1$, methanol). Anal. Calcd for $C_{22}H_{28}N_2O_8$: C, 58.9; H, 6.3; N, 6.3. Found: C, 58.5; H, 6.4; N, 6.0.

ϵ-Coumaryloxyacetyl-L-lysine Hydrochloride. Boc−ϵ-coumaryloxyacetyl-L-lysine (20.0 g, 44.6 mmol) in 400 mL of dry dioxane was treated with 217 mL of 4 M hydrogen chloride in dioxane (892 mmol) for 4 h at room temperature to give ϵ-coumaryloxyacetyl-L-lysine hydrochloride. After removal of the solvent, the crude product obtained was recrystallized from ethanol and ether and a pale pink crystal was obtained: yield 77.9%; mp 186 °C; R_f 0.53 (D); $[\alpha]^{25} = 13.1°$ ($c = 1$, methanol). Anal. Calcd for $C_{17}H_{21}N_2O_6Cl$: C, 53.0; H, 5.5; N, 7.3. Found: C, 53.0; H, 5.8; N, 7.0.

ϵ-Coumaryloxyacetyl-L-lysine *N*-Carboxyanhydride [Lys(Cou)NCA]. To a suspension of ϵ-coumaryoxyacetyl-L-lysine hydrochloride (6.86 g, 17.8 mmol) in 2000 mL of dry dioxane was first added 0.67 M phosgene in toluene (53.4 mL, 35.6 mmol). The mixture was reacted for 3 h at 60 °C. Then, after 3, 6, 8, and 9 h, 26.7 mL each of 0.67 M phosgene in toluene was successively added to the reaction mixture and the reaction temperature was raised to 70 °C. After total of 11 h, a small amount of the insoluble material was removed by filtration. A clear filtrate was evaporated to dryness under reduced pressure, and the crude NCA was twice recrystallized from dry DMF and dry ether: yield 80%; mp 130−135 °C; Ir bands 1775 and 1842 cm^{-1} (cyclic anhydride). Anal. Calcd for

Table 1. Molecular Weights of Copoly[LysLys(Cou)] Containing Coumarin

samples	[η]	Dp	M_w	solubility in water (10% w/v)
poly[Lys(Cou)]	0.230	110	36300	insol
poly[Lys^{32}Lys(Cou)^{68}]^a	0.220	110	34500	insol
poly[Lys^{49}Lys(Cou)^{51}]	0.220	110	32600	insol
poly[Lys^{67}Lys(Cou)^{33}]	0.245	120	34300	soluble
poly[Lys^{80}Lys(Cou)^{20}]	0.365	220	60700	soluble
poly[Lys^{90}Lys(Cou)^{10}]	0.455	310	83400	soluble
poly[Lys^{95}Lys(Cou)^{5}]	0.345	200	53100	soluble
poly(Lys)	0.375	230	60300	soluble

^{a} Lys(Cou) content was estimated from the protected copoly[Lys(Z)Lys(Cou)] using the ϵ value of poly[Lys(Cou)] in HFIP.

$C_{18}H_{18}N_2O_7$: C, 57.8; H, 4.9; N, 7.5. Found: C, 57.5; H, 5.3; N, 7.7.

Copoly[Lys(Z)Lys(Cou)]. Lys(Z)NCA and Lys(Cou)NCA were copolymerized at a concentration of 10% in dry DMF with TEA as an intiator (A/I = 100). The copolypeptides were precipitated with water, filtered and dried; yields 81−92%. Poly[Lys(Cou)]: yield 92.4%; Ir bands 1535 and 1645 cm^{-1} (amides). Anal. Calcd for $(C_{17}H_{18}N_2O_5)_n$: C, 61.8; H, 5.5; N, 8.5. Found: C, 61.4; H, 5.4; N, 8.2. The results including their molecular weights are shown in Table 1.

Copoly[LysLys(Cou)] Hydrobromide. The copolypeptide hydrobromides were prepared from copoly[Lys(Z)Lys(Cou)] by the usual procedure using hydrogen bromide in glacial acetic acid.[19] The crude copolypeptide hydrobromides were reprecipitated twice from DMF and ether.

Results and Discussion

Coumarin-Containing Polylysine. Copoly[Lys(Z)-Lys(Cou)] containing different amounts of coumarin moieties in the side chains were synthesized by the NCA method. The most difficult point to synthesize the polypeptides was the solubility problem. The solubility of the Lys(Cou)NCA in dioxane is very low, so when reacted with phosgene, a very large amount (about 300-fold) of dry dioxane was necessary to solubilize the Lys-(Cou)NCA. For this reason DMF was used as the solvent at the polymerization stage to prepare the polypeptides. The polymerization of Lys(Cou)NCA and Lys(Z)NCA in DMF proceeded quite well, exhibiting high molecular weights of 15000−39000 (Table 1).

Conformation of Copoly[Lys(Z)Lys(Cou)] and Copoly[LysLys(Cou)]. From the CD measurements, poly[Lys(Cou)] exhibits a negative dichroic band at 224 nm with $[\theta]_{224} = -2000$ deg cm^2 cm^{-1}, a crossover point at 221 nm, a positive peak at 216 nm with $[\theta]_{216} = 4000$ deg cm^2 cm^{-1}, and a positive trough at 212 nm with $[\theta]_{212} = 2800$ deg cm^2 cm^{-1} in HFIP. The CD spectra of copoly[Lys(Z)Lys(Cou)] containing 51−10 mol % of Lys-(Cou) exhibit two negative dichroic bands at 220−221 and 208−209 nm with the ellipticity values of −10800 and −9200 (51 mol %), −18000 and −20400 (33 mol %), and −24400 and −28400 (10 mol %), respectively, in HFIP. From the assignment of the peptide electronic transitions, copoly[Lys(Z)Lys(Cou)] containing 0−51 mol % coumarin contents take the helical conformation in HFIP. Figure 1 shows the CD spectra of copoly[LysLys-(Cou)] in water at pH 10. The polypeptides exhibit two negative dichroic bands at 221 nm and at 207 nm with the ellipticity values of −9100 and −9900 (33 mol %), −20700 and −23100 (10 mol %), and −27000 and −28300 (0 mol %), respectively, in water at pH 10. The two peptide transitions at around 222 nm due to the n−π* peptide electronic transition and at around 208 nm due to the parallel-polarized π−π* exciton transitions of the peptide groups for the typical right-handed α-helices such as poly(L-Glu) and poly(L-Lys) were used to assign the conformations.[20,21] On the basis of the above assignment of the peptide transitions, copoly-[LysLys(Cou)] containing 0−33 mol % coumarin contents take the helical conformation at pH 10, while the copolypeptides take random coil structures at pH 5.4 exhibiting the positive dichroic band at 217 nm with $[\theta]_{217} = 1600$ (33 mol %) to 3800 (0 mol %). Copoly-[LysLys(Cou)] containing 51−75 mol % Lys(Cou) are sparingly soluble in water allowing one to prepare the photoirradiated hydrogels described below.

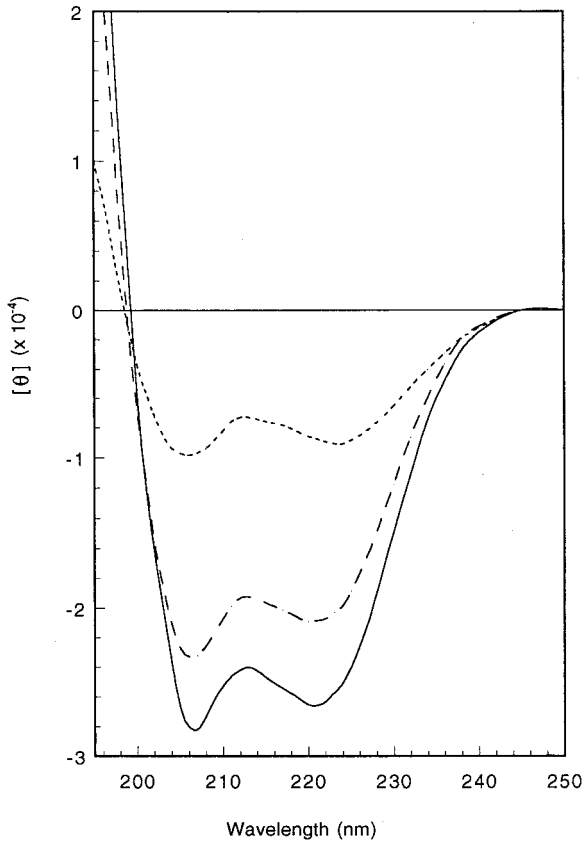

Figure 1. CD spectra of copoly[LysLys(Cou)] in water at pH 10: solid line, poly(Lys); dashed line, copoly[Lys^{90}Lys(Cou)^{10}]; dotted line, copoly[Lys^{67}Lys(Cou)^{33}].

Photo-Cross-Linking. Historically the product obtained, when a solution of coumarin in alcohol or a suspension of the solid in water was used, was reported as early as 1902 by Ciamician and Silber.[16] Then photoinduced cyclodimerization from coumarin derivatives was first correctly investigated by Schönberg et al.[22] However, the reproducibility of the two methods in water and in ethanol by this Egyptian group was invariably poor. In particular, the glacial acetic acid used as the recrystallization solvent exhibits a good solubility to recrystallization and has proven unsatisfactory in our hands. Since then many scientists have been attracted by this very interesting intermolecular cyclodimerization reaction. Later, the cyclodimerization of coumain to cis head-to-head, trans head-to-head, and trans head-to-tail stereoisomers has been investigated by Anet[23] and by Schenck et al.[24,25] The trans head-to-head (96%) and trans head-to-tail (1.5%) isomers were prepared in benzene with benzophenon as a photosensitizer,[24] while the cis head-to-head isomer was prepared in absolute ethanol (10.5% yield[24] and 1.3% yield[23]). More recently, the configurations of the photodimerized

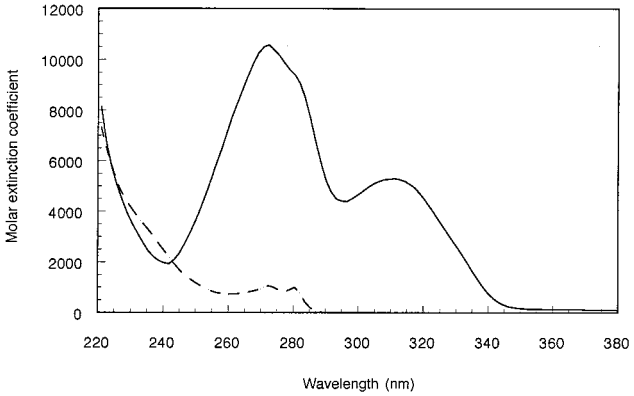

Figure 2. Absorption spectra of coumarin and cyclocoumarin in acetonitrile: solid line, coumarin; dashed line, cis head-to-head cyclocoumarin.

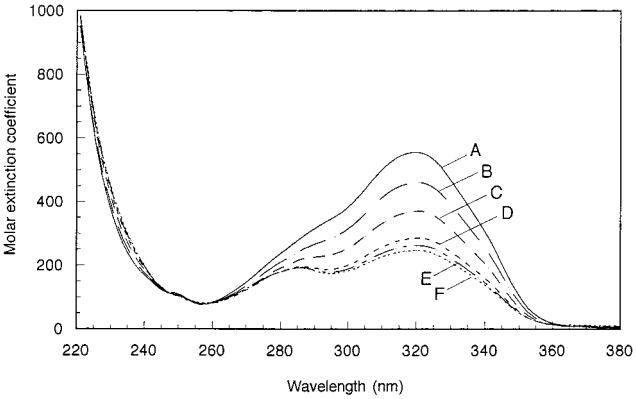

Figure 3. Change of absorption spectrum of copoly[Lys^{95}Lys(Cou)^{5}] in water upon irradiation with light. Irradiation time: A, 0 min; B, 5 min; C, 15 min; D, 60 min; E, 120 min; F, 180 min.

coumarin dimer and the mechanism have been studied by several groups.[26−29]

We describe a facile synthetic method to prepare the cis (syn) head-to-head cyclocoumarin using dichloromethane and ethanol in the Experimental Section. Figure 2 shows the absorption spectra of coumarin (monomer) and cis head-to-head cyclocoumarin (dimer) in acetonitrile. The coumarin was reported to exhibit two characteristic peaks at 311 nm with $\epsilon = 5700$ and at 273 nm with $\epsilon = 11{,}400$ in cyclohexane.[30] The absorption spectra of trans head-to-head cyclocoumarin have been reported in chloroform (estimated $\epsilon_{274} = 2530$ and $\epsilon_{283} = 2200$)[31] and in acetonitrile ($\epsilon_{215} = 25000$, $\epsilon_{271} = 2400$ and $\epsilon_{281} = 2160$; $[\theta]_{215} = -52800$ and $[\theta]_{228} = 59400$),[27] while only the ϵ value of the cis head-to-head cyclocoumarin has been reported in methanol ($\epsilon_{280} = 4000$).[26] From our synthesis of cyclocoumarin (refer to the Experimental Section), the ϵ values of the cis head-to-head dimer are 1980 at 280.4 nm and 2140 at 272.2 nm in acetonitrile (dashed line in Figure 2), $\epsilon_{274} = 2750$ in chloroform, and $\epsilon_{281} = 4850$ in methanol. Thus, the dimerization of monomeric coumarin moieties upon irradiation can be followed the decrease of the absorption bands at 311 and 273 nm (Figure 2). As anticipated,[27] a symmetric molecule, cis head-to-head dimer, exhibited no circular dichroism in the 200−300 nm range (3.2×10^{-4} M in acetonitrile). Also, the photo-cross-linked bis(α-Boc−ϵ-coumaryloxyacetyl-L-lysine) exhibited no ellipticity in the coumarin transition region, except the peptide transitions. From these reasons, we concluded that the conformation of photo-cross-linked cyclocoumarin dimer in the side chains of poly[LysLys(Cou)] is cis head-to-head as drawn the chemical structure in the Introduction.

Table 2. Conditions for Hydrogel Preparation of Poly[LysLys(Cou)]

concn of samples (w/v %)	Lys(Cou) contents (mol %)				
	5	10	20	33	51
10	○[a] (139)[b]	○ (65)	△	×	×
20	○ (45)	○ (23)	○ (19)	△	△
30	○ (23)	○ (14)	△	△	△

[a] Key: ○, hydrogel; △, partial gel; ×, liquid. [b] In parentheses is given the swelling ratio in water; see text.

The dilute aqueous copoly[LysLys(Cou)] solutions (at 10^{-3}−10^{-4} mean M residue) in quartz cells (10 mm) were irradiated at 360 nm light. For an example, Figure 3 shows the spectral changes of poly[Lys^{95}Lys(Cou)^{5}] in water upon irradiation with light. The absorption bands at 311 and 273 nm decreased with the irradiation time, exhibiting the cross-linking (dimerization) between the coumarin moieties in the side chains. This spectrometric change of poly(L-lysine) containing ϵ-7-coumaryloxyacetyl groups is similar to the change of the photogelation of polyoxazoline having coumarin moieties upon irradiation with light.[18]

Photo-Cross-Linked Hydrogels. When the 10−30% (w/v) aqueous solutions of poly[LysLys(Cou)] containing 5−20 mol % Lys(Cou) in glass capillary or glass tube were irradiated with light, the photo-cross-linked reaction proceeded slowly and after 24 h at 20 °C the solutions settled to transparent hydrogels. As listed in

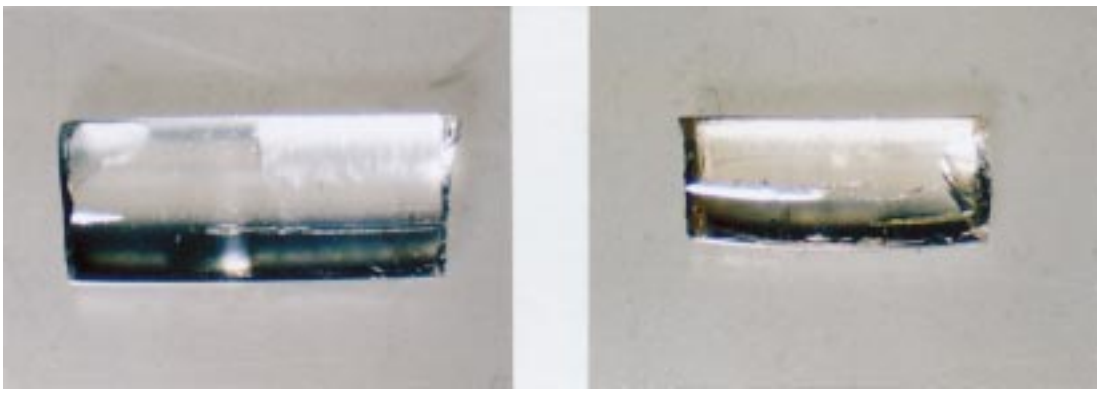

Figure 4. Photographs of the photo-cross-linked copoly[LysLys(Cou)] cylindrical gels prepared from 20% (w/v) solutions upon irradiation for 24 h: left, copoly[Lys^{95}Lys(Cou)^{5}]; right, copoly[Lys^{90}Lys(Cou)^{10}].

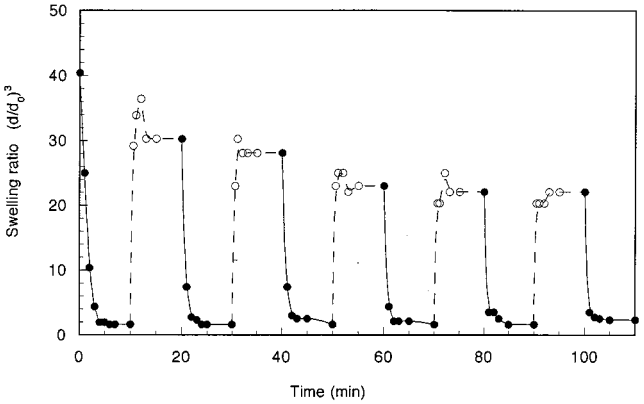

Figure 5. Solvent-induced expansision and contraction pattern of the photo-cross-linked copoly[$Lys^{95}Lys(Cou)^5$] cylindrical gel: open circle, in water; closed circle, in ethanol.

Table 2, poly[$Lys^{90-95}Lys(Cou)^{10-5}$] at 10−30% (w/v) exhibit the strong and expandable photo-cross-linked hydrogels, while the photo-cross-linked poly[LysLys(Cou)] containing more than 33 mol % Lys(Cou) are fragile. Figure 4 shows the photographs of the photo-cross-linked copoly[$Lys^{95}Lys(Cou)^5$] and copoly[$Lys^{90}Lys(Cou)^{10}$] gels, which were prepared from the 20% (w/v) solutions upon being irradiated for 24 h in water. The extent of cross-linking among the coumarin moieties was estimated to be 27.8% in the case of photo-cross-linked copoly[$Lys^{95}Lys(Cou)^5$] gels and to be 18.5% in the case of copoly[$Lys^{90}Lys(Cou)^{10}$] gels from the Flory equation (assuming that χ is 0.35).

The photo-cross-linked copoly[LysLys(Cou)] hydrogels expand when they are immersed in water and shrink when they are immersed in ethanol. As an example, Figure 5 shows the solvent induced reversible expansion and contraction patterns of the photo-cross-linked copoly-[$Lys^{95}Lys(Cou)^5$] hydrogels. After three repetition, the cylindrical gel exhibited mostly alternate behavior to swell (q, 22.2) in distilled water and to shrink (q, 1.6) in ethanol.

Enzymatic Degradation of Photo-Cross-Linked Hydrogels. Since the late 1940s the digestion of non-cross-linked PLL by a variety of proteases such as trypsin has been reported in many earlier articles, in which they reported that the main degradation process to oligopeptides occurred within 30 min.[32−35] The enzymatic degradation of cross-linked PLL and related hydrogels using organic cross-linking agents has first been reported from this laboratory.[10]

First, 1000 units of trypsin, or 350 units of protease (type XXIII from *Aspergillus*[36,37]) solution were added onto the gel disk swollen with distilled water, and the gel was allowed to stand at 25 °C. Hydrolysis of the hydrogel by proteolytic enzymes was observed as transition of the gel matrix into a liquid state. The degradation time was defined as a period required for transition of the gels into complete liquid within an observation by naked eye. Figure 6 shows a series of photographs of the transition of the gel matrix into the liquid state during hydrolytic degradation by trypsin or *Aspergillus* protease. Diameter of the disk of photo-cross-linked

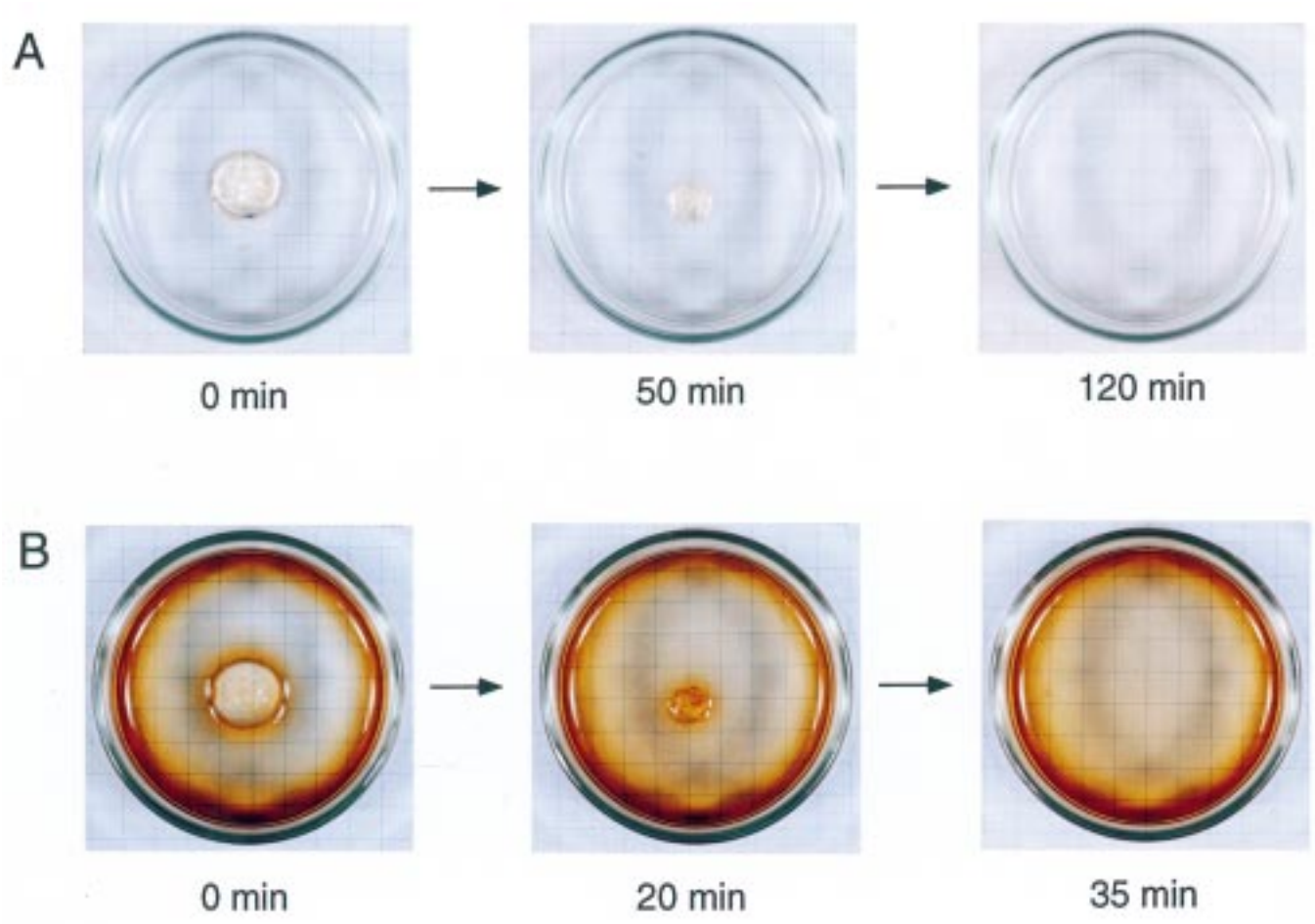

Figure 6. Photographs of the biodegradation time course of the photo-cross-linked copoly[$Lys^{90}Lys(Cou)^{10}$] gel: A, by trypsin; B, by *Aspergillus* protease.

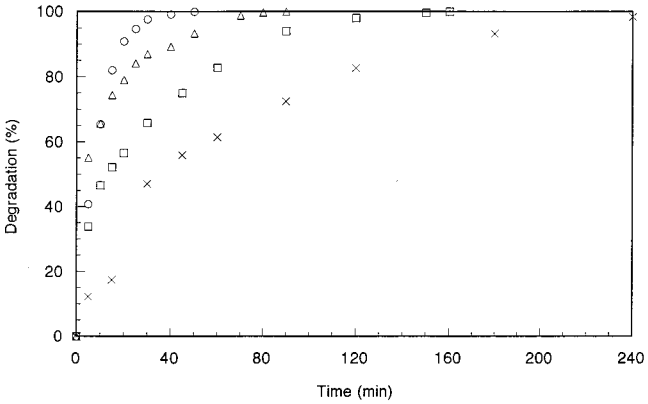

Figure 7. Biodegradation time course of the photo-cross-linked copoly[$Lys^{90-95}Lys(Cou)^{10-5}$] gels by trypsin. Key: (○) copoly[$Lys^{95}Lys(Cou)^{5}$] gel, irradiation for 24 h and trypsin 3000 units; (△) copoly[$Lys^{95}Lys(Cou)^{5}$] gel, irradiation for 6 h and trypsin 3000 units; (□) copoly[$Lys^{90}Lys(Cou)^{10}$] gel, irradiation for 24 h and trypsin 3000 units; (×) copoly[Lys^{90}-$Lys(Cou)^{10}$] gel, irradiation for 24 h and trypsin 1000 units.

copoly[$Lys^{90}Lys(Cou)^{10}$] gel was reduced, and the liquidified fraction of the gel gradually spread around the disk during the time course. After 120 min, the gel matrix was completely hydrolyzed into a homogeneous and transparent liquid by trypsin. When *Aspergillus* protease was used in the hydrolysis, it took 35 min for the complete liquidfication of copoly[$Lys^{90}Lys(Cou)^{10}$] gel. This degradation time was less than one-third that when trypsin was used, suggesting the higher susceptibility of the copoly[LysLys(Cou)] gel toward enzymatic degradation by *Aspergillus* protease than that by trypsin. This result suggests the possibility of controlled biodegradation by use of the irradiation time, which changes the cross-linking densities in the gel matrixes.

Figure 7 shows the complete biodegradation time course of photo-cross-linked copoly[$Lys^{90-95}Lys(Cou)^{10-5}$] gels. The degradation time in tryptic digestion increased with increasing the coumarin contents in the polypeptide gels. Using 3000 units of trypsin, liquidification of copoly[$Lys^{95}Lys(Cou)^{5}$] gel was completed with the fastest degradation time of 50 min among all gels, while liquidification times of copoly[$Lys^{90}Lys(Cou)^{10}$] gels were 160 min in the case of 24 h irradiation for photo-cross-linking and 90 min in the case of 6 h irradiation. Furthermore, when the amount of trypsin units was decreased to 1000 units, liquidification times of 24 h irradiated copoly[$Lys^{90}Lys(Cou)^{10}$] gels increased to 250 min.

Degradation of Hydrogels by Microorganisms. Microorganisms were added to photo-cross-linked copoly[LysLys(Cou)] hydrogel disks immersed in water under aseptic conditions. The microorganisms attached onto the hydrogels were statically cultured at 25 °C. Biodegradation of poly(amino acid) hydrogels was observed as a collapse of the disk of hydrogel accompanying the growth of microorganisms.[38,39] Figure 8 shows the time course photographs of two growing microorganisms, *A. oryzae* and *P. citrinum*, in water. The degree of growth of *A. oryzae* or *P. citrinum* was represented by formation of black sporangia, which were seen as darkish fraction of a colony in the photograph or by increasing number of the bulk colony, respectively.

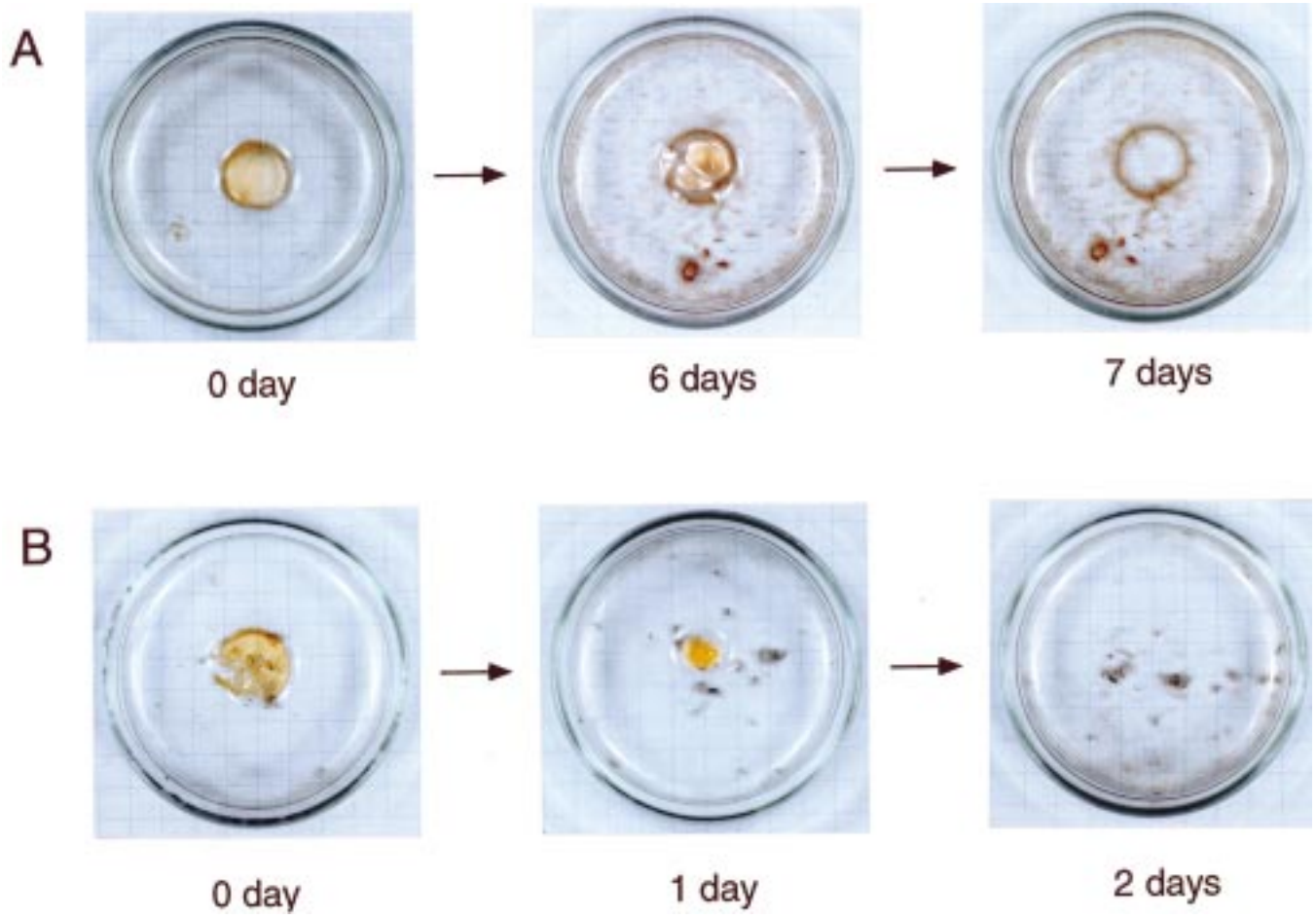

Figure 8. Photographs of the biodegradation time course of the photo-cross-linked copoly[$Lys^{90}Lys(Cou)^{10}$] gel: A, *A. oryzae*; B, *P. citrinum*.

When *A. oryzae* (21×10^6 spores) was cultured with copoly[Lys^{90}Lys(Cou)^{10}] gels, the gel matrix gradually began to collapse, accompanying the growth of the colony after 3 days. The development of brawny or darkish sporangia was observed after several days, and in this time the gel matrixes were partly liquidified. The gel mostly liquidified after 7 days. Similar but faster degradation was obtained when *P. citrinum* (34×10^6 spores) was cultured with the same hydrogel and medium. In this case, the period required for complete liquidification of the gel was much shorter than that in the case of *A. oryzae* and completely liquidified after 2 days. The relative degrees of growth of microorganisms on copoly[Lys^{90}Lys(Cou)^{10}] gels estimated by the imaging analysis system[11] are 1-fold at 0 day, 11-fold after 6 days, and 33-fold after 7 days in the case of *A. oryzae* (from Figure 8A) and 1-fold at 0 day, 8-fold after 1 day, and 13-fold after 2 days in the case of *P. citrinum* (from Figure 8B), respectively. These results suggest that the productive secretions of hydrolytic enzymes, such as proteases, from the microorganism during their growth caused the liquidification (degradation) of gel materials.

The present results of the biodegradation of photo-cross-linked copoly[LysLys(Cou)] coincide with the earlier results of the biodegradation of cross-linked films of polycaprolactone using the yeast *cryptococcus* and the fungus *Fusarium* reported by P. Jarrett et al.[40] and the more recent biodegradation of cross-linked chitosan using three fungi studied by us.[12,41]

The attempt to regulate the biodegradation of photo-cross-linked cationic poly(amino acid) hydrogels by incorporation of coumarin moieties have been achieved here. In our previous study,[11] the chemically cross-linked poly(L-ornithine) (PLO)−glutaraldehyde (GA) gels resisted against hydrolytic action of three protease, trypsin, chymotrypsin and papain, while poly(L-lysine) (PLL)−GA gels were digested by trypsin but not by chymotrypsin and papain. Difference in the susceptibilities toward proteolytic enzyme between PLL− and PLO−GA gels was explained by specificity of the three proteases toward peptide bond to be cleaved.[7,11] From the interest of the controlled biodegradation, copoly-(LysOrn)−GA hydrogels with seven ratios of ornithine from 0 to 100 mol % were subjected to hydrolysis by two kinds of protease, trypsin and *Aspergillus* protease type XIII.[11] As we expected, the degree of the biodegradation decreased depending on the increase of Orn content in the polypeptide gels,[11] suggesting a possibility for application of chemically cross-linked cationic poly-(amino acid) gels as a "controlled-biodegradable material". Likewise, photo-cross-linked copoly[LysLys(Cou)] gel matrixes degradated into liquid state during hydrolytic digestion by trypsin and *Aspergillus* protease (Figures 6 and 7) or microorganisms (Figure 8). These results suggest a possibility of controlled biodegradation by the different cross-linking densities in the gel matrixes upon the controlled irradiation.

In conclusion, in this paper it was demonstrated for the first time that a transparent cross-linked copoly-[LysLys(Cou)] hydrogel can be photochemically prepared. The reversible solvent-induced swelling properties and controlled-biodegradable material science of the photo-cross-linked poly(amino acid) gels are of scientific interest. Other anionic polypeptide coumaryl esters may also be potential candidates for protein model sources that lead to photo-cross-linked gels. The application of photo-cross-linked polypeptide hydrogel matrixes as biomedical protein formulations is currently under study in this laboratory.

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