Polymer Bulletin 46, 241–248 (2001)

Polymer Bulletin

Syntheses and antitumor activities of polymers containing 2-acrylamido-2-methyl-1-propanesulfonic acid or 5-fluorouracil

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Received: 6 September 2000/Revised version: 12 March 2001/Accepted: 22 March 2001

Summary

The polymers containing 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPA) were prepared by radical polymerizations. The polymers were identified by FT-IR, ^{1}H-, and ^{13}C-NMR spectroscopies. The contents of AMPA unit in poly(AMPA-*co*-MAH), terpoly(AMPA-MAH-FUR), and poly(AMPA-*co*-EETFU) were 67, 73 and 49 mol %, respectively. The number average molecular weights of the polymers determined by GPC were in range from 5,600 to 9,200. The IC_{50} values of the synthesized polymers against cancer cell lines were in the range of 0.02 to 127. The *in vivo* antitumor activities of polymers against Balb/C mice bearing the sarcoma 180 tumor cells were greater than those of 5-FU.

Introduction

Synthetic polyanions, such as pyran copolymer (DIVEMA) of divinyl ether and maleic anhydride, poly(ethylene-*co*-maleic anhydride), poly(acrylic acid), and poly(maleic anhydride) have been extensively studied for antitumor, antiviral, antibacterial, interferon-inducing and antifungal activities [1]. Among the polyanions, DIVEMA exhibits high antitumor activities as well as toxic side effects such as anemia, enlarged liver, and spleen *etc* [2]. Afterward, many attempts were made to obtain a polymeric drug like DIVEMA [3-5]. We have reported syntheses and biological activities of polymeric antitumor compounds such as 5-FU containing acryl derivative polymers [6-8], tetrahydrophthalic acid derivative polymers (TADP)s [9-14], poly(diallyl ether-*co*-maleic anhydride) [15], poly(glycinyl maleamic acid) derivatives [16,17], and methoxyitaconyl-5-fluorouracil [18] and described their reduced toxicity and improved antitumor activity. Among them, (TADP)s showed excellent *in vivo* antitumor activities against cancer cell lines and weak cytotoxcities against normal cell line.

In this study, the new polymers, poly(2-acrylamido-2-methyl-1-propanesulfonic acid) [poly(AMPA)], poly(2-acrylamido-2-methyl-1-propanesulfonic acid-*co*-maleic anhydride) [poly(AMPA-*co*-MAH)], terpoly(2-acrylamido-2-methyl-1-propanesulfonic acid-maleic anhydride-furan) [terpoly(AMPA-MAH-FUR)], and poly(2-acrylamido-2-methyl-1-propanesulfonic acid-*co*-α-ethoxy-*exo*-3,6-epoxy-1,2,3,6-tetrahydrophthaloyl-5-fluoro-

ouracil) [poly(AMPA-*co*-EETFU)] were prepared from corresponding monomers by the radical polymerizations at 70 °C for 48 hr under presence of benzoyl peroxide (BPO) as an initiator. The synthesized polymers were characterized by FT-IR, ^{1}H-NMR, ^{13}C-NMR, elemental analysis, and gel permeation chromatography (GPC). The *in vitro* antitumor activities and cytotoxicities were evaluated with mouse mammary carcinoma (FM3A), mouse leukemia (P388), and human histiocytic lymphoma (U937) as cancer cell lines and mouse liver cells (AC2F) as a normal cell line. The *in vivo* antitumor activities of the synthesized samples against mice bearing the sarcoma 180 tumor cell line were evaluated.

Experimental

Materials

2-Acrylamido-2-methyl-1-propanesulfonic acid (AMPA; Aldrich Co., USA), maleic anhydride (MAH; Aldrich Co.,), *exo*-3,6-epoxy-1,2,3,6-tetrahydrophthalic anhydride (ETA; Aldrich Co.,), benzoyl peroxide (BPO; Tokyo Kasei Co., Tokyo, Japan) were used without further purification. α-Ethoxy-*exo*-3,6-epoxy-1,2,3,6-tetrahydrophthaloyl-5-fluorouracil (EETFU) were synthesized by the reported method [26]. All other chemicals were reagent grade and were used without further purification. P388, FM3A, and U937 as cancer cell lines and AC2F as a normal cell line were used for *in vitro* test. Balb/C mice and sarcoma 180 cell line for *in vivo* test were purchased from the Center of Genetic Engineering (Korea Institute of Science and Technology).

Instruments

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a FT-300 MHz Varian Gemini 2000 spectrophotometer. FT-IR spectra were obtained with a Jasco FT/IR-5300 spectrophotometer by using a KBr pellet for analysis. Elemental analysis was performed by an elemental analyzer (Carlo Erba model EA1180). The number and weight average molecular weights were determined by gel permeation chromatography (GPC; Waters 410).

Homopolymerizations of AMPA

The solution of AMPA (1.0 g, 4.8 mmol) and BPO (0.033g, 0.01 mmol) as an initiator in the 10 mL of a dry DMSO was introduced into a dried polymerization tube. The tube was sealed after flushing twice with bubbling purified N_2 gas and placed in a regulated thermostat bath at 70 °C for 48 hr. The obtained polymer solution was precipitated in 150 mL of chloroform/petroleum ether (V/V; 1/1). The precipitate was collected by filtration and dried at room temperature under vacuum to a constant weight. The conversion was 55%.

Copolymerizations of AMPA with MAH, ETA, and EETFU

To prepare poly(AMPA-*co*-MAH), the solution of AMPA (1.0 g, 4.8 mmol), MAH (0.6 g, 5.8 mmol), and BPO (0.033g, 0.01 mmol) as an initiator in the 10 mL of a dry DMSO and for poly(AMPA-*co*-EETFU), the solution of AMPA (1.0 g, 9.3 mmol), EETFU (1.9 g, 5.8 mmol), and BPO (0.033g, 0.01 mmol) in the 10 mL of a dry ethanol were introduced into a dried polymerization tubes, respectively. The polymerization and the treatment procedures were the same as that described for the homopolymerization of AMPA except for the monomer pairs. The conversions were 61 % for AMPA and MAH and 53 % for AMPA and EETFU. The copolymerization of AMPA (1.0 g, 4.8 mmol), and ETA (1.0 g, 5.8 mmol) dissolved in 10 ml of a dry DMSO was carried out in a dried polymerization tube at 70 °C for 48 hr. The treatment procedures were the same as that described for

AMPA polymerization. The conversion was 48 %. The structure of the obtained polymer will be shown in results and discussion section.

Measurement of average molecular weight

The average molecular weights and polydispersity (PD=Mw/Mn) were determined by gel permeation chromatography (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 Å connected in series. The used standard was polystyrene and the eluent was DMF at a flow rate of 1 mL/min (40 °C).

Elemental analysis of copolymers

The contents of the AMPA moiety in copolymers were calculated from C, H, N, and S data obtained by elemental analysis.

In vitro cytotoxicity

The *in vitro* cytotoxicities of the monomer and the synthesized polymers were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay method [19]. The target cells such as P388, FM3A, and U937 cancer cell lines and AC2F normal cell line were cultured in RPMI 1640. The samples were dissolved in a very small amount of dimethyl sulfoxide and then diluted with phosphate-buffered saline just before use. The prepared sample solution (200 μl) was added to the $2x10^4$ cells/ml target cells in 96-well microtiter plates and cultured at 37 °C for 3 days. The cultured cell lines were mixed with 20 μL of MTT solution and incubated at 37 °C for 4 hr. The supernatant was removed from each well and 100 μL of 100 % DMSO was added to solubilize the formazan crystals which were formed by the cellular reduction of MTT. After mixing of the samples with a mechanical plate mixer, absorbance spectra were measured on ELISA Processor II Microplate Reader at the wavelength of 570 nm. The 50 % cytotoxic dose (IC_{50}) was defined as the concentration of samples that reduced the absorbance of the treated cells by 50 %.

In vivo antitumor activities test

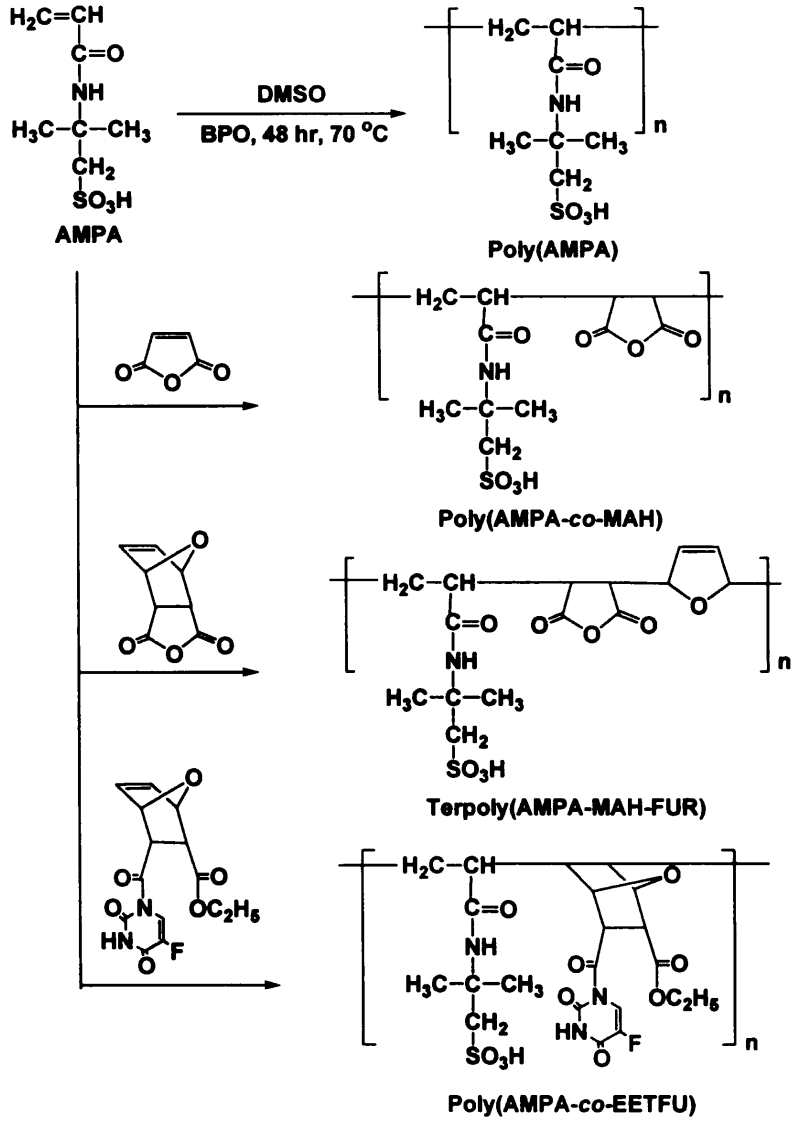
To evaluate the *in vivo* antitumor activity of synthesized samples, mice bearing sarcoma 180 tumor cells were used. Ten Balb/C mice per group were first intraperitoneally (i.p.) implanted with sarcoma 180 cells (2×10^5 cells/ml). The mice were then treated with a saline of sample on days 1 ~4. Three 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.

Results and discussions

Identification of the polymers

The polymers were synthesized according to the scheme 1.

The structures of the synthesized polymers were identified by IR, ^{1}H-NMR, and ^{13}C-NMR spectroscopies The ^{1}H-NMR spectrum (DMSO-d_6) of poly(AMPA) showed methine and methylene protons of polymer backbone at 2.1 and 2.6 ppm, and sulfonic acid proton at 7.9 ppm. The absorption peak assignable to the vinyl protons of monomeric



Scheme 1.

AMPA was not observed at 5.5 and 6.0 ppm. In the ^{1}H-NMR spectrum (DMSO-d_6) of poly(AMPA-*co*-MAH), methine protons of maleic anhydride were appeared at 3.3 ppm, and methine and methylene protons of AMPA unit were observed at 2.1 and 2.6 ppm.

The ^{1}H-NMR spectrum (DMSO-d_6) of the obtained polymer by the copolymerization of AMPA and ETA showed the olefinic protons and methine peaks of furan moiety at 6.1 and 5.2 ppm, respectively, and the methine peaks of maleic anhydride moiety at 3.2~3.4 ppm together with the peaks due to protons of AMPA moiety. In order to confirm the structure of the polymer obtained by the copolymerization of AMPA and ETA, the radical terpolymerization of AMPA, MAH, and FUR were carried out in the same conditions as the radical copolymerization. The ^{1}H-NMR peaks of the obtained polymer by the terpolymerization were the same as those of the prepared polymer by the radical copolymeization of AMPA and ETA. Thus, it can be concluded that the structure of the obtained polymers by the copolymerization of AMPA and ETA is poly(AMPA-MAH-FUR). This result was ascribed to the reported fact that ETA dissociates into MAH and FUR at above 50 °C. That is, MAH and FUR dissociated from ETA at 70 °C participate in *in situ* terpolymerization together with AMPA. Thus, terpoly(AMPA-MAH-FUR) can be

obtained by copolymerization of AMPA and ETA at 70 °C. According to the our previous reports [20], poly(AMPA-*co*-ETA) may be obtained by the photo-polymerization of AMPA and ETA at 25 °C. The ^{1}H-NMR spectrum (DMSO-d_6) of poly(AMPA-*co*-EETFU) showed -NH proton of 5-FU at 11.2 ppm, sulfonic acid proton of AMPA at 7.9 ppm, olefinic protons of 5-FU at 8.0 ppm, methyl and methylene protons of EETFU 1.2 and 2.7 ppm, respectively. The absorption peaks due to protons of AMPA moiety in copolymer were assigned to the same as those of poly(AMPA).

Solubilities of AMPA and polymers

The solubilities of AMPA and synthesized polymers were listed in Table I. AMPA and its polymers were soluble in H_2O, DMSO, and DMF except terpoly(AMPA-MAH-FUR) in H_2O. The samples were in soluble in MEK, THF, and ether except poly(AMPA-*co*-EETFU)

Table I. The Solubilities of AMPA and Its Polymers

Sample	Solvent					
	H_2O	DMSO	DMF	MEK	THF	Ether
AMPA	S[a]	S	I[c]	I	I	I
Poly(AMPA)	S	S	S	I	I	I
Poly(AMPA-*co*-MAH)	S	S	S	I	I	I
Terpoly(AMPA-MAH-FUR)	P.S[b]	S	S	I	I	I
Poly(AMPA-*co*-EETFU)	S	S	S	P.S	P.S	I

[a] S , soluble; [b] P.S, poorly soluble; [c] I, insoluble.

Average molecular weights of polymers

The average molecular weights of the fractionated polymers were listed in Table II. Ottenbrite *et al*. [21] have reported that good antitumor activities can be obtained in the range of average molecular weights from 10,000 to 30,000 depending on polymers. The average molecular weights of the synthesized polymers were in the range of medium molecular weights and narrow polydispersity, which can be exhibited good antitumor activities or low cytotoxcities. The polymers designated as medium molecular weight are in the range of number average weights from 5,000 to about 20,000 or weight average molecular weights from 10,000 to about 30,000 and polydispersities from 1 to 2.5 in our laboratory [12-14].

Table II. The Average Molecular Weights and Polydispersity of The Polymers

Polymers	M_n[a]	M_w[a]	M_w / M_n
Poly(AMPA)	7,100	17,700	2.5
Poly(AMPA-*co*-MAH)	5,600	5,900	1.3
Terpoly(AMPA-MAH-FUR)	6,200	6,700	1.1
Poly(AMPA-*co*-EETFU)	9,200	11,600	1.3

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

Contents of AMPA moiety in copolymers

The contents of AMPA in copolymers were determined by the elemental analysis as shown in Table III. The AMPA contents in poly(AMPA-*co*-MAH), terpoly(AMPA-MAH-FUR), and poly(AMPA-*co*-EETFU) were 67, 73, and 49 mol%, respectively.

Table III. The Elemental Compositions and Contents of AMPA in The Copolymers.

Sample	E.A. (%)				AMPA contents in copolymer (mol%)[a]
	C	H	N	S	
Poly(AMPA-*co*-MAH)	40.2	6.6	5.4	12.5	67
Terpoly(AMPA-MAH-FUR)	42.2	6.1	5.2	11.9	73
Poly(AMPA-*co*-EETFU)	40.3	6.7	7.6	5.9	49

[a] Calculated from elemental analysis.

In vitro cytotoxicity of AMPA and polymers

The *in vitro* cytotoxicities of the samples were measured on the three cancer cell lines such as P388, FM3A, and U937 and one normal cell line such as AC2F. As shown in Table IV, the IC_{50} values of the synthesized polymers against cancer cell lines were in the range of 0.02 to 127. For FM3A, the IC_{50} values of AMPA, poly(AMPA), poly(AMPA-*co*-MAH), terpoly(AMPA-MAH-FUR), and poly(AMPA-*co*-EETFU) were 69, 120, and 98, 37, and 0.03 μg/mL, respectively. The *in vitro* antitumor activities against P388 and U937 and cytotoxcities against AC2F of 5-FU, AMPA, and the polymers decreased in following order: poly(AMPA-*co*-EETFU) > 5-FU > terpoly(AMPA-MAH-FUR) > AMPA > poly(AMPA-*co*-MAH) > poly(AMPA). The IC_{50} values of poly(AMPA-*co*-EETFU) against all cancer cell lines were better than those of 5-FU. The lower IC_{50} value, the stronger *in vitro* antitumor activity.

Table IV. The *In Vitro* Cytotoxicities of AMPA and Its Polymers against Cancer and Normal Cell Lines

Sample	IC_{50}[a] (μg / mL)			
	Cancer Cells			Normal Cell
	FM3A[b]	P388[c]	U937[d]	AC2F[e]
5-FU	0.03	0.04	0.05	0.16
AMPA	69.00	64.00	86.00	74.0
Poly(AMPA)	120.00	110.00	127.00	112.00
Poly(AMPA-*co*-MAH)	98.00	95.00	89.00	115.00
Terpoly(AMPA-MAH-FUR)	37.00	32.00	24.00	28.00
Poly(AMPA-*co*-EETFU)	0.03	0.02	0.01	0.20

[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 line.

In vivo antitumor activity against mice bearing sarcoma 180

The *in vivo* antitumor activities of polymers containing AMPA against mice bearing the sarcoma 180 tumor cell line are listed in Table V, and 5-FU was used for comparison. The ratio *T/C* was used as index of the antitumor activity:

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

The life spans (*T/C*) of mice treated with AMPA and polymer at dose of 800 mg/kg were much lower than those of AMPA and polymers at dose of 80 and 0.8 mg/kg due to the toxic side effect of polymers at higher concentration except poly(AMPA-*co*-MAH). At a dose of 80 mg/kg, the *T/C* values of polymers containing AMPA moiety were 107 for poly(AMPA), 99 for poly(AMPA-*co*-MAH), 196 for terpoly(AMPA-MAH-FUR) and 406 for poly(AMPA-*co*-EETFU), respectively. Poly(AMPA-*co*-EETFU) which exhibits

the high antitumor activities at a dose of 80 and 0.8 mg/kg can be explained by the chemical structure designed to slowly release 5-FU and anionic groups via hydrolyses.
It is known that the polymeric prodrug enter the target cells by endocytosis mechanism and thereby the growth of target cells is inhibited [22]. In case of poly(AMPA-*co*-EETFU), 5-FU and ethanol are slowly released by the hydrolysis of the amide and ester bonds in the side part of polymers in a tumor cell membrane and the total concentration of released free 5-FU can be maintained at effective level to kill tumor cells for a long period. In order to verify the above elucidation, more experimental evidence such as the release rate of 5-FU from poly(AMPA-*co*-EETFU) and the concentration in the culture media are needed in the future. Further work on the release rate under the various conditions such as concentraion, pH, temperature are now in progress and a full account of the work will be published in the near future.

Table V. The *In Vivo* Antitumor Activity of 5-FU, AMPA and Polymers

Sample	Dosage (mg/kg)	Mean Survival Time (day)	*T/C* (%)[a]
Control	-	14.7 ± 2.3	100
	saline	15.7 ± 0.5	100
5-FU	800.0	5.9 ± 0.3	39
	80.0	21.3 ± 2.8	140
	0.8	20.3 ± 1.8	134
AMPA	800.0	10.6 ± 2.5	67
	80.0	14.1 ± 3.0	90
	0.8	19.7 ± 1.1	125
Poly(AMPA)	800.0	12.5 ±5.4	80
	80.0	16.8 ± 2.3	107
	0.8	13.3 ± 5.3	85
Poly(AMPA-*co*-MAH)	800.0	23.1 ± 5.4	147
	80.0	15.5 ± 2.8	99
	0.8	12.2 ± 3.8	78
Terpoly(AMPA-MAH-FUR)	800.0	19.1 ± 4.5	122
	80.0	30.7 ± 3.5	196
	0.8	25.0 ± 2.1	159
Poly(AMPA-*co*-EETFU)	800.0	18.5 ± 3.6	118
	80.0	63.7 ± 2.8	406
	0.8	58.5 ± 3.1	373

[a]*T/C*(%) represents percentage the ratio of the survival time of mice treated with polymer (T) to control (C) mice.

Conclusions

The new polymers, poly(AMPA), poly(AMPA-*co*-MAH), terpoly(AMPA-MAH-FUR), and poly(AMPA-*co*-EETFU), were prepared by the radical polymerization using BPO in DMSO at 70 °C for 48 hr. The structures of synthesized polymers were identified by FT-IR and ^{1}H, ^{13}C-NMR spectroscopies. The average molecular weights and polydispersity indices of the synthesized polymers were as follows: *Mn*=7,100, *Mw*=17,700, *Mw/Mn*=2.5 for poly(AMPA); *Mn*=5,600, *Mw*=5,900, *Mw/Mn*=1.3 for poly(AMPA-*co*-MAH); *Mn*=6,200, *Mw*=6,700, *Mw/Mn*=1.1 for terpoly(AMPA-MAH-FUR); *Mn*=9,200, *Mw*=11,600, *Mw/Mn*=1.3 for poly(AMPA-*co*-EETFU). The contents of AMPA unit in the

copolymers were as follows: 67 % for poly(AMPA-*co*-MAH), 73 % for terpoly(AMPA-MAH-FUR), and 49 % for poly(AMPA-*co*-EETFU). The *in vitro* cytotoxicies of the prepared samples against all cancer cell lines were much lower as compared with those of 5-FU. The *in vivo* antitumor activities of terpoly(AMPA-MAH-FUR) and poly(AMPA-*co*-EETFU) were better than those of 5-FU.

Acknowledgement. This work was financially supported by the Korea Research Foundation (1998-017-D00005) and we thank the Ministry of Education (Brain Korea 21) for the use of supporting instruments.

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