Uptake of CDDP-containing Polymeric Micelles by Cells Using Particle Induced X-Ray Emission

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Drug Delivery System/CDDP/Polymeric micelle.

Polymeric micelles loaded with cis-diamminedichloroplatinum(II), CDDP, (cisplatin micelles) enable higher accumulation in solid tumors and lower toxicities compared with CDDP alone. The combined use of cisplatin micelles with radiation is expected to enhance therapeutic effects and reduce side effects. The kinetics of cisplatin micelle uptake, however, have not been fully understood. Particle Induced X-Ray Emission has been employed in this study to measure the time transients of platinum in Chinese Hamster ovary cells. The results show that the platinum content of cells treated with cisplatin micelles increased more slowly than with CDDP alone, suggesting that cellular uptake could be controlled using micelles. The CDDP released from micelles was predominantly incorporated into the cells by diffusion. The uptake characteristics were further analyzed using micelles with different collapse rates. The results and techniques used in this study will be useful for designing an optimum treatment plan combining platinum-containing polymeric micelles and radiation in clinical applications.

INTRODUCTION

Recent progress in nanotechnology enables delivery of highly functionalized target drugs in a so-called Drug Delivery System (DDS), which attempts to regulate the kinetics of the drugs. Liposomes and polyethylene glycol (PEG) have been found to be efficient materials for DDS and some drugs using these materials are already commercially available. Polymeric micelles are one of the most promising drug carriers, composed of amphiphilic polymers that can assemble into micelles with a diameter of several tens of nanometers. The micelles can carry a hydrophobic drug inside the core, surrounded by an outer shell such as PEG. They are proven to achieve long circulation in the blood stream and effective accumulation in tumors, reducing side effects. However, they still have unfavorable distribution into normal tissues, albeit much less so than conventional drugs. To achieve further minimization of the side effects, a combination of DDS and radiation therapy has been proposed. The idea of combining physical energy and targeted drugs has been successful in photodynamic therapy (PDT), as reported by Kataoka and his group. PDT, however, utilizes visible light for excitation of the drugs and is applicable only on tumors very near the surface of the skin (1 to 3 cm). Radiation is required for deeper cancers. One of the most well-known radio sensitizers is cis-diamminedichloroplatinum(II) (CDDP), which is a key anticancer drug used in current chemotherapy for several types of cancer, including lung, esophagus, head and neck, and bladder cancers. It is hypothesized that the arrest of the cell cycle by DNA cross-linking by CDDP increases radio-sensitivity.

Similar to other anti-cancer drugs affecting DNA synthesis and function, CDDP has severe side effects, including neurotoxicity and nephrotoxicity. Aiming to reduce the side effects, Nishiyama et al. developed CDDP-containing polymeric micelles, or cisplatin micelles, which achieved a 20-fold increase in accumulation in solid tumors and prolonged circulation in the blood stream, exceeding 24 hours. It is expected that the combination of radiation and cisplatin micelles will be more effective than that of radiation and CDDP alone. To maximize their anti-cancer effect, it is necessary to understand the kinetics of cisplatin micelles, especially their incorporation through the cellular membrane. The timing of irradiation after administration of cisplatin micelles should be carefully designed to optimize the therapeutic effect, based on uptake kinetics.

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The detection of cisplatin micelles, however, is difficult due to the small molecular weight of CDDP. Modification with fluorescence dye has been attempted but this does not necessarily show the same kinetics as CDDP itself. Another approach is to use an isotope, but the synthesis of cisplatin micelles with isotopes is very difficult. To date, accumulations of cisplatin micelles in tumors, kidney and liver have been studied by inductively-coupled plasma mass spectrometry (ICP-MS). In this study, we used the particle induced X-ray emission (PIXE) analysis technique for quantification of platinum, which is a component of CDDP. The PIXE method has several advantages compared with the ICP-MS method, such as easy sample preparation and non-destruction. The aim of this study was to evaluate the uptake characteristics of cisplatin micelles by measuring the amount of platinum inside cells treated under various conditions.

MATERIALS AND METHODS

Chemicals

Both cis-diammine-dichloroplatinum(II) (CDDP) (Randa Inj., Nippon Kayaku, Tokyo) and cisplatin micelles (NC-6004, NanoCarrier Co., Chiba) were obtained from NanoCarrier Co. Cisplatin micelles are composed of an inner core of CDDP and an outer shell of polymer with an approximate diameter of 30 nm. Preparation and the properties of the cisplatin micelles were previously reported by Nishiyama et al.

Cell line and culture

Chinese hamster ovary (CHO) cells used in this study were kindly supplied by Dr. Okayasu (NIRS, Chiba), which had been gifted by Dr. Bedford (Colorado State University, USA). The cell medium was minimum essential alpha medium (MEM Alpha, 12571-063, GIBCO), supplemented with 10% fetal bovine serum (SH30396.D3, HyClone) and antibiotics (Antibiotic-Antimycotic, penicillin 10,000 unit/mL and streptomycin 10,000 μg/mL, 15240-062, GIBCO), and the cells were grown in plastic flasks at 37°C in a humidified atmosphere comprising 5% CO2 in air. The cells were regularly sub-cultured to 1/10-1/20 concentrations before the cells reached a confluent condition. During sub-culturing, the cells were rinsed with Dulbecco’s Phosphate Buffered Saline (PBS) (D8537, SIGMA) and treated with trypsin-EDTA solution (0.05% trypsin, 0.02% EDTA, T3924, SIGMA).

Sample preparation

For samples used in conventional PIXE analysis, the cells were plated onto plastic dishes with diameters of 10 cm (353003, FALCON), filled with 10 mL of media. Two dishes were prepared for each condition; one for PIXE analysis and the other for counting the number of the cells. After the cells were exponentially grown and reached 2.5 × 106 cells per dish, 250 μM of CDDP and cisplatin micelles were added to 5 mL of media and stored at 37°C in a humidified atmosphere comprising 5% CO2 in air. The exposure time was 24 hours for both CDDP and cisplatin micelles. After the exposure, the media was extracted and the cells were rinsed twice with PBS. The cells were collected by scraping from the dish into a solution. The total amount of cell solution collected was approximately 150 μL and 50 μL of it was used for PIXE analysis. This cell-containing solution was dropped onto a carbon tape (731, Electrically conductive double-sided carbon tape for SEM (5 mm × 20 m), Nissin EM Co., Tokyo) attached to a sample holder and dried under a white-hot lamp installed in a vacuum chamber. The media extracted from the dishes were also inoculated on carbon tape for PIXE analysis. All the procedures for the sample preparation were carefully conducted so as not to contaminate with substances floating in air.

To obtain the time transient of uptake of CDDP, the exposure time for CDDP was varied from 3 to 24 hours (3, 8, 12, 16, 20, 24 hours). To verify the relation between collapse of the micelles and uptake of CDDP, cisplatin micelles were mixed with fresh media at a concentration of 250 μM, 0–72 hours before exposure to the cells (pre-treatment). The exposure time of the cisplatin micelle-containing media to the cells was 24 hours. The measurements for time dependence were repeated twice.

For the micro-beam scanning PIXE (μ-PIXE) analysis, the cells were plated onto a polypropylene (PP) sheet (3399N015, Rigaku) with a thickness of 6 μm, which was fixed to a sample holder with a 5 mm diameter hole at the center. When the cells were in their exponential growth phase, they were exposed to media containing 500 μM of cisplatin micelles for 72 hours. After the exposure, the cells were rinsed twice with PBS. The sample holder with the cells were dipped in liquid nitrogen for about five seconds and then dried overnight using a freeze-dryer (FDU-1100, EYELA) at −50°C and 10 Pa. The freeze dried samples were fixed on a metal holder and installed in the vacuum chamber of the μ-PIXE analysis system.

Conventional PIXE analysis

The PIXE analysis was conducted at the National Institute of Radiological Sciences (NIRS), Chiba, Japan, using a PIXE Analysis System and Tandem Accelerator, PASTA. PASTA has one electrostatic accelerator (Tandem-type (Model 4117MC) manufactured by High Voltage Engineering Europe Co.) and three beam ports for different types of PIXE analysis. Of the three, the beam port for analysis in vacuum (conventional PIXE) was used in this study. Characteristic X-rays were measured using a HP-Ge Detector (IGX10115, Princeton Gamma-Tech) with an active area of 10 mm2, aligned at an angle of 135 degrees. In order to reduce low energy X-rays produced by low-Z elements such as Cl and K, 1 mm of polypropylene was set between the tar-
get and the detectors as an absorber. The beam size was 0.5 × 0.5 mm², regulated with a set of X-Y slits. The beam current and the irradiation dose were 30 nA and 20 μC, respectively. The beam current was monitored using a propeller, which was set in front of the sample holder. Fifteen samples could be measured semi-automatically at one time under computer control. Measurements of the time dependence of the cellular uptake were conducted at least twice, independently. Other measurements were only conducted once due to the availability of machine time.

Figure 1 shows the energy spectrum of the characteristic X-rays measured using the HP-Ge detector. The large peaks in the lower energy region are the characteristic X-rays of Cl, K and Ca, originating from the cells. Small peaks of Fe and Zn, also contained in the cells, can be observed as the sum of characteristic and continuous X-ray signals. Platinum, originating from CDDP, has three peaks in the higher energy region: Pt Lα, Pt Lβ and Pt Lγ. The area of the each peak, corresponding to the sum of the counts, was calculated using Spectrum Navigator software (Seiko EG&G). The background was estimated using trapezoidal approximation and eliminated. Because the peak of Zn Kβ (9.6 keV) overlapped with the peak of Pt Lα (9.4 keV), the original area of Pt Lα was evaluated by subtracting the area of Zn Kβ of the control sample. For the cellular samples, the area of Zn Kβ was standardized by the number of cells contained in the sample.

**Micro-beam scanning PIXE analysis**

To confirm that platinum is accumulated inside the cells, the μ-PIXE analysis was conducted at NIRS. A proton beam with a beam-spot size of about 1.5 × 1.5 μm², and a current of ca. 30 pA was irradiated for 2 hours onto CHO cells pre-treated with 500 μM of cisplatin micelles for 72 hours. The scanning area was 50 × 50 μm². The characteristic X-rays were measured using an Si(Li) detector (Sirius 80, Gresham Scientific Instruments) with a resolution of 149 eV FWHM placed at an angle of 135° with respect to the beam axis. To prevent the cumulative effect of low energy X-rays in the Si(Li) detector, a Mylar membrane with a thickness of 200 μm was used as an absorber. The sensitive area of the Si(Li) detector was 80 mm². Scanning transmission ion microscopy (STIM) images for the imaging of energy-loss at the sample were also taken using a Si surface barrier detector (BR-015-050-100, Advanced Measurement Technology, Inc.). By scanning the beam, the spatial distributions of elements in the cells were obtained. The data was analyzed using OMDAQ software (Oxford Microbeams, Ltd.).

**RESULTS**

Images of the STIM and μ-PIXE analysis of CHO cells treated with cisplatin micelles for 72 hours are shown in Fig. 2. The STIM image (Fig. 2. (a)) shows the thickness of the sample, hence cells should be located in the white area. Pt Lα signals (Fig. 2. (b)) were observed at the cell locations, indicating that the cisplatin micelles and/or released CDDP from the micelles are accumulated inside the cells. The μ-PIXE analysis is a powerful tool for capturing images of elemental distributions but the current system in NIRS requires a high concentration of platinum and a long irradiation time to obtain enough signals for quantitative analysis. Therefore, conventional PIXE analysis was used in this study to measure uptake characteristics.

The time dependence of CDDP-uptake into CHO cells is shown in Fig. 3. The samples for measurements 1 and 2 were prepared separately. The area of the Pt Lα peak increased slowly within 3 hours from the beginning and then rapidly increased. The increase in the amount of platinum from 5 to 20 hours was linear with elapsed time and was not affected by the CHO cell cycle.

Because the doubling time of CHO cells is 12 hours, we
incubated the cells with CDDP and cisplatin micelles for 24 hours to determine differences in cellular uptakes. The 24-hour incubation time is also the limit in terms of cytotoxicity because the number of attaching cells after 24-hour incubation with 250 μM of CDDP was less than 2% of the control cells. The areas of the Pt Lα peak for the cells treated with CDDP and cisplatin micelles for 24 hours were 929 and 108, respectively, while the areas of the media mixed with CDDP and cisplatin micelles were 1598 and 1550. Although the amounts of platinum contained in the media mixed with CDDP and cisplatin micelles were almost identical, the CDDP-treated cells contained 10 times more platinum than cisplatin micelle-treated cells. These results indicate that CDDP released from collapsed micelles was predominantly incorporated. To confirm this hypothesis, the platinum accumulation was compared using cisplatin micelles with different collapse rates. Media mixed with cisplatin micelles for different time periods were used to adjust the rate of the collapsed micelles.

Figure 4 shows the time dependence of the area of the Pt Lα peak for cells exposed to the media for 24 hours. The media were mixed with cisplatin micelles 0–72 hours prior to the exposure. The amount of platinum gradually increased with pre-mixture time and then saturated at around 72 hours (48 hours for pre-mixture time and 24 hours for treatment time).

**DISCUSSION**

In this study, we attempted to follow the incorporation of cisplatin micelle into cells by measuring the amounts of platinum in the cells treated by cisplatin micelles and CDDP. Information on CDDP uptake plays an important role in estimating the behavior of the micelles. The linear increase of platinum in Fig. 3 suggests that CDDP is predominantly incorporated by passive diffusion, which is the main uptake process for CDDP previously reported.17) The first 3 hours lag time could be the time necessary for the concentration gradient to reach its equilibrium. The approximate incorporation of platinum into cells could be expressed as follows:

\[
Q(t) = AC_0 (t - 3) \quad (t \geq 3) \quad (1)
\]

where Q(t) is the amount of platinum contained in the cells at exposure time t, C_0 is the concentration of CDDP in media and A is an arbitrary constant. The lag time (the first 3 hours) is included. The graph in Fig. 3 also shows that the uptake was becoming saturated at around 24 hours of incubation. The absolute platinum concentration in the cells will give information on whether the cells were saturated with CDDP. The absolute platinum concentrations can be approximately estimated as follows. Because the number of counts for 50 μL of medium with a concentration of 250 μM of CDDP was 1598, therefore 1 count corresponded to 1.2 ng of platinum. The counts of the cellular samples were 108 for cisplatin micelles and 929 for CDDP-treated cells. The platinum concentrations in the cells for these two conditions could thus be derived as 0.05 pg/cell for cisplatin micelles and 0.45 pg/cell for CDDP-treated cells. Platinum with a weight of 0.45 pg is equivalent to 1.4 × 10⁹ CDDP molecules, which is about half of the number of binding sites for 6 Gbp DNA, which has 3 × 10⁹ sites for DNA cross-linking. This estimate indicates that the cellular uptake after incubation with CDDP for 24 hours at this concentration would be enough to saturate the binding sites.

The amount of platinum in the cells treated with the same concentration of CDDP and cisplatin micelles for the same time differed greatly. We may hypothesize that the micelles did not pass through the cellular membrane or that the chance of incorporation was very low due to the large size...
of the micelles. This is confirmed by the fact that pre-mixture with the media improved the platinum content in the cells (Fig. 4). We attempted to explain this result mathematically by fitting the results of CDDP-uptake and micelle collapse rates as follows. Concentration of CDDP, \( C(t) \), which is released from the micelles, can be written as,

\[
C(t) = M_0 k(t)
\]

where \( M_0 \) is the concentration of cisplatin micelles at time 0 and \( k(t) \) is the release rate of CDDP. The amount of platinum contained in the cells after 24 hours treatment, \( Q_{24}(T) \), is

\[
Q_{24}(T) = A M_0 k_{t=44} \int_0^T dt
\]

where \( T \) is the pre-mixture time of cisplatin micelles with the media before the 24 hour treatment. The 3 hour time lag has been introduced into the integral. The estimation of \( Q_{24}(T) \) calculated from equation (3) with \( k(t) \), which is the previously reported release rate,\(^{12}\) provides good correlation with the results, supporting the hypothesis that the released CDDP is predominantly incorporated into the cells.

This finding indicates that the kinetics of cisplatin micelles should be evaluated not only by the total platinum content in tumors or organs by ICP-MS but also by the collapse rate of the micelles. This is because the detected platinum could comprise both CDDP released from the micelles and CDDP still contained within the micelles. Assuming that a cisplatin micelle is retained for 5 times longer and at 20 times higher concentration in the tumor than free CDDP,\(^{12}\) the platinum accumulation of cisplatin micelles in the cells would be higher than CDDP alone, even if non-collapsed micelles are taken into account. This is admittedly a rough estimate, in which other factors are neglected. In order to quantitatively estimate the total accumulation of CDDP within cancer cells, all information on the kinetics of cisplatin micelles, such as the accumulation in the tumor, permeability of blood vessels, distribution from blood vessels and elimination by the reticular system are required. For future studies, the platinum distribution in tumors, organs and single cells should be evaluated by fluorescent X-ray analysis techniques such as synchrotron radiation X-ray fluorescent spectrometry (SR-XRF) and micro-beam scanning PIXE (\( \mu \)-PIXE).\(^{18,19}\) These techniques will provide detailed information on the uptake kinetics of CDDP and cisplatin micelles with micrometer resolution. Information on cytotoxicity when cancer cells are treated with cisplatin micelles and irradiated for different times is also required. By integrating all of this kinetic and cytotoxicity information under various conditions, optimal treatment scheduling with the most effective outcomes and the least side effects will be obtained.

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