Available online at www.sciencedirect.com

SCIENCE DIRECT®

Journal of Controlled Release 101 (2005) 223–232

journal of controlled release

www.elsevier.com/locate/jconrel

Preparation and biological properties of dichloro(1,2-diaminocyclohexane)platinum(II) (DACHPt)-loaded polymeric micelles

Horacio Cabral[a], Nobuhiro Nishiyama[b], Soichiro Okazaki[a], Hiroyuki Koyama[b], Kazunori Kataoka[a,*]

[a]*Department of Materials Science and Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan*

[b]*Department of Vascular Regeneration, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan*

Received 27 June 2004; accepted 26 August 2004
Available online 7 October 2004

Abstract

Block copolymer micelles, containing dichloro(1,2-diaminocyclohexane)platinum(II) (DACHPt), the oxaliplatin parent complex, were prepared through polymer–metal complex formation of DACHPt with poly(ethylene glycol)–poly(glutamic acid) block copolymer [PEG–P(Glu)] in distilled water. By dynamic light scattering (DLS) measurement, the micelle size was determined to be 40 nm with narrow distribution. The release of platinum complexes from the micelle core was measured in phosphate buffer saline (pH 7.4) at 37 °C. DACHPt-loaded micelle showed a sustained release rate of platinum after an induction period of 12 h. In the same conditions, the kinetic stability of DACHPt-loaded micelle was measured. The micelle was found to be very stable, keeping the initial size, for 240 h. Against murine colon adenocarcinoma 26 (C-26) cells, DACHPt-loaded micelle exhibited considerable in vitro cytotoxicity, lower than oxaliplatin but increasing with exposure time as a result of the release of platinum complexes from the micelle. In vivo biodistribution assay performed on tumor-bearing mice demonstrated that the micelle showed prolonged blood circulation due to its high stability and high tumor accumulation for a prolonged time.

Keywords: Polymeric micelle; Drug delivery system; Dichloro(1,2-diaminocyclohexane)platinum(II); Cancer chemotherapy; Controlled release

Abbreviations: C-26, colon adenocarcinoma 26; CDDP, *cis*-dichlorodiammineplatinum(II); DACH, diaminocyclohexane; DACHPt, dichloro(1,2-diaminocyclohexane)platinum(II); DMF, *N*,*N*-Dimethylformamide; EPR, enhanced permeability and retention; FDA, Food and Drug Administration; IC_{50}, 50% inhibitory concentration; ICP-MS, ion coupled plasma-mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MWCO, molecular weight cutoff; PBLG, poly(γ-benzyl L-glutamate); PEG, poly(ethylene glycol); P(Asp), poly(aspartic acid); P(Glu), poly(glutamic acid); RES, reticuloendothelial system.

* Corresponding author. Tel.: +81 3 5841 7138; fax: +81 3 5841 7139.
E-mail address: kataoka@bmw.t.u-tokyo.ac.jp (K. Kataoka).

0168-3659/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2004.08.022

1. Introduction

Colloidal carriers have been used for many years mainly as the modified formulation of drug molecules exhibiting low aqueous solubility. The discovery of new therapeutic agents has facilitated a demand for more sophisticated carrier systems which are able to protect agents from inactivation due to chemical or enzymatic degradation, migrate, and selectively accumulate at target sites in the body, thus enhancing the performance of the delivered agents. The recent progress in polymer science and nanotechnology certainly lends a strong basis to develop such colloidal carriers with high performance and modulated targetability.

Among these colloidal carriers, polymeric micelles have received significant attention as a promising supramolecular carrier system [1–5] due to their small size and stability, which lead to a prolonged blood circulation with reduced nonspecific accumulation in normal tissues and preferential accumulation in solid tumors by the enhanced permeability and retention (EPR) effect [6]. In addition to these exceptional properties, a high loading capacity of hydrophobic drug in the micelle core establishes polymeric micelle as a unique anticancer drug delivery system [7,8].

Previously, *cis*-dichlorodiammineplatinum(II) (cisplatin, CDDP; Fig. 1), a widely used anticancer drug, was incorporated into poly(ethylene glycol)–poly(amino acid) block copolymers [poly(aspartic acid) or poly(glutamic acid)] forming polymeric micelles [9]. The physicochemical and biological properties of this micelle were extensively studied [9–13], indicating that CDDP-loaded micelles are an effective delivery system for CDDP complexes.

CDDP shows acute dose-related side effects (such as nephrotoxicity, ototoxicity, neurotoxicity, nausea, vomiting, and mielosupression) and the appearance of intrinsic and acquired resistance [14]. Thus, since the discovery of CDDP in the mid-1960s, the enormous efforts to develop improved CDDP analogs had been devoted. Nevertheless, a limited number of these analogs reached their final approval. Among these compounds, dichloro(1,2-diaminocyclohexane)platinum(II) (DACHPt; Fig. 1) has shown wide and markedly different spectrum of activity than CDDP, such as lower toxicity than CDDP and no cross-resistance with CDDP in many CDDP-resistant

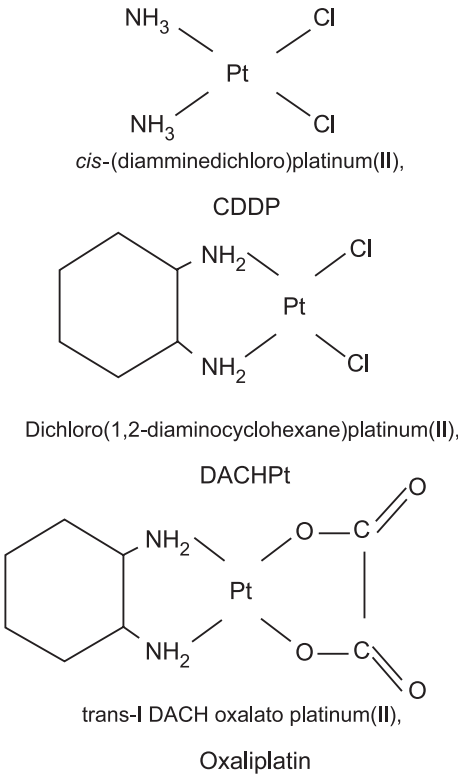

Fig. 1. Chemical structures of cisplatin, DACHPt, and oxaliplatin.

cancers [15–22]. However, the solubility of DACHPt in water is much lower than CDDP (0.25 mg/ml for DACHPt vs. 1.2 mg/ml for CDDP) [17]. With the purpose of enhancing water solubility of DACHPt, oxalate 1,2-diaminocyclohexane platinum(II) (oxaliplatin; Fig. 1) was develop by Kidani et al. in 1977 [16]. Oxaliplatin is a third generation platinum drug approved by the Food and Drug Administration (FDA) in the U.S.A. in 2002. Stability, solubility, formulation, and safety issues were more promising for oxaliplatin than for other diaminocyclohexane (DACH)-platinum compounds initially selected for preclinical testing and evaluated in early clinical trials. Oxaliplatin possesses an oxalate group, which is displaced by water and nucleophiles (such as Cl^- and HCO_3^- ions) in biological media to activate the drug, and it also has a nonhydrolysable diaminocyclohexane (DACH) ligand, which is maintained in the final active cytotoxic metabolites of the drug. From a pharmacological standpoint, the differential resistance of CDDP and oxaliplatin appears to be linked to the DACH ligand. This DACH ligand may induce DNA

lesions, which are poorly recognized by DNA recognition and repair pathways [23,24].

Although oxaliplatin shows better relative tolerability compared to other platinum compounds, a small number of side effects (like cumulative peripheral distal neurotoxicity and acute dysesthesias) restrain the range of working doses [25–28]. Recently, many drug delivery systems have been developed to increase oxaliplatin efficiency as well as to modify its pharmacokinetics. These drug delivery systems

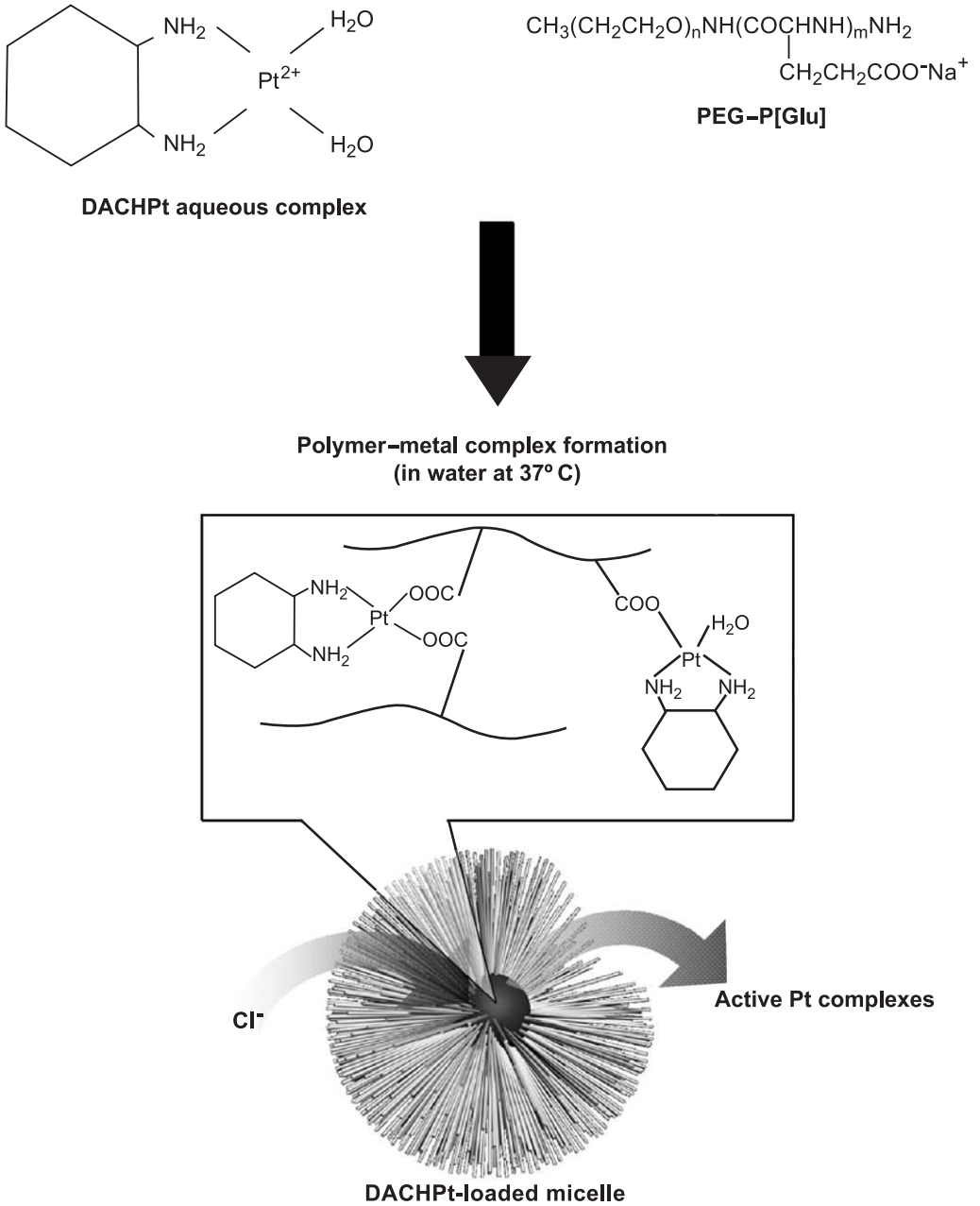

Fig. 2. Preparation of DACHPt-loaded micelles and release of drug in media containing chloride ion.

include microspheres [29], stealth liposomes [30], and macromolecular drugs [31]. The development of polymeric micelles loaded with DACHPt could lead to an oxaliplatin carrier with superior properties such as prolonged blood circulation or higher tumor accumulation. Thus, in the present study, novel polymeric micelles entrapping DACHPt in their core were prepared through the polymer–metal complex formation between DACHPt and poly(ethylene glycol)–poly(glutamic acid) block copolymers [PEG–P(Glu)] in distilled water after converting DACHPt to its aqueous complex by pretreatment with $AgNO_3$ (Fig. 2) to increase water solubility. Subsequently, the biological properties of DACHPt-loaded micelles were characterized. The kinetic stability of the carrier as well as the release rate of platinum complexes from the micelle core were evaluated in phosphate buffered saline solution. Moreover, in vivo experiments were performed on tumor-bearing mice to study the plasma clearance and the tumor accumulation of DACHPt-loaded micelles and free oxaliplatin. Finally, the in vitro cytotoxicity against murine colon carcinoma cell line was evaluated to compare the cytotoxicity of the DACHPt micellar system with that of free oxaliplatin.

2. Materials and methods

2.1. Materials

β-Benzyl L-glutamate was bought from Sigma Chemical (St. Louis, MO). Bis(trichloromethyl)carbonate (triphosgene) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). *N*,*N*-Dimethylformamide (DMF) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Wako Pure Chemical (Osaka, Japan). Dichloro(1,2-diamminocyclohexane)platinum(II) (DACHPt) and $AgNO_3$ were purchased from Aldrich Chemical (Milwaukee, WI). α-Methoxy-ω-aminopoly(ethylene glycol) (CH_3O-PEG-NH_2; M_w=12,000) was purchased from Nippon Oil and Fats (Tokyo, Japan). Murine colon adenocarcinoma 26 (C-26) cells were kindly supplied from the National Cancer Center (Tokyo, Japan). C-26 cells were maintained in RPMI 1640 medium (Sigma Chemical) containing 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.2. Preparation of PEG–P(Glu)

PEG–P(Glu) block copolymers were synthesized according to the previously described synthetic method of PEG–P(Asp) [9] with a slight modification. Briefly, the *N*-carboxy anhydride of γ-benzyl L-glutamate was synthesized by the Fuchs–Farthing method, using triphosgene. Then, *N*-carboxy anhydride of γ-benzyl L-glutamate was polymerized in DMF, initiated by the primary amino group of CH_3O–PEG–NH_2, to obtain PEG–poly(γ-benzyl L-glutamate) (PEG–PBLG) block copolymer. A narrow distribution (M_w/M_n: 1.06) of PEG–PBLG was confirmed by gel permeation chromatography [column: TSK-gel G3000HHR, G4000HHR (Tosoh, Yamaguchi, Japan); eluent: DMF containing 10 mM LiCl; flow rate: 0.8 ml/min; detector: refractive index (RI); temperature: 25 °C]. The polymerization degree of PBLG was determined to be 46 by comparing the proton ratios of methylene units in PEG (-OCH_2CH_2-: δ=3.7 ppm) and phenyl groups of PBLG (-$CH_2C_6H_5$: δ=7.3 ppm) in ^{1}H-NMR measurement [JEOL EX270 (JEOL, Tokyo, Japan) solvent: DMSO-d_6; temperature: 80 °C]. The deprotection of the benzyl group of PEG–PBLG was carried out by mixing with 0.5 N NaOH at room temperature to obtain PEG–P(Glu).

2.3. Preparation of DACHPt-loaded micelles

DACHPt (5 mM) was suspended in distilled water and mixed with silver nitrate ([$AgNO_3$]/[DACHPt]=1) to form the aqueous complex (Fig. 2). The solution was kept in dark at 25 °C for 24 h. AgCl precipitates were found after reaction. Next, the mixture was centrifuged at 3000 rpm for 10 min to eliminate the AgCl precipitates. Afterward, the supernatant was purified by passage through a 0.22-μm filter.

PEG–P(Glu) was added to the solution of DACHPt aqueous complex (polymer concentration: 2.1 mg/ml; [Glu]=5 mmol/liter; [DACHPt]/[Glu]=1.0) and reacted for 120 h to prepare DACHPt-loaded micelles. The prepared micelles were purified by ultrafiltration [molecular weight cutoff size (MWCO): 100,000]. The size distribution of DACHPt-loaded micelles was evaluated by dynamic light scattering (DLS) measurement at 25 °C using a Photal dynamic laser scattering spectrometer DLS-7000 (Otsuka Electronics, Osaka,

Japan). The Pt content in the micelles was determined by ion coupled plasma-mass spectrometry (ICP-MS, Hewlett Packard 4500).

2.4. Drug release in phosphate buffered saline

The release of the platinum from the micelles in phosphate buffered saline at 37 °C was evaluated by the dialysis method [Spectra/Por-6 (MWCO: 1000), Spectrum Laboratories, Rancho Dominguez, CA]. Briefly, a micellar solution of known platinum drug concentration was placed inside a dialysis bag and dialyzed against phosphate buffered saline (10 mM phosphate buffer, pH 7.4, plus 150 mM NaCl) at 37 °C. The released Pt outside of the dialysis bag was sampled at defined time periods and measured by ICP-MS.

2.5. Micellar kinetic stability in phosphate buffered saline

The kinetic stability of DACHPt-loaded micelles in phosphate buffered saline at 37 °C was evaluated by static light scattering measurement using a DLS-7000, according to the method reported previously [9]. Changes in the scattering light intensity (i.e., the Rayleigh ratio at 90° of the scattering angle) were measured at a defined time period. In this analysis, a decrease in the scattering light intensity is due to a decrease in the apparent molecular weight of the micelles as well as in the micelle concentration.

2.6. Platinum concentration in plasma and platinum accumulation in tumor

CDF_1 mice (female, n=6) were inoculated subcutaneously with C-26 cells (1×10^6). After 14 days, oxaliplatin and DACHPt-loaded micelles were administered i.v. at a dose of 100 μg/mouse on a platinum basis (tumor weight: 0.2±0.05 g; mean±S.D.). The mice were sacrificed after defined time periods (1, 5, 9, 27, 48, and 72 h). The tumor was excised, and the blood was collected from the inferior vena cava, heparinized, and centrifuged to obtain the plasma. The plasma and the tumor were decomposed on heating in nitric acid, evaporated to dryness, and redissolved in 2 N hydrochloric acid solution. The Pt concentration in the solution was measured by ICP-MS.

2.7. In vitro cytotoxicity

Fifty percent growth inhibitory concentration (IC_{50}) of free oxaliplatin and DACHPt-loaded micelles on the C-26 cell lines was evaluated by MTT assay. C-26 cells (5000 cells) were cultured in RPMI 1640 containing 10% fetal bovine serum in a 96-well multiplate. Then, the cells were exposed to free oxaliplatin or DACHPt-loaded micelles for 48 or 72 h, and MTT solution was added. Cell viability was measured by the formed formazan absorbance at 570 nm.

3. Results and discussion

3.1. Micelle size and size distribution

The driving force for micelle assembly is the metal–polymer complex formation between the platinum of DACHPt and the carboxylic group of the P(Glu), as seen in Fig. 2 [9]. Once the micelles were formed, free platinum complexes and block copolymer were eliminated by dialysis and ultrafiltration. After micelles purification, the cumulant diameter of DACHPt-loaded micelles was measured by dynamic light scattering measurements. The size was determined to be 40 nm for the micelle prepared at [DACHPt]/[Glu]=0.75 with a considerable low cumulant polydispersity (μ_2/T^2=0.03). Note that the size of a colloidal drug carrier is a determinant feature of its fate in blood circulation and its biodistribution. The recognition by the reticuloendothelial system (RES) is known to be the principal reason for the removal of many colloidal drug carriers from the blood compartment. The size of a colloidal carrier is favorable to be below 200 nm, and its optimization may result in the extension of blood circulation. The sub-100 nm size as well as the hydrophilic PEG palisade surrounding the core are the substantial advantages of the micelles to avoid their uptake by the RES. Solid tumors display diverse pore cutoff size for transvascular transport, depending on the microenvironment and on their type [32–34]. Therefore, the dimension of a drug carrier critically affects its extravasation efficiency, and this efficiency varies for different tumors. Nevertheless, the size of the micelles (40 nm) is small enough to reasonably assume a high extravasation efficiency

regardless of the tumor type and conditions [32,33]. Also, it has been reported that small sized carriers with sizes under 100 nm can attain deeper penetration into poorly permeable tumors, and consequently, increased accumulation [35]. Thus, the size of DACHPt-loaded micelles appears to be optimal from the standpoint of tumor-targeted therapy.

The yield of platinum loading was 75% of the initial feed amount at [DACHPt]/[Glu]=0.75. However, it could be increased by augmenting the [DACHPt]/[Glu] molar ratio, as seen in Fig. 3, obtaining approximately 90% incorporation of fed drug. The size of the micelles could also be changed by the incorporation amount of drug to the poly(-glutamic acid) backbone. Fig. 4 shows a change in the cumulant diameter for DACHPt-loaded micelles against [DACHPt]/[Glu] molar ratio. The size ranged from 25 nm for a [DACHPt]/[Glu] molar ratio equal to 0.25 to 50 nm for a [DACHPt]/[Glu] molar ratio equal to 1.5. Here, the simple size control with narrow distribution in polymeric micelles is a unique attribute that may not be seen in other colloidal carriers.

3.2. Release of DACHPt complexes

In the case of physically encapsulated drugs in polymeric micelles, the release rate is controlled by diffusion out of the micellar core or by the disintegration of the micelles. On the other hand, when the drug and the polymer form conjugates, the bond between them has to be cleaved for drug release [36,37]. Thus, in chemically conjugated polymeric micelles, the mechanism for drug release can be summarized in three steps: initially, the molecules that activate the bond cleavage penetrate into the micelle core; next, the cleavage of the bonds between drug and polymer occurs; and finally, the drug diffuses out of the micelle. Additionally, if the micellar structure is sufficiently stable, drug release could even be delayed until the carrier reaches the target. The premature drug release from the carrier is an undesired event, which can lead to toxic effects due to the presence of free drug in the blood stream.

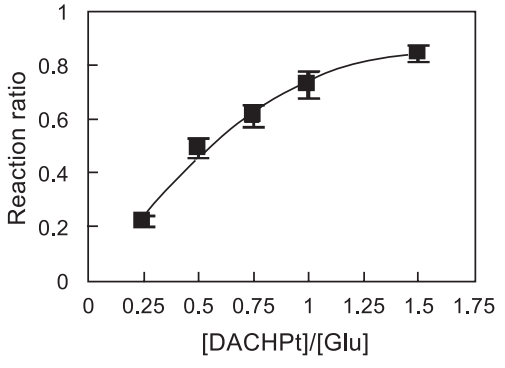

Fig. 3. Effect of [DACHPt]/[Glu] feeding molar ratios on the reaction ratio of Glu units in the block copolymer with DACHPt. Data are shown as the mean±S.D. (n=3).

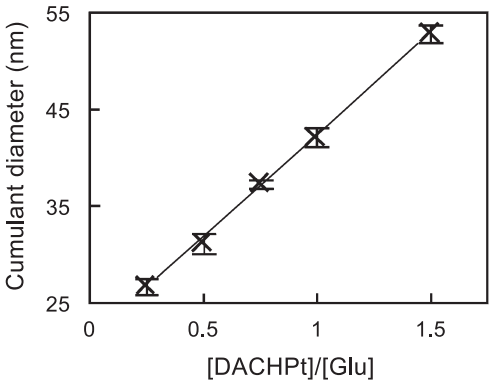

Fig. 4. Change in micelle size due to the variation of the [DACHPt]/[Glu] molar ratio measured by DLS at 25 °C. Data are shown as the mean±S.D. (n=3).

DACHPt-loaded micelles did not show any release of platinum complexes in distilled water (data not shown). However, in media containing chloride ions, platinum complexes could be released from the micelle core by exchange reaction between chloride ions and carboxylic groups of P(Glu) in the platinum complexes (Fig. 2). The release profile of platinum complexes from the DACHPt-loaded micelles is shown in Fig. 5. During the first 15 h of exposure, the micelles presented an induction period. This phase may be related to the hydrophobic nature of the core of DACHPt-loaded micelles, which may hamper the in/out diffusion of water, ions, and Pt. After the induction period, the micelles released the drug in a sustained way, and the release rate of DACHPt-loaded micelle was found to be similar to that of CDDP-loaded micelles [13]. Probably, after the induction period, enough amount of DACHPt–P(Glu) complexes has been cleaved by the chloride ions to augment the hydrophilicity of the micelle core and to facilitate the diffusion out of platinum complexes.

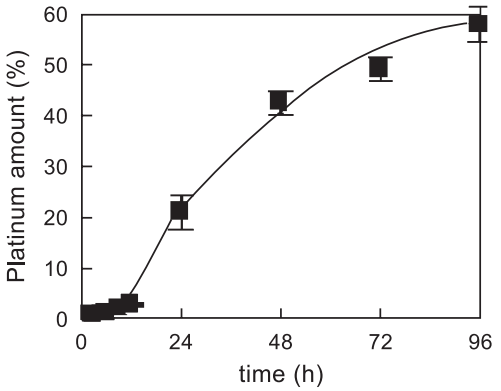

Fig. 5. Release profile of DACHPt from the micelles in phosphate buffered saline (pH 7.4) at 37 °C. The amount of platinum in the solutions was measured by ICP-MS. Data are shown as the mean±S.D. (n=4).

This unique profile of drug release owing to the induction period may still be rare in the other drug delivery systems for oxaliplatin active complexes [29,31] and might be advantageous for in vivo use because drug release is expected to be accelerated after the micelles reach the site of tumor.

3.3. Micelle kinetic stability

The kinetic stability of the micelle was measured by static light scattering. A decrease in the relative scattering intensity was considered to be owing to micellar disruption. The average diameter of the micelles was measured by DLS at the same time. The changes in the relative scattering intensity and the cumulant diameter of the micelle in phosphate buffered saline at 37 °C are shown in Fig. 6.

Inasmuch as the driving force of the formation of CDDP-loaded and DACHPt-loaded micelles is the polymer-metal complex formation, the release of platinum complexes may destabilize the micelle structure. Indeed, we previously reported that CDDP-loaded micelles were detected by DLS only for 50 h in phosphate buffer saline at 37° [13]. On the other hand, the stability of the DACHPt-loaded micelles was significantly elevated, and the size was maintained for 240 h, whereas the DACHPt-loaded micelles showed a comparable release rate to CDDP-loaded micelles (Fig. 5). This elevated stability of DACHPt-loaded micelles might be due to the bulky and hydrophobic nature of the DACH group in DACHPt [38]. Thus, once the micelle is formed, the higher hydrophobicity of the micellar core might lead to an increased kinetic stability, maintaining the micelles form for prolonged time period.

The elevated stability in biological media is a desired property of drug carriers because it is correlated with longer blood circulation; however, in many cases, increasing the stability of the carrier could signify diminishing or impeding the drug release. On the other hand, DACHPt-loaded micelles

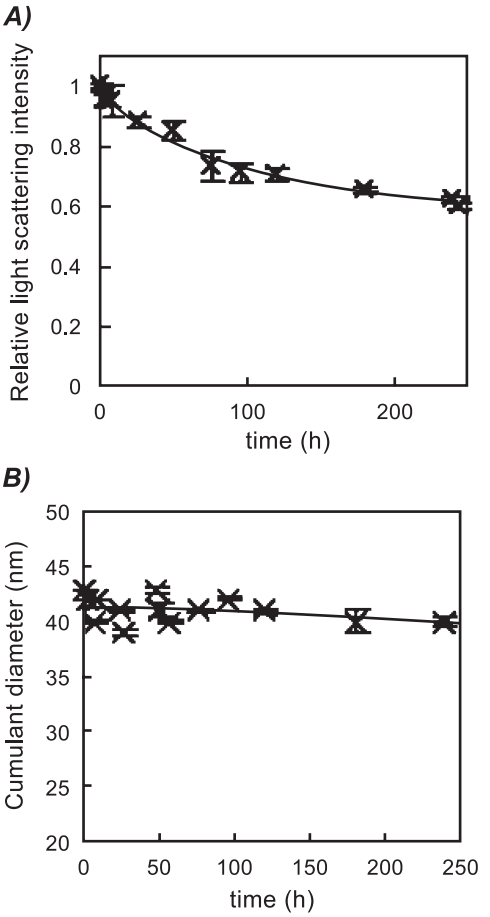

Fig. 6. Kinetic stability of DACHPt-loaded micelles in phosphate buffered saline at 37 °C. (A) Variation in the relative light scattering intensity of micellar solution (micelle concentration: 1.5 mg/ml) measured by SLS at a detection angle of 90° (phosphate buffered saline, 37 °C, pH 7.4). (B) Variation in the hydrodynamic diameter of micelle measured by cumulant approach of DLS. Data are shown as the mean±S.D. (n=3).

achieved high kinetic stability, which seems to be appropriate for systemic drug delivery, although the drug release from the micelles was not compromised.

3.4. Plasma clearance

Oxaliplatin elimination is mostly due to renal clearance. In clinical studies, it was observed that, at the end of a 2-h infusion of oxaliplatin, only 15% of the administered platinum was present in the systemic circulation. The plasma protein binding of platinum is irreversible, leading to inactivation of oxaliplatin, and is greater than 90%. Moreover, platinum binds irreversibly and accumulates in erythrocytes where it appears to have no relevant activity [22]. Thus, polymer conjugation of oxaliplatin will improve its blood circulation.

The platinum level in plasma after i.v. administration of free oxaliplatin or DACHPt-loaded micelles is shown in Fig. 7A. The DACHPt-loaded micelles showed remarkably prolonged blood circulation, whereas free oxaliplatin was cleared rapidly from circulation. Taking into account that the plasma volume in mice is 45.6 ml/kg [39], the platinum amount at 9 h for DACHPt-loaded micelle was determined to be over 80% of the injected dose and more than 16% even at 27 h after injection. We previously reported that CDDP-loaded micelles exhibited approximately 60% of the injected dose in plasma at 8 h and 13% of the injected dose at 24 h after injection [13]. The plasma Pt level of the micelles was appreciably augmented in the case of DACHPt-loaded micelles. It seems that the increased residence time of DACHPt-loaded micelles in the bloodstream is reasonably associated with the high kinetic stability of the micelles in phosphate buffered saline at 37 °C shown in Fig. 6.

3.5. Platinum accumulation in tumor

Selective and high accumulation of drug carriers at the tumor site is indispensable for drug targeting success. Accumulation of free oxaliplatin and DACHPt-loaded micelles in the solid tumor (C-26 cells) is shown in Fig. 7B. DACHPt-loaded micelles showed high and extended platinum levels at the tumor. Previously, we reported for CDDP-loaded micelles that the accumulation at tumor tissue took

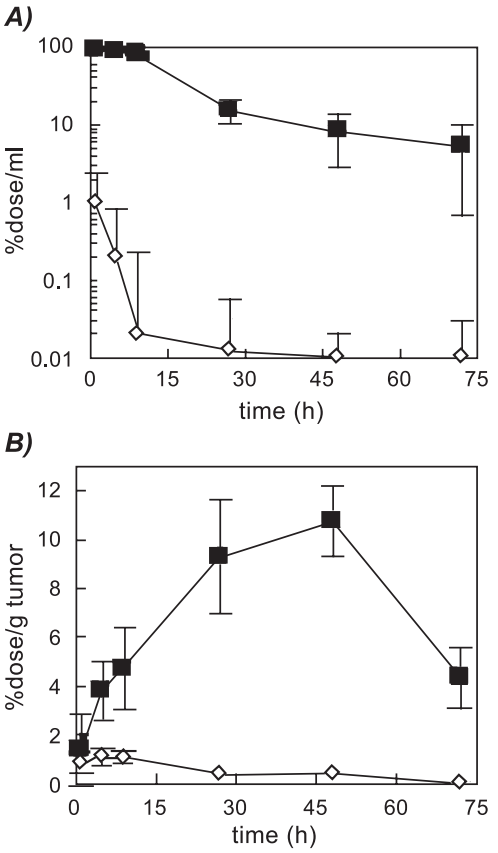

Fig. 7. Time profiles of platinum concentration in the plasma (A) and platinum accumulation in tumor (B) after i.v. administration of free oxaliplatin (◇) and DACHPt-loaded micelles (■). Each drug was administered to C-26-bearing CDF_1 mice (female, n=6) at a dose of 100 μg on Pt basis. Data are shown as the mean±S.D. (n=6).

maximum at 24 h with approximately 10% of the dose/g of tissue, and at that time, the platinum concentration in the blood was lower than 13% [13]. In the case of DACHPt-loaded micelles, the accumulation at the tumor at 24 h was similar to CDDP-loaded micelles. However, the former kept higher platinum concentration in plasma than the latter at the similar time range. Eventually, the maximum accumulation of DACHPt-loaded micelles was shifted to 48 h with 11% of the dose/g of tissue.

3.6. In vitro cytotoxicity

IC_{50} values are listed in Table 1 for DACHPt-loaded micelles and free oxaliplatin but also for CDDP-loaded micelles and free CDDP. Cytotoxicity

Table 1
In vitro cytotoxicity of free oxaliplatin, DACHPt-loaded micelles, free CDDP, and CDDP-loaded micelles against C-26 cell line

Incubation time (h)	IC_{50} (μM)[a]			
	Free oxaliplatin	DACHPt-loaded micelles	Free CDDP [12]	CDDP-loaded micelles [13]
48	8×10^{-4}	6×10^{-3}	8×10^{-3}	7×10^{-2}
72	2×10^{-4}	2×10^{-3}	2×10^{-3}	2×10^{-2}

[a] IC_{50} value obtained by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

of DACHPt-loaded micelles was approximately one order of magnitude lower than that observed for oxaliplatin, probably due to the slow release behavior of DACHPt complexes from the micelles. Nevertheless, DACHPt-loaded micelles showed comparable cytotoxicity to free CDDP and approximately 10 times higher than CDDP-loaded micelles [13] because DACHPt complexes are known to be much more cytotoxic than CDDP complexes [17]. This increased in vitro cytotoxicity of DACHPt-loaded micelles compared to CDDP-loaded micelles is promising for its in vivo application, considering that even the latter had an appreciable efficacy to cure solid tumors in experimental animals [13].

4. Conclusion

DACHPt-loaded micelles showed a size of 40 nm, which might be small enough to achieve extended blood circulation avoiding the uptake by the RES and to overcome the permeation barrier of poorly permeable tumors. DACHPt-loaded micelles showed considerable high stability, maintaining the size for 240 h in phosphate buffered saline, due to the highly hydrophobic core. Nevertheless, after an induction period of 15 h, the DACHPt complexes contained in the micelle core were released in a sustained way. In line with this release profile, the in vitro cytotoxicity assay against the C-26 cell line showed an appreciably lowered IC_{50} value after a prolonged incubation period of 72 h to expect high therapeutic index. Furthermore, prolonged blood circulation and increased tumor accumulation of DACHPt-loaded micelles compared to oxaliplatin were observed, facilitating their in vivo cytotoxicity against solid tumor, and a research in this direction is in progress in our laboratory.

Acknowledgement

This research was supported by the Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology of Japan as well as by the Core Research Program for Evolutional Science and Technology (CREST) from Japan Science and Technology (JST).

References

[1] A.V. Kabanov, V.P. Chekhonin, V.Yu. Alakhov, E.V. Batrakova, A.S. Lebedev, N.S. Melik-Nubarov, S.A. Arzhakov, A.V. Levashov, G.V. Morozov, E.S. Severin, V.A. Kabanov, The neuroleptic activity of haloperidol increases after its solubilization in surfactant micelles: micelles as microcontainers for drug targeting, FEBS Lett. 258 (1989) 343–345.
[2] M. Yokoyama, M. Miyauchi, N. Yamada, T. Okano, Y. Sakurai, K. Kataoka, S. Inoue, Polymer micelles as novel drug carrier: adriamycin-conjugated poly(ethylene glycol)–poly(aspartic acid) block copolymer, J. Control. Release 11 (1990) 269–278.
[3] K. Kataoka, G.S. Kwon, M. Yokoyama, Y. Sakurai, T. Okano, Block copolymer micelles as vehicles for drug delivery, J. Control. Release 24 (1993) 119–132.
[4] C. Allen, D. Mysinger, A. Eisenberg, Nano-engineering block copolymer aggregates for drug delivery, Colloids Surf., B Biointerfaces 16 (1999) 3–27.
[5] K. Kataoka, A. Harada, Y. Nagasaki, Block copolymer micelles for drug delivery: design, characterization and biological significance, Adv. Drug Deliv. Rev. 47 (2001) 113–131.
[6] Y. Matsumura, H. Maeda, A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent SMANCS, Cancer Res. 46 (1986) 6387–6392.
[7] G.S. Kwon, M. Naito, M. Yokoyama, T. Okano, Y. Sakurai, K. Kataoka, Physical entrapment of adriamycin in AB block copolymer micelles, Pharm. Res. 12 (1995) 192–195.
[8] K. Kataoka, T. Matsumoto, M. Yokoyama, T. Okano, Y. Sakurai, S. Fukushima, K. Okamoto, G.S. Kwon, Doxorubicin-loaded poly(ethylene glycol)–poly(β-benzyl-L-aspartate) copolymer micelles: their pharmaceutical characteristics and biological significance, J. Control. Release 64 (2000) 143–153.
[9] N. Nishiyama, M. Yokoyama, T. Aoyagi, T. Okano, Y. Sakurai, K. Kataoka, Preparation and characterization of self-assembled polymer–metal complex micelle from *cis*-dichlorodiammineplatinum(II) and poly(ethylene glycol)–pol-

y(α,β-aspartic acid) block copolymer in an aqueous medium, Langmuir 15 (1999) 377–383.
[10] N. Nishiyama, K. Kataoka, Preparation and characterization of size-controlled polymeric micelle containing *cis*-dichlorodiammineplatinum(II) in the core, J. Control. Release 74 (2001) 83–94.
[11] Y. Mizumura, Y. Matsumura, T. Hamaguchi, N. Nishiyama, K. Kataoka, T. Kawaguchi, W.J.M. Hrushesky, F. Moriyasu, T. Kakizoe, Cisplatin-incorporated polymeric micelles eliminate nephrotoxicity, while maintaining antitumor activity, Jpn. J. Cancer Res. 92 (2001) 328–336.
[12] N. Nishiyama, Y. Kato, Y. Sugiyama, K. Kataoka, Cisplatin-loaded polymer–metal complex micelle with time-modulated decaying property as a novel drug delivery system, Pharm. Res. 18 (2001) 1035–1041.
[13] N. Nishiyama, S. Okazaki, H. Cabral, M. Miyamoto, Y. Kato, Y. Sugiyama, K. Nishio, Y. Matsumura, K. Kataoka, Novel cisplatin-incorporated polymeric micelles can eradicate solid tumors in mice, Cancer Res. 63 (2003) 8977–8983.
[14] B.A. Chabner, C.E. Myers, in: V.T. DeVita, S. Hellman, S.A. Rosenberg (Eds.), Cancer: Principles and Practice of Oncology, 3rd ed., JB Lippincott, Philadelphia, 1989.
[15] T.A. Connors, M. Jones, W.C. Ross, P.D. Braddock, A.R. Khokhar, M.L. Lobe, New platinum complexes with antitumor activity, Chem.-Biol. Interact. 5 (1972) 415–424.
[16] Y. Kidani, K. Inagaki, R. Saito, S. Tsukagoshi, Synthesis and anti-tumor activities of platinum(II) complexes of 1,2-diaminocyclohexane isomers and their related derivatives, J. Clin. Hematol. Oncol. 7 (1977) 197–202.
[17] Y. Kidani, M. Iigo, K. Inagaki, A. Hoshi, K. Kuretani, Antitumor activity of 1,2-diaminocyclohexane-platinum complexes against sarcoma-180 ascites form, J. Med. Chem. 21 (1978) 1315–1318.
[18] Y. Kidani, M. Noji, T. Tashiro, Antitumor activity of platinum(II) of 1,2-diaminocyclohexane isomers, Gann 71 (1980) 637–643.
[19] O. Rixe, W. Ortuzar, M. Alvarez, R. Parker, E. Reed, K. Paull, T. Fojo, Oxaliplatin, tetraplatin, cisplatin and carboplatin: spectrum of activity in drug resistant cell lines and in the cell lines of the national cancer institute's anticancer screen panel, Biochem. Pharmacol. 52 (1996) 1855–1865.
[20] C.X. Zhang, S.J. Lippard, New metal complexes as potential therapeutics, Curr. Opin. Chem. Biol. 7 (2003) 1–9.
[21] M. Karatalou, J.M. Essigmann, Mechanisms of resistance to cisplatin, Mutat. Res. 478 (2001) 23–43.
[22] M.A. Graham, G.F. Lockwood, D. Greensdale, S. Brieza, M. Bayssas, E. Gamelin, Clinical pharmacokinetics of oxaliplatin: a critical review, Clin. Cancer Res. 6 (2000) 1205–1218.
[23] E. Raymond, S. Faivre, S. Chaney, J. Woynarowski, E. Cvitkovic, Cellular and molecular pharmacology of oxaliplatin, Mol. Cancer Ther. 1 (2002) 227–235.
[24] A.M. Di Francesco, A. Ruggiero, R. Riccardi, Cellular and molecular aspects of drugs of the future: oxaliplatin, Cell. Mol. Life Sci. 59 (2002) 1914–1927.
[25] J.L. Misset, H. Bleiberg, W. Shuterland, Oxaliplatin clinical activity: a review, Crit. Rev. Oncol./Hematol. 35 (2000) 75–93.
[26] S. Giachetti, B. Perpoint, R. Zidani, Phase III multicenter randomized trial of oxaliplatin added to chronomodulated fluorouracil-leucovorin as first line treatment of metastasic colorectal cancer, J. Clin. Oncol. 18 (2000) 136–147.
[27] J.M. Extra, M. Espie, F. Calvo, Phase I study of oxaliplatin in patients with advanced cancer, Cancer Chemother. Pharmacol. 25 (1990) 299–303.
[28] G. Mathé, Y. Kidani, M. Segiguchi, M. Eriguchi, G. Fredj, G. Peytavin, J.L. Misset, S. Brienza, F. de Vassals, E. Chenu, C. Bourut, Oxalato-platinum or l-OHP, a third generation platinum complex: an experimental and clinical appraisal and preliminary comparison with *cis*-platinum and carboplatinum, Biomed. Pharmacother. 43 (1989) 237–250.
[29] F. Lagarce, O. Cruaud, C. Deuschel, M. Bayssas, G. Griffon-Etienne, J.P. Benoit, Oxaliplatin loaded PLAGA microspheres: design of specific release profiles, Int. J. Pharm. 242 (2002) 243–246.
[30] I. Han, O.J. Kim, G.Y. Lee, Y.K. Sung, R. Song, Y.S. Sohn, Enhanced antitumor activity of *trans*(±)-1,2-diaminocyclohexane glutamatoplatinum(II) with stealth liposome, Bioorganic Med. Chem. 11 (2003) 5443–5447.
[31] A. Furin, A. Guiotto, F. Baccicheti, G. Pasut, C. Deuschel, R. Bertani, F.M. Veronese, Synthesis, characterization and preliminary cytotoxicity assays of poly(ethylene glycol)-malonato-Pt-DACH conjugates, Eur. J. Med. Chem. 38 (2003) 739–749.
[32] V. Weissig, K.R. Whiteman, V.P. Torchilin, Accumulation of protein-loaded long circulating micelles and liposomes in subcutaneous lewis lung carcinoma, Pharm. Res. 15 (1998) 1552–1556.
[33] S.K. Hobbs, W.L. Monsky, F. Yuan, W.G. Roberts, L. Griffith, V.P. Torchilin, R.K. Jain, Regulation of transport pathways in tumor vessels: role of tumor type and microenvironment, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 4607–4612.
[34] O. Ishida, K. Maruyama, K. Sasaki, M. Iwatsuru, Size-dependent extravasation and interstitial localization of polyethyleneglycol liposomes in solid tumor-bearing mice, Int. J. Pharm. 190 (1999) 49–56.
[35] A. Lukyanov, Z. Gao, L. Mazzola, V.P. Torchilin, Polyethylene glycol-dyacyllipid micelles demonstrate increased accumulation in subcutaneous tumors in mice, Pharm. Res. 19 (2002) 1424–1429.
[36] C. Allen, D. Maysinger, A. Eisenberg, Nano-engineering block copolymer aggregates for drug delivery, Colloids Surf., B Biointerfaces 16 (1999) 3–27.
[37] A. Lavasanifar, J. Samuel, G.S. Kwon, Poly(ethylene oxide)-block-poly(L-amino acid) micelles for drug delivery, Adv. Drug Deliv. Rev. 54 (2002) 169–190.
[38] J.A. Platts, D.E. Hibbs, T.W. Hambley, M.D. Hall, Calculation of the hydrophobicity of platinum drugs, J. Med. Chem. 44 (2000) 472–474.
[39] F.G. King, R.L. Dedrick, Physiological pharmacokinetic parameters for *cis*-dichlorodiammineplatinum(II) (CDDP) in mouse, J. Pharmacokinet. Biopharm. 20 (1992) 95–99.