Visible-light-induced surface graft polymerization via camphorquinone impregnation technique

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Abstract: A surface modification method that is particularly applicable to complexly shaped fabricated devices has long been awaited. In this article, we describe the visible-light-induced surface photograft polymerization technique by which an inner surface of the device is modified by visible-light irradiation through the external surface. Comphorquione, as a photoradical initiator, was impregnated on a segmented polyurethane surface by solvent soaking, followed by visible-light irradiation in the presence of monomers such as acrylamide and poly(ethylene glycol) methacrylate. The resultant surfaces were highly wettable with water, and surface chemical compositional analysis by X-ray photoelectron spectroscopy revealed that the surface was graft-polymerized with these monomers. The simple and widespread applicability of this surface modification technique to biomedical devices is discussed. © 2001 John Wiley & Sons, Inc. J Biomed Mater Res 59: 386–389, 2002

Key words: surface photograft polymerization technique; camphorquione; X-ray photoelectron spectroscopy

INTRODUCTION

It is crucial in the surface design of biomaterials intended for use as blood-contacting surfaces to address problems related to their blood compatibility.[1] Although various surface modification methods have been developed and applied to a wide spectrum of industrial fields, the use of these methods for surface modification of complexly shaped medical devices to upgrade their biocompatibility has been limited. The goal of our research was to develop a photochemical surface modification method that is directly applicable to fabricated devices in order to overcome the problems associated with the existing technology. To this end, we have developed photochemistry-based technologies enabling photografting of a blood-compatible photoreactive polymer[2] or photograft polymerization initiated from a photoreactive radical initiator that is preimmobilized on surface regions.[3] Irrespective of the type of reactions, the reactive species are generated under ultraviolet (UV)-light irradiation. The eventuality of using such blood-contacting polymers in an artificial heart device raises the question of how this could be practically achieved.

The coating of the polymer surface of a complex-shaped artificial heart with photoreactive polymers is performed by irradiation of inside part of the device with UV-light through an optical fiber [Fig. 1(A)]. The disadvantage of using UV light is that most polymeric materials absorb the UV light, which hampers the penetration of UV light into the deeper surface region or often results in no bonding between photocured polymers and the surface if the coated layer is thick. In addition, a procedure involving UV light irradiation through the external side of the device is not applicable because UV light is completely adsorbed by the external surface before it reaches the internal surface to be modified.

A more practical approach is to develop a technique of surface photograft polymerization that may be induced by visible-light irradiation from the external surface of the device, because visible light can pass through even thick transparent polymeric material.

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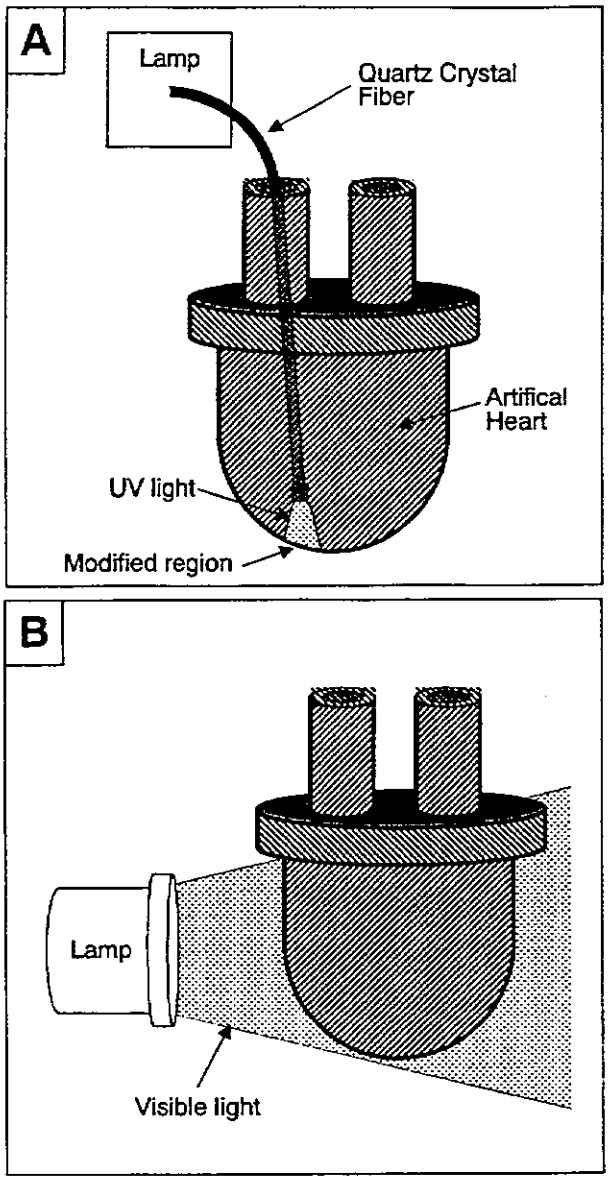

Figure 1. Schematic representation of (A) UV-light-induced polymer graft polymerization of the inner side of an artificial heart through a quartz optical fiber, and (B) visible-light-induced photograft copolymerization inside an artificial heart. Visible light was irradiated from the exertior side of the device.

One additional advantage is that all parts of the device are encompassed inside a field of visible light [Fig. 1(B)].

Design Concept and Surface Processing

The principle of the visible-light-induced photograft polymerization under development uses the photochemical reactivity of camphorquinone (CQ). Upon irradiation at an appropriate wavelength (400–500 nm), CQ, which has been used in the polymerization of dental resins for many years, generates radicals that can induce the polymerization of radical polymerizable vinyl monomers. If water-soluble, nonionic polymers such as poly(acrylamide) or poly(ethylene glycol) are produced on the photoirradiated surfaces, this surface-coating technology will prove very efficient for the surface design of complexly shaped devices. The technique consists of casting a CQ-containing solution to form a photochemically radical-producing thin layer on a polymer surface, followed by spreading with a monomer solution on the CQ-adsorbed substrate and then irradiation of the substrate with visible light through the solution of induce the graft polymerization reaction.

MATERIALS AND METHODS

Materials

Tetrahydrofuran (THF) and CQ were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Acrylamide (AAm) was purchased from Wako Pure Chemicals Industry Ltd. (Osaka, Japan). Poly(ethylene glycol) methacrylate (PEG-methacrylate) (MW 306; 5 ethylene oxide units) in a side chain is a product of Polysciences, Inc. (Warrington, PA).

Surface polymerization

A CQ-containing THF solution (0.5 wt %) was cast on a segmented polyurethane (SPU) sheet (fabricated from Elastollan; Takeda-Badish Urethane Co., Ltd., Tokyo, Japan) by Sheedom Co., Ltd. (Osaka, Japan), with an area of 20 × 20 mm and thickness of 200 μm, and air-dried overnight. An aqueous solution of a monomer (AAm or PEG-methacrylate, 5 wt %, 100 μL) was sparged with nitrogen gas for 10 min and spread on the CQ-adsorbed SPU film. The film was then irradiated for 10 min using an Xenon lamp (Super Bright 152S, wavelength 300–800 nm; San-Ei Electronic Co., Ltd., Osaka, Japan) through an optical fiber. The treated surface was washed thoroughly with water and dried overnight under a vacuum.

Physical measurement

Static advancing contact angles toward deionized water were measured using a contact angle meter (CA-D; Kyowa Kaimen Kagaku Co., Ltd., Tokyo, Japan) at 25°C by the sessile drop method. Because the data of the water contact angle were reproducible (n = 5, s.d. < 5%), only the average values were reported. ESCA spectra were recorded on a

Shimadzu ESCA750 (Kyoto, Japan) instrument. The spectra were deconvoluted into subpeaks by computer-aided processing.

RESULTS

The preliminary experiments of the visible-light-induced surface photograft polymerization of AAm and PEG-methacrylate were first performed in a direct irradiation manner to evaluate the feasibility of this newly developed technology. After visible-light irradiation of SPU sheets, in which the surfaces were preimpregnated with CQ by casting, in an aqueous solution of monomers such as AAm and PEG-methacrylate, the sheets were washed several times with water. The high wettability of the sheets was notable after washing. Table I lists the water contact angle values of the nontreated and photochemically treated sheets surfaces. A considerable change in the surface wettability was observed upon surface graft polymerization. The decrease in the contact angle indicated that the SPU surface became quite hydrophilic because of the surface fixation of the produced poly-AAm and poly(PEG-methacrylate).

ESCA data of the nontreated and photochemically treated SPU sheets is also listed in Table I. Marked differences in the surface elemental ratio and subpopulations of C1s, O1s, and N1s spectra were observed. A marked increase in the N/C elemental ratio was observed: the N/C ratio for the AAm-treated SPU surface was 16.6% (theoretical value of polyAAm, 33.3%), whereas that for the nontreated SPU was of 2.5%. The O/C ratio (29.6%) was very close to the theoretical value (33.3%). The ESCA spectra of the PEG-methacrylate-treated surface showed that the observed O/C ratio (22%) was almost one half the theoretical value (50%). On the other hand, the deconvoluted peaks for the hydrocarbon-derived C1s subpeak value (78%) and C–O bonds (20%) are quite different from the theoretical values (21.4% and 71.4%, respectively). The discrepancy between experimentally observed and theoretical values for both polymer systems may be due to (1) a low degree of polymerization, (2) surface contamination by a hydrophobic substance such as CQ due to excessive impregnation, and (3) excessive graft polymerization in deeper regions resulting in the formation of a semi-interpenetrating network of SPU and grafted polymers, which eventually deteriorates both surface and bulk integrity.

DISCUSSION

The preliminary experiments on visible-light-induced graft polymerization of AAm and PEG-methacrylate were performed. This polymerization is based on the ability of CQ, which was preadsorbed or preimpregnated on an SPU surface region, to generate radicals that induce the polymerization of AAm and PEG-methacrylate under visible light irradiation. The obtained results are very encouraging; we were able to generate a hydrophilic surface on the fabricated biomedical devices. However, improvements in this technology are required. Several parameters must be controlled for better optimization. These factors include (1) local impregnation of a photolyzable initiator, particularly on the outermost surface region and (2) a reduction in irradiation time. The first issue is particularly important to prevent graft-polymerization and/or radical-induced cleavage and crosslinking of substrate polymers on the inner surface region and in the interior bulk phase, which prevent the realization of "ideal" surface architecture in which only grafted chains are exposed to water without deterioration of surface and interior integrity. The coating of THF solution on SPU sheets resulted in spontaneous swelling, soaking, penetration, and distribution of CQ throughout the entire body of the sheet, although very hydrophobic CQ appears to be concentrated at the outermost region after air drying. Because no effort to minimize such an unfavorable effect was made in this experiment, the CQ concentration, chemical modification of CQ, irradiation condition, choice of solvent, and monomer concentration must be key subjects in future studies. The second issue is also important to prevent prolonged heating of the polymer surface,

TABLE I
Water-Contact Angles and Surface Chemical Compositions of Nontreated and Photochemically Treated SPU Surfaces

Surface	Water-Contact Angle (deg)		Carbon Subpopulation (%)[a]			Elemental Ratio[a]	
	Advancing	Receding	C–O + C–N	C=O	C–C	N/C	O/C
SPU	84	68	38	3	59	2.5	22
After irradiation in PAAm[b]	46	36	16 (25)	20 (25)	64 (50)	16.6 (33.3)	29.6 (33.3)
After irradiation in PEG-MA[b]	48	32	20 (71.4)	2 (71.5)	78 (21.4)	1.1 (0)	22 (50)

[a]XPS analysis determines carbon subpopulation and elemental ratio.
[b]Theoretical values are in parentheses.

which might result in surface degradation, and of the device, which may cause shape deformation. This problem may be eliminated by device irradiation under water. The ability to induce polymerization of AAm or PEG-methacrylate upon irradiation of the SPU sheet from the back surface is the primary goal of this technology. In other words, if CQ is to be coated on the inner surface of an artificial heart device, this technology should be able to induce the polymerization through a very thick or thin, transparent polymer surface, depending on the conditions as listed above. Therefore, it is strongly conceived that visible-light induced photopolymerization has many advantages over UV-induced photopolymerization. Experiments are in progress to achieve these goals.

A more detailed description of the surface design, leading to surface localization of the CQ derivative as mentioned above, and physicochemical surface characterization to verify the surface graft polymer using confocal scanning microscopy will be reported in the very near future. The application of this technology to artificial heart devices and testing of its *in vitro* and *in vivo* performances are in progress.

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Photocurable Liquid Biodegradable Copolymers: In Vitro Hydrolytic Degradation Behaviors of Photocured Films of Coumarin-Endcapped Poly(ϵ-caprolactone-*co*-trimethylene carbonate)

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Coumarin-endcapped tetrabranched liquid copolymers composed of ϵ-caprolactone and trimethylene carbonate (TMC), prepared using pentaerythritol or four-branched poly(ethylene glycol) (PEG) as an initiator, were ultraviolet irradiated to produce photocured solid biodegradable copolymers. The hydrolytic degradation behaviors of photocured films were determined from the weight loss of the films. The initial hydrolysis rate (determined for up to 24 h using a quartz crystal microbalance) was enhanced with aqueous solutions of higher pH. The hydrolysis rate in the early period of immersion was increased with an increase in TMC content, whereas that in the later period (week order) decreased with a increase in TMC content. This inverse relation of composition dependence on the hydrolysis rate between the early and late periods was discussed. Topological measurements using scanning electron microscopy and atomic force microscopy as well as depth profiles of the fluorescein-stained hydrolyzed layer showed that for the pentaerythritol-initiated copolymer, irrespective of copolymer composition, hydrolysis occurred at surface regions and surface erosion proceeded with immersion time. For PEG-based copolymers, both surface erosion and bulk degradation occurred simultaneously. The hydrolyzed surfaces became highly wettable with water and exhibited noncell adhesivity.

Introduction

Photocurable liquid biodegradable copolymers show promise in the development of new surface and device fabrication process technology and may be used for various biomedical applications.[1] That is, rapid liquid-to-solid-phase transformation upon photoirradiation enables surface layering, device construction and drug-immobilized device. To this end, we have designed photocurable liquid prepolymers composed of ϵ-caprolactone (CL) and trimethylene carbonate (TMC). The polymers or copolymers of CL and TMC have been used as biodegradable materials in biomedical applications.[3−7] As shown in Scheme 1, molecularly designed copolymers with a molecular weight of a few to several thousands have four-branched chains, all the terminals of which are endcapped with a coumarin group as a photoreactive center. Since an associated pair of coumarin groups in liquid film forms a dimer by cyclobutane ring formation upon ultraviolet (UV) irradiation, chain extension and cross-linking occur to form photocured biodegradable films (Scheme 1B).[8−12]

In our previous study, detailed preparation methods for photocurable liquid copolymers composed of CL and TMC and their photocuring characteristics were described.[11] In this paper, we evaluated hydrolytic degradation behaviors of photocured films immersed in aqueous solutions. The hydrolytic degradation behaviors were determined by several techniques including the use of a quartz crystal microbalance (QCM) for the measurement of weight loss, scanning electron microscopy (SEM) and atomic force microscopy (AFM) for topological measurements, and confocal laser scanning microscopy for depth profiling of hydrolyzed regions. The structure and copolymer composition dependence of hydrolytic degradation behaviors is discussed.

Experimental Section

General Procedure. All the solvents and reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), or Sigma-Aldrich Japan, Inc. (Tokyo, Japan). Diglycerol polyoxyethylene glycol ether (b-PEG) was obtained from Shearwater Polymers, Inc. (Alabama, USA). 5-[4,6-Dichlorotriazin-2-yl]amino)fluorescein was obtained from Research Organics, Inc. (Ohio, USA). TMC was prepared according to the method previously reported, and recrystallized from a mixed solvent of ethyl acetate and hexane.[2,11] Pentaerythritol was recrystallized from acetone. Other solvents and reagents were purified by distillation. ^{1}H NMR spectra were recorded on a JEOL JNM-GX270 FT-NMR spectrometer (270 MHz, Tokyo, Japan). The chemical shifts were given in δ values from Me_4Si as an internal standard. UV absorption spectra were recorded on a JASCO Ubest-

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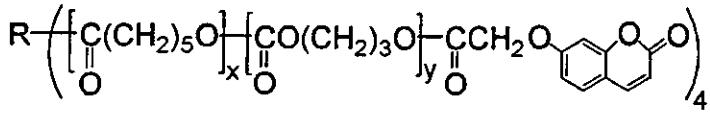
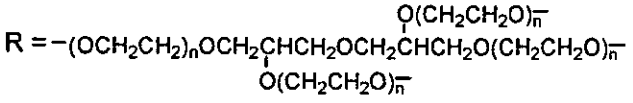
Scheme 1. Structures of Photocurable, Biodegradable Liquid Copolymers (A) and Photodimerization of Coumarin Groups (B)

A) Photocurable Liquid Copolymer



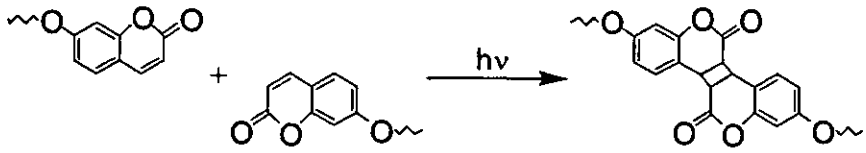
R = $C(CH_2O)_4$ (pentaerithritol)

or



(b-PEG: diglycerol polyoxyethylene glycol ether, n=10)

B) Photocuring



30 UV/VIS spectrophotometer (Tokyo, Japan). UV light (250 W Hg–Xe lamp: Hamamatsu Photonics L5662-02, Shizuoka, Japan) was irradiated through a Pyrex filter. The intensity of UV light source was measured at 250 nm (the photodimerization of coumarine groups maximally occurred at this wavelength) using a TOPCON UVR-25 (Tokyo, Japan). The surface topographic changes in photocured films were determined by scanning electron microscopy (SEM, JEOL JSM-6301F, Tokyo, Japan) and atomic force microscopy (AFM, Digital Instruments NanoScope III, California, USA), and the depth information on surface erosion upon hydrolysis was determined by confocal laser scanning microscopy (Bio-Rad μRadiance, Hertfordshire, U.K.).

Synthesis of Coumarin-Endcapped Poly(ϵ-caprolactone-*co*-trimethylene carbonate) Branched Copolymers. The preparation of coumarin-endcapped copolymers was carried out according to the method previously reported.[11] First, copolymers were prepared using trimethylolpropane, pentaerythritol, or branched poly(ethylene glycol) (PEG) (b-PEG, mol wt 2040) as an initiator and tin(II) 2-ethylhexanoate as a catalyst.[2–7] Subsequently, hydroxyl groups at terminals of the copolymers were esterificated using 7-chlorocarbonylmethoxycoumarin.[11] The composition of the copolymers was determined by ^{1}H NMR spectroscopy, and the coumarin content of the coumarin-endcapped copolymers was determined using ϵ_{max} of 1.35×10^4 (318 nm) by UV spectroscopy (Table 1).

Preparation of Photocured Films. The photocured films were prepared by UV light irradiation. A liquid film of coumarin-endcapped copolymer (0.053 g) coated on a cover glass (15 mm diameter) was prepared by casting dichloromethane solution and subsequent solvent evaporation, and UV light irradiation was carried out for 60 min at an intensity of 10 mW/cm^2 at 250 nm. The resulting photocured films (0.3 mm thickness) were subjected to determination of water adsorption and hydrolysis characteristics.

Table 1. Coumarin-Endcapped Tetrabranched Biodegradable Liquid Copolymers

polymer code	initiator[a]	copolymer composition CL:TMC[b]	coumarin content (mg/g)	M_w[c]	DW[d]
a	$C(CH_2OH)_4$	0.76:0.24	8.07×10^{-4}	5.0×10^{-3}	0.03
b	$C(CH_2OH)_4$	0.49:0.51	7.90×10^{-4}	5.1×10^{-3}	0.03
c	$C(CH_2OH)_4$	0.27:0.73	8.04×10^{-4}	5.0×10^{-3}	0.05
d	$C(CH_2OH)_4$	0.07:0.93	8.00×10^{-4}	5.0×10^{-3}	0.05
e	$C(CH_2OH)_4$	0.00:1.00	8.00×10^{-4}	5.0×10^{-3}	0.07
f	b-PEG[e]	0.49:0.51	5.71×10^{-4}	7.0×10^{-3}	0.58

[a] Molar fraction of monomer per total OH group of initiator was fixed at 6.6. [b] Copolymer composition ratio of CL and TMC. [c] Molecular weight determined based on coumarion content. [d] Degree of water adsorptivity (relative weight of water uptake to polymer) of photocured film. [e] Branched-PEG (diglycerol polyoxyethylene glycol ether) (mol wt 2040).

Water Adsorptivity. A photocured film was immersed in water for 2 days at room temperature. The degree of water adsorptivity (DW) was determined as the relative amount of water uptake against the photocured copolymer on the basis of weight.

Hydrolytic Degradation. The weight loss upon hydrolysis was determined on an hourly and weekly basis. The weekly order hydrolytic degradation behaviors were determined as follows. Photocured films were weighed and immersed into 0.001 M PBS aqueous solution (pH 7.4) at 37 °C for up to 8 weeks. After each 1-week period, the films were dried and weighed and immersed in a freshly prepared buffer solution. Three samples for each photocured film were determined. The results are shown as the average value (deviation was less than 5%). The hourly order hydrolytic weight loss was determined using a QCM, which is composed of a piezoelectric quartz crystal (9 MHz; AT cut) with gold electrodes, a dc source (5 V), and a frequency counter connected to a computer system. The change in frequency, which can be converted to weight (−1.01 ng/Hz), was monitored upon immersion of a gold-plated electrode (0.196 cm^2 area) coated

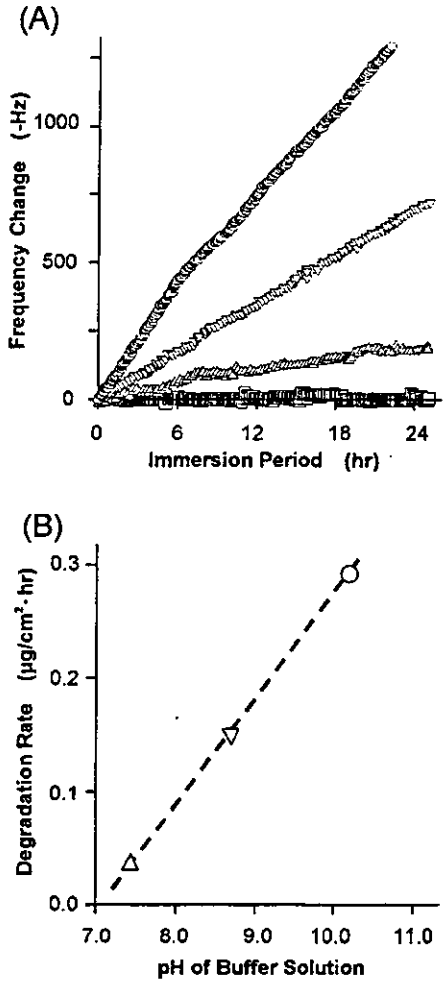


Figure 1. The frequency change (A) and hydrolytic degradation rate (B) of an electrode coated with a photocured film (**b**) upon immersion in 0.05 M tris-HCl buffer aqueous solution of pH 10.2 (○), pH 8.7 (▽), pH 7.4 (△), and pH 6.5 (deionized water, □) at 37 °C. Film thickness: 0.36 μm.

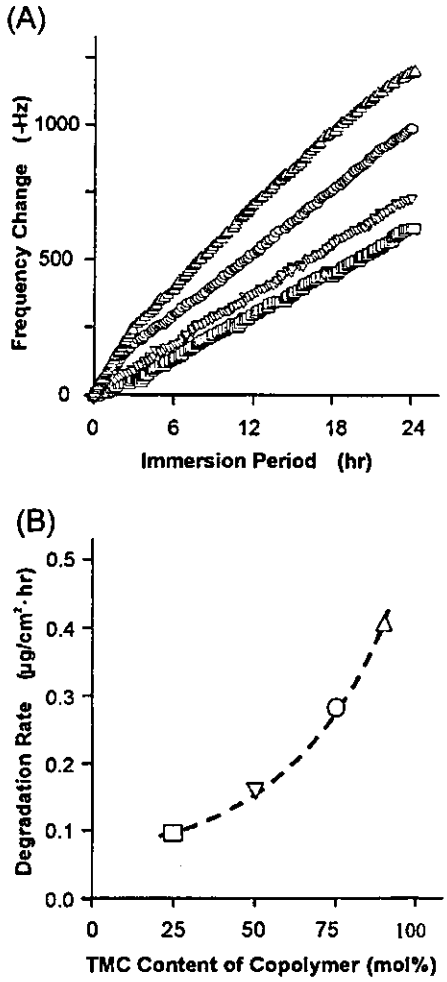


Figure 2. The frequency change (A) and hydrolytic degradation rate (B) of an electrode coated with photocured films (**a**) (□), (**b**) (▽), (**c**) (○), and (**d**) (△) upon immersion into 0.05 M tris-HCl buffer aqueous solution of pH 8.7 at 37 °C. Film thickness: 0.36 μm.

with a photocured film (7.0 μg) into 50 mL of 0.05 M tris[(hydroxymethyl)aminomethane]-tris[(hydroxymethyl)-aminomethane hydrochloride] (tris-HCl) buffer aqueous solution at 37 °C.

Surface Wettability. The wettability of the photocured films, before and after immersion in a phosphate buffer solution (PBS) of pH 8.0 at 37 °C for 24 or 60 h, was determined as follows. Static advancing and receding contact angles with deionized water were measured with a contact angle meter (Kyowa Interface Co. Ltd., Tokyo, Japan) at 25 °C by the sessile drop method.

Cell Culture Examination. Photocured films composed of copolymer (**b**) (15 mm diameter) were prepared by photoirradiation. The eroded films were prepared by immersion in a buffer solution of pH 8.0 for 60 h. All the films were washed with ethanol, air-dried, and placed on the bottom of a 24-well tissue culture dish (Corning, NY). Bovine endothelial cells (ECs, 4 × 10^4 cells/well) were seeded into the dish with Dulbecco's modified Eagle's medium (DMEM, Flow Laboratories, McLean, VA) supplemented with 15% fetal bovine serum (Hyclone Laboratories, Inc., Logen, UT), and the dish was incubated in a 37 °C incubator equilibrated with 5% CO_2–95% air. Observation was carried out using a phase microscope after 24 h of incubation.

Results

Synthesis of Photocurable Copolymers and Photocuring. Tetrafunctional photocurable liquid prepolymers were prepared by two-step reactions according to our previous method:[11] first, with a tetrafunctional polyol (pentaerythritol or four-hydroxyl-branched PEG (b-PEG)) as an initiator, CL and TMC were polymerized to produce tetra-branched copolymers under fixed conditions of monomer/initiator molar ratio. The copolymer compositions, determined by ^{1}H NMR spectroscopy, were found to be almost identical to those of the initial monomer feed composition, since the copolymer yields were almost 100%. Subsequent esterification with a coumarin acid chloride derivative resulted in complete endcapping of the hydroxyl group at the terminal ends of copolymers with coumarin groups, similar to those described in our previous paper.[11] Tetra-branched copolymers prepared using pentaerythritol have a wide spectrum of copolymer compositions: the fraction of TMC ranged from 0.24 to 0.93 (polymers **a**–**d**). Other

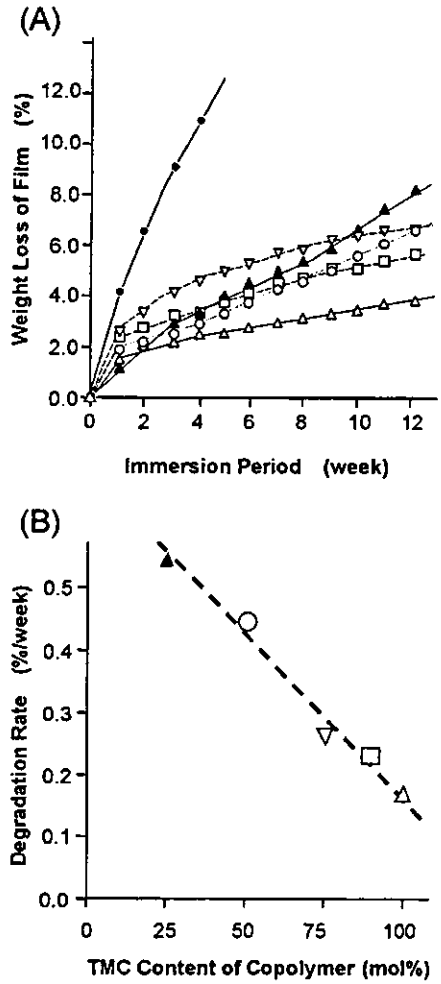


Figure 3. The weight loss (A) and steady degradation rate (B) of photocured films (a) (▲), (b) (○), (c) (▽), (d) (□), (e) (△), and (f) (●) (15 mg/cm^2) upon immersion in PBS aqueous solution (0.001 M, pH 7.4) at 37 °C.

tetrabranched liquid polymers or copolymers were poly(TMC) (e) and PEG-based copolymer (f). The coumarinated copolymers were viscous liquids, irrespective of functionality and copolymer composition. Except for copolymer (f), the coumarin contents were almost the same (8×10^{-4} mol/g). The molecular weight of a branch was found to be around 1.2×10^3 to 1.3×10^3 approximately. Considering functionality, calculated molecular weights of these copolymers ranged approximately from 5×10^3 to 7×10^3.

The liquid films (0.3 mm thickness) cast on the substrates were irradiated with UV light to produce highly cross-linked solid films. After immersion of these photocured films in acetone for 3 days, the photocured yield was determined. Irrespective of copolymers, full photocuring (~100%) was obtained in this experimental conditions. The equilibrium water uptake was very low (around several percent of initial weight) except for copolymer **f**, which adsorbed an amount of water equal to almost one-half the weight of photocured film.

Hydrolytic Behaviors. Photocured copolymers of copolymer **b** were subjected to hydrolysis, the degree of which was determined by two alternative methods. One method is in situ real-time determination of the weight loss of gold-plated QCM electrodes coated with photocured films, which were subjected to immersion into acetone solution to remove unreacted copolymers and which were immersed in buffer solutions with different pH values (pH 7.4−10.2) or deionized water (pH 6.5). A continuous change in frequency (Hz) with an immersion time for up to 24 h was observed (Figure 1). At higher pH values, more rapid hydrolysis occurred. From the fairly good linear change in frequency against immersion time, the degradation rate, which is defined as the hourly weight loss per unit area (μg/cm^2·h), was calculated using the conversion factor (−1.01 ng/Hz), and plotted against the pH of the immersion solutions. Figure 1B shows that the degradation rate linearly increases with a pH above the physiological pH. On the other hand, the hydrolytic behaviors of the photocured films composed of different copolymer compositions (polymers **a**−**d**) in buffered solution of pH 8.7 were also subjected to QCM measurements for up to 24 h of incubation. The accelerated hydrolysis condition at higher pH instead of physiological pH was used to differentiate hydrolytic behaviors of photocured copolymers tested. The initial hydrolysis rate, deter-

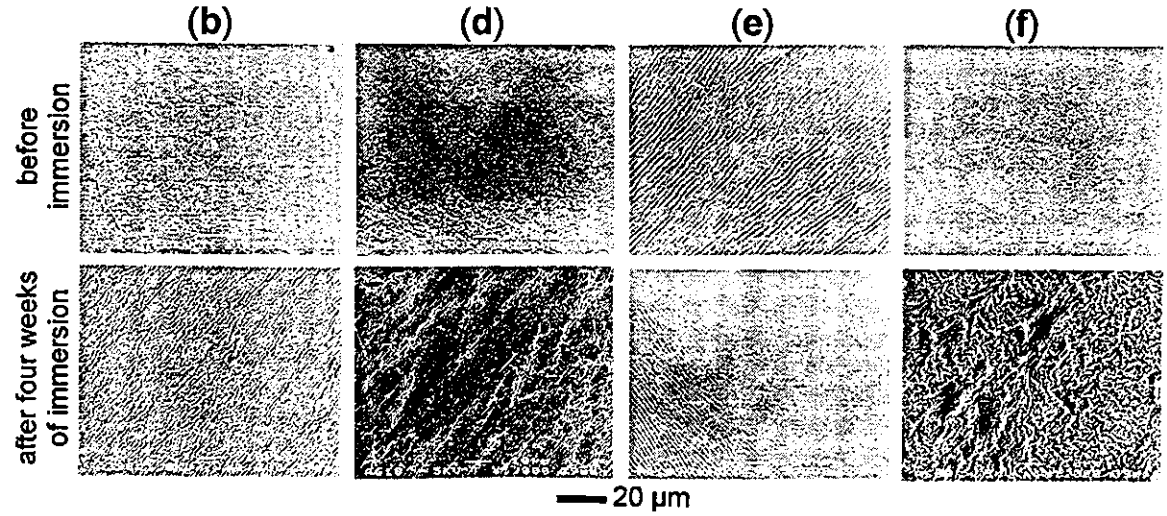


Figure 4. SEM images of photocured films (b), (d), (e), and (f) before and after 4 weeks of immersion in 0.001 M PBS aqueous solution at 37 °C.

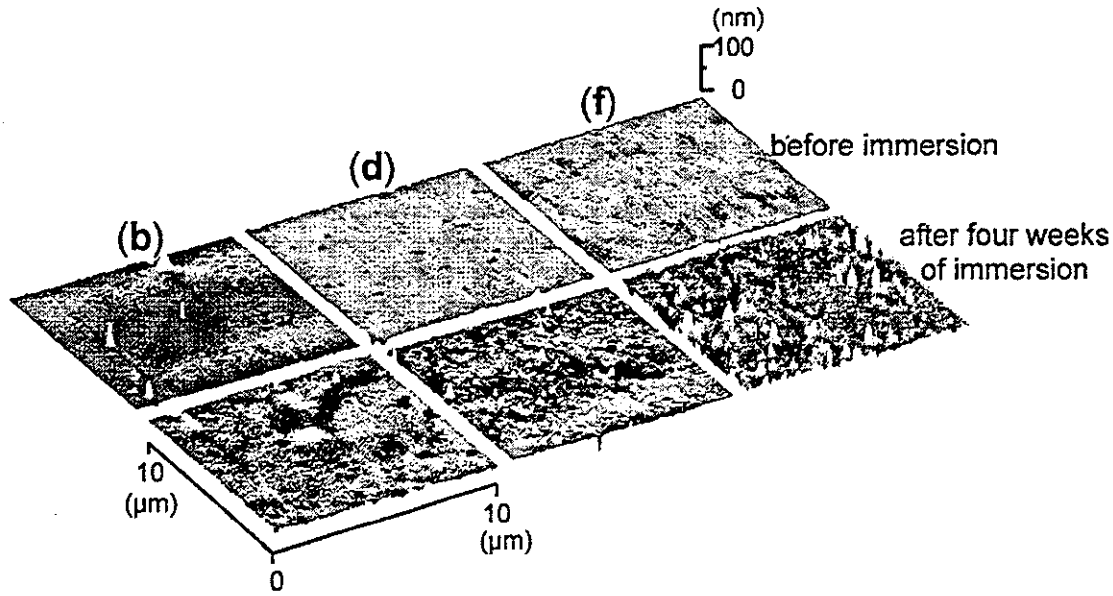


Figure 5. AFM images of photocured films (b), (d), and (f) before and after 4 weeks of immersion in 0.001 M PBS aqueous solution at 37 °C.

mined at linear regions in Figure 2A in manner similar to the method described above, was plotted against the TMC content of the copolymers (Figure 2B). With an increase in TMC content, a higher hydrolysis rate was observed during the early period of incubation (24 h).

On the other hand, week-order hydrolysis behaviors were determined by direct measurement of the weight change of film immersed in buffer solution (pH 7.4), which was replaced with freshly prepared solution every week for up to 12 weeks. PEG-based photocured film f exhibited extremely high hydrolytic capability. The general trend showed that a rapid weight loss was observed during the early period of incubation (within a few weeks after immersion), followed by slower hydrolysis during the later period of incubation, irrespective of the photocured films (Figure 3A). The hydrolysis rate, defined as the weight loss (%) per week calculated from the constant linear weight loss per week during the later period of incubation (a few weeks to 12 weeks), decreased with an increase in the TMC content in the photocured copolymers, as shown in Figure 3B.

Figure 4 shows scanning electron micrographs of photocured surfaces before and after 4 weeks of immersion. Before immersion, relatively smooth surfaces were observed irrespective of the types of photocured films used. Upon immersion, the surfaces became rough. The PEG-based surface was most roughened, probably due to hydration of PEG segments as well as degradation, whereas the least rough surface was poly(TMC) (e). The topological images determined by AFM clearly differentiated the degree of surface erosion among various photocured films, as shown in Figure 5. Before immersion, relatively smooth surfaces were noticed irrespective of copolymers. Upon immersion for 4 weeks, the poly(TMC) (e) surface remained smooth, whereas the surfaces of CL-rich copolymers became quite rough. The PEG-based copolymer provided an extremely roughened surface (Figure 6). Confocal laser scanning microscopic observation of films stained with the fluorescent dye 5-(4, 6-dichlorotriazin-2-ly)aminofluorescein (DTAF),

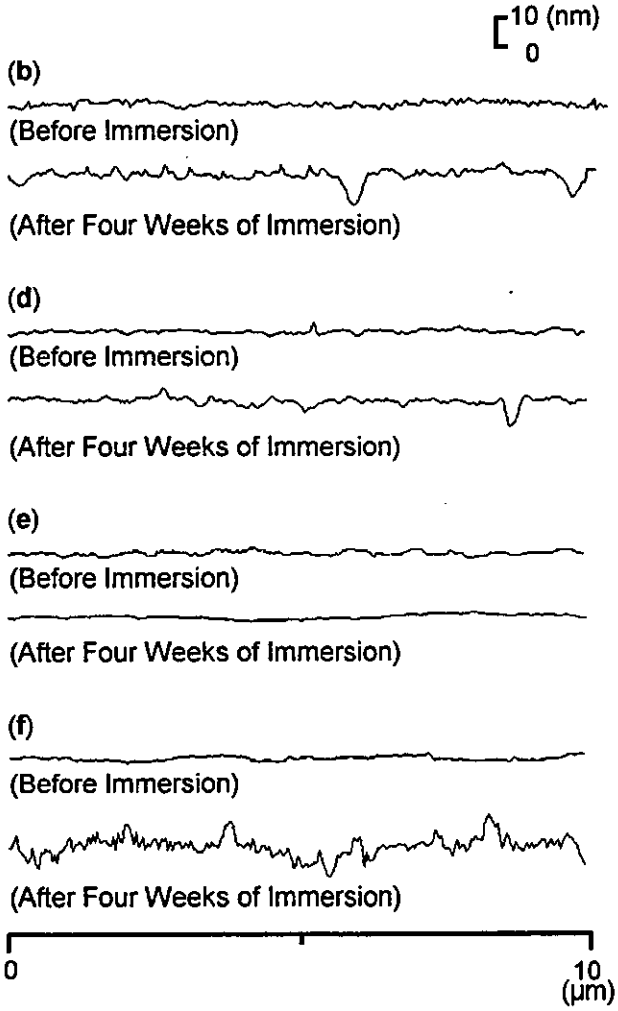


Figure 6. AFM section analysis of photocured films (b), (d), (e), and (f) before and after 4 weeks of immersion in 0.001 M PBS aqueous solution at 37 °C.

which was subjected to conjugation with a hydroxyl group in the presence of a condensation agent, showed that

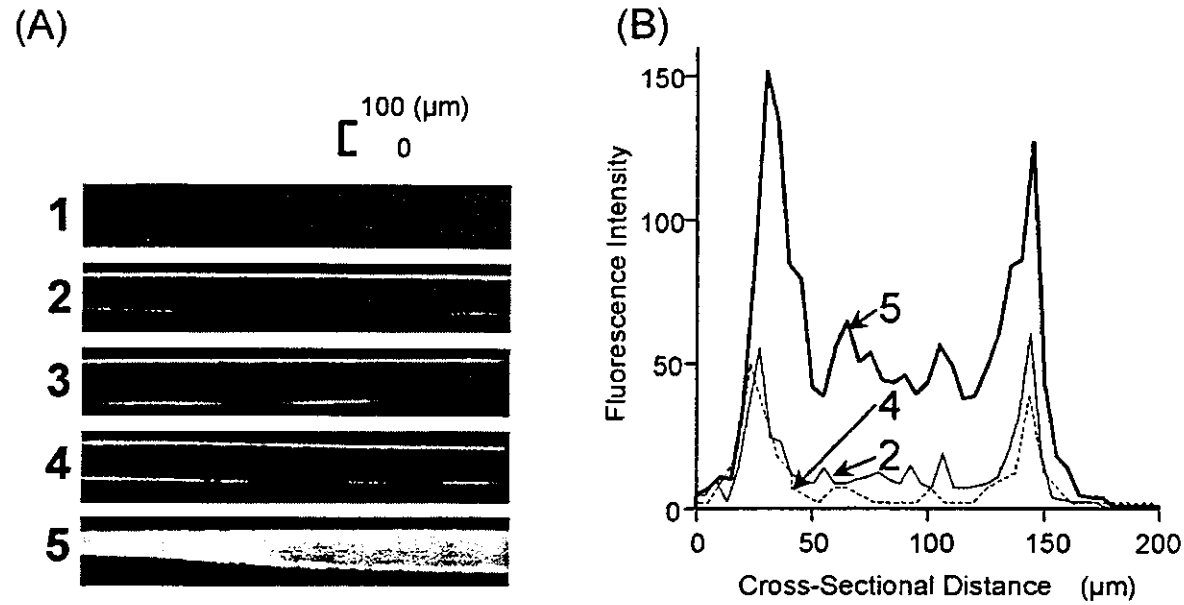


Figure 7. (A) Distribution of fluorescence at the cross section of films which were stained by the fluorescent dye DTAF: nontreated (**b**), 1; 1 day immersed (**b**), 2; 1 month immersed (**b**), 3; 3 months immersed (**b**), 4; and 1 day immersed (**f**), 5. (B) The fluorescence intensity of 2 (thin line), 4 (dashed line), and 5 (bold line).

Table 2. The Surface Wettablilites of Photocured Films

base polymer				water contact angle[a] advancing/receding (deg)		
code	initiator	CL:TMC[b]	M_w	nontreated film	24 h immersion	60 h immersion
b	$C(CH_2OH)_4$	49:51	5.1×10^3	65/44	60/<5	60/<5
c	$C(CH_2OH)_4$	27:73	5.0×10^3	60/36	54/<5	54/<5
e	$C(CH_2OH)_4$	0:100	5.1×10^3	52/24	33/<5	27/<5
f	b-PEG[c]	49:51	7.0×10^3	45/10	18/<5	14/<5

[a] Experimental error was ±5%. [b] Copolymer composition ratio of CL and TMC. [c] Branched PEG (diglycerol polyoxyethylene glycol ether, mol wt 2040).

fluorescent staining was only limited to the surface regions even at 3 months of immersion for nonswellable photocured copolymer (**b**), whereas both surface and bulk regions were highly stained for PEG-based swollen polymer (**f**) (Figure 7).

Surface Wettability and Cell Adhesion. Table 2 lists the water contact angles of photocured films (advancing and receding angles) as a function of immersion time for up to 60 h in aqueous solutions. Except for PEG-based photocured film, which has quite low angles, these photocured films gave medium angles (around 45−65°), exhibiting polar characteristics. Upon immersion in aqueous solution, surfaces became highly wettable. Low receding angles upon immersion indicate that the surfaces were very hydrophilic, irrespective of the types of photocured films used. When ECs were cultured on photocured films, they adhered and spread well in the initial period, but few cells were observed on surface-eroded films subjected to 60 h of immersion (Figure 8).

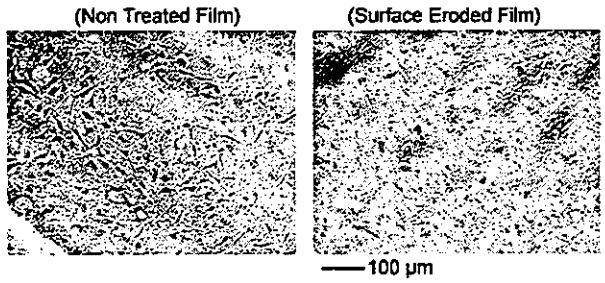


Figure 8. Micrographs of nontreated (A) and 60 h immersed (B) (**b**) films on which endotherial cells were cultured for 48 h ($n = 3$, typical micrographs are shown here).

Discussion

As an extension of a series of studies on designs of photocurable biodegradable copolymers based on CL and TMC, we have thoroughly evaluated the hydrolytic degradation behaviors of photocured films in aqueous solutions at physiological and higher pHs. The QCM, which is extremely very sensitive to weight loss, was employed for hydrolysis of initial period. Since the photocuring was found to be completed in this experimental conditions, the possibility of leaching-out of nonphotocured copolymer (which is not water soluble) in buffered solution is ruled out. The initial weight loss (for up to 24 h) increased linearly with incubation time, and the hydrolysis rate was linearly increased with both an increase in pH of aqueous solutions (Figure 1B) and an increase in TMC content of the copolymer composition (Figure 2B). On the other hand, at longer immersion time (a few to 12 weeks), the hydrolysis rate was decreased with an increase in the TMC content of the copolymer (Figure 3B). Although there is no supportive evidence to explain the

discrepancy in the copolymer−composition dependence of hydrolysis between early and long periods, the authors attempt to explain as follows. The intrinsic hydrolysis capability of carbonate linkage is much poorer than that of the ester linkage.[13] On the other hand, the crystallization capability of poly(CL) is much better than that of poly(TMC). For example, the literature showed that poly(CL) is crystalline with a melting point of 63 °C and low glass transition temperature (T_g) of −60 °C,[17] whereas poly(TMC) is amorphous and elastic rubbery material with T_g of −38 to −17 °C, depending on the molecular weight.[18] In general, hydrolysis preferably occurs at the amorphous region rather than at crystalline region.[13−16] Therefore, if the surface crystallization of the CL segment is enhanced with an increase in CL content of the copolymer, the higher content of TMC may facilitate hydrolysis. On the other hand, once the crystalline CL regions were hydrolyzed after a longer period, which eventually accelerates decrystallization, higher CL content of the copolymer due to its inherent hydrolytic capability may facilitate hydrolysis. This may be a plausible explanation to describe the inverse relation of the copolymer-composition dependence on hydrolysis between early and late periods. No attempt to determine surface enrichment of hydrophobic CL units as compared with TMC units and surface crystallization of CL units was made. Further study will be needed for validation of the hypothetical explanation.

Topological SEM and AFM images and depth profiles of fluorescent staining by confocal laser scanning microscopy clearly demonstrated that surface erosion except for PEG-based copolymers proceeded with incubation time. Apparently, eroded surfaces became rough and very hydrophilic. This hydrophilic characteristic may be derived from the generation of carboxyl and hydroxyl groups, both of which are produced by the hydrolysis of ester and carbonate groups in the copolymers. A large hysteresis between advancing and receding contact angle, observed for hydrolyzed photocured films, indicates that these hydrophilic functional groups produced by hydrolysis are preferentially orientated at water/material interface, whereas nonhydrolyzed hydrophobic parts are surface-enriched upon drying. Thus, in addition to liquid-to-solid phase transformation, our photocurable copolymer system has another advantage: the hydrolysis rate can be controlled by the molecular parameter of the copolymer system. CL-rich photocured films were more vulnerable to the nucleophilic attack of hydroxide ions during prolonged immersion in water. Since the CL and TMC copolymers had the least water adsorptivity, hydrolytic degradation occurs only at the surface regions, whereas water-adsorptive PEG-based copolymers appear to degrade at both the surface and bulk regions.

Therefore, the combined features of phase transformation and controlled hydrolytic degradation characteristics may be most suitable for surface design, device construction, and drug loading. Irrespective of types of the photocured copolymers used, hydrolytically degraded surfaces became very hydrophilic and exhibited noncell adhesivity. This noncell adhesivity is not due to the coumarinate group of hydrolyzed form but to a carboxylated coumarin, and there is no toxic nature of these compounds (data not shown). Such characteristics may be suited for use as a non-tissue-adhesive coating for implant devices, since tissue adhesion of implanted devices often causes adverse biological effects on the body.

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Thermoresponsive artificial extracellular matrix: N-isopropylacrylamide-graft-copolymerized gelatin

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Abstract—Poly(N-isopropylacrylamide)-graft-copolymerized gelatin (PNIPAM-gelatin) was prepared by iniferter-based photopolymerization of multiply derivatized dithiocarbamylated gelatin. PNIPAM-gelatins exhibited low critical solution temperature (LCST) immediately below the physiological temperature. PNIPAAm-gelatin-coated dishes induced cell adhesion at 37°C but incomplete detachment at room temperature, whereas dishes coated with PNIPAM or a mixture of PNIPAAm and gelatin showed little cell adhesion. The mixture of PNIPAAm-gelatin and PNIPAAm induced cell adhesion at 37°C and detachment at 20°C: the degrees of cell adhesion and detachment depended on the mixed ratio of PNIPAAm-gelatin and PNIPAAm. Complete thermoresponsive adhesion and detachment were found for the mixture containing a small fraction of PNIPAAm-gelatin (approximately 5 wt% with respect to PNIPAAm; gelatin content in the mixture is 2.7 wt%). Such a mixture may serve as thermoresponsive cell matrix for fabrication of a tissue-engineered device.

Key words: Poly(N-isopropylacrylamide); gelatin; thermoresponsiveness; cell adhesion; cell detachment.

INTRODUCTION

Much attention has been paid to the development of thermoresponsive cell-adhesive matrix on which cells adhere, spread, and proliferate well at physiological temperature; however, adhered cells are detached or cellular sheets formed on these surfaces are delaminated upon standing at room temperature. Poly(N-isopropylacrylamide) (PNIPAAm) is insoluble in water above the low critical solution temperature (LCST; approximately 32°C) and reversibly soluble below the LCST. This inverse temperature phenomenon of PNIPAAm was first reported by Heskins and Guillet [1]

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more than three decades ago, and it is only recently that this unique temperature-dependent characteristic has been extensively utilized for various biomedical applications where the thermoresponsiveness of the solubility of the polymer in water plays a major role in its function.

Thermoresponsive polymer surfaces using PNIPAAm were developed almost simultaneously by Takezawa *et al.* [2] and Yamada *et al.* [3] in 1990. The surface preparation method of the former group involved coating of a mixture of PNIPAAm and type I collagen. On such a coated dish, fibroblasts adhered, spread, and proliferated well, resulting in the formation of a monolayer cell sheet with time. By simply decreasing the solution temperature below LCST, adhered cells or formed cell sheets could be recovered from the substrates without using any conventional detachment agent such as trypsin or Ca ion chelating agent. In addition, the detached cells tended to form multicellular aggregates called spheroids. They demonstrated that hetero-spheroids composed of fibroblasts and hepatocytes were also prepared on mixed collagen-PNIPAAm coating [4].

On the other hand, the thermoresponsive surface developed by Okano *et al.* was based on electron beam irradiation of dishes coated with NIPAAm-containing isopropyl alcohol solution, and subsequent thorough washing with water [5]. On such treated dishes, endothelial cells (ECs) and hepatocytes grew well at physiological temperature. This technique has been utilized in the production of organized monolayered or bilayered tissues.

The authors prepared artificial fibrin glue [6] and two- and three-dimensional (2D and 3D) artificial extracellular matrices (ECMs) [7], which is a copolymer composed of an NIPAAm unit and a vinyl monomer unit with a cell-adhesion peptidyl moiety (Arg–Gly–Asp; RGD), the minimal amino acid sequence of adhesive sites being common to adhesive proteins such as fibronectin (PNIPAAm partially derivatized with RGD-containing peptide). The buffered solution is viscous and transparent at room temperature, but on elevation to physiological temperature, spontaneous precipitation occurs owing to a thermoresponsive phase transition of the NIPAAm unit. In the case of artificial fibrin glue, thermal sol-precipitate phase transition and subsequent cell adhesion via cell-adhesive moiety resulted in the formation of thrombus, possibly due to interaction with platelets. When the copolymer was coated on dishes, cells adhered and proliferated on the treated dishes to form a 2D tissue. When a mixed buffered solution of the copolymer and cells were elevated to physiological temperature, 3D hybrid tissues were formed in which smooth muscle cells embedded and proliferated. Upon standing at room temperature, cells adhering on 2D substrate were spontaneously delaminated and 3D hybrid tissues were dissolved.

In this paper, our study was extended to the preparation of a thermoresponsive bioconjugate as a thermoresponsive artificial ECM. The designed conjugate was PNIPAAm-gelatin that was prepared by quasi-living photo-graft polymerization initiated from a photoinitiator multiply derivatized on gelatin molecules. Temperature-

dependent cell adhesion and detachment were attained using PNIAAm-grafted gelatin.

MATERIALS AND METHODS

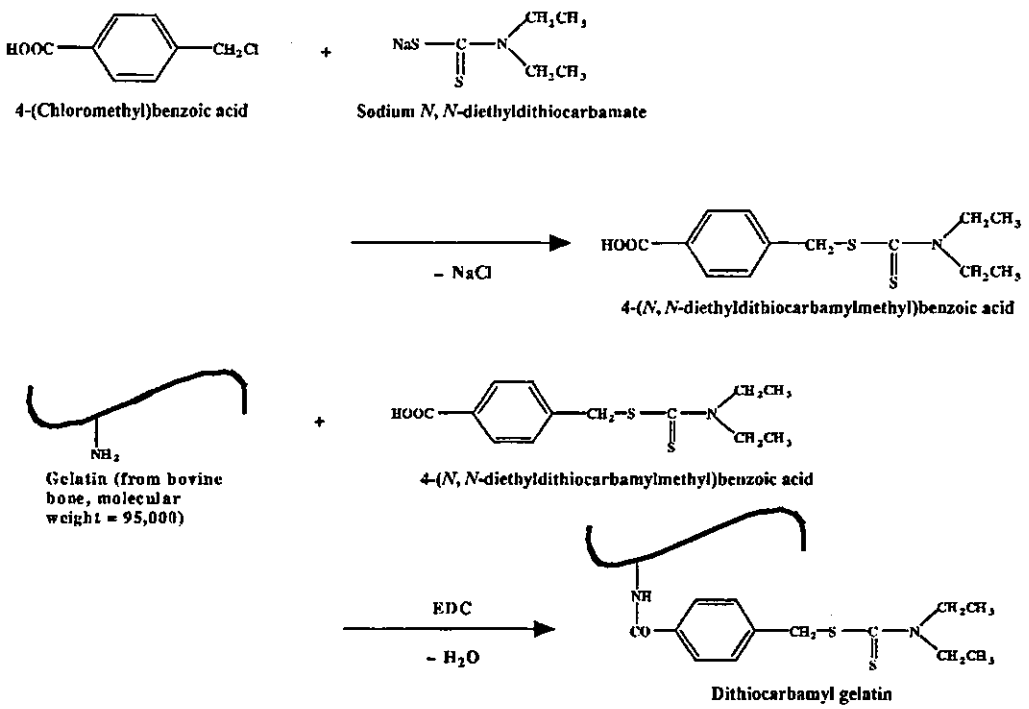
Materials

Solvents, all of which were of special reagent grade, were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and used without further purification. N-isopropylacrylamide (NIPAAm) (Eastman Kodak Co., Rochester, NY, USA) was purified by recrystallization from toluene and *n*-hexane. Gelatin (molecular weight = 9.5×10^4 g/mol, extracted from bovine bone), sodium N,N-diethyldithiocarbamate trihydrate, ammonium persulfate, N,N,N′,N′-tetramethylethylenediamine, and 2,4,6-trinitrobenzenesulfonic acid sodium salt dihydrate (TNBS) were purchased from Wako Pure Chemical Industries, Ltd., while 4-(chloromethyl)benzoic acid was from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), a water-soluble carbodiimide, was obtained from Dojindo Laboratories (Kumamoto, Japan).

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Laboratories, Life Technologies, Inc. (Grand Island, NY). Antibiotics (penicillin, streptomycin, and amphotericin B) were from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Phosphate-buffered saline (PBS) was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Dialysis membrane (cut-off molecular weight = $1.2 \times 10^4 - 1.4 \times 10^4$ g/mol) was purchased from Viskase Sales Corp. (Chicago, IL, USA). Cell culture dishes made of polystyrene (35-mm tissue culture dish and 35-mm non-treated dish) were obtained from Iwaki Glass Co., Ltd. (Chiba, Japan).

Synthesis of PNIPAAm

PNIPAAm was prepared by dissolving 10 g of NIPAAm in 100 ml of deionized water. Following degassing of the monomer solution for 10 min, 100 mg of ammonium persulfate and 1.0 ml of N,N,N′,N′-tetramethylethylenediamine were added. Polymerization proceeded at 25°C for 12 h. The polymer was obtained by precipitation upon warming the solution to 60°C: then, it was purified by repeated precipitation from methanol solution into diethyl ether and dried under reduced pressure for one day. Gel permeation chromatography (GPC) using a series of columns of a-5000 and a-3000 (TOSOH, Tokyo, Japan) was carried out with N,N-dimethylformamide as eluent at 40°C. The number-average molecular weight of PNIPAAm was estimated from the elution curve, referring to the calibration curve of standard poly(ethylene glycol)s.



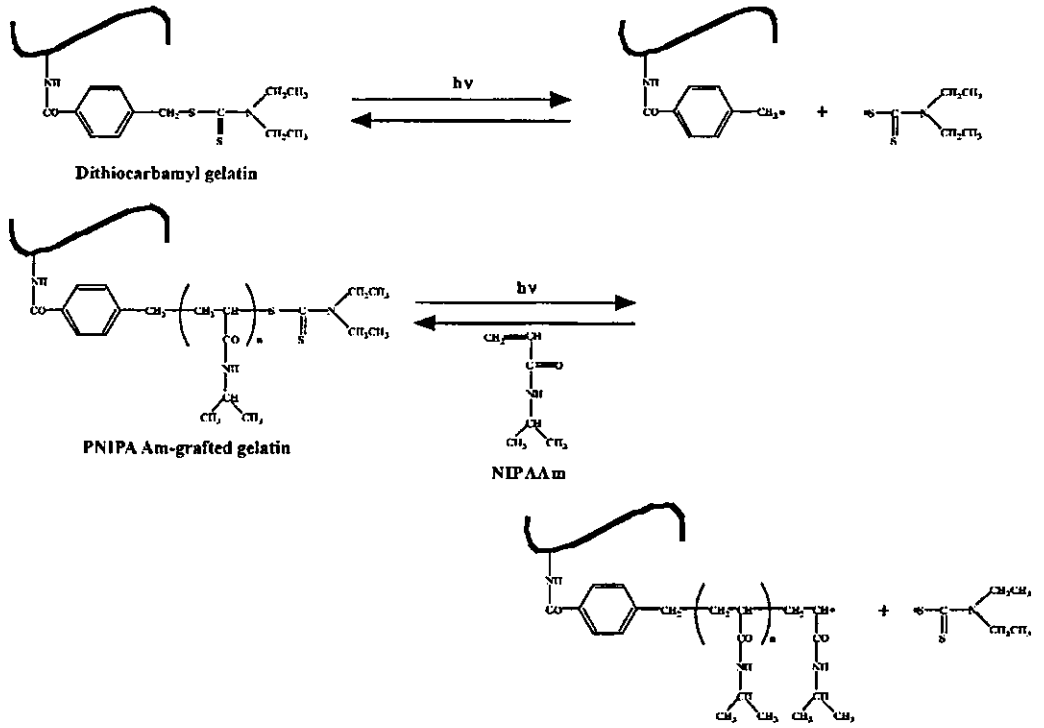
Scheme 1. Preparation route of multiply dithiocarbamylated gelatin (iniferter-derivatized gelatin).

Synthesis of 4-(N,N-diethyldithiocarbamylmethyl)benzoi c acid

4-(N,N-diethyldithiocarbamylmethyl)benzoi c acid was synthesized by reacting of 4-(chloromethyl)benzoi c acid with sodium N,N-diethyldithiocarbamate as a photoiniferter (Scheme 1). In a 300-ml three-necked flask equipped with a magnetic stirrer and a dropping funnel were placed 12.4 g (0.055 mol) of sodium N,N-diethyldithiocarbamat e trihydrate and 50 ml of ethyl alcohol. A solution of 7.85 g (0.046 mol) of 4-(chloromethyl)benzoi c acid in 100 ml of ethyl alcohol/toluene mixture (50/50 in volume) was added dropwise at room temperature. The reaction proceeded under vigorous stirring for 12 h at room temperature. The solution was then poured into 300 ml of deionized water and extracted with diethyl ether. The extract was washed with deionized water and dried on anhydrous magnesium sulfate. After concentration of the dried solution under reduced pressure, the residue was identified by a ^{1}H NMR spectrometer (JNM-GX270, JEOL, Ltd., Tokyo, Japan) as 4-(N,N-diethyldithiocarbamylmethyl)benzoi c acid [yield: 11.0 g (84.6%); ^{1}H NMR (DMSO-d_6 with $Si(CH_3)_4$) d 7.91 and 7.52 (dd, 4H, C_6H_4), 4.61 (s, 2H, CH_2S), 3.99 and 3.76 (qq, 4H, NCH_2), and 1.21 (t, 6H, CH_2CH_3)].

Synthesis of PNIPAAm-grafted-gelatin

The gelatin multiply derivatized with photoiniferters was synthesized as follows (Scheme 2). 4-(N,N-diethyldithiocarbamylmethyl)benzoi c acid (2.18 g, 7.69 mmol) was dissolved in 20 ml of water at pH 9 by adding sodium hydroxide solution under cooling in an ice bath. A solution of EDC (2.94 g, 15.3 mmol) in 120 ml of PBS



Scheme 2. Preparation route and mechanism of quasi-living radical photopolymerization of multiply grafted PNIPAM gelatin.

(pH 7.4) was added to the solution under stirring and cooling in an ice bath. After the reaction continued under stirring for 1 h, a solution of gelatin (4.0 g) in 40 ml of PBS (pH 7.4) was added to the mixture under stirring at room temperature. The reaction mixture was allowed to stand overnight with continuous stirring. Extensive dialysis using a dialysis membrane was conducted in water at approximately 40°C for three days. After freeze–drying *in vacuo*, the product was washed with ethyl alcohol, acetone, and diethyl ether. Dithiocarbamylated gelatin was collected by filtration and dried under reduced pressure for one day. The content of photoiniferter in the dithiocarbamyl gelatin molecule was determined from the absorbance at 281.5 nm using the extinction coefficient of N,N-diethyldithiocarbamate, $e_{281.5} = 13\,300\ \text{mol}^{-1}\ \text{cm}^{-1}$.

Dithiocarbamylated gelatin and NIPAAm were dissolved in distilled water to concentrations of 1.0 and 10 w/v%, respectively, and then a stream of dry nitrogen was introduced through a gas inlet into the mixture solution for 10 min. Photopolymerization was carried out in the presence of the mixture solution in a quartz glass tube of 17.5 mm diameter under irradiation with UV light from a 250 W Hg lamp (Spot Cure, Ushio, Inc., Tokyo, Japan) in a nitrogen atmosphere. The light intensity, measured with a photometer (UVR-25, Topcon, Tokyo, Japan), was adjusted to 5 mW/cm^2. Following polymerization at room temperature for 15 or 30 min, extensive dialysis using a dialysis membrane was conducted in water at approximately 40°C for three days. The dialyzed polymer was freeze–dried *in*

vacuo to obtain a white solid. The synthetic route used to prepare PNIPAAm-grafted gelatin is shown in Scheme 2.

The amine content of the polymer was determined using the TNBS method reported by Fields [8]. Briefly, the polymer was dissolved in water to a concentration of 1.0–10 mg/ml. The polymer solution (0.5 ml) was added to a solution of 0.1 M $Na_2B_4O_7$ in 0.1 M NaOH (0.5 ml), followed by the addition of 1.1 M TNBS aqueous solution (0.02 ml). After 5 min incubation, the reaction was stopped by adding 0.1 M NaH_2PO_4 aqueous solution containing 1.5 mM Na_2SO_3 (2 ml). The absorbance of the solution was measured at 420 nm using a 1-cm cuvette, and the amino concentration was determined using the extinction coefficient of trinitrophenyl-α-amino group conjugates, $e_{420} = 22\,000$ mol^{-1} cm^{-1} [13]. The total amine group of lysine residue of non-treated gelatin was 36.8 per molecule. The calculated dithiocarbamyl group in gelatin was 29.4 per molecule.

The molecular weight of dithiocarbamyl gelatin was calculated as follows. Since the molar number of the lysine residue of gelatin remains constant regardless of modification or not, the following relation should hold.

$$MW_{D\text{-gelatin}} \times ([Amine]_{D\text{-gelatin}} + [Dithiocarbamyl]_{D\text{-gelatin}}) = MW_{Gelatin} \times [Amine]_{Gelatin}, \quad (1)$$

where $MW_{D\text{-gelatin}}$ is the molecular weight of dithiocarbamyl gelatin, $MW_{Gelatin}$ is the molecular weight of gelatin (= 95 000), $[Amine]_{D\text{-gelatin}}$ is the content of amine group of dithiocarbamylated gelatin [mol/g], $[Dithiocarbamyl]_{D\text{-gelatin}}$ is the content of dithiocarbamyl group of dithiocarbamylated gelatin [mol/g], and $[Amine]_{Gelatin}$ is the content of amine group of gelatin [mol/g]. The molecular weights of PNIPAAm-gelatins were also calculated as follows. Since the molar number of free amine groups of dithiocarbamate-derivatized gelatin remains constant regardless of photograft polymerization or not, the following relation should be hold.

$$MW_{N\text{-gelatin}} \times [Amine]_{N\text{-gelatin}} = MW_{D\text{-gelatin}} \times [Amine]_{D\text{-gelatin}}, \quad (2)$$

where $MW_{N\text{-gelatin}}$ is the molecular weight of PPNIPAAm-gelatin and $[Amine]_{N\text{-gelatin}}$ is the content of amino group of PPNIPAAm-gelatin [mol/g].

Determination of LCST

PNIPAAm or PNIPAAm-grafted gelatin was dissolved in deionized water to a concentration of 1.0 mg/ml and the transmittance at 600 nm of each solution was measured with increasing temperature at a rate of 10°C/h using a UV-VIS spectrophotometer (Ubest-30, JASCO, Tokyo, Japan). The temperature at which the transmittance dropped from 100 to 90% was referred to as the LCST of that polymer [14].

Cell culture

PNIPAAm and PNIPAAm-grafted gelatin (polymerization time: 30 min) were dissolved in deionized water and sterilized by filtration using a microfilter with a pore size of 0.22 μm (Millipore Corp., Bedford, MA). PNIPAAm and PNIPAAm-grafted gelatin solution were uniformly mixed at approximately 4°C. Then, 0.4 ml of the mixture solution was poured into a 35-mm non-treated dish (9.62 cm^2) to uniformly coat the surface and then air-dried in a clean bench for 7 h [2]. ECs, harvested from bovine thoracic aorta by collagenase digestion method, were cultured in DMEM supplemented with 15% FBS, 50 IU/ml of penicillin, 50 μg/ml of streptomycin, and 2.5 μg/ml of amphotericin B at 37°C in a fully humidified atmosphere of 5% CO_2/95% air. ECs used for experiments had several passages and were identified by positive staining with factor-VIII-specific antigen specific to EC. The detailed harvesting and culture was described in our previous papers [15, 16]. ECs were seeded onto the polymer-coated dish at a seeding density of 1×10^5 cells/cm^2 and then incubated 37°C in a fully humidified atmosphere of 5% CO_2/95% air. As a control, ECs were also cultured on a 35-mm tissue culture dish (9.62 cm^2) under the same conditions. After culture for one day, the dishes were allowed to stand at room temperature (20°C). The cell sheet that was detached from polymer-coated dishes was then collected and cell number was determined under a phase-contrast microscope (OPTIPHOT2-POL, Nikon, Tokyo, JAPAN) after trypsin treatment. The non-detached cell number was also determined by the same procedure.

RESULTS

Synthesis of PNIPAAm and PNIPAAm-grafted gelatin

The photoiniferter, 4-(N,N-diethyldithiocarbamylmethyl)benzoi c acid, was synthesized according to the method shown in Scheme 1, and was characterized by NMR (spectral measurement) (see Experimental section). PNIPAAm-grafted gelatin was prepared as follows. First, the photoiniferter was derivatized on gelatin via condensation between the amine group of lysine residues of gelatin and the carboxyl group of the photoiniferter using a condensation agent in aqueous solution. Then, photograft polymerization of NIPAAm on photoiniferter-derivatize d gelatin (photoiniferter content determined by titration of amine group: 2.54 mol/g, degree of derivatization per amine group: 87.6%) was carried out in an aqueous solution as shown in Scheme 2. The polymerization time was set at 15 and 30 min. The obtained polymers were designated as the PNIPAAm-gelatin (I) for 15-min polymerization and PNIPAAm-gelatin (II) for 30-min polymerization. PNIPAAm was prepared by conventional redox polymerization.

The characteristics of bioconjugated gelatin are summarized in Table 1. The number-average molecular weight of PNIPAAm, estimated by GPC, was approximately 4.8×10^5 g/mol. The molecular weight of dithiocarbamyl derivatized

Table 1.
Characteristics of PNIPAAm-grafted gelatins

Polymer	LCST[c] (°C)	Amino content[d] ($\times 10^{-4}$ mol/g)	Dithiocarbamyl content[e] ($\times 10^{-4}$ mol/g)	Molecular weight	Molecular weight (per graft chain)[h]
Gelatin	—	3.16	0	9.5×10^4	—
Dithiocarbamyl gelatin	—	0.393	2.54	1.0×10^5[f]	—
PNIPAAm-grafted gelatin (I)[a]	34.3	0.284	—	1.4×10^5[f]	1.5×10^3
PNIPAAm-grafted gelatin (II)[b]	33.4	0.105	—	3.8×10^5[f]	1.1×10^4
PNIPAAm	33.1	—	—	4.8×10^5[g]	—

[a] Polymerization time: 15 min.
[b] Polymerization time: 30 min.
[c] Determined by turbidity measurement in aqueous solution.
[d] Determined by TNBS method.
[e] Determined from the extinction coefficient of N,N-diethyldithiocarbamate, $e_{281.5} = 13\,300$ mol^{-1} cm^{-1}.
[f] Calculated from the contents of amino group and dithiocarbamyl group.
[g] Number-average molecular weight determined by GPC in DMF at 40°C.
[h] Number-average molecular weight of graft chain, assuming that all the derivatized dithiocarbamyl groups initiated graft polymerization.

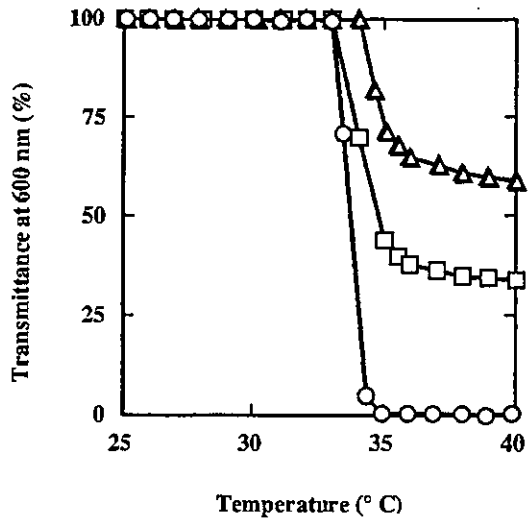


Figure 1. Temperature dependence of optical transmittance at 600 nm for (○) PNIPAAm, (△) PNIPAAm-grafted gelatin (I), and (□) PNIPAAm-grafted gelatin (II) aqueous solution.

gelatin, calculated according to equation (1) using the molecular weight of non-modified gelatin (9.5×10^4 g/mol), was 1.0×10^5 g/mol. Molecular weights of PNIPAAm-gelatin (I) and (II) were calculated as 1.4×10^5 g/mol and 3.8×10^5 g/mol, respectively, according to equation (2). Assuming that all the dithiocarbamyl groups derivatized on gelatin initiated photograft polymerization, the number of graft chains was estimated as 25.9 per gelatin molecule, and the number-average molecular weight of graft chain was estimated as 1.5×10^3 g/mol for PNIPAAm-gelatin (I) and 1.1×10^4 g/mol for PNIPAAm-gelatin (II). In other words, PNIPAAm contents in PNIPAAm-grafted gelatin (I) and (II) were calculated as 73.0 wt% and 29.3 wt%, respectively.

Optical transmittance changes of PNIPAAm and PNIPAAm-gelatin aqueous solutions are shown in Fig. 1. The optical transmittance of PNIPAAm solution sharply decreased to zero upon increasing solution temperature to physiological temperature, whereas for the two PNIPAAm-grafted gelatin solutions, optical transmittance gradually decreased with an increase in temperature and remained constant at physiological temperature; the transmittance was approximately 60% for PNIPAAm-gelatin (I) and 35% for PNIPAAm-gelatin (II). LCSTs determined at the optical transmission of 90% were as follows: PNIPAAm, 33.1; PNIPAAm-grafted gelatin (II), 33.4; and PNIPAAm-grafted gelatin (I), 34.3°C. These indicate that upon grafting PNIPAAm on gelatin, the LCSTs of PNIPAAm-grafted gelatins were shifted slightly to a higher temperature than that of PNIPAAm. The increase in the molecular weight of graft chain (PNIPAAm-gelatin (I)) appears to provide an LCST closer to that of PNIPAAm.

Cell culture

ECs were seeded at a high cell density (1×10^5 cells/cm^2) and cultured on dishes coated with PNIPAAm gelatin or PNIPAAm-gelatin (II) at 37°C for one