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Liposome-encapsulated Carboquone. I. Method of Preparation and Carboquone Release

KAZUHIRO TSUKADA,* RYUZO OKADA, MASAFUMI HISAOKA and TADASHI MORIOKA

Product Development Laboratories, Sankyo Company Ltd., Hiromachi 1-2-58, Shinagawa-ku, Tokyo, 140, Japan

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Encapsulation of carboquone (CQ) in liposomes was investigated by applying a reverse-phase evaporation vesicle (REV) method, and the *in vitro* release of CQ from the liposomes was also studied. The mean diameter of the liposomes was 0.2 μm. The encapsulation efficiency in liposomes was greatly affected by the materials used for the membrane of the liposomes. The efficiency increased with a rise in the phase transition temperature of the phospholipids, the maximum encapsulation of CQ in liposomes being obtained with distearoyl phosphatidylcholine. The results of drug release showed that CQ remained in CQ-liposomes for a long time at 5°C but was released considerably faster at around the phase transition temperature (58°C) of distearoyl phosphatidylcholine.

Keywords——liposome; carboquone; liposome preparation method; reverse phase evaporation vesicle; release from liposomes; particle size distribution of liposomes

Recently, liposomes have received considerable attention as potential carriers for antitumor agents, and animal experiments have been carried out.[1] Liposome-mediated drug transport can produce an altered retention and distribution of the drugs.[2] Although many reports have been published on liposomes as drug carriers, there are very few reports on encapsulation techniques, especially encapsulation of lipophilic drugs in liposomes. *In vivo* studies on the behavior of drugs encapsulated in liposomes have been carried out. However, the properties of the liposomes themselves used in those experiments were not always well defined. The present study has been undertaken to establish the techniques for encapsulation of 2,5-bis(1-aziridinyl)-3-(2-hydroxy-1-methoxyethyl)-6-methyl-*p*-benzoquinone carbamate, carboquone (CQ), in liposomes and to investigate several factors which influence the degree of CQ encapsulation. CQ is an anticancer agent which was developed by Nakao *et al.*[3] The solubility of CQ is so poor that the efficiency of CQ encapsulation in egg phosphatidylcholine liposomes by the method developed by Bangham *et al.*[4] was only 0.2—0.5%. Neither the ethanol injection method[5,6] nor the ether infusion method[7] was successful for CQ encapsulation. Recently, however, Szoka and Papahadjopoulos[8] reported a reverse-phase evaporation vesicle (REV) procedure. The report described the encapsulation efficiency in REV of cytosine arabinoside, sucrose and some other water-soluble materials into the large internal aqueous spaces of liposomes. The REV method was also successfully applied in the case of CQ. This report presents details of the preparation, the conditions for optimum encapsulation of CQ in liposomes and the *in vitro* release of CQ from CQ-liposomes.

Experimental

Materials——Carboquone (CQ, Sankyo Co., Ltd.) was used. Distearoyl phosphatidylcholine (DSPC), dipalmitoyl phosphatidylcholine (DPPC), dimyristoyl phosphatidylcholine (DMPC) and dioleoyl phosphatidylcholine (DOPC) were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Egg phosphatidylcholine (egg PC) was purchased from Funakoshi Pharmaceutical Co., Ltd. Cholesterol was purchased from Wako Pure Chemical Ind., Ltd. All other chemicals were of reagent grade or analytical grade.

Preparation of CQ-Liposomes——CQ-liposomes were prepared by a method based on that of Szoka and Papahadjopoulos[8] with some minor modifications. Liposomes were usually prepared by dissolving 15.6 μmol of CQ and 330 μmol of DSPC in 35 ml of chloroform in a 300 ml beaker. Then 35 ml of isopropyl

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ether and 10 ml of phosphate-buffered saline were added. This phosphate-buffered saline (PBS) contained 0.64 mM Na_2HPO_4, 0.14 mM KH_2PO_4, 13.7 mM NaCl and 0.26 mM KCl, the pH being 7.2. The mixture was stirred continuously with a magnetic stirrer, then sonicated (25 kHz×160 W) at room temperature for 5 min using a horn type sonicator (Choonpa Kogyo Co., Ltd., model USH-150N-25) until the mixture became a homogeneous opalescent w/o emulsion. The emulsion was transferred to a 500 ml round-bottomed flask and organic solvents were evaporated off under reduced pressure at 60°C. During the evaporation of solvents, phase inversion occurred and simultaneously liposomes were formed. This crude liposomal suspension was subjected to gel chromatography at room temperature on a Sephadex G-50 column to separate CQ-liposomes from unencapsulated CQ. The fractions containing CQ-liposomes were pooled and centrifuged at 15000 rpm (27000×*g*) for 20 min at 4°C. The clear supernatant liquid was discarded and precipitated CQ-liposomes were collected and resuspended in fresh PBS. The encapsulation efficiency (%) was expressed as a percentage of the ratio of encapsulated CQ to the total amount of CQ used for the preparation. Similar experiments were carried out to prepare CQ-liposomes with other phospholipids. The compositions of various CQ-liposomes are shown in Table I. Several encapsulation techniques employed for making CQ-liposomes are listed in Table II.

Particle Size Determination of CQ-Liposomes——The particle size distribution of CQ-liposomes was determined from negatively stained electron micrographs by measuring the diameter of individual particles and assigning them to a specific size interval. For each determination, at least 200 particles were measured. Samples for negatively stained electron micrographs were prepared by the following procedure. CQ-liposomes were diluted with PBS and incubated in phosphotungstate solution (pH 7.0) for 3 min at room temperature. Collodion-coated grids were floated onto the surface of a drop of CQ-liposomes for 3—4 min. After removal of excess fluid, the grids were allowed to dry and then viewed on a Hitachi electron microscope.

***In Vitro* Release Experiment**——The *in vitro* release experiments were carried out using Amicon Centriflo CF-25 ultrafiltration membrane cones (Lexington, U.S.A.). Aliquots of 20 ml of liposomal suspension, containing 120 μg of CQ in 1.0 ml, were each transferred into a flask and immersed in a water bath maintained at 5, 15, 25, 37 or 50°C. At appropriate intervals, 2 ml portions of incubated suspension were withdrawn and transferred into an Amicon Centriflo CF-25 ultrafiltration membrane cone and centrifuged at 1500 rpm (500×*g*) for 5 min. The filtrate containing CQ released from CQ-liposomes was then assayed.

Analytical Method——An appropriate volume (0.1—0.5 ml) of liposomal suspension containing CQ-liposomes was transferred to a centrifuge tube and CQ was extracted with 10 ml of chloroform, by shaking for 30 min. After centrifugation, 5 ml of the chloroform layer was withdrawn with a pipette and the chloroform was removed with a rotary evaporator. This residue was redissolved with an adequate volume of methanol and the concentration of CQ was determined by high performance liquid chromatography (HPLC) using a Waters HPLC system (6000/440 U) with a μ-Bondapak C_{18} column (Waters). CQ was detected by measuring the absorbance at 340 nm.

Phase Transition Temperature (T_c)——The T_c values for phospholipids were estimated using a Mettler FP-5+FP-52 melting point apparatus. The phospholipids were suspended in PBS, then sonicated for 5 min at 50°C. The suspensions were cooled and viewed under a microscope as the temperature was increased. The T_c was determined as the temperature at which the solid-like gel transformed into a fluid-like sol.

Partition Coefficient——The partition coefficient of CQ was determined by the usual shake-out method at temperatures of 5, 15, 25 and 37°C.

Results

Encapsulation of CQ in Liposomes

Liposomal encapsulation of lipophilic drugs is greatly influenced by the encapsulation techniques and the phospholipids selected, because the lipophilic drug would be mainly retained in the liposome membrane matrix. The solubility of CQ in PBS at 37°C was 130 μg/ml. The values of partition coefficient in the chloroform/water system at 5, 15, 25 and 37°C were 29.2, 27.5, 26.0 and 25.3, respectively. Considering the properties of CQ, it might be suggested that the major portion of CQ is encapsulated in the membrane matrix, composed of phospholipids. In order to select the most appropriate phospholipid for CQ encapsulation, several kinds of phospholipids were examined (Table I). Different kinds of encapsulation techniques were applied to prepare CQ-liposomes (Table II). Encapsulation of CQ in liposomes was found to be greatly influenced by the phospholipid selected. There is some relationship between the amount of CQ encapsulation and the T_c of the phospholipid, that is, the amount of CQ encapsulation in liposomes increases with a rise in the T_c of the phospholipid used. The T_c values were in good agreement with the results reported previously.[2] On the basis of these results, DSPC

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Table I. Encapsulation Efficiency of CQ in Liposomes

Phospholipid	T_c (°C)	Encapsulation (%)
DSPC	58	21.7±4.0
DPPC	42	16.6±4.5
DMPC	23	5.9±1.4
Egg PC	−15	0.4±0.2
DOPC	−20	2.3±1.3

The amounts of CQ and phospholipids used for the preparation of the liposomes were 15.6 μmol and 330 μmol, respectively. Each result is the mean ±S.D. of 4 experiments.

Table II. Encapsulation Efficiency of CQ in Liposomes

Preparation method	Phospholipid	Encapsulation (%)
Bangham method[4]	Egg PC	0.3±0.2
	DSPC	0.5±0.3
Ethanol injection method[5,6]	Egg PC	0.1±0.0
	DSPC	0.1±0.0
Ether infusion method[7]	Egg PC	0.0±0.0
	DSPC	0.0±0.0
REV method	Egg PC	0.4±0.2
	DSPC	21.7±4.0

The amounts of CQ and phospholipids used for the preparation of the liposomes were 15.6 μmol and 330 μmol, respectively. Each result is the mean ±S.D. of 3 experiments.

was selected as the best material for CQ encapsulation in liposomes. The mean percentage of CQ encapsulation was 21.7±4.0% (mean±S.D., n=4).

Regular liposomal suspensions, 1 ml of which contained approximately 400 μg of CQ in 43 mg of DSPC in dry weight, were typically obtained by our procedure for each preparation, and subsequent experiments were carried out using these CQ-liposome preparations.

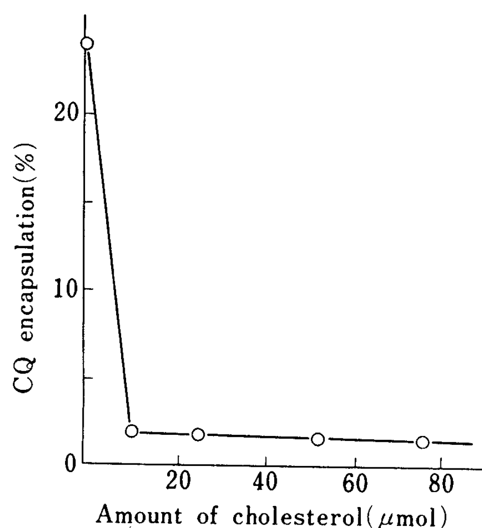

Fig. 1. Effect of Cholesterol on CQ Encapsulation

The amounts of CQ and DSPC used for the preparation of the liposomes were 15.6 μmol and 330 μmol, respectively. Results are expressed as the means of 3 experiments

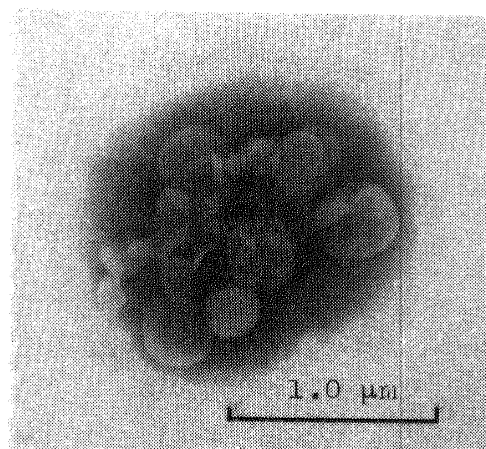

Fig. 2. Negatively Stained Electron Micrograph of CQ-Liposomes

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It is well known that cholesterol is easily incorporated into a membrane matrix of liposomes, and strengthens the matrix.[9] CQ encapsulation was investigated in the presence of cholesterol and DSPC using the same procedure (Fig. 1). CQ encapsulation was greatly limited by the presence of cholesterol. The results suggest that cholesterol interferes with the encapsulation of CQ in the membrane matrix of liposomes. In fact, encapsulation of CQ in liposomes was markedly affected by differences in the applied techniques and it was found that only the REV method was successful in preparing CQ-liposomes efficiently, at least in the present experiments (Table II). Thus CQ-liposomes were very sensitive to the preparation method and the material used for the liposome membrane.

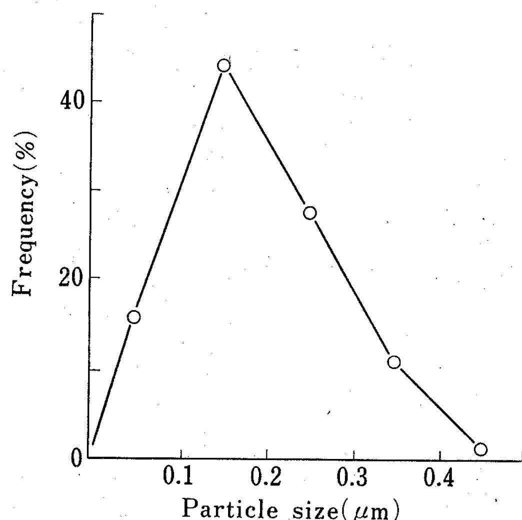

Fig. 3. Primary Particle Size Distribution of CQ-Liposomes from the negatively stained Electron Micrograph

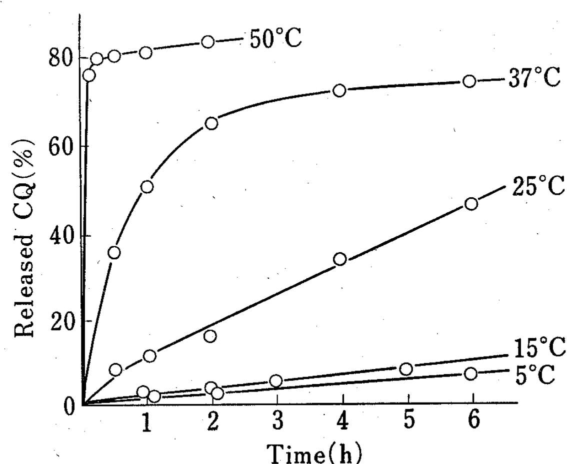

Fig. 4. *In Vitro* Release Patterns of CQ from CQ- Liposomes

Results are expressed as the means of 3 experiments.

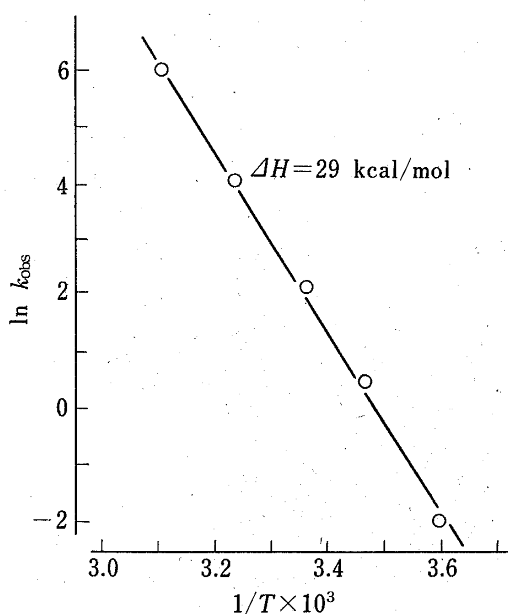

Fig. 5. Arrhenius Plot of CQ Release from CQ-Liposomes

T, k_{obs} and ΔH are the absolute temperature, the release rate constant and the activation energy for the initial release phase, respectively.

Particle-Size Analysis

Fig. 2 shows a negatively stained electron micrograph of a typical CQ-liposome preparation. The size distribution of CQ-liposomes, obtained from an electron micrograph, is shown in Fig. 3. The arithmetic mean diameter for primary particles was 0.2 μm.

In Vitro Release of CQ from CQ-Liposomes

The *in vitro* release patterns of CQ from CQ-liposomes are illustrated in Fig. 4. The amount of CQ released was plotted against time. *In vitro* release of CQ was apparently influenced by temperature, that is, the release rate increased with a rise in temperature. At 50°C about 80% of CQ was released within 2 h after the start of the experiment. In contrast, little CQ was released after 2 h at 5°C. The observation was continued at 5°C for 7 d, and the percentages released after 1, 3 and 7 d were 3.4, 7.4 and 9.7%, respectively. At both 50 and 37°C the

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release patterns showed a fast release phase in the earlier stages, followed by a slower release phase. The rate constants for the initial release phases at different temperatures were calculated from the results in Fig. 4 and plotted against the reciprocal of absolute temperature using an Arrhenius equation (Fig. 5). The activation energy calculated from the slope of the Arrhenius plot was 29 kcal/mol.

Discussion

Szoka and Papahadjopoulos[8] reported that large unilamellar and oligolamellar liposomes could be obtained by the REV method. The mean diameter of our CQ-liposomes was the same as that of their liposomes.

As shown in Table II, the encapsulation efficiency of CQ was very sensitive to the actual techniques applied to the preparation of CQ-liposomes. In contrast, liposomal encapsulations of other anticancer agents such as actinomycin D,[9] bleomycin,[10] cytosine arabinoside[8] and methotrexate[1] were relatively easy, regardless of the technique used for encapsulation. This difficulty of encapsulation of CQ in liposomes might be due to its relatively low solubility in water or organic solvents, and its lipophilicity. According to the Bangham method,[4] a mixed membrane containing CQ and phospholipid was produced by evaporating the organic phase. However it was observed that considerable amounts of CQ precipitated in the process of the dispersion; the sonication process, particularly, accelerated the precipitation. Thus little CQ was encapsulated in the liposome particles. In the case of both the ethanol injection method[5,6] and the ether infusion method,[7] CQ has such a low solubility in those systems that encapsulation of CQ did not take place but precipitation occurred during infusion of the organic phase into the aqueous phase. As found by Szoka and Papahadjopoulos,[8] initial sonication of the buffered aqueous phase in organic solvents in the presence of amphiphilic phospholipid and CQ might produce small water droplets stabilized by a phospholipid monolayer. CQ might condense in the interfacial membrane matrix when the organic phase is removed by evaporation. Then, the inverted emulsion may collapse into a viscous gel-like state, resulting in the formation of liposome particles without crystallization of CQ. Thus, considerable amounts of CQ were encapsulated in the liposomes by using the REV method. A typical suspension of CQ-liposomes contains 400 μg of CQ in 43 mg of DSPC per 1.0 ml of PBS. In relation to the partition coefficient and solubility in the aqueous system, a large amount of CQ seemed to be encapsulated in a membrane matrix of CQ-liposomes. Therefore, the encapsulation efficiency of CQ in liposomes was significantly influenced by the phospholipids used as the membrane material. Encapsulation of CQ increased with a rise in T_c of the phospholipids (Table I). It was shown that cholesterol interferred with the encapsulation of CQ in the membrane matrix of liposomes. This is considered to be caused by the decrease of CQ solubility in the liposome membrane matrix upon addition of cholesterol. The result also shows that CQ is encapsulated in the membrane matrix. As shown in Fig. 4, the *in vitro* release patterns of CQ from CQ-liposomes were greatly influenced by the temperature. It is evident that the drug was released more rapidly at higher temperature. From the change of partition coefficient of CQ with increasing temperature, it seems likely that the partition of CQ from the liposome membrane matrix into water is one of the main factors controlling the drug release.

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