Korea Polymer Journal, Vol. 9, No. 5, pp 277 – 284 (2001)

Synthesis, Antitumor Activity and Release Rate of Polymers Containing Anionic Group and 5-Fluorouracil

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Received July 28, 2001

Abstract : Poly(*exo*-3,6-epoxy-1,2,3,6-tetrahydrophthalic anhydride)s [poly(ETA)s] and poly(α-ethoxy-*exo*-3,6-epoxy-1,2,3,6-tetrahydrophthaloyl-5-fluorouracil)s [poly(EETFU)s] with various average molecular weights were prepared by photopolymerizations. The number average molecular weights of the fractionated poly(ETA)s and poly(EETFU)s determined by GPC were in the range of 3,600~21,000 and 3,600~33,400, respectively. The release rate of 5-FU from poly(EETFU) decreased with increasing average molecular weight. The *in vitro* cytotoxicity of poly(ETA) against a normal cell line was lower than that of 5-fluorouracil(5-FU). The *in vivo* antitumor activities of the synthesized samples at dosage of 0.8 mg/kg against mice bearing sarcoma 180 tumor cell line decreased in the following order: poly(EETFU) > poly(ETA) > EETFU > ETA > 5-FU. The antiangiogenic activities of the poly(ETA)s were better than those of 5-FU.

Introduction

Although the 1 : 2 alternating copolymer of divinyl ether and maleic anhydride (DIVEMA) reported by Butler has activities against Friend leukemia, Moloney sarcoma, vesicular stomatitis and interferon inducing abilities,[1-3] its toxic side effects such as anemia, enlarged liver, and spleen *etc.*[4,5] have been challenges to polymer scientists and biologists. 5-Fluorouracil (5-FU) is a low molecular weight antitumor agent, which has also remarkable antitumor activities but at the same time strong side effects such as gastrointestinal toxicity and delivery problems.[6-9] Therefore, in order to reduce the toxic side effects of both DIVEMA and 5-FU, many attempts have been made to obtain the polymers like DIVEMA and 5-FU derivatives in our laboratory.[10-24] Especially, polymeric antitumor compounds having 5-FU have been widely studied for their syntheses and antitumor activities, since they are expected to maintain an effective concentration of 5-FU in blood and/or tissue for a long time by slow release. If the concentration is too high, various toxic side effects arise, while if the level is too low the drug is no longer effective in treating the disease. Akashi *et al.* and Ouchi *et al.* have reported the release rate of 5-FU from various polymeric matrices.[25-29]

This paper deals with the antitumor activities of [poly(ETA)] and poly(α-ethoxy-*exo*-3,6-epoxy-1,2,3,6-tetrahydrophthaloyl-5-fluorouracil) [poly(EETFU)] with various average molecular weights and the release rate of 5-FU from the synthesized poly(EETFU) at several pH using high performance liquid chromatography (HPLC), because the structure of the hydrolyzed poly(EETFU) is similar to that of poly(ETA). The number and weight average molecular weights of the polymers were determined by gel permeation chromatography (GPC). The *in vitro* cytotoxicities of the prepared polymers were evaluated with mouse mammary carcinoma (FM3A), mouse leukemia (P388), and human histiocytic lymphoma (U937) as cancer cell lines and mouse liver cell (AC2F) as a normal cell line. The *in vivo* antitumor activities of the prepared samples against mice bearing sarcoma 180 tumor cell line were evaluated. The antiangiogenic activities of poly(ETA)s were examined by the

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embryo chorioallantoic membrane (CAM) assay. The release rate of 5-FU from poly(EETFU)s were measured by HPLC.

Experimental

Materials. *Exo*-3,6-epoxy-1,2,3,6-tetrahydrophthalic anhydride (ETA) [Aldrich] was used without further purification. α-Ethoxy-*exo*-3,6-epoxy-1,2,3,6-tetrahydrophthaloyl-5-fluorouracil (EETFU) were synthesized by the reported method.[23] P388, FM3A, and U937 as cancer cell lines and AC2F as a normal cell line were used. Balb/C mouse and sarcoma 180 cell line were purchased from the Center of Genetic Engineering (Korea Institute of Science and Technology). Fertilized chick eggs were obtained from Han-shin Farm (Kimhae, Korea). Fat emulsion (10%) was purchased from Green Cross Pharm. Co. (Seoul, Korea). Thermanox coverslips were purchased from Nunc Inc. (Naperville, IL, U.S.A). All other chemicals were reagent grade and were used without further purification.

Instruments. ^{1}H-NMR spectra were recorded on a FT-300 MHz Varian Gemini 2000 spectrophotometer. IR spectra were obtained with a Jasco FT/IR-5300 spectrophotometer by using KBr pellet for analysis. Elemental analysis was performed by elemental analyzer (Carlo Erba: Model EA1180). The number and weight average molecular weights were determined GPC (Waters 410). The hydrolysis rates of 5-FU were measured by HPLC (Young Lin M 930).

Syntheses of Poly(ETA)s and Poly(EETFU)s. The solution of ETA (1.0 g, 6 mmol) and 2,2-dimethoxy-2-phenylacetophenone(DMP) (0.04 g, 0.156 mmol) as a photoinitiator in the 10 mL of a dry mixed solvent of 2-butanone and acetone (1 : 1, v/v) was introduced into a dry Pyrex polymerization tube. To prepare the polymers with four different average molecular weights, the amount of DMP was adjusted. The tube was sealed after flushing twice with purified N_2 gas bubbling. The tube was irradiated by 313 nm U.V lamps in a photochemical chamber at 25°C for 48 hr. The obtained polymer solution was precipitated in 200 mL of *n*-hexane. The precipitate was collected by filtration and dried at room temperature under reduced pressure to constant weight.

Poly(EETFU)s were synthesized by the similar method as described for poly(ETA)s except that EETFU(1.5 g, 4.6 mmol) and DMP (0.06 g, 0.234 mmol) were used. The conversions of ETA and EETFU are shown in Table I.

Measurement of Average Molecular Weights: The average molecular weights and polydispersity index (M_w/M_n) were determined by GPC using Waters GPC 410 with a refractive index detector and four μ-styragel columns with pore sizes of 10^5, 10^4, 10^3, and 500 connected in series. The used standard was polystyrene and the eluent was DMF. The flow rate was 1 mL/min at 40°C.

In order to obtain the polymers with narrow molecular weight distribution, the polymers were fractionated three times using THF as a soluble solvent and ether as a non-solvent. The average molecular weights of synthesized polymers are listed in Table I.

Ottenbrite *et al.*[30] has reported that a good antitumor activity can be obtained in the range of

Table I. The Average Molecular Weights and Polydispersity of Polymers

Sample	Conversion (%)	M_n[a]	M_w[a]	M_w/M_n
Poly(ETA) I	66	3,600	3,900	1.1
Poly(ETA) II	73	9,900	11,900	1.2
Poly(ETA) III	76	14,900	15,100	1.0
Poly(ETA) IV	79	21,000	22,300	1.1
Poly(EETFU) I	54	3,600	3,700	1.1
Poly(EETFU) II	60	9,200	20,700	2.3
Poly(EETFU) III	67	14,300	16,700	1.2
Poly(EETFU) IV	76	33,400	35,600	1.2

[a]The number (M_n) and weight (M_w) average molecular weights of polymers were determined by GPC in DMF.

average molecular weights from 10,000 to 30,000 depending on polymers.

Hydrolysis of 5-FU from Poly(EETFU)s: In order to evaluate the release rate of 5-FU residue from poly(EETFU), a given amount of poly (EETFU)s was hydrolyzed at 37±0.2°C with shaking for a given time under several pH such as pH 1.5 (a mixture of 10 mL of 2 M citric acid and 0.3 mL of 0.2 M NaOH) of stomach, pH 6.8 (a mixture of 50 mL of 0.2 M Na_2HPO_4 and 24.5 mL of 0.2 M KH_2PO_4) of tumor cell, pH 7.4 (a mixture of 50 mL of 0.2 M Na_2HPO_4 and 40.5 mL of 0.2 M KH_2PO_4) of blood, and pH 7.8 (a mixture of 50 mL of 0.2 M Na_2HPO_4 and 45.75 mL of 0.2 M KH_2PO_4) of small intestine, respectively. The extent of hydrolysis was determined by measuring the amount of 5-FU that had been released by HPLC (column: Shodex Ohpak B-803). The flow rate was 1.0 to 1.5 mL/min and the column effluent was monitored at 266 nm which is the maximum absorption wavelength of 5-FU. Under these conditions the 5-FU derivatives showed elution times of 3-4 min. Quantitation of the compounds was done by measuring the peak heights in relation to those of standards measured under the same conditions. The reproducibility of the release rate was checked by three independent measurements and deviation was confirmed to be less than 5%.

Biological Activity Test.

***In Vitro* Antitumor Activities and Cytotoxcities Test:** The *in vitro* cytotoxicities of the prepared samples were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay method.[31] In case of the insoluble polymers in water, the samples were dissolved in a very small amount of dimethylsulfoxide and then diluted with phosphate buffered saline just before use. The prepared sample solution was added to P388, FM3A, U937 cancer cell lines, and AC2F normal cell line in 96-well microtiter plates, and cultured for 3 days at 37°C. The cultured cell lines were mixed with 20 μL of MTT solution and incubated for 4 hr at 37°C. The supernatant was removed from each well and 100 μL of 100% dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals which were formed by the cellular reduction of MTT. After mixing with a mechanical plate mixer, absorbance spectra were measured on ELISA Processor II Microplate Reader at the wavelength of 570 nm. The percentage of cytotoxicity was defined with treated and untreated cell lines. The 50% cytotoxic dose (IC50) was defined as the concentration of samples that reduced the absorbance of the treated cells by 50%.

***In Vivo* Antitumor Activities Test:** To evaluate the *in vivo* antitumor activity of the synthesized samples, mice bearing sarcoma 180 tumor cells were used. Ten Balb/C mice per group were first intraperitoneally (i.p.) implanted with sarcoma 180 cells (2×10^5 cells/mL). The mice were then treated with a saline of sample on days 1~4. Three different dosages such as 0.8, 80, and 800 mg/kg were tested. For comparison, antitumor activities of 5-FU also were tested by the same method. A control group was divided into two groups. One group was treated with sarcoma 180 cells along with the same volume of saline and the other group was treated with only sarcoma 180 cells. The ratio (*T*/*C*) obtained by survival time of mice treated with polymer (*T*) to that of mice in control groups (*C*) was used as the index of the antitumor activity.

Embryo Chorioallantoic Membrane (CAM) Assay: The fertilized chicken eggs used in this study were kept in a humidified incubator at 37°C. After 3-days incubation, about 1 mL of albumin was aspirated from the eggs with an 18-gauge hypodermic needle through the small hole drilled at the narrow end of the eggs, allowing the small CAM and yolk sac to drop away from the shell membrane. On day 4, the shell covering the air sac was punched out and removed by forceps, and the shell membrane on the floor of the air sac was peeled away.[32] Embryos with chorioallantois of 35 mm in diameter were employed for the assay of antiangiogenetic activity. Five μL of an aqueous, salt-free solution of each sample, were applied to sterile Thermanox 15-mm disks and allowed dry under laminar flow conditions.[33] The loaded-disks were inverted and applied to the CAM surface of 4.5-day-old embryos through the windows. The air sac ends of the embryo with shells were covered with scotch tape. Two days

later, an appropriate volume of a 10% fat emulsion was injected using a 33-gauge needle into the 6.5-day embryo chorioallantois so that the vascular network of CAM stood out against the white background of lipid. At least 20 eggs were used for each dose of agent. Finally, the chorioallantois was microphotographed.

Results and Discussion

Identification of Monomer and Polymers. The FT-IR spectrum (KBr pellet) of poly(ETA) [anhydride of unit in Scheme I] showed characteristic absorption peaks at 2930 (-CH stretching), 1860, 1790 (anhydride C = O) and 1235 cm^{-1} (-C-O-C stretching). The absorption peaks at 1630 cm^{-1} assignable to the C = C stretching of furan ring was not observed for the ETA monomer. In ^{1}H-NMR spectrum (DMSO-d_6) of poly(ETA), the methine protons of anhydride ring and adjacent to oxygen in cyclic ether ring appeared at 3.4 and 4.7 ppm, respectively. The peak at 2.9 ppm was assigned to the methine protons of the polymer backbone and the peak at 6.2 ppm assigned to the protons attached double bond in ETA was disappeared [reactant structure in Scheme I].

The FT-IR spectrum (KBr pellet) of poly(EETFU) indicated characteristic absorption peaks at 1720 cm^{-1} (C = O) with disappearance of absorption peaks of protons attached to double bond at 1650 and 990 cm^{-1} which appeared in the EETFU monomer. In ^{1}H-NMR spectrum (DMSO-d_6) of poly(EETFU), the methine protons in polymer backbone and methyl protons in ethyl group exhibited at 1.1 and at 1.2 ppm, and olefinic and N-H protons in 5-FU showed at 7.7 and at 11.5 ppm, respectively, with disappearance of absorption peak of protons attached to double bond at 6.7 ppm which appeared in the EETFU monomer.

Solubility of Prepared Monomers and Polymers. The solubilities of monomers and their polymers were listed in Table II. The prepared samples were soluble in tetrahydrofuran(THF), methyl ethyl ketone(MEK), DMSO, and *N,N*-dimethylformamide(DMF) and poorly soluble in H_2O except EETFU. The samples were insoluble in diethyl ether.

Hydrolysis of 5-FU from Poly(EETFU)s. The hydrolyses of 5-FU from poly(EETFU)s with several number average molecular weights were studied in aquous solution at 37°C under several pHs. The main products of hydrolyzed poly (EETFU) were found to be 5-FU, poly(ETPA) and ethanol as shown in Scheme I.

As can be seen in Figure 1 and 2, the hydroysis rates of poly(EETFU)s increased with time. Poly (EETFU)s showed similar hydrolysis rates for the first 1 hr but the hydrolysis rates vary afterward depending on pH and molecular weights. The polymers with average molecular weights from 9,200 to 33,400 showed slower release rates in comparison to the polymer with number average molecular weight of 3,600. This result was ascribed to the higher steric hindrance by chains of high molecular weights(M_n = 33,400) than low molecular weight(M_n = 3,600). The hydrolysis rates of poly(EETFU)s at pH 1.5 of the stomach, pH 7.4

Scheme I

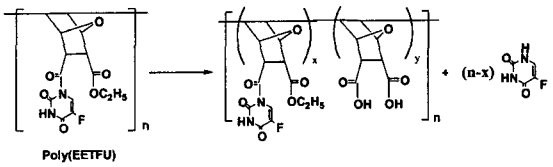

Table II. The Solubility of the Monomers and Polymers

Sample	Solvent					
	H_2O	THF	MEK	DMSO	DMF	Ether
ETA	S	S[a]	S	S	S	[c]IS
Poly(ETA)	S	S	S	S	S	IS
EETFU	S	S	S	S	S	IS
Poly(EETFU)	PS[b]	S	PS	S	S	IS

[a]S = Soluble, [b]PS = Poorly soluble, [c]IS = Insoluble.

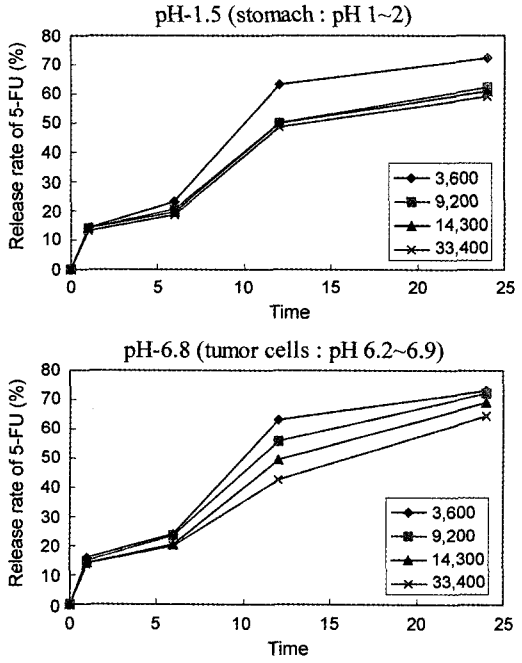

Figure 1. The release rates of 5-FU from poly(EETFU)s in phosphate buffer solutions at 37 under pH; 1.5 and pH; 6.8.

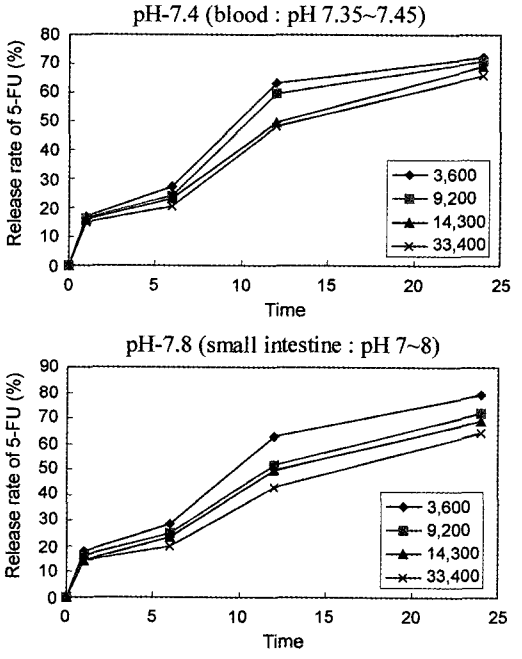

Figure 2. The release rates of 5-FU from poly(EETFU)s in phosphate buffer solutions at 37 under pH; 7.4 and pH; 7.8.

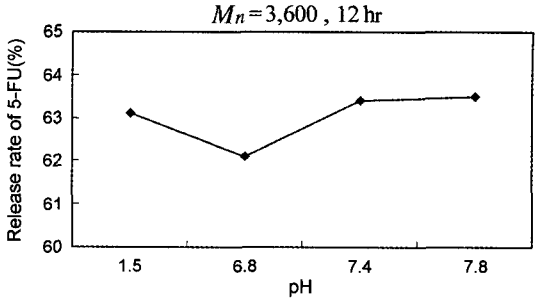

Figure 3. The release rate of 5-FU from poly(EETFU) [M_n = 3,600] in phosphate buffer solutions at 37.

of the blood, and pH 7.8 of the small intestine were higher than those of pH 6.8 of the tumor cells as shown in Figure 3. This means that the hydrolysis of poly(EETFU) is catalyzed by both acid and alkali conditions. The higher hydrolysis rate under pH 7.8 than pH 6.8 is in accordance with the result of the Kim *et al.*[34]

***In Vitro* Cytotoxicity.** The *in vitro* cytotoxicities of the samples were measured on the three cancer cell lines such as P388, FM3A, U937, and one normal cell line (AC2F). The lower IC_{50} value of a compound means the stronger *in vitro* antitumor activity. Thus, the *in vitro* antitumor activities of the polymers were greater than those of monomers. The *in vitro* antitumor activities of EETFU and poly(EETFU) II were similar to those of 5-FU. As shown in Table III, the IC_{50} values of ETA and poly(ETA)s against several tumor cell lines decreased in the following order: poly(ETA) III > poly(ETA) IV > poly(ETA) I > poly(ETA) II > ETA for FM3A, and poly(ETA) III > poly(ETA) IV > poly(ETA) II > poly(ETA) I > ETA for P388 and U937. The cytotoxicities of 5-FU and the prepared samples against a normal cell line increased in the following order: EETFU = poly(EETF) II > 5-FU > poly(ETA) III > poly(ETA) IV > poly(ETA) I > poly(ETA) II > ETA.

***In Vivo* Antitumor Activity against Mice Bearing Sarcoma 180.** The *in vivo* antitumor activities of 5-FU, the monomer and the polymers against mice bearing sarcoma 180 tumor cell line are listed in Table IV, and 5-FU was used for comparison. The ratio, *T/C*, was used as the index of the antitumor activity :

$$T/C(\%) = \frac{\text{Survival time of mice treated with polymer } (T)}{\text{Survival time of mice in a control group } (C)} \times 100$$

As can be seen in Table IV, antitumor activities of related compounds decreased with increasing dosage concentration. This result was attributed to the revelation of toxicity at high concentration. The *in vivo* antitumor activities of synthesized polymers were greater than those of 5-FU and monomers at the same dosage. The highest antitumor activity (*T/C* value) was 828% for poly (EETFU) at 0.8 mg/kg. This value is about 19

Table III. The Cytotoxicity of the Synthesized Samples against Cancer Cell Lines

Sample	IC_{50} (μg/mL) for Cell Line[a]			
	Cancer Cell			Normal Cell
	FM3A[b]	P388[c]	U937[d]	AC2F[e]
5-FU	0.03	0.04	0.05	0.16
ETA	12.00	32.00	27.00	14.60
Poly(ETA) I	2.30	2.60	3.50	9.80
Poly(ETA) II	2.70	2.40	3.45	10.90
Poly(ETA) III	1.10	1.10	1.60	5.80
Poly(ETA) IV	1.30	1.80	2.50	7.60
EETFU	0.04	0.04	0.05	0.01
Poly(EETFU) II	0.07	0.08	0.05	0.01

[a]The 50 % growth inhibition concentration (IC_{50}). [b]Mouse mammary carcinoma cell.
[c]Mouse leukemia cell. [d]Human histiocytic lymphoma cell. [e]Mouse liver cell.

Table IV. The *In Vivo* Antitumor Activity of the Synthesized Samples

Samples	Dosage (mg/kg)	Mean Survival Time (day)[a]	*T/C* (%)[a]	*S/E*[c]
Control	-	14.7 ± 2.3	100	0/10
	Saline	15.7 ± 0.5	100	0/10
5-FU	800.0	5.9 ± 0.3	39	0/10
	80.0	21.3 ± 2.8	140	0/10
	0.8	20.3 ± 1.8	134	0/10
ETA	800.0	4.2 ± 0.7	29	0/10
	80.0	12.7 ± 1.2	84	0/10
	0.8	20.6 ± 0.8	136	0/10
EETFU	800.0	9.6 ± 1.1	61	0/10
	80.0	74.2 ± 8.9	463	2/10
	0.8	31.0 ± 6.0	197	0/10
Poly(ETA) I	800.0	11.1 ± 2.6	73	0/10
(M_n = 9,900)	80.0	20.3 ± 0.8	134	0/10
	0.8	32.6 ± 5.9	251	0/10
Poly(EETFU) II	800.0	29.2 ± 6.6	186	0/10
(M_n = 9,200)	80.0	109.6 ± 9.9	698	6/10
	0.8	130.0 ± 0.0	828	10/10

[a]Mean survival time of animals dying within experiment period of 130 days.
[b]*T/C* (%) represents the ratio of the survival time of treated (*T*) to control (*C*) animals × 100.
[c]*S/E* denotes the ratio of the number of survival mice (*S*) to number of experimental mice (*E*) after experimental period of 130 days.

times higher than the same dosage of free 5-FU. This means that poly(EETFU) has excellent antitumor activity and low toxcity. Poly(EETFU) showed higher antitumor activities than poly(ETA) in spite of similar average molecular weights. This result is attributed to the 5-FU moiety in poly(EETFU).

Antiangiogenesis of ETA and Polymers. In 1996, J. Folkman[35] reported that the inhibition of angiogenesis might lead to inhibition of tumor growth and metastasis. Because antiangiogenic compounds prevent the formation of new blood vessels which supply nutrients to tumor cells.

In Table V, Poly(ETA) with number average molecular weights from 9,900 to 21,000 showed higher antiembryogeneses and antiangiogenesis than those of 3,600. As shown in Figure 4, the number of formed blood vessels for poly(EETFU) was lesser than that for control and poly(ETA). This means that poly(EETFU) has an antiangiogenesis activity. This result agreed well with the *in vivo* antitumor activity shown in Table IV.

Table V. The Antiangiogenic Activities of the Prepared Samples

Sample	Antiembryogenesis[a] Inhibition (%)	Antiangiogenesis[b] Inhibition (%)
Control	16.7	30.0
5-FU	52.3	50.0
ETA	31.1	39.4
Poly(ETA) I (3,600)	32.8	42.8
Poly(ETA) II (9,900)	50.0	50.0
Poly(ETA) III (14,900)	51.6	58.9
Poly(ETA) IV (21,000)	50.0	50.0

[a]Number of antiembryogenic eggs/Number of total eggs × 100. [b]Number of antiangiogenic eggs/Number of total eggs × 100.

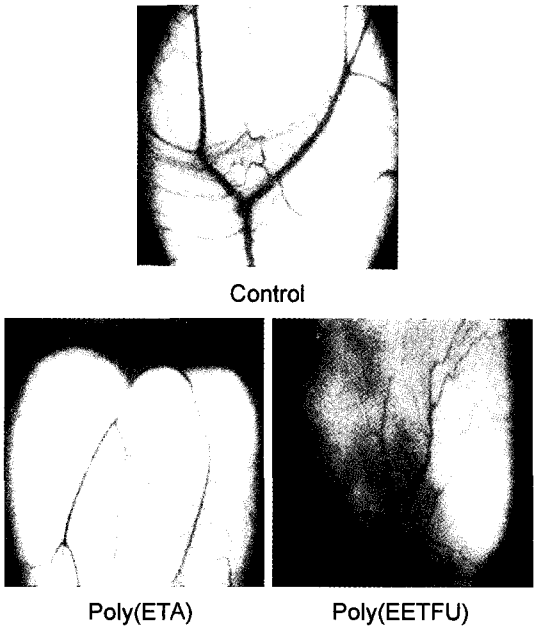

Figure 4. The microphotographs of control, poly(ETA), and poly(EETFU) on embryonic angiogenesis in CAM(100).

Conclusions

Poly(*exo*-3,6-epoxy-1,2,3,6-tetrahydrophthalic anhydride)s and poly(α-ethoxy-*exo*-3,6-epoxy-1,2,3,6-tetrahydrophthaloyl-5-fluorouracil)s with several average molecular weights were prepared by photopolymerizations. The number average molecular weights of the fractionated poly(ETA)s and poly(EETFU)s determined by GPC were in the range of 3,600~21,000 and 3,600~33,400, respectively. Poly(EETFU) showed the highest *in vitro* antitumor activities against cancer cell lines. The *in vivo* antitumor activities of the synthesized samples at dosage of 0.8 mg/kg against mice bearing sarcoma 180 tumor cell line decreased in the following order: poly(EETFU) > poly(ETA) > EETFU > ETA > 5-FU. The antiangiogenic activities of the poly(ETA)s were similar to those of 5-FU except poly(ETA)I. The release rate of 5-FU from poly (EETFU) was slower with increasing average molecular weight regardless of pH. Poly(EETFU) was suitable for a polymeric drug from the viewpoint of the release rate of 5-FU.

Acknowledgement. This work was financially supported by the Korea Research Foundation (1998-017-D00005).

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