Efficient Intraliposomal Entrapment of Hydrophilic Platinum Oligonuclear Complexes

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ABSTRACT: A simple method for intraliposomal entrapment of platinum complexes is presented, where hydrophilic platinum oligonuclear complexes, 1-methyluracil green (MeUG), uridine green (UdG) and uridine blue (UdB), are included inside liposomes and allowed to react with bilayer lipids. The liposomes prepared in this method exhibit higher entrapment efficiency and higher distribution to organs (liver, kidney, spleen, lung) and blood (but not B16 cancer cells) than those prepared from mononuclear Pt complexes [*cis*-diamminedichloroplatinum, *cis*-diammine-1,1′-dicarboxylatocyclobutaneplatinum, and *cis*-dichloro-*cis*-dihydroxy-*trans*-bis(isopropylamine)platinum)].

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KEY WORDS: Intraliposomal entrapment, liposomes, platinum oligonuclear complex, tissue uptake of platinum complexes.

Liposomes are one of the promising delivery vehicles of drugs, where the most challenging target may be anticancer drugs. The use of conventional liposomes is, however, limited by instability in the bloodstream and removal by cells of the reticuloendothelial system (RES) largely in liver and spleen (1). Instability of liposomes in the bloodstream is thought to be due to the interaction with plasma high-density lipoproteins (HDL) (2,3). This problem can be solved, however, by some modification such as linking of poly(ethylene glycol) (PEG) on the surface of liposomes (4). On the other hand, Perez-Sole and Khokhar (5) studied so-called "intraliposomal drugs," where lipophilic *cis*-platin derivatives were incorporated to lipid bilayers instead of inner aqueous phase, and accomplished the depression of the leakage of platinum complexes.

As mentioned above, an intraliposomal platinum complex is intriguing as a drug delivery system where platinum is carried in a stable state. In the present study, we present a simple method to prepare intraliposomal platinum complexes. Our novel idea in this method is to use hydrophilic platinum oligonuclear complexes: *viz.*, uridine green (UdG) (6), uridine blue (UdB) (6), and 1-methyluracil green (MeUG) (7). It was reported that these complexes are relatively large in size [MeUG contains four platinum atoms (7) and UdG and UdB have more than four Pt atoms (8)] and quite hydrophilic owing to their high cationic charges. It is expected from these properties that the platinum oligonuclear complexes cannot pass through lipid bilayer membranes and thus do not leak from liposomes, even if the membrane is partly damaged by serum proteins. It is predicted, moreover, that the platinum oligonuclear complexes pooled in the inner aqueous phase of liposomes can be converted to lipophilic platinum complexes by the reaction with nucleophilic functional groups (e.g., carboxyl group) of bilayer lipids, since the leaving groups of the platinum oligonuclear complexes are known to be substituted by nucleophiles (9). This would further suppress the leakage of platinum complexes from liposomes.

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EXPERIMENTAL PROCEDURES

Materials. Phosphatidylcholine (PC), cholesterol (Ch), and phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabaster, AL). *cis*-Diamminedichloroplatinum (**I**) (CDDP) was commercially available from Aldrich Chemical Co. (Milwaukee, WI). *cis*-Diammine-1,1′-dicarboxylatocyclobutaneplatinum (**II**) (CBDCA) was from Strem Chemicals (Newburyport, CT). *cis*-Dichloro-*cis*-dihydroxy-*trans*-bis-(isopropylamine)platinum (**IV**) (CHIP) was from Johnson Matthey Technology Center (Royston, Hertfordshire, United Kingdom). Synthesis of oligonuclear platinum complexes, *viz.*, UdG, UdB, and MeUG, were reported previously (6,7). Sephadex G-50 was the product of Pharmacia Biotech (Uppsala, Sweden). Fetal calf serum (FCS) was obtained from Cell Culture Technology (Rexdale, Ontario, Canada). All other reagents were of the highest grade commercially available and used without further purification.

Tumor tissue (B16) was donated by Japan Foundation of Cancer Research, Tokyo. Female C57 BL/6 mice (6 wk) were obtained from Clea Japan, Inc. (Tokyo, Japan).

Preparation of liposomes. PC/Ch/PS (6:3:1 molar ratio) (30 μmol total) mixture was dissolved in chloroform in a test tube and dried under an N_2 gas stream and then under vacuum for 5 h to form a thin lipid film. PBS (phosphate buffer saline, pH 7.2) including the Pt complex (10 mg/mL) was prepared in another vessel. In using the above materials, we prepared liposomes by the following four methods.

Sonication (10). The lipid film was hydrated by vortexing

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on a vortex mixer with 1.0 mL of PBS including the Pt complex. The suspension was sonicated in a bath sonicator for 20 min at 65°C.

Reverse-phase evaporation (11). In this method, a thin lipid film was prepared in a round-bottomed flask. The thin lipid film was dissolved in 3.0 mL of diethyl ether and added to the 1.0 ml of PBS containing the Pt complex. The mixture was sonicated in a bath sonicator under N_2 for 5 min at 20°C and then diethyl ether was evaporated in a rotary evaporator. Through the above operations, liposome was formed in the aqueous solution.

Freeze-thaw (12). Multilamellar vesicles (MLV) were prepared by vortexing the dry lipid film in the presence of PBS including the Pt complex. Five freeze-thaw procedures were undertaken to achieve homogeneous mixtures. The MLV were extruded 10 times through two polycarbonate filters (pore size: 100 nm) to produce large unilamellar vesicles (LUV).

Dialysis (13). The lipid film was suspended again in 0.5 mL of a detergent solution (17.8 mg/mL cholic acid, pH 7.0) and added to the 0.5 mL of PBS including the Pt complex. The resultant micelle solution was dialyzed against PBS (500 mL × 3) for 20 h.

The liposomes prepared in the four methods were separated from nonencapsulated materials by gel filtration on Sephadex G-50 (column size, 1.0 × 25 cm; elution buffer, PBS, pH 7.2). The fraction corresponding to liposomes was collected and the amount of Pt entrapped in liposomes was measured by atomic absorption spectrometry. The percentage of entrapment efficiency was calculated as:

$$\%\text{ of entrapment efficiency} = \frac{\text{Pt entrapped in liposome}}{\text{total initial Pt}} \times 100 \quad [1]$$

Effect of serum on entrapment efficiency. FCS (500 μL) was added to the liposome solution (500 μL) prepared by the freeze-thaw procedure, and the mixture was incubated for 8 h at 37°C. After gel filtration by Sephadex G-50, the amount of Pt entrapped by liposomes was measured. The percentage of relative entrapment efficiency was calculated as follows:

$$\%\text{ of relative entrapment efficiency} = \frac{\text{Pt entrapped in liposome after incubation in serum}}{\text{Pt entrapped in liposome before incubation in serum}} \times 100 \quad [2]$$

Extraction of Pt to ether phase. Into the thin lipid layer (PC/Ch/PS = 6:3:1, total 34 μmol) prepared in a similar method to that described above, the PBS (1.0 mL) including 1.5 mg CDDP/mL, 1.0 mg UdG/mL, or 1.0 mg UdB/mL was added. The solution (0.8 mL) of liposomes containing the Pt complex, which was prepared by sonication and gel filtration, was mixed with ether (0.8 mL) using a vortex mixer for 3 min (1000 rpm). The ether layer was isolated and washed with PBS (0.7 mL) in a similar method. This washing operation was repeated two more times. The amount of Pt in the ether phase and in the total water phase was measured by atomic absorption spectrometry. The percentage of extracted fraction into an ether phase was calculated as:

$$\%\text{ of extraction to ether} = \frac{\text{Pt extracted into ether phase}}{\text{Total Pt extracted into ether and aqueous phases}} \times 100 \quad [3]$$

Biodistribution. Tumor implantation. The B16 tissue was cut into 5-mm pieces and rinsed once in cold culture medium, and then two randomly selected pieces were implanted in the right and left posterior flanks through incisions in the skin of mice using a biopsy trocar. The mice were used for biodistribution study on day 7 after implantation.

Biodistribution of Pt. The liposome solution (20–100 μL, PBS buffer) prepared by the freeze-thaw procedure was administered into the veins of mice with transplanted B16 under the skin. All urine was collected during the experiment. After 60 min, the whole amount of blood was estimated from the body weight of mice, and the organs (liver, kidney, spleen, and lung) including blood were excised and weighed. The blood and organs were decomposed in 10.0 mL of 60% nitric acid for 3–5 h at 180°C. The resultant nitric acid solution was concentrated to less than 1 mL, and the amount of Pt was measured. The percentage of biodistribution was calculated as:

$$\%\text{ of biodistribution} = \frac{\text{Pt distributed to tissue}}{\text{total initial Pt administered}} \times 100 \quad [4]$$

The platinum amount in urine, which is represented by Equation 5, was used as the relative distribution to urine:

$$\%\text{ of relative distribution to urine} = \frac{\text{Pt in urine on administration in liposome} - \text{entrapped form}}{\text{Pt in urine on administration in free form}} \times 100 \quad [5]$$

RESULTS

To investigate the dependence of the leakage of the Pt complexes on their size and charge, we measured the amount of Pt entrapped in liposomes which were prepared by the abovementioned four methods. Figure 1 indicates that, as a whole, the entrapment efficiency is higher in the oligonuclear complexes (UdG, UdB, MeUG) than in the mononuclear ones (CDDP, CBDCA, CHIP). Another noticeable feature is that the reversed-phase evaporation and the freeze-thaw procedures tend to give higher efficiency than the sonication and the dialysis techniques, irrespective of the type of Pt complexes. For example, in the former methods UdG, UdB, and

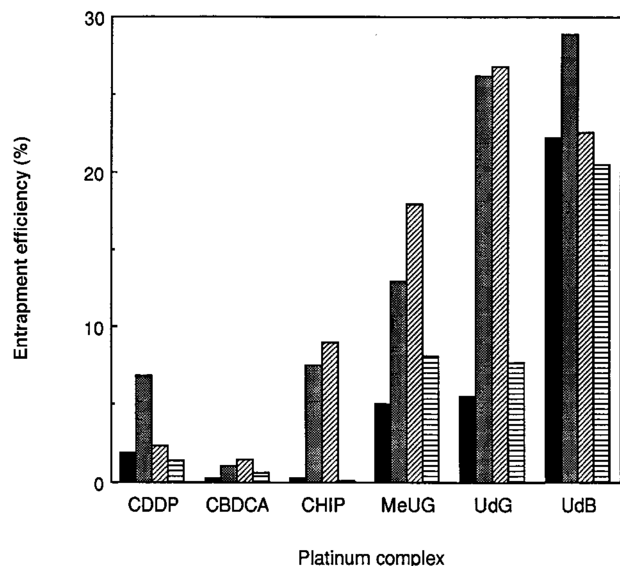


FIG. 1. Entrapment efficiency of platinum complexes in liposomes prepared by (solid box) sonication, (cross-hatched box) reversed-phase evaporation, (diagonally lined box) freeze-thaw, (horizontally lined box) dialysis. CDDP, diamminedichloroplatinum; CBDCA, *cis*-diammine-1,1′-dicarboxylatocyclobutaneplatinum; CHIP, *cis*-dichloro-*cis*-dihydroxy-*trans*-bis(isopropylamine)platinum; MeUG, methyluracil green; UdG, uridine green; UdB, uridine blue.

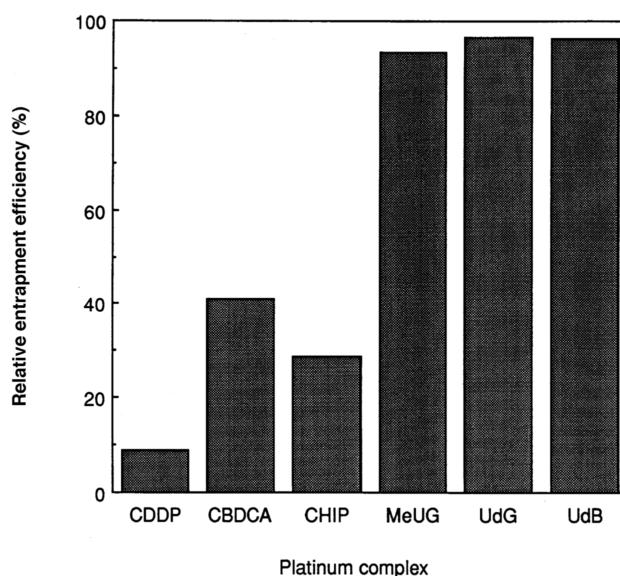


FIG. 2. Relative entrapment efficiency of platinum complexes in liposomes, which reflects the effect of serum proteins. For abbreviations see Figure 1.

MeUG, respectively, gave entrapment efficiencies 26–27, 23–29, and 13–18%, while in the latter methods the corresponding values were 6–8, 21–22, and 5–8%. In contrast to the oligonuclear complexes, the entrapment efficiencies for the mononuclear complexes were very low; namely, the reverse phase evaporation and the freeze-thaw procedures gave 2–7% (CDDP), 8–9% (CHIP), and 1–2% (CBDCA), and the sonication and the dialysis techniques 1–2% (CDDP), 0% (CHIP), and 0–1% (CBDCA).

The effect of serum proteins on the entrapment efficiency of the platinum complexes was investigated, and the results are depicted in Figure 2. We see that apparently the incubation in serum has almost no effect on the leakage of the platinum oligonuclear complexes, but it significantly decreases the relative efficiency of the mononuclear complexes. Namely, the relative efficiency remains more than 93% in MeUG, UdG, and UdB, while the efficiency is remarkably reduced in the mononuclear platinum complexes: 41% for CBDCA, 29% for CHIP, and 9% for CDDP.

The fraction of platinum complexes extracted into an ether phase was also examined, as shown in Figure 3. It should be noted here that platinum was extracted into an ether phase in a considerably high percentage when UdG and UdB were used. The extraction fractions were 93% for UdB, 67% for UdG, and 32% for CDDP. Since liposomes cannot be formed in an ether phase, the above results indicate that platinum should be extracted as some kind of hydrophobic complexes. Platinum atoms are probably coordinated by lipids composing bilayer membranes.

In order to apply this type of intraliposomal hydrophobic platinum complex to actual drug delivery systems, it is necessary to examine the biodistribution of Pt complexes. The percentage fraction of platinum accumulated in four organs (liver, kidney, spleen, lung), blood, and B16 cancer of mice was measured. In Figure 4 we compare the results obtained at 60 min after intravenous injection of three Pt complexes (UdB, UdG, CDDP) either in the liposome-entrapped form or in the free form. The ratio of platinum amount liberated to

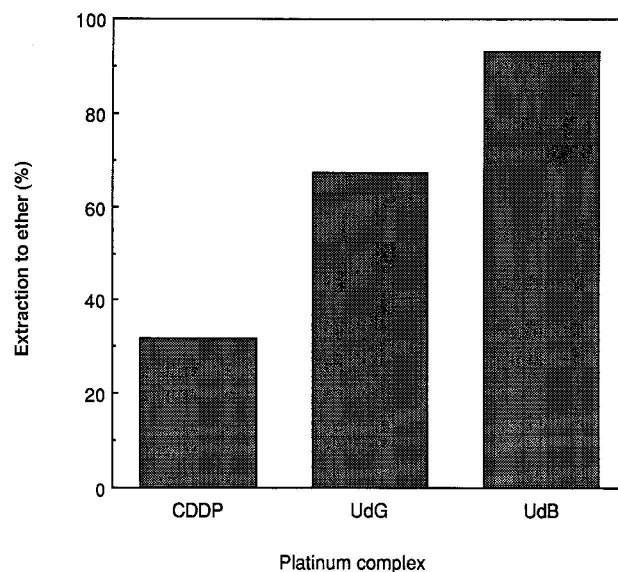


FIG. 3. Extraction of platinum complexes into ethyl ether. For abbreviations see Figure 1.

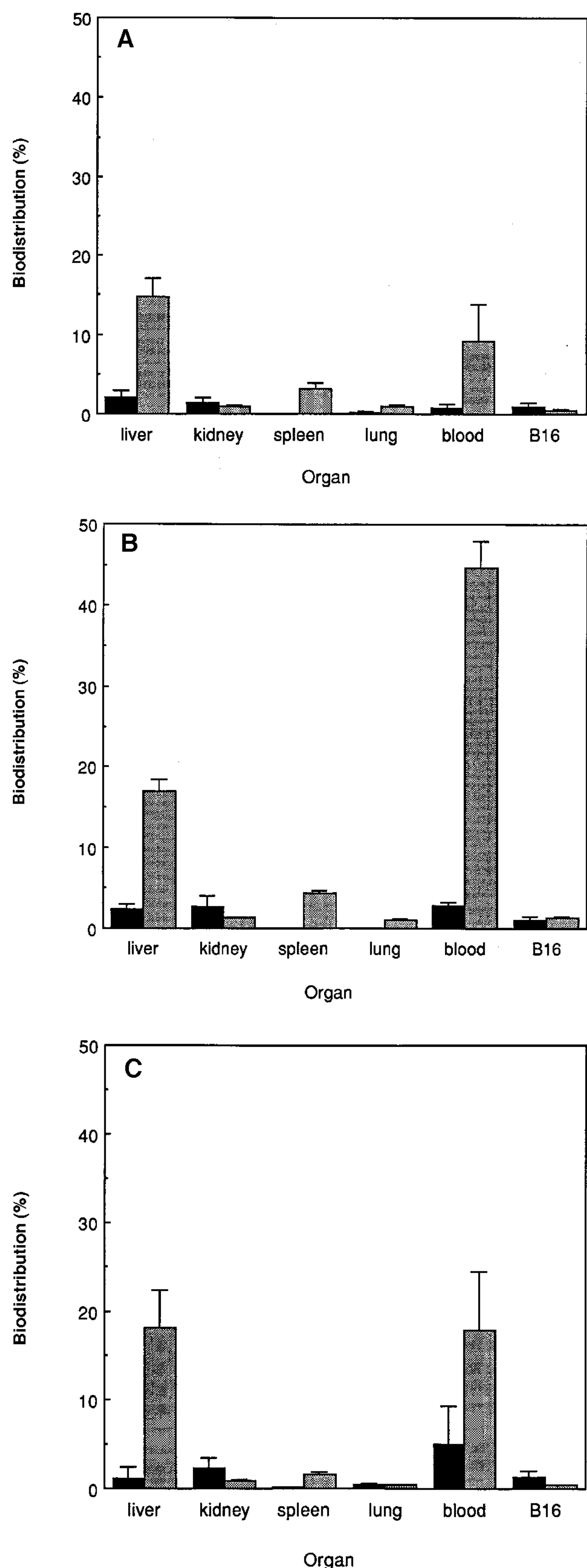


FIG. 4. Biodistribution of platinum complexes; (A) CDDP, (B) UdG, (C) UdB: (solid box) free platinum complexes; (cross-hatched box) liposome-entrapped platinum complexes. For abbreviations see Figure 1.

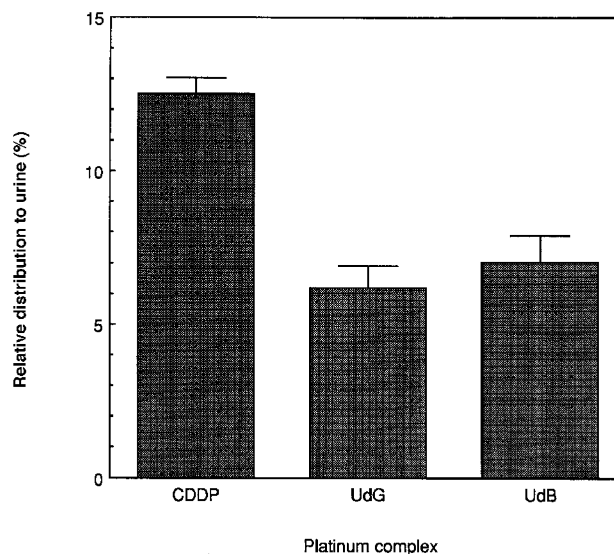


FIG. 5. Relative distribution of platinum complexes into urine. For abbreviations see Figure 1.

urine (liposome-entrapped complex vs. free complex) was also measured (Fig. 5). There is an obvious tendency that as a whole the liposome-entrapped Pt complexes are taken up by liver, spleen, lung, and blood more than the free forms, regardless of the type of platinum complex. It is also recognized that UdG and UdB are accumulated in a higher degree than CDDP. The distribution to blood is especially remarkable (45%) in the UdG-liposome system compared to the CDDP-liposome system (9%) and the UdB-liposome system (17.8%). This property is favorable for the application of liposome-entrapped drugs *in vivo*. In contrast to these organs, there are no prominent effects of the liposome entrapment on the uptake to B16 cancer cells, that is, the ratios of accumulated platinum (liposome-entrapped complex vs. free complex) are 0.54 for CDDP, 1.27 for UdG, and 0.31 for UdB. The overall suppression of the platinum liberation to urine by the effect of liposome-entrapment (Fig. 5) is compatible with the distribution to the organs and blood as shown in Figure 4.

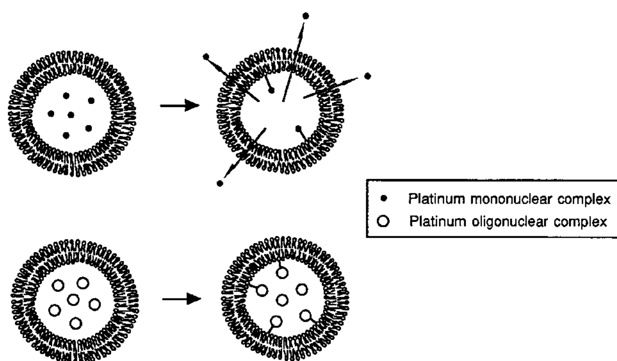
DISCUSSION

The most striking finding in this study is that the oligonuclear Pt complexes are intraliposomally entrapped in a higher yield than mononuclear ones. This probably can be explained from the standpoint of the size and charge of the Pt complexes, as follows. The molecular weights of UdG and UdB were estimated from the retention time in gel filtration (TSK gel G2500PWXL; eluted with a solution mixture of 10 mM H_2SO_4 and K_2SO_4; pH 6.0). Pt-α-pyrrolidone tan, which is a tetranuclear complex with molecular weight 1252 (14), was used for the calibration of molecular weight. The retention time of UdG, UdB, and Pt-α-pyrrolidone tan are, respectively, 15.3, 16–17.5, and 19.3 min, suggesting that UdG and UdB are larger than Pt-α-pyrrolidone tan and that UdB is larger

than UdG. The structure of MeUG was determined by X-ray structural analysis, which showed that it has four Pt atoms aligned in a zigzag chain like Pt-α-pyrrolidone tan, and the molecular weight of the tetranuclear unit is 1416 (7). Since the molecular weights of CDDP, CBDCA, and CHIP are, respectively, 300, 372, and 418, the molecular sizes are CDDP < CBDCA < CHIP < MeUG < UdG < UdB. The total charge of platinum complexes is another important factor affecting the leakage from liposomes. All the mononuclear platinum complexes investigated here do not have any charge, while MeUG has +5 charge and UdG and UdB are thought to have higher positive charges. This means that the magnitude of charge is CDDP = CBDCA = CHIP < MeUG < UdG < UdB. It is quite reasonable to consider that compounds with larger size and higher charge are more difficult to pass through hydrophobic lipid bilayer membrane. Probably this partly accounts for the experimental results of trapping efficiency (Fig. 1), effect of serum (Fig. 2), and biodistribution (Fig. 4).

If there is no interaction between the platinum complexes and the lipid components of liposomes, they should be extracted into an aqueous phase because of their high hydrophilicity. The extraction to an ether phase (Fig. 3), however, gave reverse results, which means that the platinum complexes included in the inner aqueous phase of liposomes do react with the nucleophilic functional groups of lipids and form hydrophobic platinum complexes. The carboxyl group of PS may be one of the possible candidates of nucleophiles. A proposed mechanism of the formation of intraliposomal platinum complexes is shown in Scheme 1. Most probably, in the mononuclear complexes without charge (upper case in Scheme 1) the complexes entrapped inside liposomes are appreciably leaked from liposomes, and only a small portion reacts with lipids. In contrast to the mononuclear complexes, the oligonuclear complexes with high positive charge (lower case in Scheme 1) probably remain inside liposomes more and thus react with lipids in a higher yield.

It has been revealed in the above discussion that the leakage of Pt complexes entrapped by liposomes can be strongly suppressed by converting the Pt complexes to intraliposomal forms. We next investigated biodistribution of platinum complexes and found the considerably pronounced increase in blood residence time by using liposome-entrapped platinum complexes compared to the free forms. This effect is especially prominent in UdG (Fig. 4B), where the percentage of biodistribution is, respectively, 2.5 and 4.8 times larger than those of UdB (Fig. 4C) and CDDP (Fig. 4A). The *in vivo* measurement, however, shows that there is not an expected effect of intraliposomal platinum complexes on the uptake to B16 cancer. This may be ascribed to the lack in recognition parts on the surface of liposomes and, consequently, the poor selectivity of binding to cancer cells. If some recognition parts toward cancer cells can be equipped on the surface of liposomes, they may open a new way to the development of cancer-targeting drug delivery systems. One of the interesting substances for such recognition parts may be oligopeptides, which have a possibility to bind selectively to cancer cells. Study along this line is now in progress and will be reported elsewhere.



SCHEME 1

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