In Vitro Degradation and Cytotoxicity of Alkyl 2-Cyanoacrylate Polymers for Application to Tissue Adhesives

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ABSTRACT: To investigate the *in vitro* degradability and cytotoxicity of long alkyl cyanoacrylate polymers [polycyanoacrylates (PCAs)], we synthesized five kinds of alkyl cyanoacrylates (ethyl, 2-octyl, *n*-octyl, ethylhexyl, and ethyl cyanoacryloyllactate). *In vitro* degradation in buffer solutions and cell cultures for cytotoxicity were performed with PCAs prepared by various polymerization methods. Lower alkyl homologues such as ethyl cyanoacrylate revealed a higher tissue toxicity than higher alkyl homologues. The amounts of formaldehyde released from various PCAs were not proportional to the rate of degradation. The apparent form of the cyanoacrylate polymers greatly affected the degradation rate, as the powdery polymers degraded much more quickly than the films. A new biodegradable polymer, prepared from ethyl 2-cyanoacryloyllactate, degraded more quickly than the others. The amount of formaldehyde released from the polymer degradation was high because it degraded rapidly. It was observed from cell culture experiments that the viability of the cells was higher with a lower release of formaldehyde because the alkyl side groups were bigger. Therefore, octyl cyanoacrylate polymers demonstrated lower amounts of formaldehyde by degradation and higher cell viability, and these monomers may be desirable for use as tissue adhesives. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 89: 3272–3278, 2003

Key words: adhesives; degradation; biomaterials

INTRODUCTION

Alkyl 2-cyanoacrylates (ACAs) were first synthesized in 1949 by Alan Ardis[1] and were developed in the early 1950s; they became commercially available in 1958. They are called instantaneous adhesives because the adhesion between two adherends is formed instantly. Cyanoacrylate (CA) monomers are reactive enough to initiate anionic polymerization in the presence of even a weak base because of the electron-withdrawing CN and COOR groups.[2]

The capability of rapidly polymerizing monomeric ACAs to adhere firmly to moist surfaces has evoked considerable medical interest for their potential as hemostatic agents and tissue adhesives for the closure of wounds in place of or as adjuncts to conventional surgical sutures.[3] ACAs have been exploited extensively as a tissue adhesive for joining human tissues and in healing wounds.[4] Of these CAs with different alkyl side groups, a new skin adhesive called Dermabond, which is a 2-octyl cyanoacrylate (2-OCA), has been developed and approved for human use in the United States and Europe.[5] It protects the wound during the normal healing process and then sloughs off gradually and painlessly. It is hardly bioabsorbable.[6] If the adhesive stays intact, it is unlikely to be toxic. However, some studies have shown that polycyanoacrylates (PCAs) are biodegradable and that the formaldehyde produced by degradation irritates the adjacent tissues.[7] In such a case, if they are not used topically, CAs could induce inflammation, tissue necrosis, and infection risks.[8]

Two primary mechanisms of degradation of PCAs have been proposed up to now: an inverse Knoevenagel reaction producing formaldehyde and cyanoacetate[9] and the hydrolysis of the ester group yielding poly(cyanoacrylic acid) and alcohol.[10] The products degraded from the former are potentially harmful to the human body. The poisonous effects of PCAs attracted a great deal of interest because of their potentiality as medical and surgical adhesives.[11] In the case of the lower alkyl homologues, such as methyl and ethyl, their applications have been restricted because

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of tissue toxicity. However, the higher alkyl homologues exhibit lower tissue toxicity because the rate of degradation is slower. It has been speculated that the main cause of less degradation is steric hindrance due to the size of the alkyl side chains.[12] As long as the rate of degradation is slow enough, the products of degradation may not affect the body, and so biodegradability is an important factor in tissue adhesives. A number of studies have sought a correlation between degradation and toxicity. Nevertheless, there is room for controversy on the cause of the toxicity of PCAs.

The purpose of this study was to examine the toxicity and degradation of some alkyl PCAs based on ethyl, 2-octyl, *n*-octyl, ethylhexyl, and ethyl cyanoacryloyllactate. The various PCAs were prepared from each monomer, and their degradation behaviors, the amount of released formaldehyde, and the cytotoxicity were evaluated. The characteristics of long alkyl PCAs were compared with those of ethyl cyanoacrylate (ECA), which was used as a control.

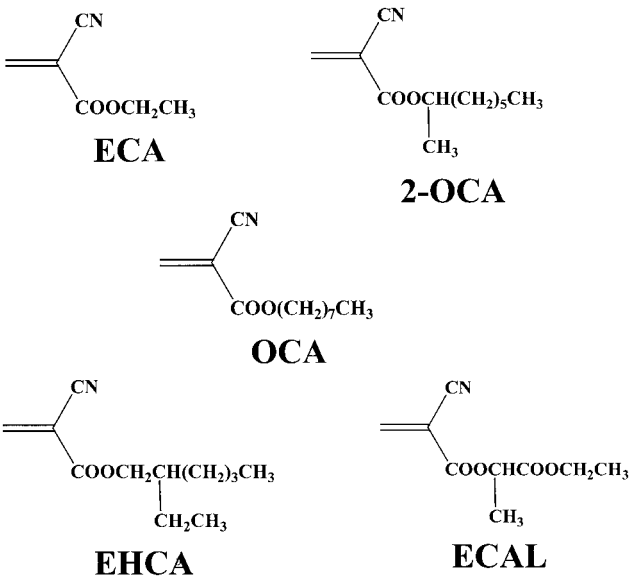

Figure 1 Chemical structure of the CA monomers.

EXPERIMENTAL

Materials

ECA, 2-OCA, *n*-octyl cyanoacrylate (OCA), and ethylhexyl cyanoacrylate (EHCA) were synthesized in our laboratory with methods similar to those previously described.[13] In addition, a novel biodegradable cyanoacrylate, ethyl 2-cyanoacryloyllactate (ECAL), was synthesized by the Knoevenagel reaction of formaldehyde and ethyl 2-cyanoacetyl lactate (ECAcL).[14] In brief, ECAcL was first synthesized by dicyclohexylcarbodiimide esterification of ethyl lactate and cyanoacetic acid in tetrahydrofuran (THF). Subsequently, to a refluxing solution of paraformaldehyde and a small drop of piperidine in benzene, the synthesized ECAcL was added dropwise and refluxed for 25 h. The polymeric ECAL was cracked at 150–200 °C and 0.4 mmHg, and the monomer was fractionally redistilled to obtain pure ECAL. The chemical structures of the CA monomers used in this study are shown in Figure 1. All of the chemicals were reagent-grade and were used without further purification.

Preparation of the ACA polymers

The polymerization of ACAs was simply performed by a normal anionic polymerization method to obtain poly(ethyl cyanoacrylate) [P(ECA)], poly(2-octyl cyanoacrylate) [P(2-OCA)], poly(*n*-octyl cyanoacrylate) [P(OCA)], poly(ethylhexyl cyanoacrylate) [P(EHCA)], and poly(ethyl 2-cyanoacryloyllactate) [P(ECAL)]. The polymerization was carried out as follows. All ACA monomers were polymerized at room temperature for more than 24 h without any initiator. After the polymerization, the solidified products were dissolved in acetone and precipitated into an excess of methanol for the removal of the monomers and other low molecular weight compounds. They were filtered off and dried *in vacuo* to obtain the ACA polymers. In addition, two kinds of other polymerization methods were used in this study. Methanol was used as a polymerization medium to give polymers with low molecular weight polymers and narrow distributions. Polymerization was also performed in aqueous solutions, including sodium bicarbonate, to increase the molecular weights of the polymers. In general, P(ECA) is hard and brittle,[15] and so it is difficult to make films if the molecular weight of the polymer is low. In a preliminary experiment, it was found that normal anionic polymerization method was preferable for obtaining high molecular weight polymers. The polymers (1 g) were dissolved again in THF (10 mL) and poured into a petri dish attached to two-sided Teflon-coated tape to prepare thin polymerized films with the method of solvent casting. It is not easy to detach an adhesive film intact from a substrate such as glass or aluminum. The use of the tape enabled us to remove the polymerized films as they were. The PCA films were dried in a vacuum oven at 40 °C for the complete removal of the solvent. Furthermore, the polymerized films were transformed into a powder form so that we could investigate the influence of the apparent form of the tested polymers. The polymer films were dissolved in acetone and reprecipitated with an excess of methanol. In the case of P(ECAL), it was precipitated with distilled water. The molecular weights of these polymers were determined in THF solutions by gel permeation chromatography calibrated with polystyrene (PS).

TABLE I
Molecular Weight Changes of CA Polymer Films After Degradation for 3 Days in a pH 7.4 PBS Solution

	M_n		M_w		PDI	
Polymer	Before	After	Before	After	Before	After
P(ECA)	93,400	90,900	339,700	269,800	3.6	3.0
P(2-OCA)	119,800	100,200	437,400	324,800	3.7	3.2
P(OCA)	244,300	164,900	855,600	449,700	3.5	2.7
P(EHCA)	175,300	128,400	587,300	368,300	3.4	2.9
P(ECAL)	55,300	40,300	90,500	59,300	1.6	1.5

Degradation and quantitative analysis of formaldehyde

A piece of polymerized film was used for *in vitro* degradation tests. The cast film (0.5 g), 200–300 μm thick, was placed in a phosphate buffered saline (PBS; pH 7.4, 25 mL) solution. The experiments were carried out at 37 °C and at an accelerated temperature (85 °C) with a 100 rpm shaking rate. One of the samples was immersed at 37 °C for 1, 3, 5, 7, 10, 14, and 30 days. The other samples were immersed at 85 °C for at 1, 2, 3, 5, and 7 days. The partially degraded film was separated from the PBS solution with the passage of a fixed time, filtered, dried *in vacuo* at 40 °C for 1 day, and weighed for the determination of the weight loss with time. After each weighing, the remaining polymer was returned to a fresh PBS solution. The incubated PBS solution containing degradation products was centrifuged, and the obtained clear solution was then used to determine quantitatively the formaldehyde released because formaldehyde is the most likely toxic substance in the degradation products of PCAs.[16] The amount of formaldehyde released during the degradation of the polymer films was determined by means of an ultraviolet–visible spectrophotometer with Nash's reagent. The reagent was prepared by the dissolution of 150 g of ammonium acetate, 3 mL of acetic acid, and 2 mL of acetylacetone in 200–300 mL of water. This method is similar to method 964.21 in *Official Methods of Analysis of the Association of Official Analytical Chemists*.[17]

Cell culture

The fibroblast cells for the cell cultures were aseptically isolated from a rabbit trachea. The rabbit fibroblast cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL streptomycin and penicillin, and a 1% antibiotic/antimycoic solution. The cells were incubated in an atmosphere of 5% CO_2 and 95% air at 37 °C in a humidified incubator. When needed, the cells were lifted from tissue culture dishes with a trypsin/ethylenediaminetetraacetic acid (EDTA) blend (0.05% trypsin and 0.53 m*M* EDTA) and resuspended in fresh media. All materials containing Dulbecco's PBS were purchased from Gibco BRL (Gaithersburg, MD). Neutral red (0.33%, 20 mL) and sodium bicarbonate were acquired from Sigma Co. (St. Louis, MO). For the cytotoxicity test, each CA monomer (50 μL) was dropped in DMEM (5 mL) in a sterile Falcon polypropylene tissue culture tube and immersed at 37 °C for 24 h. The extracts were centrifuged, filtered, and stored at a refrigerator until the testing. For the cell cultures, the cells (1×10^5) were seeded into 24-well PS microplates and incubated for 24 h at 37 °C in a 5% CO_2/95% air atmosphere. The cells grown on PS wells were used as controls. After incubation for 1 day, 1 mL of the extract and 1 mL of the medium were added again to the dish and reincubated under the same conditions for 24 h. Neutral red, initially depicted by Finter,[18] was used to evaluate the viability of the cells. The staining methods were the same as those described previously by Ciapetti et al.[19]

RESULTS AND DISCUSSION

Degradation of the CA polymers

All the monomers and polymers were prepared, and their chemical structures, physical properties, and adhesion characteristics were evaluated.[14] In brief, the chemical structures of the synthesized ACA monomers were confirmed by ^{1}H-NMR; the signals of the NMR spectra for the CA polymers were broader and not split into multiplets, as seen in the spectra of the monomers. The glass-transition temperature of P(ECAL) was the lowest of the tested polymers, whereas P(OCA) and P(EHCA) showed a higher half decomposition temperature ($T_{d1/2}$) than P(ECA). In addition, OCA had the highest bonding strength of the four long ACA adhesives (2-OCA, OCA, EHCA, and ECAL).

The *in vitro* degradation behavior in PBS solutions was examined with the CA polymers. An experiment was performed at 85 °C so that we could observe fast degradation behavior under accelerated conditions, in addition to the test at 37 °C. The molar masses of the various PCAs were likely to change during degradation. First, the molecular weights of the PCAs were measured before degradation because the degradation rate of the CA polymers depended not only on the

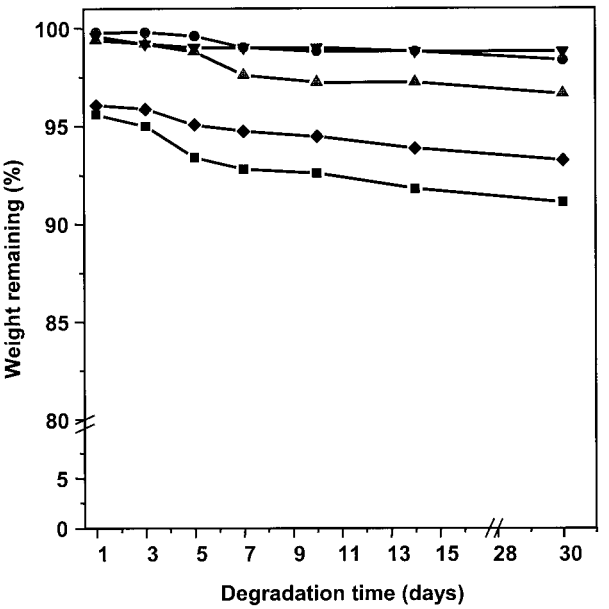

Figure 2 *In vitro* degradation behavior of PCAs at 37 °C: (■) P(ECA), (●) P(2-OCA), (▲) P(OCA), (▼) P(EHCA), and (♦) P(ECAL).

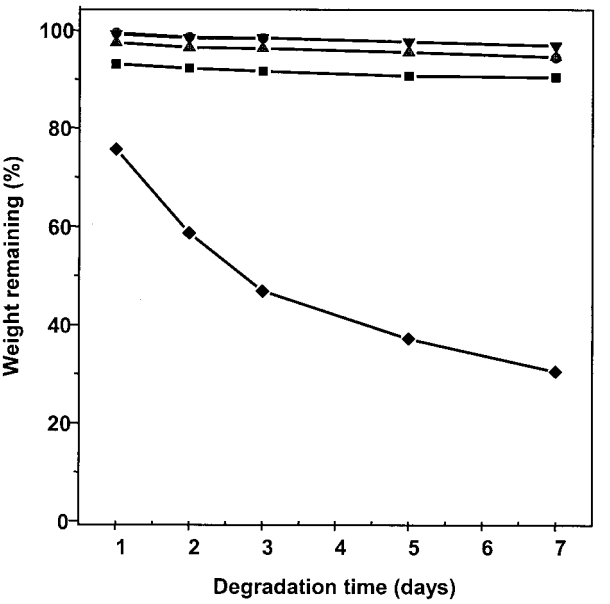

Figure 3 *In vitro* degradation behavior of PCAs at 85 °C: (■) P(ECA), (●) P(2-OCA), (▲) P(OCA), (▼) P(EHCA), and (♦) P(ECAL).

kind of side chain of CA but also on the molecular weight of the polymers used.[20] Table I indicates the changes in the molecular weights of the solution-cast PCA films after 3 days of degradation. The polydispersity index (PDI) of the CA polymer films was fairly broad (>3) on average. The number-average molecular weight (M_n) and weight-average molecular weight (M_w) of the PCAs decreased as the PCA films gradually degraded.

Figures 2 and 3 show the results of the *in vitro* degradation of CA polymer films in PBS solutions at 37 and 85 °C, respectively. As reported previously, the degradation rate increased with the decreasing size of the alkyl side group. The degradation rate for the polymers of the higher alkyl esters, such as P(2-OCA), P(OCA), and P(EHCA), was considerably slower with the degradation time. As for P(ECAL), it decomposed violently at 85 °C and transformed into a sticky material after 7 days. It is thought that an additional attack site for hydrolysis by the incorporation of another ester group into the existing molecular structure may increase the biodegradability. Such a trend was clearly demonstrated in the degradation tests at 85 °C.

Formaldehyde release

As the CA polymers were hydrolyzed, their molecular weights decreased, and they released formaldehyde by degradation. Figures 4 and 5 illustrate the amount of formaldehyde released during the degradation of the CA polymer films at 37 and 85 °C, respectively. As expected, the degradation of the CA polymer films at 37 °C generated much less formaldehyde than that at 85 °C. The release of formaldehyde increased with the decreasing size of the alkyl side groups and also increased because of the additional hydrolyzable ester side groups in P(ECAL) at 85 °C. However, as shown in Figure 4, the amounts of formaldehyde released from the P(ECA) and P(ECAL) films were relatively very small and less than those of the other PCAs. Meanwhile, the released amounts of formaldehyde in the octyl homologues were higher and increased with

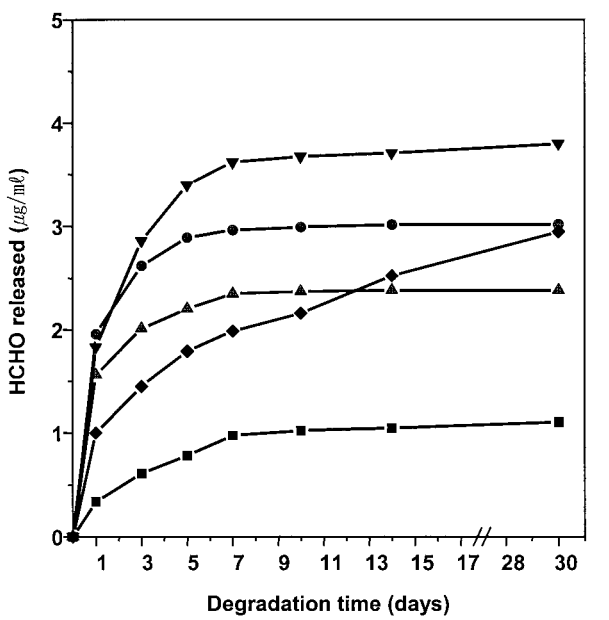

Figure 4 Release profile of formaldehyde from CA polymer films at 37 °C: (■) P(ECA), (●) P(2-OCA), (▲) P(OCA), (▼) P(EHCA), and (♦) P(ECAL).

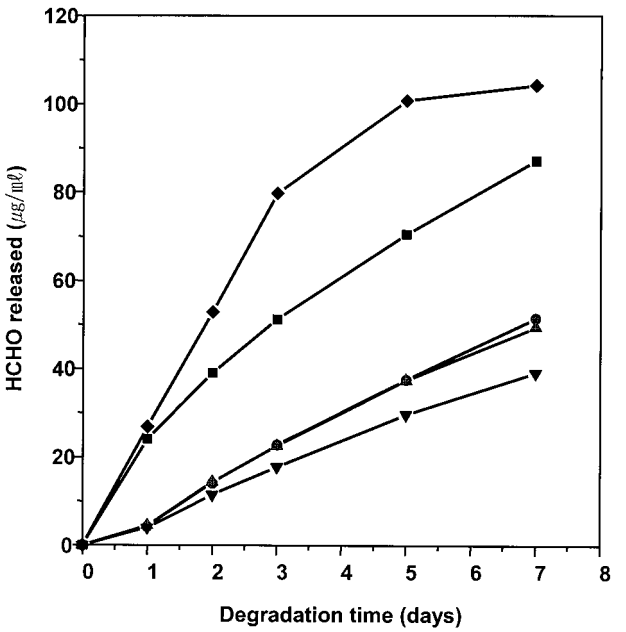

Figure 5 Release profile of formaldehyde from CA polymer films at 85 °C: (■) P(ECA), (●) P(2-OCA), (▲) P(OCA), (▼) P(EHCA), and (♦) P(ECAL).

time; they leveled off after 7 days. In the case of P(ECAL), formaldehyde was released continuously, although the value indicated an immaterial increase. This behavior may be explained as follows: P(ECAL) was expected to degrade very rapidly because of the existence of another ester group, and alcohol produced by ester cleavage accelerated the degradation as well. Experiments on degradation with the films were repeatedly carried out, and the same unexpected results were revealed, as shown in Figure 4. Millet[7] reported that higher alkyl PCAs exhibited less tissue toxicity because they degraded and released formaldehyde slowly with longer alkyl side chains. There is doubt concerning the origin of this degradation behavior. In degradation experiments for PCA film degradation, it is known that there are a number of factors affecting degradation, such as the molecular weight, molecular weight distribution, form, pH of the medium, temperature, wettability, shaking speed, and film thickness.[21] Some different polymerization methods for CAs were tried to confirm whether such a behavior was caused by a certain factor.

In addition to anionic polymerization for film preparation, a bulk anionic polymerization method was first adopted for uniform polymerization by the addition of each monomer in an excess of HPLC-grade methanol, except for ECAL. ECAL was polymerized anionically in deionized water. The molecular weights and molecular weight distributions of the polymers produced by solution polymerization are presented in Table II. When the CAs were polymerized in methanol, the molecular weights of all the polymers were relatively lower, and the PDIs displayed narrow values under 2. This was probably due to the uniform anionic polymerization of the CA monomers. The number-average molecular weight of P(OCA) showed a considerably lower value of 4300, whereas that of other PCAs ranged from only 3400 to 3900. This was because the linear alkyl side group of OCA did not influence the polymerization greatly in comparison with the bulky group of EHCA and ECAL with steric hindrance. Figure 6 shows the release profile of formaldehyde from CA polymer powders prepared in methanol. The lower alkyl CA polymers released more formaldehyde, markedly so for P(ECA). As expected, the PCA samples with lower molecular weights and PDIs revealed higher degradability along with the release of formaldehyde.

TABLE II
Molecular Weights of CA Polymers Prepared in Methanol

Polymer	M_n	M_w	PDI
P(ECA)	3400	4000	1.2
P(2-OCA)	3900	5600	1.4
P(OCA)	4300	7800	1.8
P(EHCA)	3900	5200	1.3
P(ECAL)[a]	3600	6500	1.8

Instantaneous polymer precipitation by the dropping of CAs into stirred methanol at room temperature.

[a] P(ECAL) was obtained in deionized water instead of methanol.

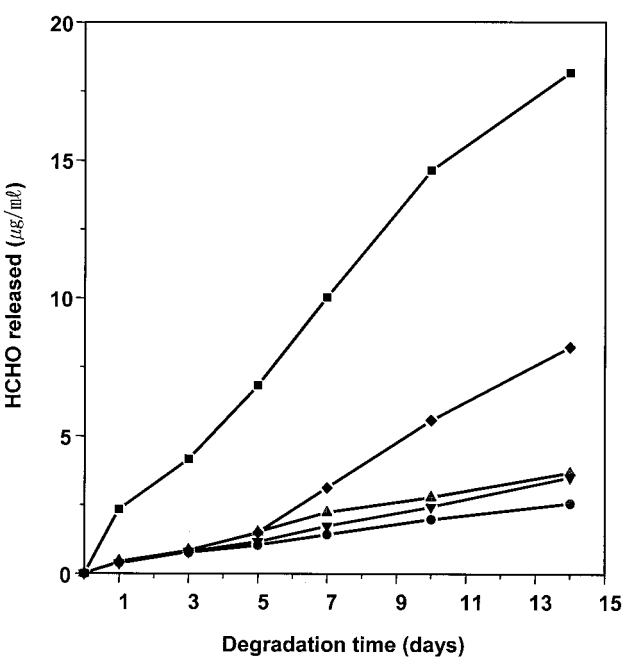

Figure 6 Release profile of formaldehyde at 37 °C from CA polymer powders prepared in methanol: (■) P(ECA), (●) P(2-OCA), (▲) P(OCA), (▼) P(EHCA), and (♦) P(ECAL).

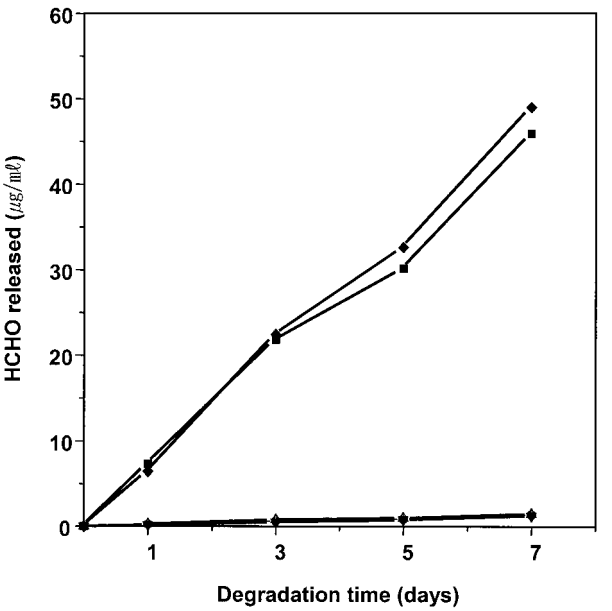

Figure 7 Release profile of formaldehyde at 37 °C from CA polymer powders prepared with sodium bicarbonate: (■) P(ECA), (●) P(2-OCA), (▲) P(OCA), (▼) P(EHCA), and (♦) P(ECAL).

The CA monomers were polymerized in aqueous solutions with sodium bicarbonate as a catalyst to evaluate the degradation behavior of the higher molecular weight polymers. The molecular weights of the polymer powders prepared with sodium bicarbonate were greater than 100,000, in contrast to the lower molecular weights and narrower PDIs of the polymers prepared in methanol. Their degradation behavior is shown in Figure 7. A comparison with Figure 6 shows there was somewhat of a difference only in P(ECAL), but the degradation behavior of the high molecular weight polymer was similar to that of the low molecular weight polymers.

Figure 8 illustrates the degradation behavior of the PCA powders. Compared with the polymer films, P(ECA) and, in particular, P(ECAL) powders released much formaldehyde, whereas other PCAs hardly degraded for 7 days.

Vezin and Florence[20] reported that degradation appeared to occur throughout the volume of the polymer particles and that the release rate of formaldehyde relied not on the surface area but on the mass of the polymer. On the basis of our degradation results from four different methods, it may be suggested that the apparent form of the CA polymers greatly affected the release of formaldehyde from the PCAs among the many factors mentioned before. In addition, it may be pointless to compare the amounts of formaldehyde because the amount of formaldehyde released in the film form was very small compared with that released in the powder form. Long-term degradation studies with various PCAs are currently ongoing to clarify this proposition.

Jaffe et al.[22] demonstrated that alkyl 2-cyanoacryloyl glycolates not only had enough bonding strength to join or hold tissue but also were both biodegradable and nontoxic. Accordingly, we expected our homologous ECAL to also be biodegradable and nontoxic. This is a requirement for the ideal tissue adhesives. However, our results did not thoroughly agree with their results. The polymer of ECAL was degraded more rapidly than the other PCAs, but the amount of released formaldehyde was still more in proportion to it. In a film experiment at 37 °C, P(ECAL) released a small quantity of formaldehyde in the beginning in comparison with the others, but the amount of released formaldehyde became higher and higher as time went by. Therefore, it is highly likely that PCAs degrade according to the mechanism of degradation proposed by Leonard et al.[9]

Cytotoxicity

We tried to culture cells on PCA films for toxicity testing, but it was difficult to estimate the cytotoxic effect because of poor cell adhesion to the film surface. Therefore, the cytotoxicity test of Ciapetti et al.[23] was used. Testing the cytotoxic effect did not matter even though it was an indirect method for cytotoxicity. The effect of the polymeric materials on cell viability and attachment was assessed through microscopic observation and dye exclusion.

Fibroblast growth in extracts from all the PCAs for 24 h is shown in Figure 9. The healthy cells were manifestly revealed by their ability to absorb neutral

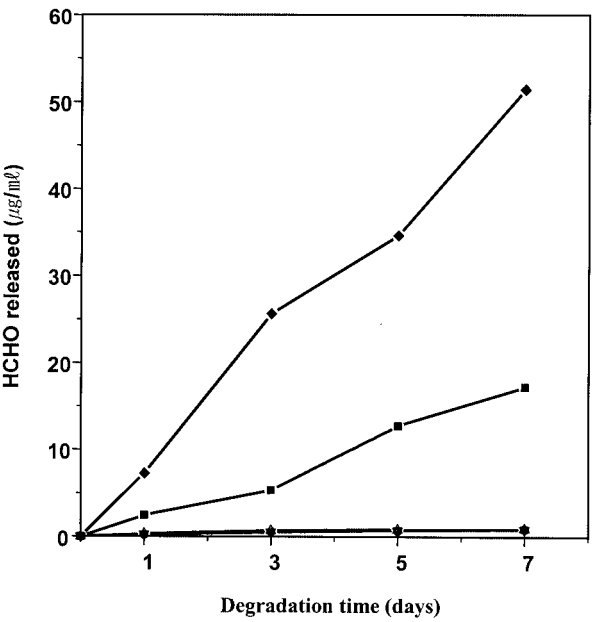

Figure 8 Release profile of formaldehyde at 37 °C from CA polymer powders transformed from films: (■) P(ECA), (●) P(2-OCA), (▲) P(OCA), (▼) P(EHCA), and (♦) P(ECAL).

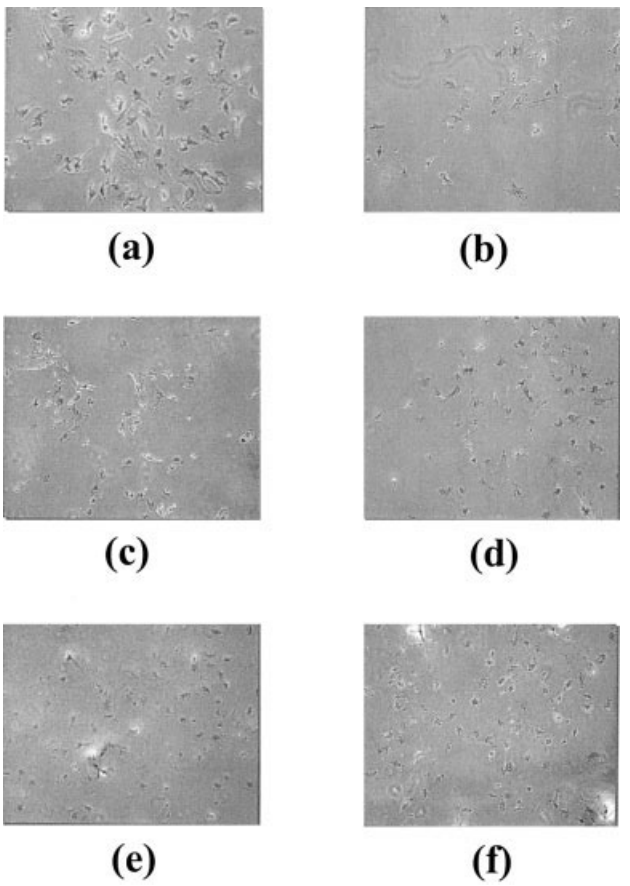

Figure 9 Microphotographs of fibroblasts stained with neutral red: (a) PS control, (b) P(ECA), (c) P(2-OCA), (d) P(OCA), (e) P(EHCA), and (f) P(ECAL).

red dye. In the case of the control (PS for the tissue cultures), the cells were well attached onto the surface of the microplate and grew normally. Meanwhile, the cells were partially killed, or the cell growth was inhibited, during the exposure of suspensions of cells and extracts of PCAs. P(ECA) clearly exhibited the poorest growth of fibroblasts in a comparison of the control and PCAs. This result was in agreement with results reported by other investigators.[24] In addition, for P(ECA) and P(ECAL), the cells were rapidly killed, probably because of an increased amount of formaldehyde after cell contact for 5 days. Therefore, it may be concluded that the cellular toxicity of the CA polymers was linearly related to the release rate of formaldehyde.

CONCLUSIONS

Five kinds of CA monomers (ECA, 2-OCA, OCA, EHCA, and ECAL) were synthesized by the Knoevenagel reaction to evaluate the *in vitro* cytotoxicity of the CA polymers. On the basis of the results of degradation and cytotoxicity experiments, it was verified that the apparent form of the CA polymers influenced greatly their degradation rate, among many factors. The CA polymers were biodegradable and toxic because of the formaldehyde released during degradation, although there was a difference according to the sizes of the introduced alkyl side groups. The higher ACAs, such as 2-OCA, OCA, and EHCA, degraded at slower rates than the lower ones, such as ECA, and this indicated the improved biocompatibility. A newly synthesized ECAL was thought to be relatively nontoxic because of the existence of an additional biodegradable ester group, but it showed a release behavior of formaldehyde similar to that of ECA with fast degradation. Therefore, it is expected that octyl cyanoacrylates such as 2-OCA, OCA, and EHCA may be useful for application to tissue adhesives.

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