**Regular Article**

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**Rapid Analysis of DOXIL Stability and Drug Release from DOXIL by HPLC Using a Glycidyl Methacrylate-Coated Monolithic Column**

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In recent years, nanomedicines have received growing attention in a range of medical applications, including selective drug delivery technology. In this context, the analysis of liposome stability and drug release from liposomes is of particular importance, as the efficacy of a nanomedicine is determined by the release of the encapsulated drug. We investigated the influence of the surrounding environment on the stability and release of the encapsulated drug (i.e., doxorubicin) from DOXIL. Thus, for the purpose of this study, we selected the liposomal anticancer drug, DOXIL, as a typical nanomedicine, and investigated the influence of the surrounding environment on release of doxorubicin from DOXIL. We found that two pathways existed for doxorubicin release, namely the collapse of DOXIL, and an increase in the permeability of the lipid bilayer. DOXIL collapse occurred upon the addition of high concentrations (>60%) of a methanol solution, while an increase in permeability occurred at temperatures above the phase transition temperature of the DOXIL lipid bilayer, under basic conditions, and in the presence of membrane-permeable bases (e.g., Tris). As DOXIL is particularly stable and limited collapse of DOXIL occurred under physiological conditions, it is expected that doxorubicin release within the body took place through permeability changes in the lipid bilayer of the DOXIL structure.

**Key words** nanomedicine; drug release; liposome; HPLC; monolith column; DOXIL

Nanomedicines are generally composed of a nanoparticle capsule and a pharmaceutical drug species, where the capsule selectively delivers the drug to its target.\(^1\)\(^-\)\(^3\) Although nanomedicines tend to exhibit good efficacy upon delivery to the target, the detailed mechanisms surrounding drug release from nanomedicines remain unclear. As such, a precise analytical method for the determination of nanoparticles is required for the quality assurance of nanomedicines.\(^4\) Ideally, this analytical method should also determine the concentration of free drug, as drug efficacy can only be measured following release of the encapsulated drug from the nanoparticle.\(^5\) Hence, the determination of both the nanoparticle and the free drug is essential to allow evaluation of the quality, efficacy, and safety of such nanomedicines.

Currently, 16 liposomal drugs consisting of nanoparticles composed of lipid bilayers are approved for use, and these are some of the most popular nanomedicines in use.\(^6\)\(^-\)\(^8\) As the outer layer of the liposome consists of lipids bearing both hydrophilic and hydrophobic moieties, lipids can be easily adsorbed onto various materials, thus rendering the liposome difficult to analyze.\(^9\) In terms of their respective sizes, liposomal drugs measure ca. 100 nm in diameter, which is tens times larger than the size of the released drug.\(^6\)\(^-\)\(^8\) The simultaneous analysis of these nanoparticles and the free drug is therefore extremely challenging, and current methods tend to involve initial separation of the nanoparticle and the released drug prior to the individual analysis of each component.\(^10\)

To address this issue, we previously developed a monolithic column coated with polymers for the simultaneous analysis of the liposome and the free drug, where the polymer coating on the surface of the monolith reduced adsorption of the liposome to the silanol group on the monolithic surface.\(^11\) Using these coated columns, we succeeded in the rapid separation of liposomes and free drugs using aqueous mobile phase conditions. As the monolithic column has a bimodal structure, i.e., pores measuring a few micrometers and mesopores measuring a few tens of nanometers, the liposome can easily penetrate the larger pores. However, the mesopores are too small for the liposome to enter, and therefore, only the free drug can enter. As such, the elution time of the small molecules can be controlled by interactions between the analytes and the surface functional groups within the mesopores.\(^12\) This new separation method was named nanoparticle exclusion chromatography (nPEC).

Building on the development of this rapid analytical method for the determination of both liposomes and free drug molecules, we herein evaluate the stability and drug release from a liposomal drug using a glycidyl methacrylate (GMA)-coated column. More specifically, DOXIL (liposomal doxorubicin) will be used as a typical liposomal drug,\(^13\) and the effects of alcohol addition, buffer composition, pH, and temperature on the stability and release of doxorubicin will be examined.

**Experimental**

**Chemical** 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). Formic acid, ammonium formate, sodium dihydrogenphosphate dehydrate, disodium hydrogenphosphate 12-water, boric acid, hydrochloric acid, 8-phosphate buffered saline (PBS) (−) and tris(hydroxymethyl)aminomethane (Tris) were pur-
chased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sodium hydroxide was from Kanto Chemical Co., Inc. (Tokyo, Japan). Glycidyl methacrylate (GMA) was purchased from Kishida Chemical Co., Ltd. (Osaka, Japan). Water was purified with a Milli-Q apparatus (Millipore, Bedford, MA, U.S.A.).

**HPLC** The HPLC system (Hitachi, Tokyo, Japan) used in this study was described in our previous studies. The analysis was performed with two L-2160U LaChrom Ultra pumps, an L-2200U LaChrom autosampler, an L-2485U LaChrom fluorescence detector, and an HPLC system organizer. GMA coated column (3×10 mm) was used. Mobile phase was 200 mM formic acid and ammonium formate (pH 3.6) at the flow rate of 0.5 mL/min. The injection volume was 10 μL, and a fluorescence detector (Ex. 480 nm, Em. 575 nm) was used for detection.

**Column Preparation** The GMA coated column was prepared using a method described in our previous studies. Monolithic silica gel (3 mm i.d.×30 mm long) having a through pore size of 2 μm, mesopore size of 18 nm, and surface area of 200 m²/g was obtained from GL Sciences Inc. (Tokyo, Japan). The monolithic silica gel was modified with a spacer, N-(3-triethoxysilylpropyl)methacrylamide that was prepared from a mixture of 3-aminopropyltriethoxysilane and triethylenylene in toluene and addition of methacryloyl chloride in toluene dropwise under stirring for 0.5 h at 0°C. A 1:1:1 mixture of N-(3-triethoxysilylpropyl)methacrylamide, pyridine, and toluene permeated the monolithic silica gel and reacted for 12 h at 80°C under nitrogen for modification of the spacer on the monolith surface. Subsequently, it was washed with methanol and methanol. Then GMA solutions and 2,2′-azobisisobutyronitrile in methanol permeated the monolith, and were allowed to react at 70°C for 2 h. After the polymer coating reaction, the monolith was washed with methanol and acetone and then dried. The GMA-coated monolithic was cladded with a chemically inert polymer layer, then embedded with a second polymeric material in a stainless-steel tube (6 mm o.d., 1.2 mm thickness). This format was used as a cartridge of the HPLC column (3 mm i.d.×10 mm) using a column holder.

**Analysis of Doxorubicin Release from DOXIL** A DOXIL solution (10 μL) was diluted using either Milli Q water, a mixture of water and methanol (0–90 vol%), or a buffer solution (90 μL), and stored between room temperature and 60°C. The various solutions were then analyzed by HPLC following the addition of PBS solution (400 μL).

**Dynamic Light Scattering (DLS) Measurements** The Nanotrac Wave DLS instrument (Microtrac BEL Corp., Osaka, Japan) employed in this study was previously described elsewhere.

**Nanoparticle Tracking Analysis (NTA) Measurements** The number of nanoparticles present was measured using NTA with a ZetaView Particle Tracking Analyzer (MicrotracBEL Corp.). All samples were diluted 1000 times using 1×PBS buffer. The NTA measurements were recorded and analyzed at 11 positions, and the temperature was maintained at 23°C. A minimum of three replicate measurements was performed for each sample.

**Results and Discussion**

**Separation of DOXIL and Doxorubicin Using the GMA-Coated Column** We previously reported that a GMA-coated column successfully separated DOXIL and doxorubicin within 2 min under aqueous conditions, and therefore, we selected the GMA-coated column for use in this study. As shown in Fig. 1, using the GMA-coated column, DOXIL and the free doxorubicin gave retention times of 0.16 and 0.66 min, respectively. In addition, due to the relatively slow flow rate of the mobile phase (i.e., 0.5 mL/min), peak broadening was observed to a greater extent for doxorubicin than for DOXIL. It should also be noted that in the presence of strong interactions between the stationary phase and doxorubicin, a narrow peak shape would be expected, and so the observation of a broad signal suggests the presence of weak interactions. Indeed, to obtain the required strong interactions, gradient elution of the mobile phase would be necessary, and this would significantly lengthen the run time required for DOXIL analysis, and so is essentially undesirable. The signals corresponding to the DOXIL and the free doxorubicin species were generated by the measurement of fluorescence originating from the doxorubicin structure. Thus, the signal corresponding to DOXIL was detected due to the presence of doxorubicin in the DOXIL structure. The presence of both of these signals in the commercially obtained DOXIL sample indicated that this sample contained small amounts of the free doxorubicin. Furthermore, the peak area reproducibilities for both DOXIL and doxorubicin were ca. 4%, indicating that the GMA-coated column is suitable for analyzing both DOXIL stability and doxorubicin release. As such, we employed this column to evaluate these factors under a range of conditions.

**Effect of Methanol Addition** Liposomes are globular structures composed of lipid bilayers. These structures are stabilized by a specific orientation of the lipid molecules, with the hydrophilic moieties being found on the outer layer of the liposome where contact with water occurs. However, upon the addition of an organic solvent, the lipid bilayer becomes unstable, inducing DOXIL collapse and doxorubicin release. We therefore examined the effect of the addition of methanol to DOXIL, where DOXIL solutions that had previously been
diluted using different ratios of a water/methanol mixture were separated using the GMA-coated column. Although a slight decrease in the peak intensity of the DOXIL signal was observed upon the addition of small quantities of methanol (0–50 vol%), a significant decrease was observed at methanol contents >60 vol% (Fig. 2). Similarly, almost no change was observed in the peak intensity of doxorubicin upon the addition of small quantities of methanol, while a rapid increase in peak intensity was observed at methanol contents >60 vol% (Fig. 2). These results indicate that the lipid bilayer was stable in the absence of high concentrations of methanol. We then examined the effect of methanol addition on the DOXIL particles using DLS and NTA. In the absence of methanol, 80 nm-diameter monodispersed DOXIL particles were observed. However, upon the addition of 50 and 90 vol% methanol, larger particles, measuring approximately 1 and 10 µm in diameter were observed, respectively (Supplementary Fig. S1).

In addition, the quantities of DOXIL present in the various solutions were compared using the loading index (LI), which is an instrument-specific parameter based on the scattering intensity. This index was selected here as it has been shown that the logarithm of the LI is proportional to the logarithm of the nanoparticle concentration. Although no significant difference was observed between the LIs of DOXIL in the presence of 0 and 50 vol% methanol, the LI value decreased significantly in the presence of 90 vol% methanol (Table 1). A similar tendency was observed for the nanoparticle count obtained by NTA. Although DOXIL collapse did not occur upon dilution with small quantities of methanol, the size and quantity of the DOXIL particles began to change upon the addition of 50 vol% methanol, with essentially no DOXIL remaining at a methanol content of 90 vol%.

It would be expected that if all released doxorubicin was detected as free doxorubicin, the variation in intensity of the DOXIL and doxorubicin signals should be similar. However, the increase in the doxorubicin peak area was ca. 40 times greater than the corresponding decrease in the DOXIL peak area. This difference could be explained by differences in the quantum yields of the doxorubicin molecules present within the DOXIL structure. More specifically, as doxorubicin likely precipitated in its crystal form in the DOXIL core (i.e., \((\text{doxorubicin-NH}_3)_2\text{SO}_4\)) a reduction in the fluorescence intensity (quenching) occurred due to the fluorescent compounds being in close proximity to one another. In addition, the released doxorubicin exhibited a large fluorescence signal compared to that of the crystal form. Thus, as we confirmed that the doxorubicin signal intensity increased significantly upon release of the free drug from DOXIL, we expect that the proposed method will be suitable for application in the highly sensitive detection of released doxorubicin.
Effect of Buffer Ion and Concentration  We then examined the effect of the buffer solution on DOXIL stability and on the release of doxorubicin. For this purpose, four different buffers were employed (i.e., borate, HEPES, phosphate, and Tris buffers) at pH 8 and at concentrations of 100–400 mM. The various solutions diluted using the different buffer solutions were analyzed using the GMA-coated column after storage at 40°C for 2 h. Upon variation in the ion concentrations of the borate, HEPES, and phosphate buffers, no significant changes in the peak areas of DOXIL and doxorubicin were observed (Fig. 3). We expect that the release of doxorubicin did not occur because the ions that composed these buffers could not penetrate the bilayer. However, when a Tris buffer was employed, a significant decrease in the intensity of the DOXIL signal was observed, accompanied by a corresponding increase in that of doxorubicin. In addition, doxorubicin release was accelerated upon increasing the Tris buffer concentration. Subsequently, we examined the effect of the Tris concentration on the size and number of DOXIL particles using DLS and NTA. As indicated in Table 2, the size of the DOXIL particles remained relatively constant at ca. 80 nm for all Tris concentrations. To determine the quantity of DOXIL particles present under the various conditions, two different techniques were employed, as described previously above. More specifically, the LI value was measured by DLS, while the number of particles was measured by NTA. Again, no significant differences were observed between the samples prepared using different Tris concentrations. These results therefore suggest that DOXIL collapse did not occur, as the size and quantity of the DOXIL particles remained constant. It therefore appears that in the absence of DOXIL collapse, doxorubicin release took place through movement of the Tris ions into the DOXIL particles to increase the internal pH values. Subsequently, doxorubicin was converted from its crystal (ionic) form to its molecular form. Finally, the molecular doxorubicin was distributed to the lipid bilayer and was released from the DOXIL particle. Indeed, it was previously reported that the Tris ion penetrates the lipid bilayer and reaches the interior of the liposome and the cell.18–20

Effect of pH As the Tris ion appears essential for doxorubicin release, we subsequently examined the effect of pH on doxorubicin release in the presence of Tris buffer between pH 7 and 9. At pH 7, no significant difference in the DOXIL peak area was observed upon increasing the Tris concentration, although a small increase in the doxorubicin content was observed at pH 7 (Fig. 4). Upon increasing the pH to 8 and 9, a decrease in the DOXIL peak intensity was accompanied by an increase in the doxorubicin peak intensity. Indeed, higher concentrations of the Tris buffer accelerated doxorubicin release under all pH conditions examined. These results therefore indicate that basic conditions are essential to promote doxorubicin release.

Effect of Temperature Finally, we examined the effect of temperature on doxorubicin release from DOXIL. As temperature affects the fluidity of the lipid bilayer, it was expected that doxorubicin release would be accelerated at temperatures above the phase transition temperature of the bilayer, at which point the fluidity of the bilayer would effectively increase. As the phase transition temperature of DOXIL is ca. 37°C,21) we examined doxorubicin release over 30 min at 30, 40, 50, and 60°C (Fig. 5). Although a small decrease in the DOXIL peak intensity was observed at 40°C, large decreases were observed at 50 and 60°C (Fig. 5). Indeed, similar results were observed for doxorubicin, with a small increase in peak intensity being observed at 40°C and a large increase occurring at 50 and 60°C (Fig. 5), where almost all doxorubicin was released within 30 min. As the transition midpoint ($T_m$) of DOXIL was determined to be ca. 50°C by differential scanning calorimetry, it appeared that the doxorubicin was released rapidly.22) Furthermore, the diameters and quantities of the DOXIL particles remained constant at all temperatures, thereby indicating that doxorubicin release occurred due to the increasing membrane fluidity upon its transformation from a gel to a liquid crystal. Hence, heating to temperatures above the phase transition temperature had a significant effect on doxorubicin release. Moreover, as the phase transition temperature of DOXIL is similar to body temperature, doxorubicin release would be expected to occur in target organs within the body.

We could therefore conclude that the rapid release of doxorubicin in the presence of high Tris buffer concentrations (i.e., 400 mM), under basic conditions, and at temperatures above the phase transition temperature of the lipid membrane (i.e., >37°C) could be accounted for as follows: 1) Higher concentrations of Tris buffer increased the Tris ion concentration in the liposome and accelerated doxorubicin conversion to its molecular form; 2) higher pH values increased the stability of the molecular form of doxorubicin; and 3) higher temperatures increased the fluidity of the lipid bilayer. Our results therefore indicate that doxorubicin release from DOXIL was largely affected by the type and concentration of the buffer ion used.

Table 2. Size and Nanoparticle Quantities as Measured by DLS and NTA

| Tris concentration (mM) | Diameter (nm) | Loading index | Particle number ($\times 10^9$ particles/mL) |
|--------------------------|---------------|---------------|-------------------------------------------|
| 100                      | 78.7          | 0.193         | 2.8                                       |
| 300                      | 79.5          | 0.192         | 2.7                                       |
| 400                      | 80.6          | 0.195         | 2.6                                       |

$a$ and $b$ indicate the measurement by DLS and NTA, respectively.
and the pH of the dispersed solution, while temperature was also key to ensuring suitable bilayer fluidity.

Conclusion

It was previously assumed that the separation and analysis of a liposomal drug and its released (free) drug were tedious and time-consuming processes due to the requirement for pretreatment mainly by ultracentrifugation and subsequent individual determination of the two components. However, we herein described the use of a GMA-coated monolithic column for the simultaneous separation and analysis of DOXIL, a liposomal anticancer drug, and the free drug doxorubicin within 2 min by HPLC. Investigations into the influence of the surrounding environment on the release of the encapsulated drug indicated that two pathways existed for doxorubicin release, namely the collapse of the DOXIL structure and an increase in the permeability of the lipid bilayer. To increase the bilayer permeability and accelerate doxorubicin release, temperatures above the phase transition temperature of the lipid bilayer are expected to promote DOXIL collapse. We therefore investigated two factors that can penetrate the lipid bilayer in vivo and alter the pH inside the organism, thus leading to potential new treatments on a shorter timescale.

Although collapse of the DOXIL structure is effective for releasing high concentrations of the encapsulated drug over a short timeframe, the organs and tissues within the human body do not tend to contain the high concentrations of organic solvents required to promote DOXIL collapse. We therefore expect that doxorubicin release within the body occurs through an increase in membrane permeability, or degradation and metabolism of the DOXIL structure. Moreover, as the increased levels of ammonia within tumors have been reported to be responsible for the release of doxorubicin, it is expected that this process is controlled by chemicals that can penetrate the lipid bilayer in vivo and alter the pH inside the liposome. However, further studies are required to clarify the detailed mechanism of doxorubicin release from DOXIL within the body. As our developed method constitutes a more rapid method for the analysis of nanomedicines, we believe that it will contribute to the rapid development of nanomedicines, thus leading to potential new treatments on a shorter timescale.

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Conflict of Interest

Eiichi Yamamoto is an employee of Eisai Co., Ltd.

Supplementary Materials

The online version of this article contains supplementary materials.

Supporting Figure S1. DLS analyses of DOXIL diluted using 0, 50, and 90 vol% aqueous methanol solutions.

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