Regular Article

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A Simple and Easy Method of Monitoring Doxorubicin Release from a Liposomal Drug Formulation in the Serum Using Fluorescence Spectroscopy

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Introduction

Liposomal drugs are about 100-nm sized liposomes containing drugs; liposomes have become popular in the field of medicine as they allow selective delivery of drugs to the disease target.1) DOXIL®, a liposomal formulation of doxorubicin, an anti-cancer drug, is one of the most successful liposomal formulation that reduce the side effects of doxorubicin. Because DOXIL has saved the lives of millions of patients,2) U.S. Food and Drug Administration (FDA) approved a generic drug of DOXIL®, LIPODOX, by a priority review.3) However, the release mechanisms of doxorubicin in the human body have not yet been clarified. DOXIL formed a protein-DOXIL complex with blood proteins called corona after intravenous injection.4–6) The kinds and quantities of proteins that form corona change depending on the time, and the adsorbed proteins change the properties of DOXIL. Hence, many previous studies have been conducted to study the dynamics and efficacy of the protein-DOXIL complex.7–9) However, popular analytical methods (ultracentrifugation, gel electrophoresis, and circular dichroism) are laborious and time-consuming.10–12) Analytical methods (ultracentrifugation, gel electrophoresis, and selective detection method. Fluorescence fingerprint (excitation and emission matrix), which measures excitation and emission wavelengths of emitted fluorescence over a wide range, is an effective technique for the measurement of samples that contain various interfering compounds.14–18) In this study, for the rapid and simple analysis of doxorubicin release, we analyzed DOXIL in different conditions using fluorescence spectrometry because doxorubicin shows fluorescence. Effect of corona formation on doxorubicin release from DOXIL was investigated using fluorescence analysis.

Experimental

Chemicals DOXIL® was obtained from Janssen Pharmaceutical K.K. (Tokyo, Japan). Doxorubicin hydrochloride and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Tokyo Chemical Industry (Tokyo, Japan). Sodium dihydrogen phosphate dihydrate, disodium hydrogen phosphate 12-water, boric acid, hydrochloric acid, bovine serum albumin, and tris(hydroxymethyl)aminomethane (Tris) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Normal mouse serum was from Chemicon International, Inc. (Temecula, CA, U.S.A.). Sodium hydroxide was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Water was purified by a Milli-Q apparatus (Millipore, Bedford, MA, U.S.A.).

Fluorescence Analysis A mixture of DOXIL (3 µL), buffer (135 µL), and 20 mg/mL of albumin (150 µL) was added to each well of a 96-well microplate (Thermo Fisher Scientific, Waltham, MA, U.S.A.), and the total volume of sample solution in each well was increased to 300 µL by adding water. Then, the plate was incubated at 40°C with shaking.

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Key words liposomal drug; fluorescence fingerprint; doxorubicin release
Results and Discussion

Calibration Curve of Free Doxorubicin  
First, we compared the fluorescence intensities of both doxorubicin encapsulated within the liposome and the free form. Although the amount of doxorubicin was the same, the fluorescence intensity of the encapsulated form was approximately 10 times lower than that of the free form. Because encapsulated doxorubicin formed a crystal in the core of liposome and the encapsulated doxorubicin was in close proximity to one another, a reduction in fluorescence intensity (quenching) was observed. Hence, it is considered that encapsulated doxorubicin does not largely affect the fluorescence detection of free doxorubicin. Figure 1 shows the calibration curve of free doxorubicin, and the horizontal axis shows the ratio of free doxorubicin to total doxorubicin in DOXIL (6 µg) used in this study. The linear calibration curve was obtained when the amount of doxorubicin was less than 2 µg; this result is similar to that reported previously. From the calibration curve, the limit of quantitation was found to be less than 0.2 µg and the relative standard deviation of 1–4 µg of doxorubicin was approximately 1.5%. Although the slope of the calibration curve was small in the high-quantity region of doxorubicin, the amount of doxorubicin (released amount) could be measured by fluorescence intensity. The reason for the decrease in the slope in the high-quantity region was that the fluorescence of one doxorubicin molecule is quenched by another doxorubicin molecule. Then, we monitored doxorubicin release from DOXIL using fluorescence analysis.

Doxorubicin Release from DOXIL without Albumin  
Although Tris is a popular chemical in life science experiment, it is known that Tris is permeable to lipid membrane. In our previous study, doxorubicin release was accelerated by a basic solution (above pH 8) containing Tris, at a temperature more than the phase transition temperature (37°C). In this study, the fluorescence intensity of DOXIL solution diluted with water or buffers (pH 8; 450 mM Tris, 450 mM HEPES, 450 mM borate, or 100 mM phosphate) kept at 40°C was measured. The fluorescence intensity of the solution diluted with water, HEPES, borate, and phosphate buffer at 580 nm changed negligibly within 3 h, while that of the solution diluted with Tris increased within 1.5 h and strong fluorescence was observed after 3 h (Fig. 2). Tris permeated the lipid bilayer and converted doxorubicin from the ionic form to the molecular form. The molecular form of doxorubicin was released through the DOXIL membrane to the outside. Based on the fluorescence intensity, it was estimated that about 90% doxorubicin was released from DOXIL. The acceleration of doxorubicin release by Tris was also shown by fluorescence analysis.

Doxorubicin Release from DOXIL with Albumin  
Following administration, DOXIL circulates in the body through blood vessels and finally reaches and accumulates in cancer tissues by the enhanced permeability and retention (EPR) effect, wherein approximately 100-nm particles leak out from the blood vessels near the cancer tissue. DOXIL forms corona with blood proteins before DOXIL reaches the cancer tissue. Albumin is the most abundant protein in the blood, and more than half of the blood proteins is albumin. Therefore, albumin is an important component of the protein–DOXIL complex is important to understand doxorubicin release in cancer tissues. Albumin does not largely affect the release in the Tris buffer, a large change was observed in the HEPES buffer by the addition of albumin. The release in the HEPES buffer started quickly (within 30 min), but the release was small when compared with that in Tris buffer. To examine the effect of albumin in the HEPES buffer in detail, we compared the release of doxorubicin in the HEPES buffer at different albumin concentrations (0–150 µg). The release of doxorubicin was increased by the increasing albumin concentration (Fig. 3b). This result indicated that corona formation
by adsorption of albumin changed the lipid membrane permeability of DOXIL and thus, HEPES ions entered DOXIL and changed doxorubicin from the ionic form to the molecular form. However, this increase was not observed in DOXIL solution diluted with water, borate buffer, and phosphate buffer containing albumin. Because both Tris and HEPES have an amine group and their $pK_a$ are about 8.1 and 7.6, respectively, these basic moieties facilitate doxorubicin release from DOXIL.25)

Because albumin shows fluorescence derived from the amino acid tryptophan, fluorescence emission of around 330 nm by excitation at around 280 nm was observed. The drug and albumin complex is important for evaluation of drug activity within the body, because the bound drug does not show efficacy and only the free (unbound) drug shows efficacy. Therefore, many studies on drug-albumin complex have been performed.18,22,23) The studies showed that the fluorescence of albumin was decreased by complex formation with doxorubicin. The fluorescence of albumin at 330 nm was compared among the diluted DOXIL solutions. The fluorescence of albumin in all solutions decreased within 30 min and a further decrease in fluorescence was observed in diluted solutions after 1.5 h (Fig. 4). The fluorescence of albumin in DOXIL diluted with water, where doxorubicin release did not occur, was decreased within 1.5 h. This could be due to corona formation, because many studies have reported that albumin fluorescence decreases by corona formation.26) It is appropriate that albumin fluorescence changed within 30 min, because it has been reported that corona was formed just after intravenous administration of the liposomal drug.8,9) Therefore, it is important to evaluate doxorubicin release from DOXIL that formed corona, and fluorescence spectrometry is a useful technique for this evaluation.

**Doxorubicin Release from DOXIL in Serum** Finally, doxorubicin release from DOXIL in the blood was examined by measuring the fluorescence fingerprint of the mixture solution of serum (150 $\mu$L), DOXIL (3 $\mu$L), and water (47 $\mu$L) without any pretreatments. Figure 5 shows the fluorescence fingerprint of the serum, serum containing DOXIL, and serum containing doxorubicin (from left to right). The fingerprints of samples just after preparation and those after 3 h are shown in the top and second rows, respectively. The fingerprints in the third and fourth rows are magnified images of fingerprint of area of doxorubicin fluorescence (from 400 to 600 nm for excitation, from 500 to 700 nm for emission). Three strong peaks were observed in the fingerprint of serum at 0 h (top left): the first one was fluorescence emission of around 330 nm, with excitation at around 280 nm; the second one was fluorescence emission of around 440 nm with excitation at approximately 350 nm; and the third one was fluorescence emission of around 650 nm, with excitation at around 280 nm. The first one was derived from proteins including albumin, the second one was derived from the reduced form of nicotinamide adenine dinucleotide (NADH),27) and the third one was derived from the double emission peak of proteins. The fluorescence spectrometer used a diffraction grating for excitation and emission light. The emission signals were observed not only at the same wavelength of excitation light, but also at 2 times longer wavelength (double emission) of the excitation light. In contrast, no signal similar to doxorubicin fluorescence at excitation of around 480 nm and emission of around 550 nm was detected in the serum sample before and after 3 h incubation. Hence, the serum components did not hinder the detection of doxorubicin.
release from DOXIL by fluorescence fingerprints. The small fluorescence of doxorubicin was detected in the serum sample containing DOXIL, and the signal increased when the sample was left for 3 h at 40°C. The release amount of doxorubicin was estimated to be around 5% by signal intensity, and this value was similar to the value measured using HPLC in our previous report. The fluorescence fingerprint of the mixture of serum and 6 µg doxorubicin, which is the total amount of doxorubicin in DOXIL sample, is shown in the right lane. Although strong fluorescence of doxorubicin was detected immediately after mixing, no significant change was observed at 3 h.

Because protein concentration in serum was high (65–80 g/L), the fluorescence signal of protein in all samples was out of range and only the double emission peak (emission around 650 nm) was in the calibration range. The double emission peak of serum containing doxorubicin decreased after 3 h. This could be because of the decrease in fluorescence of albu-
min caused by complex formation between doxorubicin and albumin. The decrease in the fluorescence by the complex formation has been reported in many studies.\textsuperscript{5,11,29} A large decrease in the fluorescence signal of protein was not observed in serum containing DOXIL after 3 h. A possible reason for this could be that the released amount of doxorubicin was too small for decreasing the protein signal. The decreasing in the NADH signals were observed in all samples after 3 h. Thus, we succeeded in evaluating not only doxorubicin release, but also changes in protein and NADH using fluorescence fingerprint.

Fluorescence fingerprint analysis of doxorubicin release from DOXIL in the serum has the following advantages: no pretreatment is required; no contamination; and many samples could be analyzed simultaneously when a microplate is used for analysis. Hence, fluorescence fingerprint is an effective tool for monitoring drug release from a liposome and corona formation of fluorescent drugs like doxorubicin.

**Conclusion**

In this study, we developed a rapid and simple monitoring method of doxorubicin release from DOXIL using fluorescence analysis. The method is applicable for monitoring doxorubicin release in real biosamples such as serum using fluorescence fingerprint. We examined the doxorubicin release under different conditions and identified two release routes of doxorubicin from DOXIL: 1) gradual release (hour-scale) of all doxorubicin molecules by the addition of Tris buffer; 2) rapid release (minute-scale) of a part of doxorubicin molecules by the addition of albumin. These findings suggest that it is important to understand doxorubicin release from the protein–DOXIL complex before the complex was formed before the complex reached the disease target. Thus, fluorescence analysis is a useful technique for monitoring doxorubicin release, and it will ensure the effective and safe usage of DOXIL.

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**Conflict of Interest** The authors declare no conflict of interest.

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