# Biodistribution of Micelle-Forming Polymer–Drug Conjugates

**Glen S. Kwon,[1,2] Masayuki Yokoyama,[1,2] Teruo Okano,[1,2] Yasuhisa Sakurai,[1,2] and Kazunori Kataoka[1,3,4]**

*Received July 14, 1992; accepted December 18, 1992*

Polymeric micelles have potential utility as drug carriers. To this end, polymeric micelles based on AB block copolymers of polyethylene oxide (PEO) and poly(aspartic acid) [p(Asp)] with covalently bound Adriamycin (ADR) were prepared. The micelle forming polymer–drug conjugates [PEO-p(Asp(ADR)] were radiolabeled and their biodistribution was investigated after intravenous injection in mice. Long circulation times in blood for some compositions of PEO-p[Asp(ADR)] conjugates were evident, which are usually atypical of colloidal drug carriers. This was attributed to the low interaction of the PEO corona region of the micelles with biocomponents (e.g., proteins, cells). Low uptake of the PEO-p(Asp(ADR)] conjugates in the liver and spleen was determined. The biodistribution of the PEO-p[Asp(ADR)] conjugates was apparently dependent on micelle stability; stable micelles could maintain circulation in blood, while unstable micelles readily formed free polymer chains which rapidly underwent renal excretion. Long circulation times in blood of PEO-p(Asp(ADR)] conjugates are thought to be prerequisite for enhanced uptake at target sites (e.g., tumors).

**KEY WORDS:** polymeric micelles; drug delivery systems; cancer therapy; Adriamycin.

## INTRODUCTION

We have been investigating micelle-forming polymer–drug conjugates as drug carriers (1–5). PEO-p[(Asp(ADR)] conjugates were synthesized, and because of their amphiphilicity they were shown to adopt micellar structures (1,2). Micelles based on PEO-p[Asp(ADR)] conjugates are approximately 30 to 50 nm in diameter and exhibit apparent thermodynamic stability. For effective drug delivery, each block of the AB block copolymer fulfills a distinct role as part of the micellar structure. The corona region of the micelle (i.e., PEO) interacts with the biological milieu. PEO is known to impart protein and cellular stealth properties to surfaces and interfaces (6). Indeed, PEO-modified proteins (7), liposomes (8,9), and nanoparticles (10) have been shown effectively to inhibit reticuloendothelial system (RES) sequestration and prolong circulation times in blood. The poly(amino acid) block with bound ADR forms the inner core of the micelle and acts as a drug reservoir. Within the micellar core, drugs may be protected from inactivation. While in this case ADR was covalently bound, drugs may also be physically loaded within polymeric micelles.

The PEO-p[Asp(ADR)] conjugates exhibit a high activity against a variety of cancers relative to free ADR (2,3). We hypothesized that the high *in vivo* anticancer activity of the PEO-p[Asp(ADR)] conjugate was a result of stable circulation in blood which allowed for uptake at the target site. Previously, we have shown that the pharmacokinetic behavior of the PEO-p[Asp(ADR)] conjugates is drastically altered compared to free ADR (3). To establish mechanisms of the high *in vivo* anticancer activity, the biodistribution of PEO-p[Asp(ADR)] conjugates was investigated in mice. Several compositions of the conjugate were studied; the detailed description of the synthesis of PEO-p[Asp(ADR)] conjugates is given elsewhere (4). For some compositions of the PEO-p[Asp(ADR)] conjugates, stable circulation in blood and low sequestration by the RES system occurred.

## MATERIALS AND METHODS

### Materials

PEO was purchased from NOF, Japan. $^{14}$C-Benzylamine hydrochloride was purchased from Amersham, The Netherlands. ADR was kindly provided by Nippon Kayaku Co., Japan. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was purchased from Peptide Institute, Japan. Cellulose acetate dialysis membranes [molecular weight cutoff (MWCO), 1000] were obtained from Gelman, Ann Arbor, MI. Disposable ultrafiltration units (MWCO, 100,000) were obtained from Millipore, Bedford, MA. Sodium pentobarbital was purchased from Abbott, Chicago, IL. Liquid scintillation cocktail, Aquasol-2, was purchased from New England Nuclear, Tokyo. All other chemicals were of reagent grade.

### Methods

PEO-p(Asp(ADR)] conjugates of varying composition were synthesized in exactly the same manner as described elsewhere (4). Briefly, the benzyl L-aspartate *N*-carboxyanhydride was polymerized using α-methyl-ω-amino-PEO as an initiator to form the AB block copolymer. The AB copolymer was deprotected by alkaline hydrolysis to form the AB block copolymer of PEO and p(Asp). ADR was conjugated to the p(Asp) block via activation by EDC. The chemical structure of the PEO-p[Asp(ADR)] conjugate is shown in Fig. 1.

PEO-p[Asp(ADR)] conjugates were radiolabeled by $^{14}$C-benzylamine using EDC to activate the free carboxyl groups on the p(Asp) segment. Activation via EDC was done in the presence of the external nucleophile, $^{14}$C-benzylamine, to preclude *N*-acylurea formation. Briefly, 20 μCi of $^{14}$C-benzylamine hydrochloride (54 mCi/mmol) was added to 800 μL of dimethylformamide. Triethylamine, 1.3 equiv, was introduced with stirring. PEO-p[Asp(ADR)] conjugate, 200 μl of a 20 mg/mL equivalents of ADR solution, was

[1] International Center for Biomaterials Science, Research Institute for Bioscience, Science University of Tokyo, Yamazaki 2669, Noda-shi, Chiba 278, Japan.
[2] Institute of Biomedical Engineering, Tokyo Women's Medical College, Kawada-cho, Shinjuku-ku, Tokyo 162, Japan.
[3] Department of Materials Science and Technology and Research Institute for Bioscience, Science University of Tokyo, Yamazaki 2641, Noda-shi, Chiba 278, Japan.
[4] To whom correspondence should be addressed (see footnote 1).

0724-8741/93/0700-0970$07.00/0 

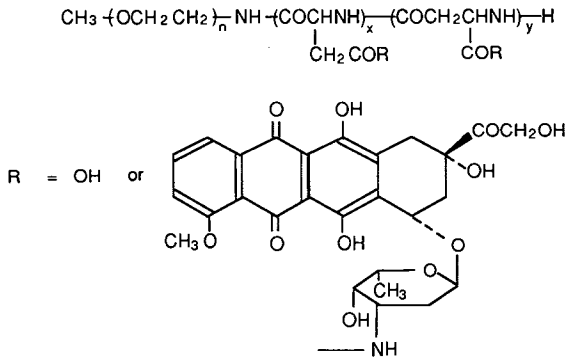


Fig. 1. Chemical structure of PEO-p(Asp(ADR)] conjugates.

added. Then 1.0 equiv of EDC was added to $^{14}$C-benzylamine; and the reaction allowed to proceed at 25°C for 4 hr, at which time an additional 1.0 equiv of EDC was added. The reaction was allowed to proceed overnight at 25°C with stirring. The reaction mixture was then dialyzed against 150 mL 0.067 *M* acetate buffer, pH 4.5, and subsequently with deionized water using a cellulose acetate dialysis membrane (MWCO, 1000). To ensure removal of free benzylamine, control experiments were carried out without the addition of EDC. The radiolabeled conjugate was then concentrated by ultrafiltration (MWCO, 100,000) and added to unlabeled conjugate to obtain a solution with 4.0 mg ADR equiv/mL and associated radioactivity of approximately 6.8 × $10^5$ dpm/mg ADR equivalents.

Biodistribution experiments were carried out using female ddy mice that were 6 to 7 weeks old. The mice were injected in the tail vein with $^{14}$C-PEO-p[Asp(ADR)] conjugate at a dose of 40 mg ADR equivalents/kg. The toxicity expressed at this dose was previously shown to be very low (3). Mice were sacrificed at 1, 4, and 24 hr. The mice were given 20-μL intraperitoneal injections of sodium pentobarbital (50 mg/mL) and approximately 1.0 mL blood collected from the abdominal aorta. A blood volume of 2.18 mL/25 g was assumed for the mice. Subsequently, major organs (i.e., liver, spleen, kidneys, heart, lungs, small intestine, stomach, and muscle) were excised. For this study, corrections were not made for residual blood in organs. The tissues were oxidized using an auto sample combustion instrument (Aloka ASC-113, Japan) and added to approximately 10 mL liquid scintillation medium composed of 9.0 mL liquid scintillation solution and 1.0 mL ethanolamine in methanol (1:2, v/v) prior to liquid scintillation counting. Radiolabeled conjugate was used as control to establish counting efficiency. Each composition of PEO-p[Asp(ADR)] conjugate was tested in four mice.

## RESULTS AND DISCUSSION

The compositions of the PEO-p[Asp(ADR)] conjugates used for the study are summarized in Table I. Evidence for micelle formation for the PEO-p[Asp(ADR)] conjugates was obtained by dynamic light scattering and gel permeation chromatography (GPC) (4). For the conjugate designated 1-40, two peaks were observed in the GPC elution profile; this was attributed to the presence of a considerable amount

Table I. Properties of Micelle-Forming PEO-p[Asp(ADR)] Conjugates

| Sample | MW PEO | MW p(Asp) | ADR substitution ratio (%)[a] | Diameter (nm)[b] | Yield of $^{14}$C-benzylamine (%)[c] |
|---|---|---|---|---|---|
| 1-40 | 1,000 | 4,800 | 12 | —[d] | 27 |
| 5-20 | 5,000 | 2,100 | 30 | 30 | 31 |
| 5-80 | 5,000 | 8,700 | 46 | 36 | 23 |
| 12-20 | 12,000 | 2,100 | 104 | 40 | 44 |

[a] With respect to aspartic acid residues.
[b] Determined by dynamic light scattering (number average).
[c] With respect to the initial amount of $^{14}$C-benzylamine used for coupling.
[d] Not detected above 10 nm.

of free polymer chains as well as micelles. Only one peak, at the void volume, was observed in the GPC elution profiles for the other compositions, and for 1-40 this indicated poor micelle stability. The PEO-p[Asp(ADR)] conjugates were radiolabeled at a high efficiency (Table I). The radiolabeled PEO-p[Asp(ADR)] conjugate contained approximately one benzylamine for every 40 ADR molecules. The protocol used to bind $^{14}$C-benzylamine to the PEO-p[Asp(ADR)] conjugates via amide bonds and form micellar structures is the same used for the conjugation of ADR to PEO-p(Asp) block copolymers. Amide bonds between molecules (e.g., drugs) and soluble polymers are hydrolytically stable and very slowly cleaved enzymatically unless spacer groups are introduced (11). For ADR, cleavage from the conjugate may not be a prerequisite for cytotoxicity (12). Without the addition of EDC, negligible incorporation of $^{14}$C-benzylamine (ca. <1%) in the PEO-p[Asp(ADR)] conjugates was determined. Retention of the PEO-p[Asp(ADR)] conjugates during ultrafiltration (MWCO, 100,000) suggested an elevated molecular weight of the micelle; this was consistent with the conjugates retaining a micellar structure after radiolabeling.

An initial biodistribution study of radioiodinated PEO-p[Asp(ADR)] conjugate, 5-20, ascertained 1 hr following intravenous injection, was reported previously (3). In this study, the biodistribution of several compositions of the PEO-p[Asp(ADR)] conjugate was investigated. For clarity, the biodistribution results of the PEO-p[Asp(ADR)] conjugates are presented in three classifications: (i) blood, (ii), liver and spleen, and (iii) other organs. The liver and spleen may be considered to approximate the RES. Figure 2 shows the level of the PEO-p[Asp(ADR)] conjugates in blood as a function of time. Long circulation times were determined for some compositions of PEO-p[Asp(ADR)] conjugate. Indeed, for 12-20, approximately 68 and 10% of the injected dose were present in blood at 4 and 24 hr, respectively. These results are consistent with long circulation times of PEO-modified proteins, liposomes, and nanoparticles. Low liver and spleen uptake (ca. 17 and 34% of injected dose/g organ at 24 hr, respectively) was determined for 12-20 (Fig. 3A). Alternatively, these values represent 28 and 3.5% of the injected dose, respectively. The polymeric micelles are retained in the vascular compartment due to the low interaction of the PEO corona region of the micelle with biocomponents and the elevated molecular weight of the mi-

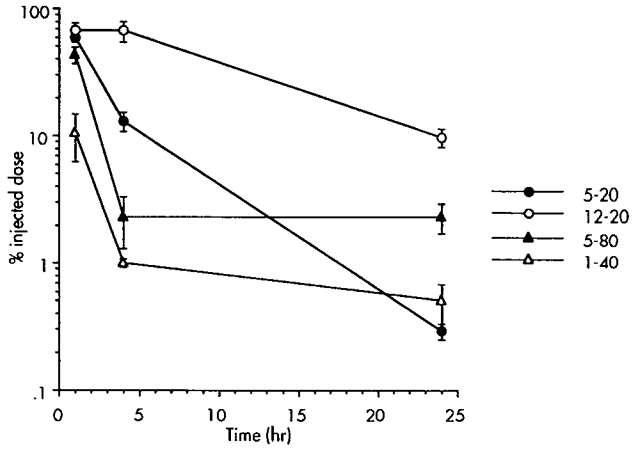


Fig. 2. The level of micelle-forming PEO-p[Asp(ADR)] conjugates in blood as a function of time. Mean ± SD.

celle, which inhibits renal excretion. For the other organs examined, other than the liver, spleen, and kidneys, 12-20 exhibited the highest tissue accumulation of the compositions examined, and the peak accumulations were attained at 4 or 24 hr (Fig. 3A). For the other compositions of the conjugate, maximum tissue concentrations were attained at 1 hr (Figs. 3B–D).

The stability of the micelle-forming PEO-p[Asp(ADR)] conjugates is in apparent contrast to low molecular weight amphiphiles. Polymeric micelles exhibit low critical micelle concentrations that can be determined by fluorescent probe techniques (13). On the other hand, critical micelle concentrations are equilibrium values, and perhaps more pertinent for the polymeric micelle behavior is the rate of dissociation of the polymeric micelle to free polymer chains. While low molecular weight amphiphiles typically exhibit liquid-like cores, chains within the core of polymeric micelles may exhibit high intermolecular interactions and a low mobility and, therefore, solid-like cores. For example, PEO–polystyrene block copolymers may exhibit cores which are glassy (14); the glass transition temperature of polystyrene is 80°C. For PEO-p[Asp(ADR)] conjugates, the inner core is stabilized by hydrophobic interactions and $\pi$–$\pi$ interactions of the ADR residues. For polymeric micelles, the rate of relaxation (i.e., dissociation to free polymer chains) may be slow and explain the stability of polymeric micelles in blood. Previously, the retention of the micellar form by sample 5-20 after circulation in the blood for 1 hr was evidenced by GPC (3). Further evidence for stable micelle circulation in the blood of sample 5-20 was obtained by ultrafiltration (MWCO, 100,000) of plasma samples taken from mice 4 hr after injection. The plasma was diluted 10-fold with isotonic phosphate buffer, pH 7.4, and the solution concentrated 10-fold twice by ultrafiltration. The solution retained above the membrane contained 90 ± 9% ($n$ = 4) of the $^{14}$C-labeled conjugate of the plasma, and the ultrafiltrate had 1.7 ± 1.1% ($n$ = 4). The results indicated that the conjugate circulated in blood predominately as micelles even after 4 hr and, further, that the radiolabel was bound to the conjugate.

As shown in Figs. 2 and 3C, 5-20 exhibited similar biodistribution behavior and blood concentrations at 1 hr as reported previously (3). At 4 hr, 13 and 2.3% of the injected dose in blood, for 5-20 and 5-80, was determined, respectively (Fig. 2). Concurrent with a faster decay in blood within 4 hr of the PEO-p[Asp(ADR)] conjugates 5-20 and 5-80, an increased uptake was seen by the liver and spleen and decreased uptake in other organs (Figs. 3A–C). Such a trend has been noted for specific liposomal formulations which exhibited prolonged circulation times in blood and enhanced tumor accumulation relative to most liposomes (15). For PEO-p[Asp(ADR)] conjugates, the balance in the block lengths affects micelle stability and interactions with biocomponents with relatively longer PEO block segments and shorter p(Asp) block segments being more favorable for long circulation times and low RES uptake. After longer time periods (ca. 24 hr); the micellar state of the PEO-p[Asp(ADR)] conjugates in blood was not clearly detectable.

For 1-40, relatively low blood circulation times were observed (Fig. 2); this is consistent with the poor micellar stability of this conjugate (4). This PEO-p[Asp(ADR)] conjugate is presumed readily to form free polymer chains in blood, circulate largely as free polymer chains, and undergo quick renal excretion; this was suggested by the observed red urine, indicative of ADR, excreted within 1 hr for mice injected with this sample, low liver and spleen uptake (ca. 10 and 4.5% of injected dose/g organ at 24 hr, respectively), and very low organ accumulation (Fig. 3D).

The mechanisms of extravasation of PEO-p[Asp(ADR)] conjugates and interaction with cells are not well understood. It is thought, because of the small size of the polymeric micelles, that extravasation of the micelle could occur directly. On the other hand, the possibility of transport of the free polymer chain across the vascular endothelia cannot be excluded. The interaction of PEO-p[Asp(ADR)] conjugates with cells is in progress.

## CONCLUSIONS

The prolonged circulation of PEO-p[Asp(ADR)] conjugates in blood was evidenced. Low sequestration of the PEO-p[Asp(ADR)] conjugates by the RES system was determined. PEO-p[Asp(ADR)] conjugates which could maintain their micellar form resulted in stable circulation; PEO-p[Asp(ADR)] conjugates which could not maintain their micellar form in blood underwent rapid renal excretion. Stable circulation in blood, by the micellar form, may result in enhanced uptake of the PEO-p[Asp(ADR)] conjugates at target sites. Future experiments will study the biodistribution of PEO-p[Asp(ADR)] conjugates in tumor-bearing mice and preparation of polymeric micelles bearing targeting moieties on the outer surface of corona region.

## ACKNOWLEDGMENTS

The first author would like to acknowledge support from the JSPS postdoctoral fellowship program. The authors would like to thank Shuji Kojima, Ph.D., and Yoshiyuki Koyama, Ph.D., Research Institute for Bioscience, Science University of Tokyo, Japan, for helpful discussions.

## REFERENCES

1. M. Yokoyama, M. Miyauchi, N. Yamada, T. Okano, Y. Sakurai, K. Kataoka, and S. Inoue. Polymeric micelle as novel carrier:

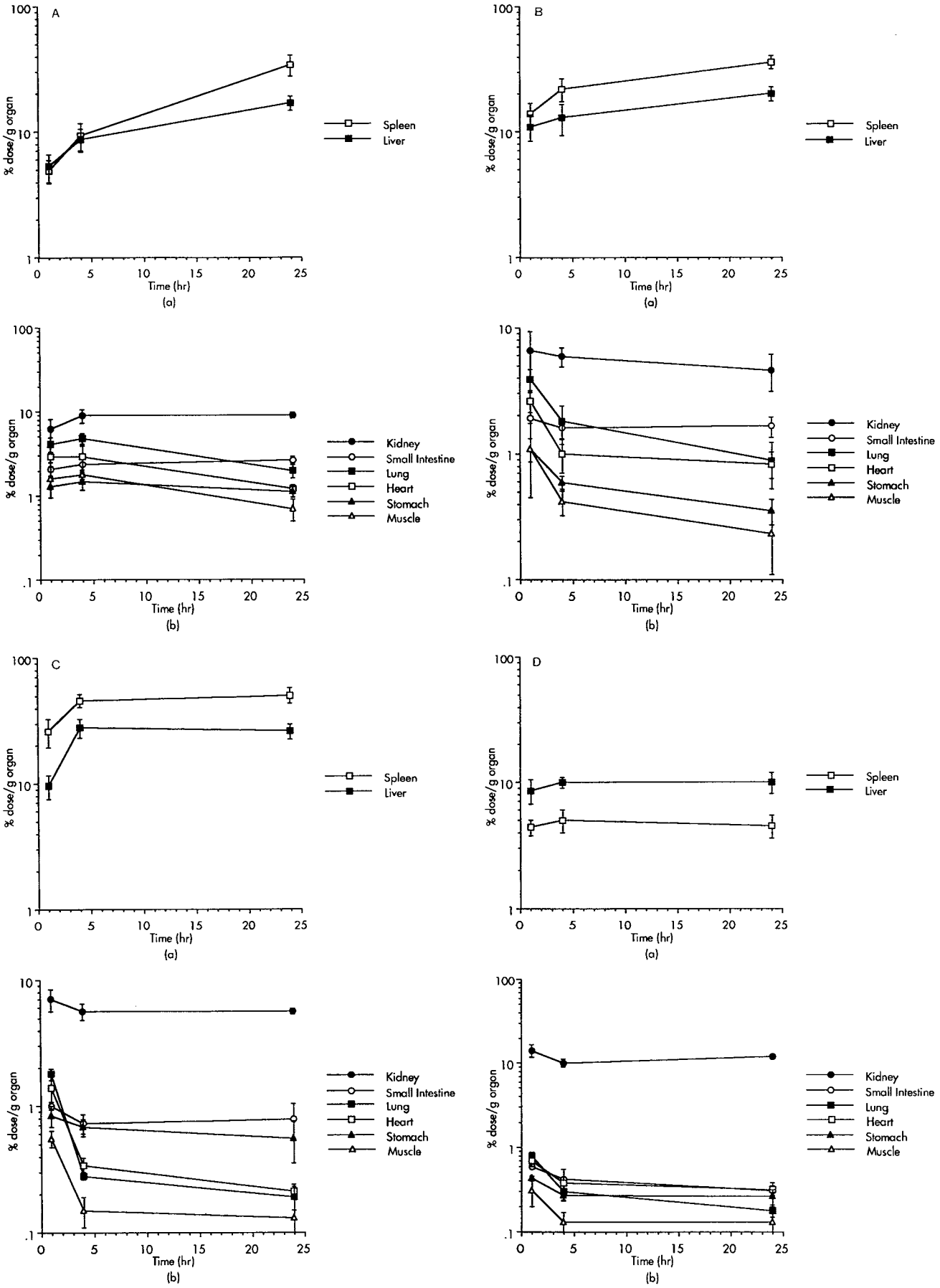


Fig. 3. The biodistribution of micelle-forming PEO-p[Asp(ADR)] conjugates as a function of time. A, B, C, and D are 12-20, 5-20, 5-80, and 1-40, respectively. (a) Liver and spleen; (b) other organs. Mean ± SD.

Adriamycin-conjugated poly(ethylene glycol)-poly(aspartic acid) block copolymer. *J. Control. Release* **11**:269–278 (1990).
2. M. Yokoyama, M. Miyauchi, N. Yamada, T. Okano, Y. Sakurai, K. Kataoka, and S. Inoue. Characterization and anticancer activity of micelle-forming polymeric anticancer drug adriamycin-conjugated poly(ethylene glycol)-poly(aspartic acid) block copolymer. *Cancer Res.* **50**:1693–1700 (1990).
3. M. Yokoyama, T. Okano, Y. Sakurai, H. Ekimoto, C. Shibazaki, and K. Kataoka. Toxicity and antitumor activity against solid tumors of micelle-forming polymeric anticancer drug and its extremely long circulation in blood. *Cancer Res.* **51**:3229–3236 (1991).
4. M. Yokoyama, G. Kwon, T. Okano, Y. Sakurai, T. Seto, and K. Kataoka. Preparation of micelle-forming polymer-drug conjugates. *Bioconjug. Chem.* 3:295–301 (1992).
5. M. Yokoyama, G. Kwon, T. Okano, Y. Sakurai, H. Ekimoto, K. Okamoto, T. Seto, and K. Kataoka. Optimization of micelle-forming polymeric drug. *Drug Target. Deliv.* (in press).
6. E. W. Merrill and E. W. Salzman. Polyethylene oxide as a biomaterial. *ASAIO J.* 6:60–64 (1983).
7. A. Abuchowski, T. van Es, N. C. Palczuk, and F. F. Davis. Alteration of the immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. *J. Biol. Chem.* **252**:3578–3581 (1977).
8. A. L. Klibanov, K. Maruyama, V. P. Torchilin, and L. Huang. Amphipathic polyethylene glycols effectively prolong the circulation time of liposomes. *FEBS Lett.* **268**:235–237 (1990).
9. M. C. Woodle, G. Storm, M. S. Newman, J. J. Jekot, L. R. Collins, F. J. Martin, and F. C. Szoka. Prolonged systemic delivery of peptide drugs by long-circulating liposomes: Illustration with vasopressin in the brattleboro rat. *Pharm Res.* 9:260–265 (1992).
10. L. Illum and S. S. Davis. The organ uptake of intravenously administered colloidal particles can be altered using a non-ionic surfactant (poloxamer 338). *FEBS Lett.* **167**:79–82 (1984).
11. R. Duncan and J. Kopechek. Soluble synthetic polymers as potential drug carriers. *Adv. Polym. Sci.* 57:51–101 (1984).
12. L. B. Wingard, T. R. Tritton, and K. A. Egler. Cell surface effects of adriamycin and carminomycin immobilized on crosslinked poly vinyl alcohol. *Cancer Res.* 45:3529–3536 (1985).
13. C. Zhao, M. A. Winnik, G. Riess, and M. D. Croucher. Fluorescence probe technique used to study micelle formation in water-soluble block copolymers. *Langmuir* **6**:514–516 (1990).
14. K. Prochazka, D. Kiserow, C. Ramireddy, Z. Tuzar, P. Munk, and S. E. Webber. Time-resolved fluorescence studies of the chain dynamics of naphthalene-labeled polystyrene-block-poly(methacrylic acid) micelles in aqueous media. *Macromolecules* **25**:454–460 (1992).
15. A. Gabizon and D. Papahadjopoulos. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. *Proc. Natl. Acad. Sci.* 85:6949–6953 (1988).