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# Effects of Molecular Weights on the Physico-pharmaceutical Properties of Poly-L-glutamic acid-cytarabine Conjugates

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**Abstract**□In order to obtain some informations about the effect of molecular weight on the release rate of drug from drug carrier, two types of poly-L-glutamic acid (PLGA)-cytarabine (ara-C) conjugates, PLGA-ara-C:I and PLGA-ara-C:II, were synthesized using two types of PLGA having different average molecular weight, 43,000 and 77,800, respectively. The PLGA-ara-C conjugates were synthesized by mixed anhydride method and found to be covalently linked. Both types of conjugates charged negatively at biological pH. The pH-dependent release rate of ara-C was observed in both cases, and the release rate was accelerated in basic, acidic conditions (the k values were 0.015 $day^{-1}$ at pH 7.0, 0.024 $day^{-1}$ at pH 5.0, and 0.059 $day^{-1}$ at pH 9.0 in the case of PLGA-ara-C:I) and in the presence of protease. The time required for the release of 16.5% of ara-C from PLGA-ara-C:I were 8 hr and 144 hr in the presence and absence of protease, respectively. Although both types of conjugates showed similar drug substitution ratio, they showed different release rates. Between the two types of conjugates, PLGA-ara-C:II showed the faster release rate (0.030 vs 0.042 $day^{-1}$ in pH 7.4 phosphate buffer solution at 37 °C) and the smaller activation energy for the release of drug (12.5 vs 7.7 Kcal/mol) than PLGA-ara-C:I. The characteristic effect of molecular weight on the release rates of PLGA-ara-C conjugates suggests that the drug release rate might be effectively controlled over a prolonged period of time by the combined use of the different types of PLGA-ara-C conjugates having different molecular weights.

**Keywords**□Poly-L-glutamic acid-cytarabine conjugates, drug carrier, effect of molecular weights, drug substitution ratio, molecular charge, drug release rate.

One of the major problems of chemotherapy is to deliver the proper amounts of anticancer drug to the target site at a proper rate. To solve this problem, many attempts have been centered to develop the drug delivery systems such as albumin microspheres[1,2], liposomes[3], and the conjugates of anticancer drugs with macromolecules including synthetic polypeptides[4-7] and natural proteins[8].

Among these systems, poly-L-glutamic acid (PLGA) conjugates, one of the synthetic polypeptide conjugates, have received wide attention. Because PLGA is biocompatible, has a lot of carboxyl groups which could be coupled with anticancer drugs, and is found to be one of the most effective inducers of pinocytosis[9]. PLGA has been used as a carrier for cytarabine (ara-C) and mitomycin C, and their greater effects against certain types of tumors than the parent drugs were reported[5,10].

It is expected that physicochemical properties of drug carriers such as charge, solubility, molecular weight and the nature of the chemical linkage play an important role in anticancer activity and *in vivo* fate of the macromolecule-drug conjugates. PLGA has an important advantage that is could be obtained with different molecular weight distributions, but little has been known about its molecular weight effects on the physico-pharmaceutical properties of the resultant conjugates.

Therefore, in this study, two types of PLGA-ara-C conjugates constituted with two types of PLGA having different average molecular weight were synthesized, and their physico-pharmaceutical properties were examined.

## EXPERIMENTAL METHODS

### *Materials*

Ara-C was kindly supplied by Choong Wae Pharm. Co. (Seoul, Korea). Two types of PLGA sodium salts having different molecular weight

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(average M.W. of 43,000 and 77,800, respectively and corresponding degree of polymerization of 285 and 515), and Amberite® IR 120-P cation exchange resin were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Amberite® IRA-67 anion exchange resin and Spectrapor® membrane tube (M.W. cut off 6,000-8,000) were purchased from Rohm and Haas (Philadelpia, USA) and Spectrum Medical Industries Inc. (Los Angeles, USA), respectively. Protease (activity 31,400 unit/g, at pH 8.0) was supplied by Dong A Pharm. Co. (Seoul, Korea). All other chemicals were of analytical reagent grade.

*Synthesis of PLGA-ara-C conjugates*

Two types of PLGA-ara-C conjugates, PLGA-ara-C:I(M.W. 43,000) and PLGA-ara-C:II (M.W. 77,800), were prepared by "mixed anhydride method"[10] using the two types of PLGA. Isobutyloxy carbonyl chloride (IBC) and triethylamine(TEA) were added to a solution of PLGA in dry dimethyl formamide(DMF) at $-7 \pm 1$ °C, and the mixture was stirred for 1 hr at $-7 \pm 1$ °C. To the resultant solution, a solution of ara-C in dry DMF and TEA were added, and allowed at 4 °C for 3 days and then, at room temperature for 4 hrs. The reaction mixture was poured into cold pH 8.0 phosphate buffer (0.4 M), and any insoluble materials were removed by filtration. The filtrate was dialyzed against 3% sodium chloride solution, and distilled water, respectively, using dialysis tube. The internal solution was collected, lyophylized, and stored in desiccator at below 0 °C.

*Measurement of molecular charge*

The molecular charge was estimated with the anion and cation exchange resins. Several buffer solutions were used in a concentration of 20 mmol/l: Bis(2-hydroxyethyl)amino tris(hydroxymethyl) methane(BIS-TRIS), pH 5.0 and 6.0; N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid (HEPES), pH 7.0; tris(hydroxymethyl)amino-2-ethane(TRIS), pH 8.0 and 9.0. 80 mg of an ion exchange resin was suspended in 5 ml of buffer solution having PLGA-ara-C conjugates (equivalent to 2 mmol of ara-C). After 30 min, the samples were centrifuged for 1 min at 2,000 rpm, and the amounts of the conjugates remained in the supernatant were measured spectrophotometrically at 300 nm.

*Determination of drug substitution ratio*

The ratio of the number of drug-substituted glutamic acid units to the total number of the glutamic acid units in the PLGA-ara-C conjugates was determined spectrophotometrically. Absorption maxima of the conjugated N-acylcytosine moiety[11] was measured in pH 7.0 phosphate buffer solution (0.1M) at 300nm ($\varepsilon = 8 \times 10^3 M^{-1}cm^{-1}$).

*Effect of pH on in vitro drug release*

PLGA-ara-C conjugates (equivalent to 4 mmol of ara-C) were dissolved in 10 ml of phosphate buffer solution (0.1M, $\mu = 0.1$), pH 5.0, 7.0, 7.4 and 9.0, respectively, and the mixture was incubated at $37 \pm 1$ °C. At periodic time intervals for 5 days, the release of ara-C was monitored spectrophotometrically at 300 nm.

*Effect of temperature on in vitro drug release*

PLGA-ara-C conjugates (equivalent to 4 mmol of ara-C) were dissolved in pH 7.0 phosphate buffer solution (0.1M, $\mu = 0.1$), and the mixture was incubated at $24 \pm 1$ °C, $37 \pm 1$ °C, and $50 \pm 1$ °C, respectively. At periodic time intervals for 5 days, the release of ara-C was monitored spectrophotometrically at 300 nm.

*Effect of protease on in vitro drug release*

30 mg of protease was dissolved in 25 ml of pH 7.4 phosphate buffer solution, and 5 ml of PLGA-ara-C conjugates solution in phosphate buffer (equivalent to 1.12 mmol of ara-C) was added. The resultant mixture was incubated at $37 \pm 1$ °C. At periodic time intervals for 8 hrs, the release of ara-C was monitored spectrophotometrically at 300nm.

## RESULTS AND DISCUSSION

*Synthesis and molecular charge of PLGA-ara-C conjugates*

Carboxyl group of PLGA was activated with IBC in the presence of base to form mixed anhydride, then was conjugated with amino groups of ara-C[10]. Unconjugated ara-C was removed by dialysis. The formation of the conjugates was confirmed by means of ultraviolet spectra, and thin layer chromatography. As shown in Fig. 1, $\lambda_{max}$ was shifted from 270nm to 300nm, which is characteristic for N-4-acyl-ara-C derivatives[11]. The TLC (0.25mm silica gel GF, water saturated with 1-butanol: propanol (3:1)) showed the different $R_f$ value of the product(0.00) from that of ara-C (0.33). Lyophilized PLGA-ara-C conjugates were white powder and soluble in water. As shown in Fig. 2, both types of the conjugates were adsorbed to the anion exchange resin, in the range of pH 5.0 to 9.0.

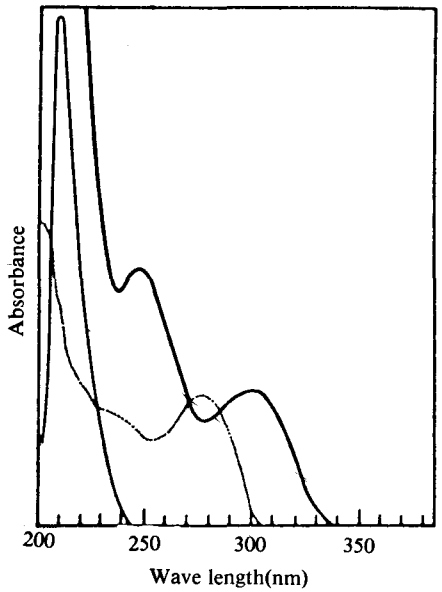


**Fig. 1. Ultraviolet spectrum of the PLGA-ara-C conjugates in comparison with that of ara-C in pH 7.0 phosphate buffer solution.**
key; ---: ara-C, —: PLGA, —: PLGA-ara-C conjugates.

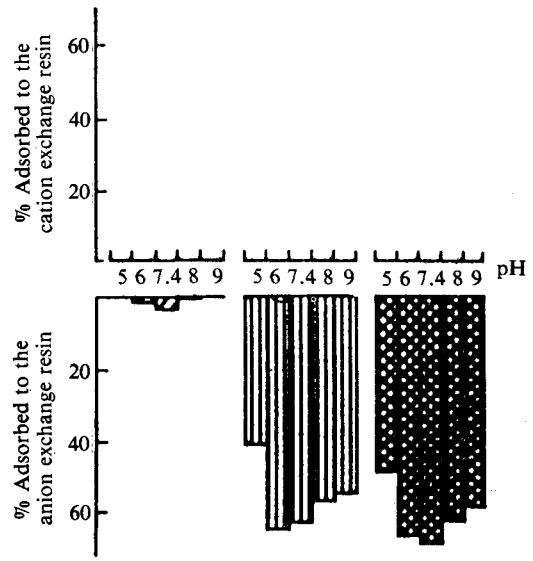


**Fig. 2. The percentage adsorbed to the cationic (upper panel) and anionic (lower panel) resines as a function of pH.**
key; ▨ : ara-C, ▥ : PLGA-ara-C:I, ▩ : PLGA-ara-C:II.

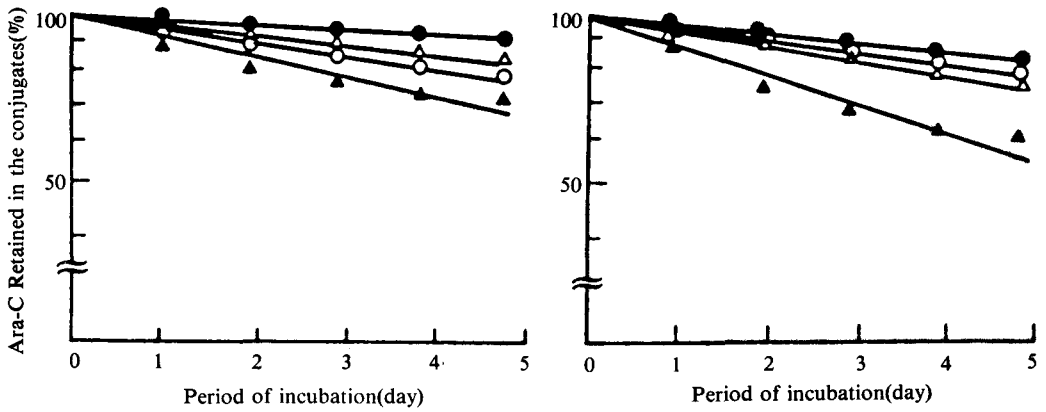


**Fig. 3. Effect of pH on *in vitro* release of ara-C from PLGA-ara-C:I (left) and PLGA-ara-C:II (right), respectively, incubated in phosphate buffer at 37 ± 1°C.**
key; ○ – ○ : pH 5.0, ● – ● : pH 7.0, △ – △ : pH 7.4, ▲ – ▲ : pH 9.0.

*Molecular weight effect on drug substitution ratio*

Both types of the conjugates showed similar drug substitution ratio, about 11%, which means that only 11% of carboxyl groups of PLGA participated in the coupling reaction with ara-C. We suppose that this might be due to the three dimensional conformation of PLGA in the non-aqueous reaction mixture. And the similar drug substitution ratio of both types of the conjugates suggestes that molecular weight of PLGA has little effect, if any on the drug substitution ratio in this case.

*Effect of pH on in vitro drug release*

Fig. 3 shows the semilogarithmic plot of the percent of ara-C retained in the both conjugates as a function of time after incubation at 37 ± 1 °C, in several pH solutions. The release of ara-C from the PLGA-ara-C conjugates was pH-dependent; the

**Table I. Release rates(k) of ara-C from PLGA-ara-C:I and PLGA-ara-C:II and half lives($t_{1/2}$) of the PLGA-ara-C conjugates incubated in the phosphate buffer solutions of several pHs, at 37 ± 1°C**

| pH | PLGA-ara-C:I | | PLGA-ara-C:II | |
|---|---|---|---|---|
| | k ($day^{-1}$) | $t_{1/2}$ (day) | k ($day^{-1}$) | $t_{1/2}$ (day) |
| 5.0 | 0.024 | 28.6 | 0.040 | 17.3 |
| 7.0 | 0.015 | 47.8 | 0.031 | 21.5 |
| 7.4 | 0.030 | 23.0 | 0.042 | 16.6 |
| 9.0 | 0.059 | 11.8 | 0.097 | 7.2 |

release rate was accelerated in basic (pH 9.0) and acidic (pH 5.0) conditions. Approximately 11.4, 13.2 and 25.0% of ara-C was released from PLGA-ara-C:I in 4 days at pH 7.0, 5.0 and 9.0, respectively.

Table I suggests that the release rates of ara-C from PLGA-ara-C:II were faster than those of PLGA-ara-C:I. The time required for the release of 50% of ara-C from PLGA-ara-C:I and PLGA-ara-C:II in pH 7.4 phosphate buffer solution were extrapolated to be 16.6 and 23.0 days, respectively.

*Effect of temperature on in vitro drug release*

The effect of temperature on the release rates of two types of conjugates is shown in Fig. 4 and Table II. As the temperature(T) increases, the drug release rates(k) were increased. The release rate of ara-C from PLGA-ara-C:I was increased from 0.014 $day^{-1}$ at 24°C to 0.075 $day^{-1}$ at 50°C. And also, the release rates of ara-C from PLGA-ara-C:II were higher than those of PLGA-ara-C:I; the k values were 0.042 and 0.030 $day^{-1}$, respectively at 37°C. From the plot of log k against 1/T (Fig. 5), the activation energy for the release of drug from the conjugates (Ea) was calculated. PLGA-ara-C:I has larger Ea (12.5 Kcal/mol) than that of PLGA-ara-C:II (7.7 Kcal/mol)(Table III).

**Table II. Release rates(k) of ara-C from PLGA-ara-C:I and PLGA-ara-C:II and half lives($t_{1/2}$) of the PLGA-ara-C conjugates incubated in pH 7.4 phosphate buffer solution at several temperatures(T)**

| T (°C) | PLGA-ara-C:I | | PLGA-ara-C:II | |
|---|---|---|---|---|
| | k ($day^{-1}$) | $t_{1/2}$ (day) | k ($day^{-1}$) | $t_{1/2}$ (day) |
| 24 | 0.014 | 50.2 | 0.023 | 30.7 |
| 37 | 0.030 | 23.0 | 0.042 | 16.6 |
| 50 | 0.075 | 9.2 | 0.065 | 10.7 |

**Table III. The activation energy for the release of the drug from PLGA-ara-C conjugates(Ea)**

| types of conjugates | Ea(Kcal/mol) |
|---|---|
| PLGA-ara-C:I | 12.5 |
| PLGA-ara-C:II | 7.7 |

*Effect of protease on in vitro drug release*

To investigate the possibility of biodissociation by the enzymatic cleavage in the body, each types of the conjugates was incubated in the presence of protease. The release of ara-C from the conjugates

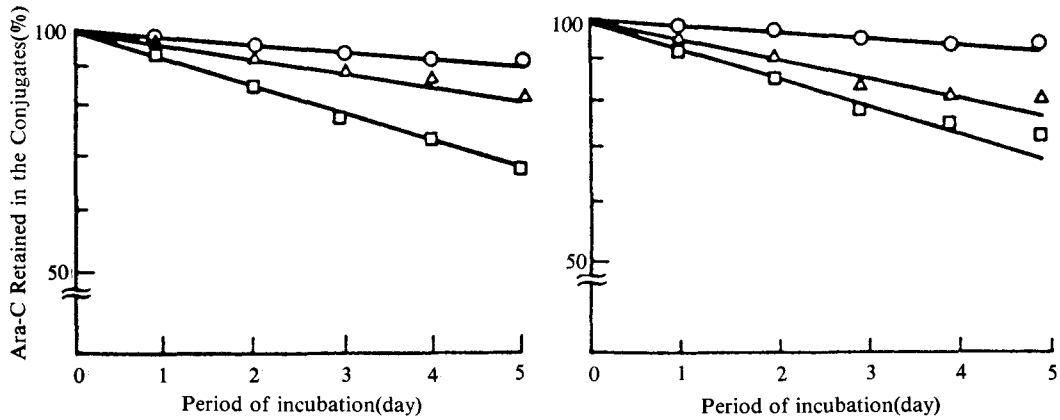


**Fig. 4. Effect of temperature on *in vitro* release of ara-C from PLGA-ara-C:I (left) and PLGA-ara-C:II (right), respectively, in pH 7.4 phosphate buffer solution.**

key; ○ – ○ : 24 ± 1 °C, △ – △ : 37 ± 1 °C, □ – □ : 50 ± 1 °C.

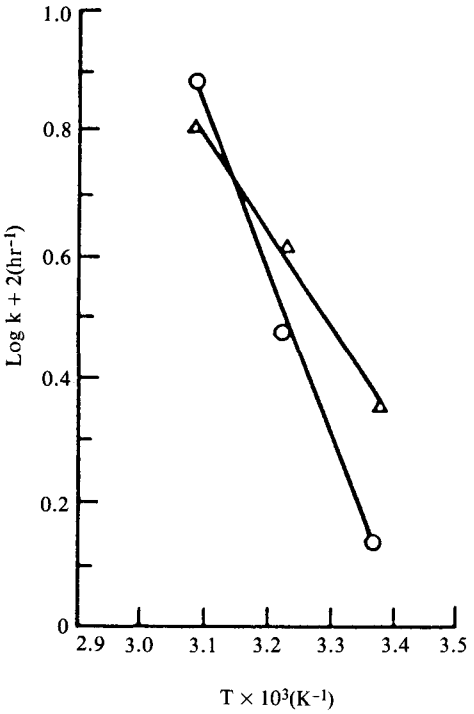


**Fig. 5. Arrhenius plot for the release of are-C from the conjugates.**
key; ○－○ : PLGA-ara-C:I, △－△ : PLGA-ara-C:II.

was significantly increased with the addition of 30 mg of protease; 16.5 and 20.1% of ara-C was released from PLGA-ara-C:I and PLGA-ara-C:II, respectively after 8 hrs of incubation (Fig. 6). From the data in Table I, the time required for the release of corresponding amount of ara-C from each types of conjugates in the absence of protease was expected to be 6.0 and 5.4 days, respectively.

From our experiments, it was revealed that PLGA-ara-C conjugates would be a potent macromolecular prodrug which release parent drug slowly by both enzymatic cleavage and hydrolysis, and the molecular weight of PLGA would be an important factor affecting the release rate. Although the PLGA-ara-C:I and PLGA-ara-C:II had similar substitution ratio, they showed different release rates. The release rates of PLGA-ara-C:II were about 2 times higher than those of PLGA-ara-C:I. This characteristic effect of molecular weight on *in vitro* release of ara-C from PLGA-ara-C conjugates suggests that the drug release rate might be effectively controlled over a prolonged period by the combined use of the different types of conjugates having different molecular weights.

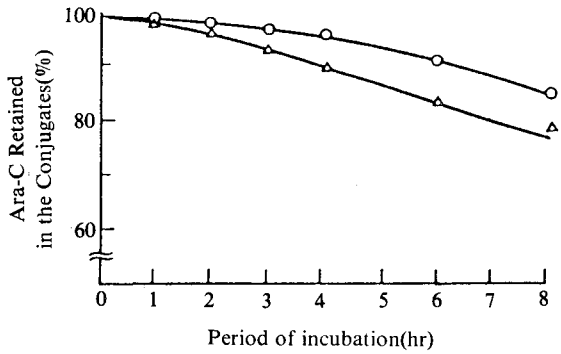


**Fig. 6. Effect of protease on *in vitro* release of ara-C from the conjugates in pH 7.4 phosphate buffer solution at 37 ± 1 °C.**
key; ○－○ : PLGA-ara-C:I, △－△ : PLGA-ara-C:II.

Futhermore, from our molecular charge experiment, the possibility of the electrostatic interaction of PLGA-ara-C conjugates with cationic surfaces in the body would be expected, and this interaction would affect the activity and *in vivo* fate of the drug. *In vivo* studies are in progress in this laboratory to evaluate the effect of molecular weight on *in vivo* fate of PLGA-ara-C conjugates.

## ACKNOWLEDGEMENTS

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