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Synthesis, Characterization and Biological Activity of Poly(diallyl ether-co-maleic anhydride)

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요 약 : Diallyl ether(DAE)와 maleic anhydride(MAH)를 용매로서 벤젠을 사용하여 60℃에서 라디칼 공중합을 하였다. 중합체들의 특성은 IR, NMR, GPC 및 TGA등으로 결정하였으며, 중합체내의 단량체조성은 원소분석으로 구하였다. 공중합체는 교호구조를 가지며, 공중합시 두 단량체간에는 UV스펙트럼에 의해 charge-transfer complex가 형성됨을 확인하였고, 이때의 평형상수값은 0.74 liter/mole이었다. 공중합체의 생물학적인 활성은 K562 세포에 대한 공중합체 자체의 세포독성과 공중합체로 처리된 림프구의 자연세포 살해능으로써 조사하였다. 그 결과 공중합체 자체는 1.0 mg/ml 농도 이하에서 세포독성이 없었으나 림프구의 자연세포 살해능을 활성화시키는 효과를 보였으며, 자연세포 살해능은 공중합체 농도가 증가할수록 증가하였다.

Abstract : Copolymerization of diallyl ether(DAE) and maleic anhydride(MAH) were carried out in benzene at 60℃ with 2,2'-Azobisisobutyronitrile(AIBN). The copolymers were characterized by IR and NMR spectroscopy, GPC and TGA. The copolymer composition was determined by elemental analysis. The copolymer had alternating structure. The formation of charge-transfer(CT) complex was identified during the copolymerization of DAE and MAH, where the equilibrium constant of the CT complexation was estimated as 0.74 liter/mole. For biological activity, the cytotoxicity of the copolymer obtained and natural killer(NK) activity of the polymer-treated lymphocytes on cells were evaluated with K562 leukemia cell line. It was found that the copolymer showed no cytotoxicity below the concentration of 1.0 mg/ml but augmented NK activity of lymphocytes. It was also observed that the cytotoxicity was increased with increasing amount of the copolymer.

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INTRODUCTION

The one to two regularly alternating copolymer (DIVEMA) of divinyl ether(DVE) and maleic anhydride(MAH) has been extensively investigated because of its biological activity.[1~3] A series of synthesized DIVEMA polyanions, of molecular weights ranging from 12,500 to 52,600, were reported to enhance macrophage tumoricidal activity and natural killer(NK) activity.[4] Treatment with the DIVEMA copolymer, of an average molecular weight of 18,000, was reported to either augment or suppress the activity of NK cells depending on timing and route of inoculation.[5]

Microstructure of the 'pyran' copolymer(DIVEMA) also attracted much interests by its charge-transfer(CT) complex strcuture. The cyclopolymerization of divinyl ether(DVE) and maleic anhydride(MAH) has been well established.[6,7] Butler and Joyce[8] proposed participation of a CT complex in the cyclocopolymerization for the 'pyran' copolymer.

Diallyl ether(DAE) has been reported to give random copolymer with acrylonitrile or 2-methyl-5-vinylpyridine.[9] It was also reported that many 1, 6-dienes cyclopolymerize to form recurring six- or five-membered rings.[10,11]

In this work, we synthesized copolymers (DAEMA) of diallyl ether(DAE) and maleic anhydride(MAH) with AIBN. We investigated the formation of CT complex during the copolymerization of MAH and DAE, its solvent effect and cyclocopolymerizability. The DAEMA was characterized by IR and NMR spectroscopy, GPC, elemental analysis and TGA. For biological activity, the cytotoxicity of poly(DAE-co-MAH) obtained and NK activity of the copolymer-treated lymphocytes on cells were evaluated with K562 leukemia cell line.

EXPERIMENTAL

Materials

Maleic anhydride (Junsei Chem.) was recrystallized from chloroform and then dried in vacuo immediately before use. Diallyl ether(Aldrich Chem.) was used after distillation. 2,2'-Azobisisobutyronitrile(AIBN) was recrystallized from ethanol. Benzene was purified by consecutive washing with concentrated sulfuric acid, dilute aqueous sodium hydroxide, and deionized water ; it was then dried over calcium chloride and metallic sodium before distillation. Chloroform was distilled by the similar method as described for benzene, except filtration through activated-alumina column. Other reagents were purified by conventional methods. Target tumor cells and effect cells used were K562 and peripheral blood lymphocytes(PBLs), respectively.

Instruments

IR spectra were taken on a Perkin-Elmer 1330 spectrophotometer using KBr pellet. UV spectra were taken on a Shimadzu 200A spectrophotometer. ^{1}H-NMR spectra were recorded on a varian A-60 spectrophotometer. Average molecular weight was determined by gel permeation chromatography(GPC ; Waters, Water-244). Elemental analysis was performed by Perkin-Elmer Model 240C Elemental Analyzer. Thermal stability was measured with a Du Pont 1090 Thermogravimetric Analyzer. The radioactivity was measured with Packard γ counter.

Copolymerization

Copolymers of DAE and MAH, poly(DAE-co-MAH), were prepared from the monomers of three different compositions in feed(1/1, 2/1 and 1/2). For the poly(DAE-co-MAH) with a feed mole ratio of 1/1, a mixture of 0.98 g(0.01 mole) of MAH, 0.98 g(0.01 mole) of DAE and 0.0164 g(1.0× 10^{-4} mole) of AIBN in 100 ml of benzene was introduced into a dry polymerization tube. The tube was sealed after degassed twice by purging with purified N_2 gas and placed in a regulated thermostat at 60℃ for 48hrs. The resulting polymer was purified by reprecipitation in diethyl ether several times and dried until a constant weight at 30℃. The other two copolymers with mole ratios of 1/2 or 2/1 were prepared by the similar method as described above, except their feed mole ratios.

Determination of Equilibrium Constant of Complexation

For the spectral studies of complexation, UV spectra were taken from a mixture of DAE as a donor and MAH as an acceptor in chloroform. The concentration of the mixture with various different compositions is fixed at 0.01 M. To determine the equilibrium constant of complexation, the absorbance of the monomer mixture dissolved in chloroform were obtained by subtrating the absorbance of MAH numerically. Here the concentration of DAE varied from 0.05 to 0.1 M but the concentration of MAH was set at 0.01 M.

Determination of Molecular Weight of Copolymer

Average molecular weight of poly(DAE-co-MAH) was measured by GPC using PS standards. THF was used as an effluent.

Analyses of Copolymer Compositions

The copolymer composition in poly(DAE-co-MAH) was determined by elemental alalysis.

Thermal Stability of Poly(DAE-co-MAH)

The thermal stability of the copolymers were measured under nitrogen by thermogravimetric analysis(TGA). Analysis was performed with a heating rate of 10℃/min over the temperature ranges of 25℃-600℃.

Cytotoxicity Tests

Preparation of Lymphocytes

Heparinized blood samples were collected from normal ones. Peripheral blood lymphocytes(PBLs) were isolated from blood by Ficoll-Hypaque density gradient centrifugation. PBLs were collected from the interface, washed twice in Hanks' balanced salt solution (Gibco, Grand Island, NY), and taken up in 15 ml RPMI-1640(Gibco) supplemented with 2 mM glutamine, 100 units penicillin and 100 micrograms streptomycin, and 10% fetal calf serum (Irvine, Santa Ana, Calif, FCS) (subsequently referred to as supplemented complete medium, SCM). Monocytes were removed by their characteristic adherence to plastic. The PBLs were incubated in 100×15 mm plastic petri dishes for 1

hr at 37℃. Nonadherent cells were recovered by repeated rinsing with SCM. Viability of the cells was greater than 95%, as determined by Trypan blue exclusion.

Culture Condition of Target Cell

K562 human erythroleukemia tumor cells were cultured as the target cell at 37℃ in a humidified air atmosphere with 5% CO_3, using SCM.

The Cytotoxicity of Poly(DAE-co-MAH)

A ^{51}Cr-release assay was used to test the cytotoxicity of poly(DAE-co-MAH). The composition of MAH in poly(DAE-co-MAH) for test was 51.96 mol%. The average molecular weight($\overline{M}_n$) of poly(DAE-co-MAH) for test was 130,000. ^{51}Cr-release assay was carried out in 96-well flat-bottom tissue culture plates. Target tumor cells used were K562 cell line. Target cells($2×10^6$) were labelled with sodium chromate (^{51}Cr : Amersham, Arlington Heights, Ⅲ) at a concentration of 100 μCi/10^6 cells at 37℃ for 1 hr. The cells were washed extensively and then suspended at $1×10^5$ cells/ml in RPMI 1640 medium containing 5% PCS. One hundred microliters of target cells and 100 μl of polymer solutions were added to each well of a 96-well microtiter plate.

The assays were run in sextuplet wells. For the maximum ^{51}Cr-release control, 0.1 ml of 0.1 M hydrochloric acid was added to the target cells, and for the spontaneous ^{51}Cr-release control, 0.1 ml of culture medium was added to the target cells. The plates were then centrifuged at 25×G for 3 min and incubated at 37℃ in a 5% CO_2 incubator for 4 hrs. At the end of the incubation period the plates were centrifuged at 400×G for 5 min. 0.1 ml of supernatants was removed and measured in a γ-counter.

The percentage cytotoxicity was determined using the following formula :

Cytotoxity(%)
$$= \frac{(\text{experimental release-spontaneous release})}{\text{maximum release-spontaneous release}} \times 100$$

The Cytotoxicity of Lymphocytes Treated with Poly(DAE-co-MAH)

4×10^6 PBLs (effector cells) pretreated with poly(DAE-co-MAH) were added to 1×10^5 target cells in effector : target(E : T) ratios of 40 : 1. These were incubated for 4 hr at 37°C in flat-bottomed microtiter 96 wells in a total volume of 0.2 ml. Supernatants were removed. The isotope release was determined by using Automated Gamma Counter.

RESULTS AND DISCUSSION

Characterization

Poly(DAE-co-MAH) was characterized by IR and ^{1}H-NMR spectroscopies. The IR spectrum of the poly(DAE-co-MAH) is shown in Fig. 1. The absorptions due to the anhydride group and the ether linkage are seen at $1850\sim1730$ cm^{-1} and 1225 cm^{-1}, respectively. The absorptions assignable to the allylic unsaturation of DAE monomer was not observed at 1636 cm^{-1}. Fig. 2 illustrates a typical ^{1}H-NMR spectrum of poly(DAE-co-MAH) with 1/1 mole ratio. The copolymer was characterized with peaks at $1.04(-CH_2-)$, $3.35(-OCH_2-)$, 1.78, 3.58, and 3.78 ppm$(-CH)$. Other copolymers, even when prepared from different monomer feed ratios, showed almost same NMR spectra as that of poly(DAE-co-MAH) with 1/1 mole ratio.

Careful inspections of the IR and NMR spectra of Figs. 1 and 2 lead to a significant results that cyclization was performed during the copolymerization of DAE and MAH because no characteristic peak due to double bond was observed in IR and NMR spectra of copolymers.
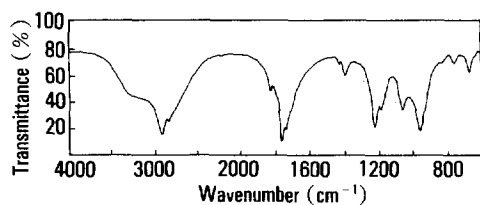
Molecular Weight Characteristics

The data of the molecular weight of copolymers were summarized in Table 1. The molecular weights of copolymers are in the order of 100,000, regardless of the monomer feed ratios.

Solubility of Polymers

Solubility data of poly(DAE-co-MAH) are listed in Table 2. Poly(DAE-co-MAH) was soluble in polar aprotic solvents and partially soluble in methanol but insoluble in toluene or cyclohexane.

Elemental Analysis

Elemental analysis data of poly(DAH-co-MAH) are listed in Table 3. The result is of great interest that the amount of MAH in poly(DAE-co-MAH) was obtained as almost near 50 mol % by elemental analysis, regardless of the monomer feed ratios. This means that the copolymer of DAE and MAH has alternating structure.

Thermal Stability

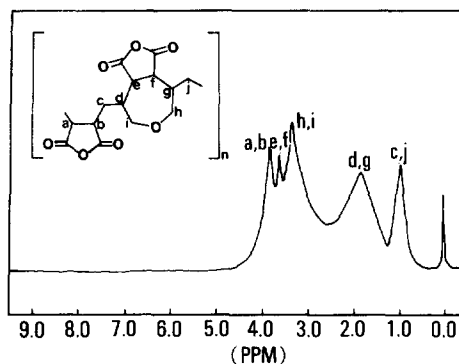All copolymers prepared from various monomer feed ratios had almost the same TGA curves,

Fig. 2. ^{1}H-NMR spectrum of poly(DAE-co-MAH) (DMSO-d$_6$).

Table 1. The Average Molecular Weight of Copolymers Synthesized in Benzene.

Polymer	$\overline{M}_n \times 10^5$	$\overline{M}_w \times 10^5$	$\overline{M}_w/\overline{M}_n$
Poly(DAE-co-MAH)	1.30	1.70	1.31[a]
	1.30	1.50	1.15[b]
	1.20	1.30	1.08[c]

[a][DAE]/[MAH] = 2/1, [b][DAE]/[MAH] = 1/1,
[c][DAE]/[MAH] = 1/2 at 60°C, 48 hrs.

Fig. 1. IR spectrum of poly(DAE-co-MAH) (Solid Phase, KBr).

Table 2. Solubility of Poly(DAE-co-MAH).

Polymer	water	THF	acetone	toluene	DMF	methanol	ethanol	cyclohexane
Poly(DAE-co-MAH)	S	S	S	I	S	PS	S	I

I : Insoluble, PS ; partially soluble, S : Soluble

Table 3. The Relation Between the Copolymer Composition and the Feed Composition ; [AIBN] = 0.001 M, at 60°C

Mole fraction of MAH in feed	Mole fraction of MAH in copolymer	composition (%)ᵃ C	H
0.67	0.52	56.75	4.80
0.50	0.50	56.29	4.69
0.33	0.50	57.04	4.70

ᵃdetermined by elemental analysis

which lends further support to the fact that the polymers are of the same structure, that is, of alternating character. Similar TGA behavior was also observed in poly(2-vinyl-1,4-hydroquinone-co-maleic anhydride).[12] Typical thermograms are displayed in Fig. 3.

The TGA curves indicate decomposition up to 4.7% at 140°C and 13.2% at 230°C. A drastic decomposition of the copolymer began around 230°C and continued to about 440°C, where the weight loss was 85%.

Charge-Transfer Complex

When a chloroform solution of maleic anhydride was added to a solution of diallyl ether, there appeared in the UV spectrum of the mixture a new band at 254 nm whose appearance and position can be attributed to the formation of an electron donor-acceptor complex.

The result implies that the copolymerization of DAE and MAH follows the mechanism of CT complex of each monomer, as suggested by the literatures.[13] The interpretation of these spectra permitted the determination of the stoichiometric composition by using the continuous variation method of Vosberg and Cooper.[14] The results are shown in Fig. 4.

The equilibrium constant(K) of complexation and the molar extinction coefficient($\in$) of a CT
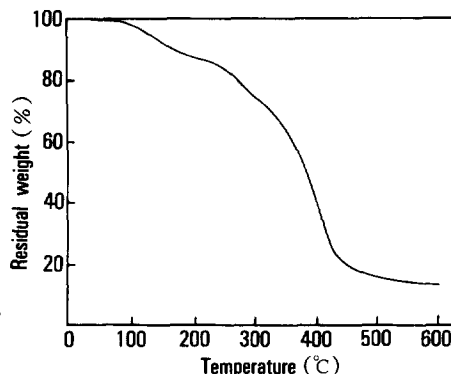
Fig. 3. TGA curves for poly(DAE-co-MAH) : Heating rate = 10°C/min under nitrogen.
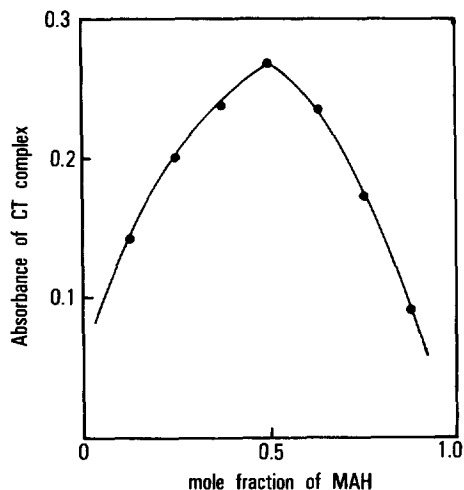
Fig. 4. Determination of stoichiometry of CT complex of DAE with MAH by UV spectrum in CHCl₃, at 20°C [DAE + MAH] = 1.0 × 10⁻² M at 254 nm.

complex can be estimated by using the Scott equation(Eq. 1),[15] which is a rearranged equation of Benesi and Hildebrand.[16]

$$\frac{[A][D]\ell}{d} = \frac{1}{\in K} + \frac{1}{\in}[D] \tag{1}$$

where, $[A]$ = electron acceptor concentration(mole /1), $[D]$ = electron donor concentration(mole/1), d = absorbancy, and ℓ = path length(cm).

In order to obtain equilibrium constant and extinction coefficients, were taken UV spectra of the monomer mixture of DAE as a donor compound and MAH as an acceptor in chloroform. The absorptions of the MAH-DAE complex and the Scott plot of MAH-DAE in chloroform are shown in Fig. 5 and Fig. 6, repectively. The equilibrium constant was estimated as 0.74 liter/mole. The other numerical results were listed in Table 4.

Fig. 7 shows the effect of the mole ratio of DAE and MAH in the monomer feed on the yield of copolymers. It is evident that the highest yield was obtained at equimolar ratio of comonomers. This again shows the alternating structure of copolymers.

Rate of Copolymerization and Solvent Effect

If the CT complex is formed during the cyclocopolymerization of DAE and MAH, the rate strongly depends on the solvent polarity, thus producing higher molecular weight polymer.[17] The point was tested by carrying out the copolymerization in several solvents of widely different dielectric constants. The results are shown in Figs. 8 and 9 and Table 5.

The polymerization was carried out in thermostatted oil bath for 24 hrs. The yield was estimated by weighing the purified copolymer. It was obser-

ved that the effect of the polarity of the polymerization medium is significant, manifesting the formation of the CT complex in this copolymerization. As shown in Table 5, the more polar solvent results in a lower yield, and also a lower rate of polymerization.
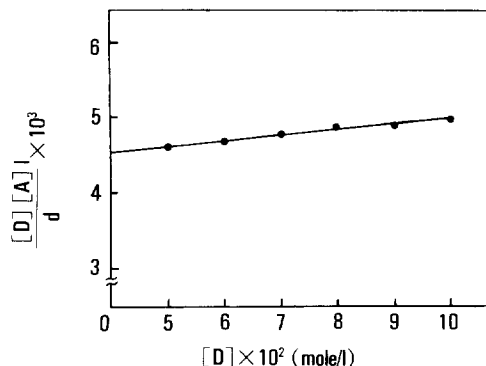

Fig. 6. Scott plot of DAE-MAH complex in CHCl₃ at 254 nm.

Table 4. Summary of the Data from UV Spectra of CT Complex of DAE and MAH in CHCl₃ at 20℃.

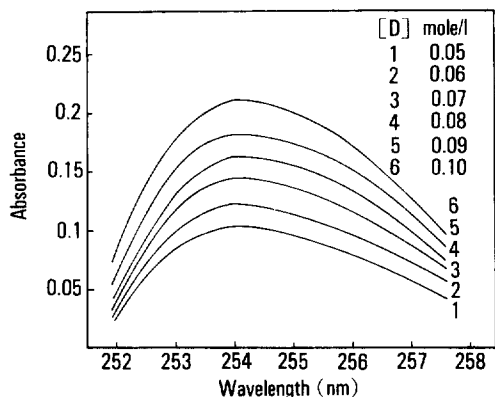| Donor	$[A] \times 10^2$ (mole/l)	$[D]$ range (mole/l)	λ_{max} (nm)	ε (l/mole cm)	k (l/mole)
DAE	1.0	0.05-0.1	254	2.94×10^2	0.74

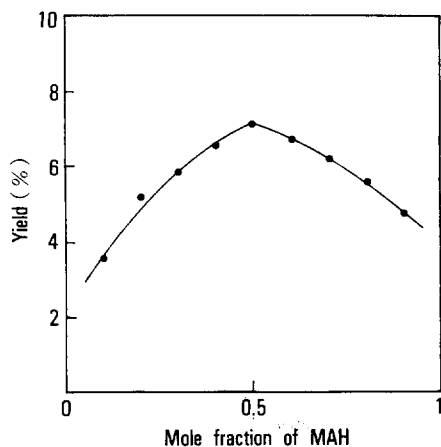**Fig. 5.** CT absorption of DAE-MAH in CHCl₃.

Fig. 7. The effect of the mole fraction of MAH in feed on the yield obtained after 24hrs at 60℃ with AIBN.
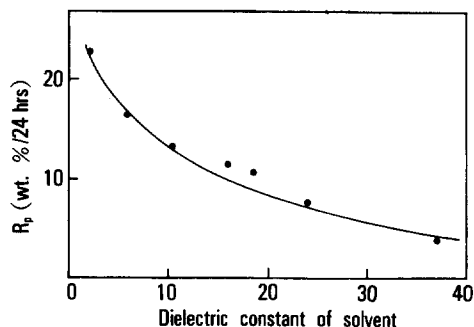
Fig. 8. Rate of copolymerization of DAE and MAH in various solvents. $[M_1]/[M_2]=1$, $[M_1+M_2]=0.2$ M, $[AIBN]=2\times10^{-3}$ M.
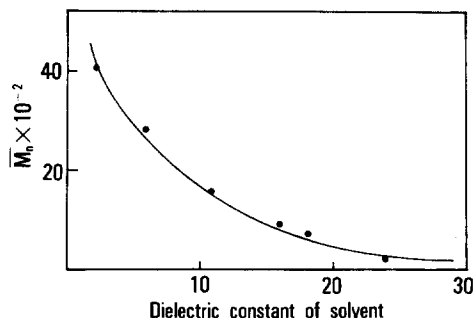

Fig. 9. Number-average molecular weight of poly (DAE-co-MAH) prepared in various solvents. $[M_1]/[M_2]=1$, $[M_1+M_2]=0.2$ M, $[AIBN]=2\times10^{-3}$ M.

Table 5. Effect of Solvents for the Conversion in Copolymerizations of DAE and MAH and the Average Molecular Weight Obtained from Poly(DAE-co-MAH).
$[M_1+M_2]=0.2M$, $[M_1+M_2]=1$, at 60℃, $[AIBN]=2\times10^{-3}M$

Solvent	Dielectric constant[18]	Yield (wt%/24hrs)	$\overline{M}_n\times10^2$
1,4-Dioxane	2.21	22.6	41.55
Ethyl acetate	6.02	16.5	29.16
1,2-Dichloroethane	10.37	13.3	15.36
Cyclohexanone	16.10	11.8	9.42
Methyl ethyl ketone	18.51	11.7	5.01
Ethanol	24.55	8.3	3.23
Dimethyl formamide	36.71	3.2	—

Table 6. The Cytotoxicity of Poly(DAE-co-MAH) on K562 Leukemia Cell Line.

Poly(DAE-co-MAH) mg/ml	% of dead cells
10	74.2
1.0	0
0.1	0
0.01	0
0.001	0

Table 7. The Cytotoxicity of Lymphocyte Treated with Poly(DAE-co-MAH) on K562 Leukemia Cell Line.

Polymer/Lymphocyte mg/ml	% of dead cells
Control	44.6
1.0	70.7
0.3	67.7
0.1	51.1
0.03	50.8
0.01	50.6

Cytotoxicity of Poly(DAE-co-MAH)

Table 6 shows the effect of treatment concentrations on the cytotoxicity of poly(DAE-co-MAH). We tested various concentrations up to 10 mg/ml. The copolymer showed no cytotoxicity until the concentration of treatment was at 10 mg/ml. The cytotoxicity of the copolymer was 74.2% when the concentration of treatment is at 10 mg/ml.

Table 7 shows the cytotoxicity of lymphocyte treated with poly(DAE-co-MAH). The concentration of the copolymer ranged from 1.0 to 0.01 mg /ml. It was found that the cytotoxicity of the lymphocyte treated with the copolymer is higher than that of the lymphocyte without the copolymer(i. e. *control* in the table). The result shows that the copolymer obtained in this work clearly augment NK activity. The augmenting effect in the cytotoxicity of the lymphocyte by the copolymer was increased with increasing the copolymer concentration.

CONCLUSIONS

Copolymerization of DAE with MAH was carried out with AIBN in benzene at 60℃. It was found that the copolymer has alternating structure from elemental analysis, TGA behavior and solvent effect on the yield of copolymer. Formation of charge transfer complex was identified from UV spectra and the equilibrium constant of complexation was estimated as 0.74 liter/mole.

For biological activities, the cytotoxic killing effect of polymers on cells was evaluated with human chronic myelogenous leukemia cell line(K 562). The cytotoxicity of poly(DAE-co-MAH) itself was not observed, except at very high concentration. It was observed that the copolymer augment NK activity. The augmenting effect in the cytotoxicity of the lymphocyte was increased with increasing concentration of the copolymer.

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