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Cubic Liquid-Crystalline Particles as Protein and Insoluble Drug Delivery Systems

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13.1 INTRODUCTION

Based on the scientific understanding of lipid particulate systems, many scientists have pursued the development of better lipid formulations for their own application fields. Numerous innovative lipid particulate systems have been formulated in recent years. One particularly interesting system is Cubosome® (10). Cubosome refers to a submicron-sized dispersed lipid particle of the bicontinuous cubic liquid-crystalline phases in an aqueous environment. A great number of studies have been performed to investigate the structure (2,3) and the mathematical description of Cubosome particles (4,5). One of the main application fields for Cubosome is drug delivery (6,7). Cubosomes have distinctly different characteristics from other conventional lipid-particulate systems including liposomes and lipid emulsions. The interior of the particles is considered the hydrated cubic phase and contains hydrophilic water channels, the hydrophobic hydrocarbon chain region, and the interfacial headgroup region (6). This somewhat complex internal structure is an ideal setting for many drugs of hydrophobic, hydrophilic, and amphiphilic nature.

A Cubosome can be formed by first forming a very viscous liquid cubic phase by adding water and an emulsifier to monoglyceride, and then by dispersing the mixture in water. A Cubosome can have an average particle size of as large as several micrometers in diameter. Since it is preferable to have submicron-sized Cubosomes to solubilize pharmaceutical compounds, submicron-sized particles have been formulated by applying mechanical forces, such as microfluidizing the coarse dispersion (6). Preparing submicron-sized Cubosome particles by means of applying mechanical force, however, may result in physicochemical instability of the constituting ingredients

or the enclosed materials due to high energy and high temperature accompanying the mechanical process.

To overcome some of the drawbacks in the conventional preparation method, we developed a homogeneous liquid formulation that can be readily dispersed in water (8,9). The liquid formulation comprises monoolein, an emulsifier, and a biocompatible organic solvent but contains no or negligible amounts of water, which can degrade drug or other components by hydrolysis; thus, the formulation is remarkably stable at room temperature. The formulation can be prepared without heat or mechanical force and can be sterilized easily by filtration. The liquid formulation can be dispersed easily in excess water by simple shaking or vortexing. By mixing the liquid formulation with an excess amount of water or phosphate-buffered saline (PBS), submicron-sized particles ranging from 200 to 500 nm in diameter can be formed. Drug encapsulation efficiency inside the dispersed particles varies mainly depending on the nature of the drug itself. While a hydrophobic or protein drug is enclosed inside the particles at a high ratio, a small hydrophilic drug resides mostly in the bulk aqueous phase.

In this chapter, we describe the preparation procedure, stability, and characteristics of our liquid formulation. One of the main application fields of our liquid formulation and its dispersion is drug delivery systems. Due to the toxicity of monoolein in blood and muscle, the dispersion could not be administered intravenously or intramuscularly (see below). The formulation, however, is an efficient oral drug delivery system without any toxicity. Oral administration of hydrophobic or protein drug will be described.

13.2 PREPARATION OF THE LIQUID FORMULATION AND SIZE DISTRIBUTION OF THE DISPERSED PARTICLES

Distilled monoglyceride (RYLO™ MG 19, NF, Danisco A/S, Denmark) consists of monoolein (ca. 77%) and other monogly-cerides, diglycerides, and triglycerides as the minor components. Monoolein refers to this distilled monoglyceride in this chapter. Pure monoolein (>99%, Nu Chek Prep, Elysian, MN) can also be used without changing most of the physical characteristics of the liquid formulation and its dispersion with or without drugs. One hundred milligrams of monoolein and 15 to 30 mg Pluronic F-127 (Lutrol F127, Poloxamer 407, BASF Corporation, Parsippany, NJ) were dissolved completely in 1 to 5 ml of absolute ethanol (Table 13.1).

Table 13.1 Size Distribution of the Particles in the Dispersions of the Liquid Formulations at Different Emulsifier Compositions in Various Solvents[a]

Weight of PF-127 per 100 mg Monoolein (mg)	Average Particle Diameter (nm)/Polydispersity in Various Solvents			
	No Solvent	Ethanol	PEG_{400}	Propylene Glycol
10	ND[b]	ND	ND	ND
15	ND	258.0/0.123	278.0/0.290	312.1/0.257
20	ND	254.4/0.069	249.3/0.219	399.1/0.996
25	ND	259.3/0.345	277.9/0.428	351.3/0.320
30	ND	262.7/0.376	246.1/0.611	336.8/0.356

[a] The liquid formulation contains 50% (w/w) of ethanol or PEG400 or 70% (w/w) of propylene glycol.
[b] Not dispersed.

In some cases, the mixture was heated to ca. 40°C to accelerate the dissolution process. Ethanol was evaporated under a stream of oxygen-free dry nitrogen for a few hours and subsequently at a reduced pressure overnight to remove any traces of the solvent. The mixture was dissolved again in absolute ethanol, 1,2-propanediol, or a low-molecular-weight polyethyleneglycol (mol. wt. 400, PEG_{400}) to form the liquid formulation, which was stored at room temperature or at 4°C for further experiments.

These liquid formations were dispersed *in situ* by adding 100 μl of the liquid formulations in 2 ml distilled water and by vortexing the mixture for 1 min. The particle size distribution was determined by quasielastic laser light scattering with a Malvern Zetasizer® (Malvern Instruments Limited, England).

The liquid formulations in water formed dispersions of lipid particles ranging from 250 to 400 nm in diameter depending on the concentrations of Pluronic F-127 and the organic solvents as shown in Table 13.1. The dispersion was the most stable with small-sized particles when more than 15 mg of Pluronic F-127 were added to disperse 100 mg of monoolein in the liquid formulation. The particle sizes did not decrease significantly when the amount of Pluronic F-127 was increased beyond 15 mg per 100 mg monoolein. When less than 15 mg of Pluronic F-127 was used, the mean particle size of the lipid dispersions was large and not reproducible. The use of Pluronic F-68 or Tween 80 instead of Pluronic F-127 also yielded a liquid formulation that dispersed well when more than 15 mg was used per 100 mg monoolein (data not shown).

Table 13.2 Size Distribution of the Particles in the Dispersions of the Liquid Formulations at Different Solvent Compositions in Various Solvents[a]

Solvent Content in liquid formulation [% (w/w)]	Average Particle Diameter (nm)/Polydispersity in Various Solvents		
	Ethanol	PEG_{400}	Propylene Glycol
0	ND[b]	ND	ND
50	253.6/0.191	278.0/0.290	ND
60	254.4/0.105	288.4/0.377	ND
70	271.9/0.175	294.2/0.393	312.1/0.257
80	296.7/0.246	268.0/0.378	295.3/0.222
90	296.8/0.191	310.3/0.394	284.8/0.224

[a] The liquid formulation contains monoolein and PF-127 at 100:15 by weight.
[b] Not dispersed.

To determine an adequate content of solvents in the liquid precursor formulations, formulations containing various amounts of solvents were also prepared (Table 13.2). Liquid formulations containing 100 mg monoolein, 15 mg Pluronic F-127, and different amounts of the solvent selected from ethanol, 1,2-propanediol, or PEG_{400} were prepared. One hundred microliters of the liquid formulations were dispersed in 2 ml distilled water to determine the mean particle size in the dispersion. Liquid formulations containing 0 to 40% ethanol or PEG_{400}, or 0 to 60% 1,2-propanediol did not disperse but formed viscous gel-like aggregates in water. With higher solvent contents, the liquid formulations dispersed easily in water. The mean particle size did not decrease significantly by increasing the amount of solvents beyond 50% (w/w) for ethanol and PEG_{400} and 70% (w/w) for 1,2-propanediol. It is worthy to mention that the liquid precursor formulations containing PEG_{400} underwent gelation in a few minutes at ambient temperature and therefore were difficult to handle. It is possible that gelation is caused by the phase transformation to the sponge or L_3 phase (10,11). The L_3 phase has been observed when the cubic phase of the monoolein/water system encounters a third component, a hydrophilic solvent such as dimethylsulfoxide, ethanol, *N*-methylpyrrolidine, PEG_{400} or 1,2-propanediol. Pluronic F-127, a nonionic amphiphile, acting as the fourth component, could also alter the gross phase behavior of the system (12).

Liquid formulations containing a drug were also prepared by mixing monoolein, emulsifier, organic solvent and the drug (Table 13.3). In case of protein drugs, monoolein was mixed homogeneously with Pluronic F-127, ethanol, and propylene glycol first. Concentrated

Table 13.3 Particle Size Distribution, Drug Loading Efficiency, and Structural Characteristics of the Dispersions of the Liquid Formulations at Different Solvent Compositions in Various Solvents

Encapsulated Molecules	Particle Size (nm)/Polydispersity	Weight of Drug/100 mg Monoolein	Loading Efficiency (%)	Space Group	Unit Cell Parameter (Å)
		Hydrophobic Drugs (50% Ethanol[a])			
Cresol red	348.0/0.351	4	86	Im3m	121[b]
Rifampicin	272.6/0.247	0.5	75	Im3m	138
Paclitaxel	196.6/0.329	0.42	100	Im3m	132
Pyrene	275.0/0.045	6	100	Im3m	123
NBD-PE[c]	290.0/0.393	1	100	ND[d]	—
Rhodamine-PE	264.4/0.306	2	100	ND	—
		Hydrophilic Molecules (50% Ethanol)			
Bromocresol green	283.1/0.583	0.5	20	Im3m	130
FITC[e]	277.8/0.194	1	3	Im3m	130
Rhodamine B	290.9/0.229	1	3.9	Im3m	130
HPTS[f]	289.8/0.312	1	5	Im3m	130
Methylene blue	282.2/0.231	0.5	0	Im3m	130
		Proteins (70% Propylene Glycol[g])			
FITC-BSA[h]	314.5/0.165	8	85	Pn3m	134
Insulin	350.7/0.170	15	87	Im3m	129
Cholera toxin B	475/0.356	2	50 to 60	Ia3d	152

Tetanus toxoid	412.1/0.143	0.23	100	Spotty[i]	—
Encapsulated Molecules	Particle Size (nm)/Polydispersity	Weight of Drug/100 mg Monoolein	Loading Efficiency (%)	Space Group	Unit Cell Parameter (Å)
Cyclosporin	424/0.133	40	100	ND	—
Lysozyme	345.8/0.182	5	85	Spotty	—
Calcitonin	319.0.260	0.6	0	Im3m	130
		Other Macromolecules (70% Propylene Glycol)			
HPTS-dextran[j]	332.0/0.455	3	67	ND	—
Blue dextran[k]	398.0/0.482	1	75	ND	—

Note: The liquid formulation contains monoolein and PF-127 at 100:20 by weight.

[a] The composition of ethanol in the liquid formulation was 50% (w/w).

[b] The error in the unit cell parameter was ca. ± 5 Å.

[c] 1,2-Dioleyl-sn-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl).

[d] Not determined.

[e] Fluorescein isothiocyanate.

[f] Pyranine, 1,3,6-pyrenetrisulfonic acid, 8-hydroxy-, trisodium salt.

[g] The composition of propylene glycol in the liquid formulation was 70% (w/w).

[h] Fluorescein isothiocyanate conjugated bovine serum albumin.

[i] Spotty diffraction patterns indicating the coexistence of different cubic phases.

[j] HPTS conjugated dextran (molecular weight 10,000).

[k] Molecular weight 600,000.

protein aqueous solution was added to the liquid mixture and stirred until clear. Ethanol in the mixture was evaporated completely under low vacuum to prepare the liquid formulation containing a protein drug. Since propylene glycol does not evaporate under this pressure, the final formulation contains monoolein, emulsifier, propylene glycol, protein and a trace of water. Liquid formulations containing different model drugs and proteins are listed in Table 13.3. All of the liquid formulations containing drugs were clear homogeneous liquids when freshly prepared. In case of rifampicin and paclitaxel, however, drugs precipitated out irreversibly with time. Precipitation of rifampicin could be prevented by adding antioxidants such as ascorbic acid to the dispersion. When dispersed in water, most of the liquid formulations produced dispersions containing finely dispersed particles. In case of paclitaxel, precipitation occurred within a few hours after preparation.

13.3 ENCAPSULATION EFFICIENCY

Two hundred microliters of the liquid formulation were dispersed in 1 to 2 ml water. The dispersion was transferred into a retentate vial of Centricon® (MWCO 100,000 or 300,000 depending on the molecular weight of the encapsulated molecules) and centrifuged at $1000 \times g$ for 30 min. After centrifugation, only the solution (filtrate) passed through the membrane, leaving the particles in the retentate vial. The concentration of the drug in the filtrate was determined spectrophotometrically (UV-VIS or fluorescence) or by ELISA. Aqueous drug solutions were prepared to examine whether the drug in the bulk aqueous phase releases out completely through the membrane. Also, aqueous drug solution was mixed with the dispersion of the liquid formulation without drug to examine the interaction between the drug molecules and the particles. In the cases of hydrophobic drugs including paclitaxel, pyrene, and phosphatidylethanolamine derivatives, control experiments could not be performed since aqueous drug solutions could not be prepared due to the low solubility of these molecules in water. Low-molecular-weight drugs solubilized in the aqueous solution passed through the membrane completely in the absence of dispersed particles proving the validity of the measurements. Approximately 5% of the protein in the aqueous solution remained inside the retentate vial after centrifugation, probably since it is adsorbed on the membrane of the retentate vial. The presence of dispersed particles had no influence on the release of drugs into the bulk water phase.

Cresol red and rifampicin, which are slightly soluble in water, were not encapsulated completely in the particles. More hydrophobic molecules, paclitaxel or pyrene, and phosphatidylethanolamine derivatives, NBD-PE or Rhodamine-PE, were encapsulated completely inside the particles. As mentioned above, paclitaxel precipitated out with time even though the drug was completely loaded in the particles when freshly prepared. The encapsulation efficiency of hydrophilic drugs was lower than that of hydrophobic drugs. Most of the proteins were encapsulated inside the particles at high ratios. Calcitonin, a small hydrophilic peptide, was not encapsulated inside the particles at all, however. Dextrans, which are soluble macromolecules, were loaded efficiently in the particles.

13.4 SMALL-ANGLE X-RAY DIFFRACTION

Since the dispersed particles were composed of monoolein, emulsifier, organic solvent, and water, it is expected that the structure of the particles would be different from that of conventional lipid particulate systems such as liposomes or oil-in-water–type lipid emulsions. The structure of the dispersed particles was investigated by small angle x-ray diffraction. One hundred microliters of the liquid formulation was dispersed in 1 ml distilled water and transferred into 1-mm diameter quartz x-ray capillaries using an 18-gauge needle. To obtain a clear x-ray diffraction pattern, the particles in the dispersion were concentrated by centrifugation at 3000 r/min for 10 min. We confirmed that the centrifugation process did not alter the physical nature of the dispersion by redispersing the concentrated lipid particles in water. Redispersed particles retained the original size distribution. The capillary was flame-sealed and glued to prevent water leakage. X-ray diffraction data was obtained by using an x-ray diffractometer with general area detector diffraction system (GADDS, Bruker, Karlsruhe, Germany). The CuK radiation (1.542 Å) was provided by an x-ray generator (FL CU 4 KE, Bruker, Karlsruhe, Germany) operating at 40 kV and 45 mA. The sample-to-detector distance was 300 mm, and exposure time was 3 h. To avoid air scattering, the beam path was filled with helium.

Fully hydrated bulk mixtures containing monoolein, Pluronic F-127, and water were prepared by mixing the contents in two syringes, each containing lipids and aqueous phases, coupled with a three-way stopcock (Discofix®, B. Braun, Emmen-brücke, Switzerland) using a method slightly modified from that described previously (13,14). Without organic solvents, the bulk mixture of monoolein and

PF-127 at 5:1 by weight formed an Im3m cubic phase with the lattice parameter of 120 Å in excess water. The small-angle diffraction pattern was also collected for the concentrated dispersion of the liquid formulations. The diffraction peaks observed at spacing ratios of 2:4:6:10:12:14 indicate that the particles of the dispersion, regardless of the amount or kind of the organic solvent, have the internal structure of the Im3m cubic phase (15). The lattice parameter, however, was 130 Å, which was different from that of the bulk cubic phase formed by the mixture of the same composition in excess water (120 Å) but identical to that of Cubosome (1). It is highly likely, therefore, that our liquid formulation is a precursor or a preconcentrate of Cubosome (6). The fact that the lattice parameter of the dispersed particles differs from that of the bulk cubic liquid-crystalline phase may also be indirect evidence that there exist interior and exterior whose compositions are different in a single particle. It is highly probable that PF-127 acts as an emulsifier and distributes mainly on the exterior similar or identical to Cubosome.

Author: Interior and exterior what?

When a hydrophobic drug was encapsulated, the type of the cubic phase did not change, but the lattice parameter was lower for pyrene and cresol red while it was higher for rifampicin. For hydrophilic drugs, the structure and the lattice parameter were virtually identical to those of Cubosomes without any drugs, probably due to low drug encapsulation efficiency. The structure and the lattice parameter changed to a great extent when proteins were encapsulated inside the particles. Dispersion containing insulin remained as an Im3m cubic phase with a lattice parameter of 129 Å. Pn3m and Ia3d cubic phases were observed for dispersions containing FITC-BSA and cholera toxin B, respectively. Dispersions with tetanus toxoid or lysozyme yielded spotty patterns that could not be indexed as a single cubic phase.

13.5 CUBOSOMES AND LIPID CUBIC PARTICLES

Since the components and the composition of the dispersion of our liquid formulation were similar to the Cubosome dispersion except for the presence of the organic solvent, it was highly likely that the particles in the dispersion are also similar to Cubosomes. If they are different from Cubosomes, the difference could be instigated by the different preparation procedures and/or the presence of the organic solvent in our system. The preparation procedure of the original Cubosome includes a step to cool the microfluidized particles slowly to room temperature to form a well-defined internal cubic

lattice (1,16]) The preparation procedure of our lipid cubic particles is lacking this step (17). We can make a scientific guess on how the particles are formed from our liquid formulation by thinking through the sequence of events that may have happened when the liquid formulation was submerged in water.

When the liquid formulation is placed in water, the lipid, emulsifier, and organic solvents will feel the presence of water suddenly and will try to form an equilibrium phase, which may be a homogeneous viscous cubic phase. The system, however, will have to undergo a dramatic change since we either shake or vortex it. Since the bulk of the liquid formulation still remains as free-flowing liquid at this time, they can be broken easily into smaller pieces, whose surface will subsequently face surrounding water. Many events may happen in and on these small particles. Water-friendly polyethyleneoxide chains of PF-127 are highly likely to be located at the interface between bulk water and the particles and form the exterior of the particles. Some of the monoolein molecules, which are more hydrophobic than PF-127, may still be mixed with PF-127 on the interface. Most of monoolein and some PF-127 molecules will comprise the internal structure of the particles along with the organic solvent that was not able to escape to the bulk aqueous phase. The organic solvent, however, would not stay inside the particles. Since solvent molecules are small in size and can be mixed easily with water, most of them will diffuse out of the particles into the bulk water phase. Also, water will rush into the particles. The rate of the molecular exchange between the organic solvent and water may be a factor to control the formation of the well-defined cubic lattice. Whether the events happen as narrated above has yet to be investigated experimentally.

Author: It is not clear what "they" refers to.

The liquid formulation contains 50 to 70% (w/w) of organic solvent. Since the dispersion contains 5 to 20% (w/w) of the liquid formulation, the content of the organic solvent becomes 2.5 to 14% (w/w) in the final dispersion prepared from our liquid formulation. Since the organic solvents used in the liquid formulation are miscible with monoolein and PF-127, they can partition into the particles as well as into the bulk aqueous phase. How the organic solvent changes the characteristics of the dispersed particles needs further investigation. Despite the possible factors that may create differences between the original Cubosome and our lipid cubic particles, our particles will be referred to as Cubosomes hereafter since they are lipid particulate systems with the internal structure of the cubic liquid-crystalline phase satisfying the necessary and sufficient conditions for the definition of Cubosome.

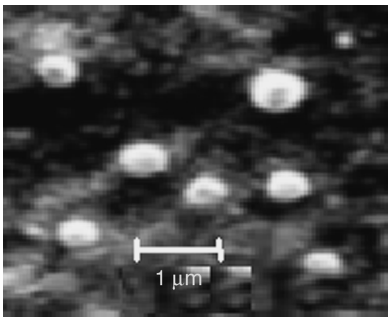


Figure 13.1 Low-temperature scanning electron microscope image of the dispersion of the liquid formulation.

13.6 LOW-TEMPERATURE SCANNING ELECTRON MICROSCOPY

Cubosomes prepared from the liquid dispersion were observed by using a low-temperature scanning electron microscope. To prepare a dispersion of Cubosomes, 10 μl of the liquid formulation was dispersed in 1 ml water by shaking. One drop (ca. 3 μl) of the Cubosome dispersion was mounted on a stub and immersed rapidly into nitrogen slushing chamber of the cryo-transfer system (CT 150 Cryotrans, Oxford Instruments Ltd., U.K.). The sample was transferred into the cryo-preparation chamber and cooled to –170°C under vacuum. The sample was transferred onto the cryo-stage of a scanning electron microscope (JSM 5410 LV, JEOL, Japan), and the temperature was raised to –70°C for 5 min to sublimate water. The sample was withdrawn to the cryo-preparation chamber again, coated with gold, and imaged at an accelerating voltage of 15 kV. Discrete submicron-sized particles were observed, but accurate structure was not visible due to the low resolution of the microscope (Figure 13.1).

13.7 PHYSICAL AND CHEMICAL STABILITY OF THE LIQUID FORMULATION

Immediately after preparation, the liquid formulation was a clear single-phase liquid. When stored at 4°C, the formulation became an opaque semisolid. Depending on the temperature, the bulk viscosity of the formulation changed. For instance, the formulation was a free-flowing liquid at 30°C whereas it was fairly viscous at 10 to 15°C.

The liquid formulations containing ethanol had a tendency to become more viscous than those with propylene glycol. Since PF127 alone precipitates in ethanol with time, the high viscosity of the liquid formulation made with ethanol may originate from the decreased solubility of the pluronics at lower temperature. The formulation containing PEG_{400} underwent gelation with time as described above. Sometimes, a small amount of white semitransparent aggregation formed in the ethanol-based liquid formulation with time, especially at or below 25°C. The heterogeneous formulation, however, became transparent liquid again when heated to ca. 35 to 40°C for a few minutes. There were no apparent changes in the physicochemical properties of the reheated sample when compared to the freshly prepared liquid formulation. There were no chemical degradations of the components in the formulation for at least 2 years when stored at 4 or –70°C. At room temperature, however, monoolein had a tendency to dissociate into glycerol and oleic acid (data not shown).

Liquid formulations containing drugs were also stable physically for at least 1 year at 4°C. For formulations containing rifampicin, however, an aggregation formed irreversibly within a few days after preparation. Pyrene and paclitaxel in the liquid formulation did not degrade for at least one year at 4°C. In the case of protein drugs, the situation was a little different. Since the liquid formulation contains a small amount of water, it is possible that the proteins can undergo a variety of destabilization processes including the conformational change, hydrolysis, or oxidation. The liquid formulation containing insulin was physically and chemically stable for at least 1 month when stored at 4°C. However, prolonged storage was not possible since the protein degraded with time. The stability was improved greatly when the liquid formulation was prepared in the absence of oxygen and stored at –70°C subsequently.

13.8 STABILITY OF CUBOSOME DISPERSION PREPARED FROM LIQUID FORMULATION

Good storage stability is crucial to a successful drug delivery system. To have commercial value, the formulation must be stable for more than 1 year at room temperature (17,18). The original Cubosome is known to be very stable at room temperature (1). We have tested the stability of Cubosomes freshly prepared from our liquid formulation and compared it with the stability of Cubosome prepared

by microfluidization. Since our Cubosome dispersion has a wider size distribution function (see below), we expected that our Cubosome system would be less stable than the original Cubosome, which has a narrow size distribution. As anticipated, the Cubosome prepared from the liquid formulation was stable for only a few days to a few weeks depending on the composition. Therefore, Cubosome prepared from our liquid formulation would not be suitable for commercialization in the form of the dispersion. Our liquid formulation, a precursor to Cubosome, however, has greater advantages that can more than compensate for the stability problems of the dispersion. Unlike its dispersion, the liquid formulation was very stable for a long period of time. Many hydrophobic drugs can be dissolved in the liquid formulation and be spontaneously encapsulated at a high rate in the Cubosome particles when dispersed in water without using mechanical devices. When mechanical force was used to disperse particles, many drugs would be chemically destabilized. In many systems, water is often the cause of physical instability and chemical degradation. Water can host reactive oxygen species, proton or hydroxide ions, and free radicals. Since our liquid formulation is a thermodynamically stable system containing no or a minimum amount of water, the sources of physicochemical destabilization were completely taken away. Even when the liquid formulation was prepared in the presence of oxygen, it was stable for more than a year without degradation of components including the drugs when stored at low temperature. For protein drugs, it was important to remove air from the precursor to prevent inactivation or conformational change of the protein since the liquid formulation contains water.

13.9 ABILITY TO FORM CUBOSOME FROM LIQUID FORMULATION AFTER STORAGE

As mentioned above, the liquid precursor formulation can be dispersed readily in excess water by mere shaking. As a drug delivery system, the liquid formulation is an ideal environment for drugs that might be oxidized and hydrolyzed in the presence of water. When the particulate dispersion system is needed, one just has to disperse the liquid formulation in water for immediate use. With this point of view, therefore, it is important to evaluate the physicochemical storage stability and the ability to form dispersion after storage of the liquid precursor formulation. To this end, liquid formulations comprising 100 mg monoolein, 20 mg Pluronic F-127, and 120 mg ethanol or PEG 400 or 280 mg 1,2-propanediol were prepared

Figure 13.2 Photograph of the dispersion of the liquid formulation after storage at 4°C for 3 days.

and stored at ambient temperature (10 to 30°C) or at 4°C for more than 500 days. There were no apparent visual changes in these liquid formulations. As mentioned earlier, white aggregates were sometimes found in the formulation but disappeared again when it was warmed to ca. 40°C. An important characteristic of the liquid formulation is the ability to form a fine dispersion of lipid particles in water. The particle size distribution was measured immediately after dispersing the stored liquid precursor formulation at different time points. These liquid formulations formed lipid dispersions of small mean particle sizes (<300 nm) and polydispersity (<0.3) for 18 months.

One of the advantages of the precursor system is that it can be stored at low temperature. The liquid formulation can be frozen or refrigerated for a long period of time. When frozen rapidly, the solution solidified without undergoing phase separation. If the liquid precursor formulation was refrigerated, however, phase separation was observed. Pluronic F-127 solidified before other components turned solid. We have to bear in mind that Cubosome is a thermodynamically unstable system, unlike the liquid formulation. The dispersion of the precursor, for instance, did undergo irreversible phase separation in a few days and generated bulk cubic phase when stored at 4°C (Figure 13.2).

13.10 *IN VITRO* DRUG RELEASE

The *in vitro* release experiments were performed with Cubosome dispersions containing four model drugs: methylene blue, bromocresol green, rifampicin, and insulin. Lipid formulations containing model drugs were dispersed in excess water. One milliliter of the dispersion was put into a dialysis membrane with molecular weight

cutoff values of 6000 for methylene blue, bromocresol green, and rifampicin and 100,000 for insulin. The bags containing the dispersion were sealed and immersed in 10 ml of 0.1 *M* Na_2HPO_4/citric acid buffer solution at pH 7.4 prewarmed at 37°C. In case of rifampicin, we were cautious to prevent oxidation of the drug by replacing the aqueous medium containing 0.5% sodium ascorbate at pH 7.4 during the experiment (19). The fact that rifampicin was not oxidized was confirmed by identical UV-VIS spectra of the released rifampicin to the freshly made one (the spectrum changes upon oxidation). If rifampicin was oxidized, it precipitated out and could not be released through the membrane. Aqueous drug solutions were used for controls. The samples in triplicate were placed in a shaking incubator at 37°C with a shaking frequency of 2 Hz. The dissolution media were replaced completely with 10 ml of fresh media at preset time intervals.

The release patterns of methylene blue, bromocresol green, rifampicin, and insulin from Cubosome are shown in Figure 13.3 (open circles). Aqueous drug solutions were used as controls (solid circles).

The release patterns of methylene blue from Cubosome and from the aqueous solution were virtually identical. Methylene blue was released completely in ca. 3 h.

An initial burst of bromocresol green release was followed by a plateau. Fifty percent of the drug was released during the first 5 h from the Cubosome dispersion. Bromocresol green in the aqueous solution was released quickly and completely in 2 h.

Rifampicin from Cubosome was released in a more sustained manner than bromocresol green from Cubosome. About 10% of the drug was released during the first 2 h, and about 30% was released during the following 20 h at zero-order rate. Complete release of the drug was not observed for the duration of the experiment. Rifampicin from the aqueous solution was released completely in 3 h. The difference in the release rate between bromocresol green and rifampicin may originate from the differences in the hydrophobicity of the drugs.

In the case of insulin, ca. 20% of the protein was released in 5 h. Insulin from the aqueous solution also showed an initial burst of release for the first 5 h. Therefore, it is possible that the initially released insulin from the Cubosome may represent those located in bulk water, not inside the Cubosome particles in the beginning. After the initial burst, insulin was not released at all from Cubosome, indicating the difficulty of diffusing a bulky protein out of the

Author: Those what?

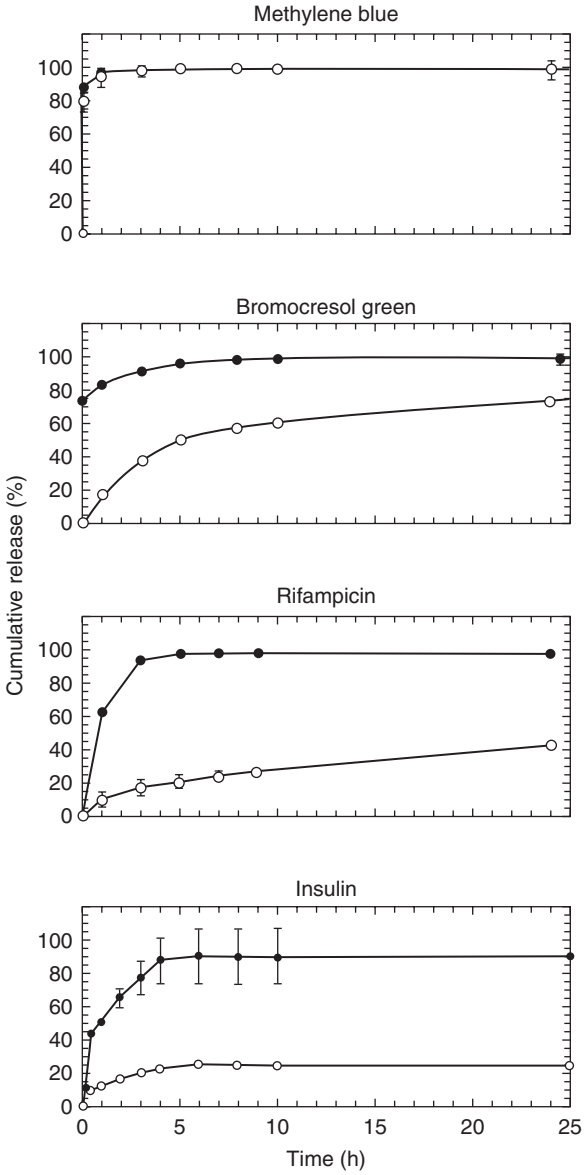


Figure 13.3 *In vitro* release profiles of methylene blue, bromocresol green, rifampicin, and insulin from aqueous solution (solid circles) and Cubosomes (open circles).

Cubosome particles to the buffer solution. Also, complete release was not observed even for the aqueous insulin solution, probably since some of the insulin molecules bind to the interior of the semipermeable membrane (MWCO 100,000).

For small and relatively hydrophilic drugs such as methylene blue, Cubosome does not act as a release barrier as it cannot encapsulate these drugs. In the case of hydrophobic drugs, however, Cubosome can help sustain the release of the encapsulated drug. Encapsu-ated protein, a macromolecule, was not released at all from Cubosomes. The resistance against release can be advantageous in some aspects. For instance, Cubosome can protect the loaded proteins from proteases until they reach the target cells when administered into the body. For this reason, the Cubosome system has a potential to become an effective drug delivery system for hydrophobic or protein drugs.

13.11 *IN VITRO* AND *IN VIVO* TOXICITY OF CUBOSOME PREPARED FROM A LIQUID FORMULATION

Since Cubosome prepared from the liquid formulation is a submicron-sized particle, it is natural to consider using it as a drug delivery system via the intravenous route. To be injected through a vein, however, Cubosomes must not be toxic or hemolytic. Since monoolein causes severe inflammation in muscle and subcutaneous tissues (Unpublished data, personal communication with T. Landh and M. Caffrey, 2002), it cannot be used as an injectable formula.

Hemolytic activity was evaluated for Cubosome prepared from the liquid formulation and the oil-in-water–type emulsion of the egg phosphatidylcholine (PC)/soybean oil system. The egg PC/soybean oil emulsion, prepared by sonicating a mixture of soybean oil and egg PC at 5 : 1 by weight in water, did not cause hemolysis even at 50 μg of total lipids/ml, while Cubosome was hemolytic at 6 μg of total lipids/ml (Figure 13.4). Cubosome, therefore, is not suitable to be injected intravenously.

The *in vitro* cytotoxicity of Cubosome in Caco-2 cells was evaluated by incubating the cells with various concentrations of Cubosomes for 1 day at 37°C, as shown in Figure 13.5 (9). Cubosome was highly toxic at concentrations equal to or greater than 500 μg of total lipids in 1 ml culture medium. AT concentrations of up to 100 μg of total lipids/ml, however, cell viability did not decrease for 3 days.

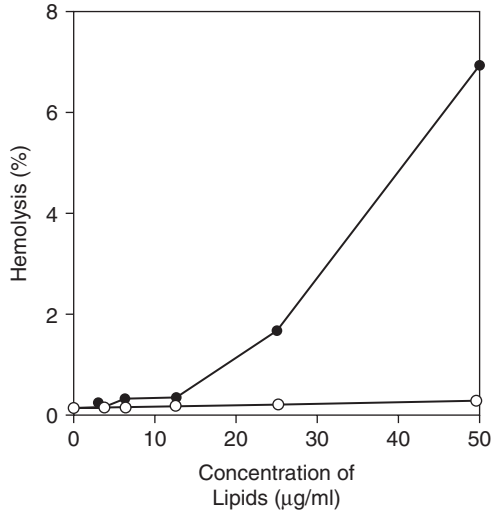


Figure 13.4 Hemolytic activity of Cubosomes (solid circles) and o/w emulsion of egg phosphatidylcholine/soybean oil (open circles). The concentration represents the total weight of the lipids and oil in the system per unit volume.

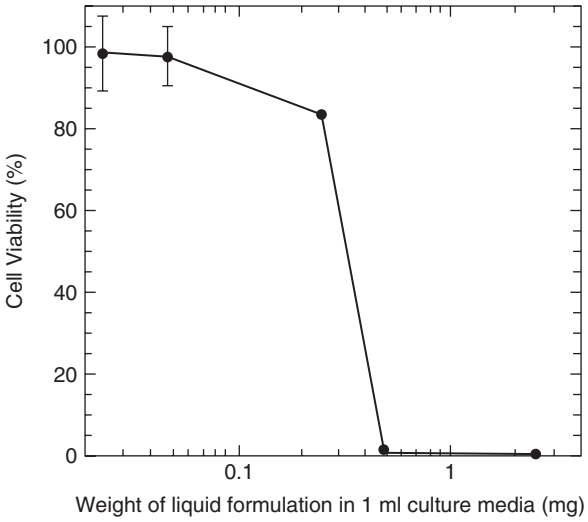
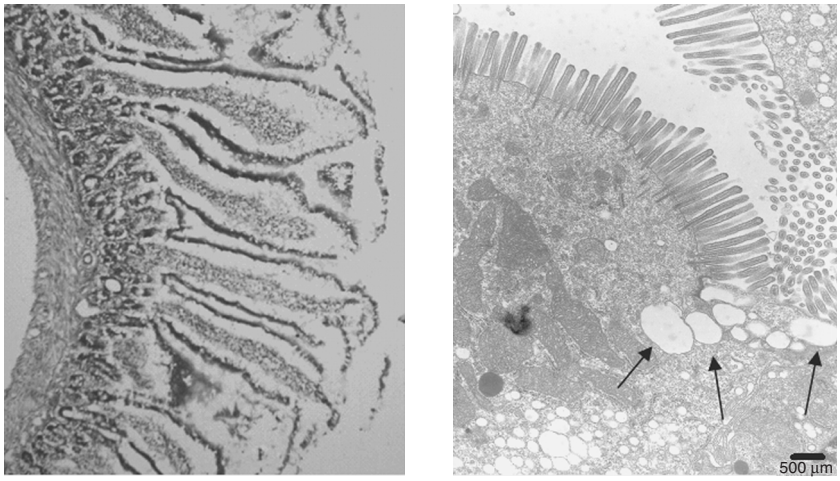


Figure 13.5 *In vitro* cell viability of Caco-2 cells upon incubation with Cubosomes made by dispersing the liquid formulation in culture media. The cells were incubated with Cubosomes for 1 day at 37°C.

To evaluate the *ex vivo* cytotoxicity of the Cubosome dispersion, rat everted jejunum was incubated with Cubosomes at a concentration of 10 mg of total lipid in 1 ml Krebs-Ringer bicarbonate buffer solution at 37°C for 1 h and observed with light microscopy and transmission electron microscopy (Figure 13.6).

There was no evidence of damage in the cells when observed by light microscopy (Figure 13.6A). Although many droplet structures were formed inside the periphery of the cells upon incubation with Cubosomes (arrows in Figure 13.6B), the cells functioned well as demonstrated by their ability to exclude trypan blue. Caco-2 cells in the *in vitro* culture system were much more susceptible to the toxic effects of Cubosomes, while the morphology of rat jejunum did not change even when a concentration of the Cubosome dispersion 50 times higher than the concentration that had shown *in vitro* toxicity was applied to the tissue. The vacuole-like structures formed after incubation with Cubosome may be similar to those formed after consuming a large amount of neutral lipids. These droplets seem to be analogous to the large lipid bodies formed during lipid digestion and absorption in intestinal absorptive cells (20). Since monoolein can enter the enterocyte by simple diffusion with or without the aid



(a) (b)

Figure 13.6 Light microscopy (A) and transmission electron microscopy (B) of rat small intestine after incubation of Cubosome made by dispersing 20 mg of the liquid formulation in 1 ml KBR solution for 1 h at 37°C.

of bile salts, constituents of these structures could be triglycerides synthesized from monoolein after being absorbed by the cells.

One of the main components of Cubosome is 1-monoolein, and one of the major digestion products of triglycerides in intestine is 2-monoolein. 2-Monoolein can convert to 1-monoolein and vice versa with an equilibrium composition of ca. 90% 1-monoolein and ca. 10% 2-monoolein at room temperature (21). Also, the equilibrium mixture of monoolein is in the GRAS (Generally Regarded As Safe) category for oral consumption (22). Single or repeated oral feeding every day for 2 weeks also showed that Cubosomes did not have any toxicity in mice (in preparation). Therefore, while Cubosome may not be a good drug delivery system for intravenous injection, it can be taken orally without exhibiting toxicity at high concentrations.

Author: Regarded or Recognized?

13.12 *IN VITRO* CELLULAR ASSOCIATION OF DRUGS FROM CUBOSOME

Since THE oral tract is one of the promising administration routes for Cubosomes, *in vitro* and *ex vivo* uptake experiments were performed with Caco-2 cells and rat everted sac, respectively (9). A hydrophobic fluorescence probe, pyrene, was encapsulated in Cubosomes to visualize the location of lipid absorption (23,24). The encapsulation efficiency of pyrene in Cubosome was ca. 100% (Table 13.3). When the liquid formulation was dispersed in PBS or DMEM, the particle size was 278.9 nm (polydispersity 0.267) and 267.8 nm (polydispersity 0.261), respectively. Pyrene was selected as a probe to follow the location of the lipids, particularly monoolein due to its hydrophobic nature. Pyrene was not released at all into the aqueous phase over 24 h in PBS at 37°C, indicating that the dye is located in the Cubosome, probably due to the hydrophobicity of pyrene and the stability of cubic particles.

The localization of pyrene and the morphology of the cells were visualized by fluorescence and phase contrast microscopy, respectively (Figure 13.7A and Figure 13.7B). We did not observe any blue fluorescence in and on the untreated cells (data not shown). When cells were incubated with Cubosome containing pyrene for 3 h at 37°C, the blue fluorescence of pyrene was observed clearly in the cells (Figure 13.7A). Intense punctual fluorescence as well as diffused blue fluorescence were observed in most of the fields. The number of dotted structures and the intensity of the diffused fluorescence increased with time, analogous to the increase of the vacuole-like structures by phase contrast microscopy (Figure 13.7B).

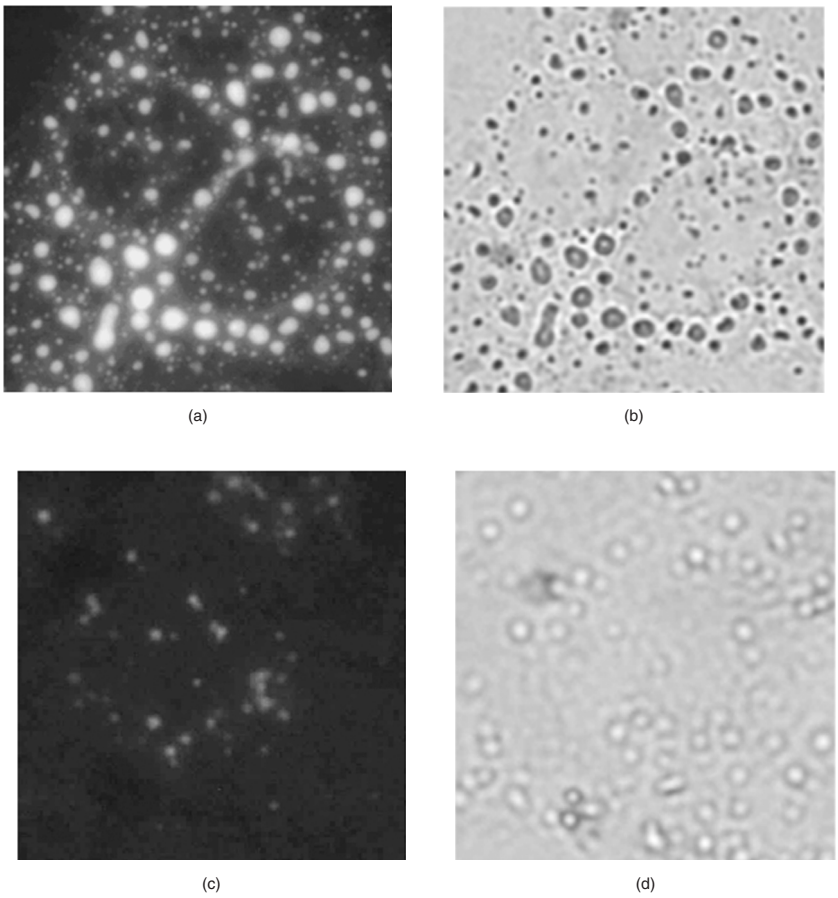


Figure 13.7 Fluorescence (A, C) and phase contrast microscopy (B, D) of Caco-2 cells incubated with Cubosomes encapsulating pyrene (A, B) and FITC-BSA (C, D).

Cubosome containing FITC conjugated bovine serum albumin (FITC-BSA) was incubated with Caco-2 cells for 3 h at 37°C. The protein was visualized by fluorescence microscopy and phase contrast microscopy (Figure 13.7C and Figure 13.7D). When FITC-BSA–loaded cubic particles were incubated with cells, droplet structures were observed by fluorescence microscopy (Figure 13.7C). Diffused fluorescence was not observed. The level of green fluorescence of FITC was similar in cells that were incubated with FITC-BSA aqueous solution or with Cubosome containing FITC-BSA. The fact that the

droplet structure inside the cells is not fluorescent may indicate that molecular diffusion of the components in the Cubosome, not endocytosis, would be the main uptake mechanism under *in vitro* conditions. We note that the lack of protein uptake *in vitro* may not imply that the same phenomenon would be observed under *in vivo* conditions; it would be risky to extrapolate the *in vitro* data to *in vivo* conditions.

To study the time-dependent cellular association of Cubosome prepared from liquid formulation, Caco-2 cells were incubated for up to 8 h with Cubosomes encapsulating pyrene in DMEM containing 10% fetal bovine serum at 37°C (Figure 13.8).

An emulsion comprising pyrene, egg phosphatidylcholine, and soybean oil (1:12:100 by weight) was also prepared for comparison. The particle size of the emulsion was ca. 250 nm. The amount of cellular-associated pyrene increased smoothly with time at 37°C upon incubation with Cubosome. After an 8-h incubation at 37°C, the cellular-associated pyrene was ca. 20% for Cubosome (Figure 13.8).

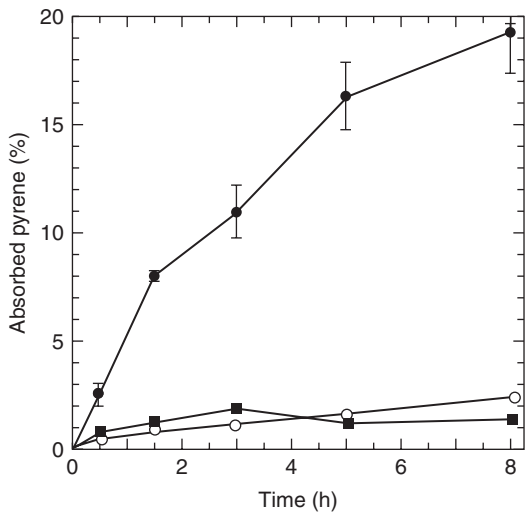


Figure 13.8 Absorption of Cubosomes by 1×10^6 Caco-2 cells as a function of incubation time. Caco-2 cells were incubated at 37°C (closed circles, n = 3) or at 4°C (open circles, n = 3) for up to 8 h with Cubosomes and at 37°C with egg PC emulsion (closed squares, n = 2). Data are represented as mean ± S.E.M. (Reproduced from JY Um, H Chung, KS Kim, IC Kwon, SY Jeong. *Int J Pharm* 253:71–80, 2003 with slight modifications with permission from Elsevier Science B.V.)

The absorption of pyrene for the emulsion formulation was only ca. 2% after 8 h of incubation. The number of associated pyrene was approximately ten times higher for Cubosomes than for the emulsion. When Caco-2 cells were incubated with Cubosomes (ca. 250 nm) at 4°C, only 2% of the pyrene in the medium was absorbed on the cells after incubation. This result coincides well with that observed by phase contrast microscopy, where the interaction between Cubosomes and cells at 4°C seems to be much weaker than at 37°C (unpublished data). Since it is highly probable that Cubosomes do not retain their internal structure at 4°C, the phase behavior of the particles must be studied further to understand the differences in the absorption efficiency at the two temperatures.

Author: Number of associated pyrene what? Molecules?

13.13 SOLUBILIZATION OF CUBOSOME BY BILE SALT AND ABSORPTION OF MIXED MICELLES

In the gastrointestinal tract, lipid particles can be digested by many enzymes and solubilized by bile salts. Since the main component of Cubosome is monoolein, bile salts secreted to the intestine can become a good solubilizer of Cubosome. Various concentrations of sodium taurodeoxycholate micelle systems were prepared to test the solubilizing power of the bile salt. Coarsely dispersed Cubosomes with an average size of ca. 495 nm were prepared. Since the concentration of the bile salts in human is ca. 5 and 10 to 20 m*M* in the fasted and at fed states, respectively, taurocholate solutions ranging from 0 to 20 m*M* were prepared to encompass the physiological range (25). The size distribution of the particles was measured using dynamic light scattering (Figure 13.9).

Author: In the human what?

As taurodeoxycholate solution was added to Cubosomes, the size distribution of the particles changed from a unimodal peak at 495 nm to bimodal peaks at 114 nm and 34 nm at 10 m*M* taurocholate (Figure 13.9A and Figure 13.9B). When the concentration of taurodeoxycholate was 15 m*M*, mixed micelles of the taurocholate/monoolein/Pluronic system with a size of 8 nm were produced (Figure 13.9C). The size of the taurocholate micelle (15 m*M*) without any other components was ca. 3 nm (Figure 13.9D).

Since the bile salt can destroy the Cubosome structure completely under physiological conditions, the absorption of the loaded drug, pyrene, by cells can be altered greatly in the presence of bile salts. To study how the addition of the bile salt changes the absorption of pyrene by intestinal absorptive cells, the mixtures of Cubosome encapsulating pyrene and sodium taurocholate at different

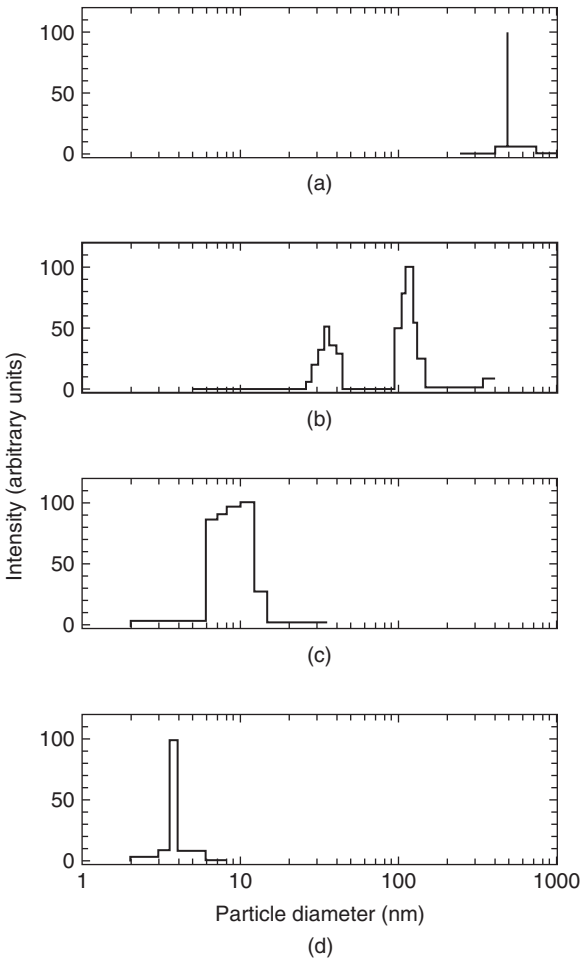


Figure 13.9 The diameter change of Cubosome upon mixing with sodium taurodeoxycholate (Na-TDC) measured with dynamic light scattering. Cubosomes were mixed with 0 mM (a), 10 mM (b) and 15 mM (c) taurodeoxycholate solution. The final concentration of Cubosomes was 20 mg of the liquid formulation in 1 ml bile salt solution. The diameter of bile salt micelle at 15 mM was measured in the absence of Cubosomes (d). (Reproduced from JY Um, H Chung, KS Kim, IC Kwon, SY Jeong. *Int J Pharm* 253:71–80, 2003 with slight modifications with permission from Elsevier Science B.V.)

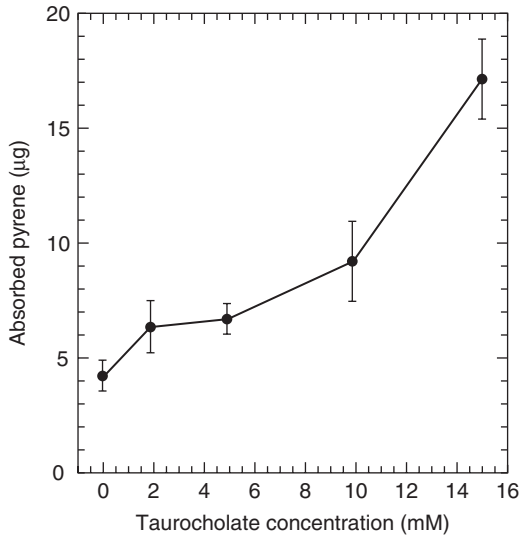


Figure 13.10 The amount of pyrene absorbed by 100 mg everted rat jejunum as a function of sodium taurodeoxycholate concentration. One milliliter of the medium contains 0.5 mg pyrene and 20 mg of the dispersed liquid formulation, Cubosomes. Vertical bars denote ± S.E. of the mean value for three separate determinations. (Reproduced from JY Um, H Chung, KS Kim, IC Kwon, SY Jeong. *Int J Pharm* 253:71–80, 2003 with permission from Elsevier Science B.V.)

concentrations were added to media containing the rat jejunum everted sac (Figure 13.10). After a 1-h incubation, pyrene concentration in the jejunum was quantified by extraction. There was a small increase in pyrene absorption when bile salt solutions at low concentrations (2 to 5 m*M*) were added to the Cubosome dispersion. As the bile salt concentration increased, the concentration of absorbed pyrene inside the cells increased by at least three times. Therefore, the bile salt can help the absorption of the drug encapsulated in Cubosome via the formation of mixed micelles.

Cubosome can be solubilized by the bile salts in the small intestine into mixed micelles and absorbed into the intestinal absorptive cells when administered orally. It is possible that ethanol used to prepare the precursor liquid formulation can also enhance the absorption. The monoolein–bile salt mixed micelle is known to penetrate into cells better than the bile salt micelle (26). Absorption of

Cubosome taken by oral administration can be further enhanced by the bile salts secreted into the intestine.

13.14 ORAL DELIVERY OF CUBOSOME ENCAPSULATING PYRENE

Monoolein and Cubosome containing 2 mg/ml of pyrene were prepared for an oral delivery experiment. Balb/c mice were fed 0.5 ml of monoolein or Cubosome corresponding to a dose of 1 mg pyrene per mouse. Monoolein containing pyrene was melted, super-cooled to room temperature, and fed to the animals as a liquid. Two or four hours after the feeding, the mice were sacrificed to obtain blood and the organs to determine the concentrations of pyrene (Figure 13.11). For mice fed with monoolein, pyrene was detected mainly in liver, kidney, and blood. In case of Cubosomes, pyrene concentration in each tissue was higher than that for monoolein. The concentration of pyrene was ca. 300 μg/g blood, which was at least 10 times higher than that of the monoolein group. Since monoolein forms the cubic phase upon contact with body fluid, the results indicate that micronization of the bulk cubic phase into cubic particles can indeed help the absorption of drugs in the oral tract.

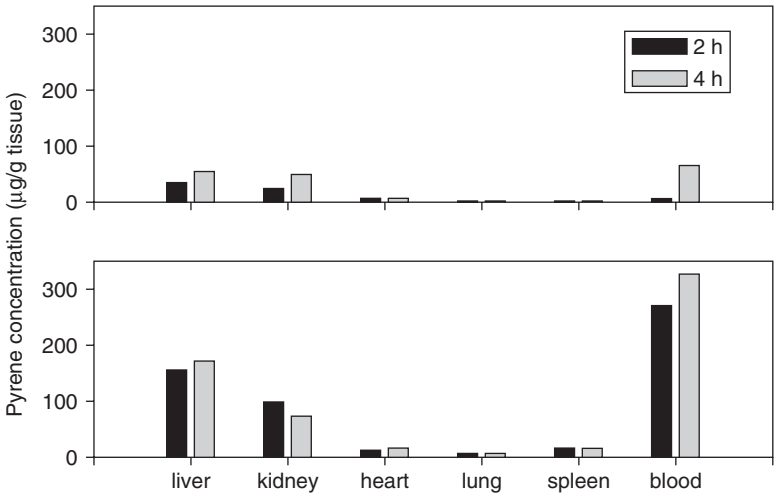


Figure 13.11 *In vivo* organ distribution profile of pyrene after oral administration of 1 mg pyrene in monoolein (A) and in Cubosome (B).

13.15 ORAL DELIVERY OF CUBOSOME ENCAPSULATING INSULIN

Since our liquid formulation or Cubosome can encapsulate protein drugs at a high ratio and therefore could be a good oral drug delivery system, a liquid formulation containing monoolein, PF-127, propylene glycol, and insulin was prepared as a peroral insulin formulation (8).

Male Wistar rats, age 5 weeks, weighing 120 to 150 g, obtained from Charles River Japan Inc. (Yokohama, Japan), were housed in groups of three in separate cages with free access to food and water for 1 week in a temperature-controlled room under a 12-h light/dark cycle prior to the experiments. Diabetes was induced by three consecutive intraperitoneal injections of streptozotocin (45 mg/kg). After 2 weeks, rats were considered diabetic if their blood glucose level was above 300 mg/dL in the fasting state. No insulin treatment was performed until the experiment.

Baseline blood glucose level was determined in normal and diabetic rats fasted for 4 h. During the experiment the rats were kept in a fasting state but allowed water *ad libitum*. Normal (Figure 13.12A) or streptozotocin-induced diabetic (Figure 13.12B)

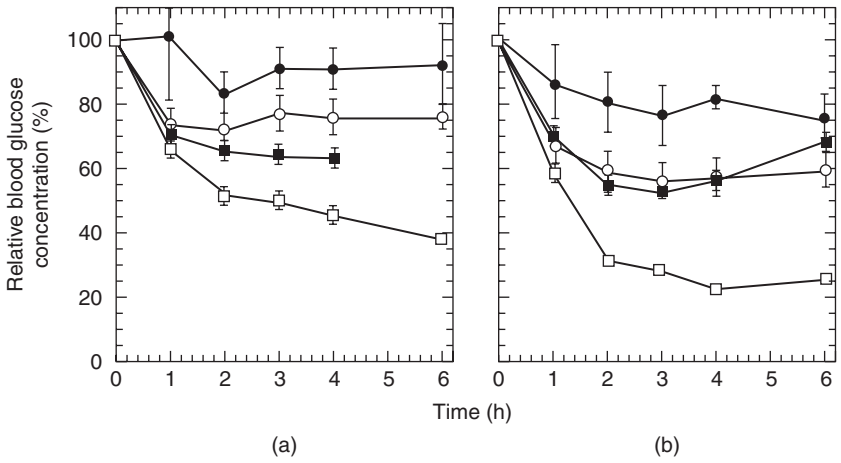


Figure 13.12 Relative blood glucose concentration after oral administration of 30 (open circles), 50 (closed squares), and 100 IU/kg (open squares) encapsulated in Cubosome in normal (A) and diabetic (B) rats. Relative blood glucose concentration of untreated rats (closed circles) was also measured as a control.

rats were fed 30 (open circles), 50 (solid squares), and 100 IU/kg (open squares) insulin-encapsulated Cubosome dispersion. Untreated rats were used as controls (solid circles).

Relative blood glucose level decreased for up to 2 h for the normal mice fed 30 or 50 IU/kg, but decreased continuously in those fed 100 IU/kg. The reduction of blood glucose concentration in normal rats was dose dependent. There was essentially no change in blood glucose concentration in the untreated rats. In diabetic rats, blood glucose concentration decreased slightly for untreated animals since fasting can lower the blood glucose concentration rather effectively. The blood glucose level was similar when 30 and 50 IU/kg of insulin in Cubosome were administered. When 100 IU/kg of insulin in Cubosome was administered, low glucose level was reached rapidly and maintained for at least 6 h.

The fact that insulin loaded inside Cubosome has a superior effect to control hyperglycemia indicates that Cubosome could be widely applied in oral protein delivery systems. It would be interesting to examine whether the particles themselves increase intestinal penetration or merely protect insulin from the attack of proteolytic enzymes.

13.16 ORAL VACCINATION BY ANTIGEN LOADED IN CUBOSOME

Another class of proteins that can be administered orally is antigens for vaccination. Oral vaccination is an attractive and convenient alternative to injection. However, oral immunization has not been widely used since antigen uptake is inefficient in the intestine and oral tolerance could also be induced. Antigens could also be degraded by proteolytic enzymes before they reach the immune cells. To overcome these problems, many scientists have developed polymer- and lipid-based delivery systems to encapsulate antigens for oral delivery. To test whether Cubosomes can be used as an oral vaccine carrier, we have encapsulated cholera toxin B subunit (CTB) inside Cubosome to administer orally into Balb/c mice three times at intervals of 2 weeks. The sera were collected 2, 4, and 6 weeks after the first immunization to follow the increase in the systemic antibody response (Figure 13.13).

Sera collected from the untreated mice and mice fed with Cubosome without CTB were used as negative controls. CTB alone or mixed with Cubosomes showed elevated systemic IgG and IgA concentrations when compared to negative controls. IgG and IgA

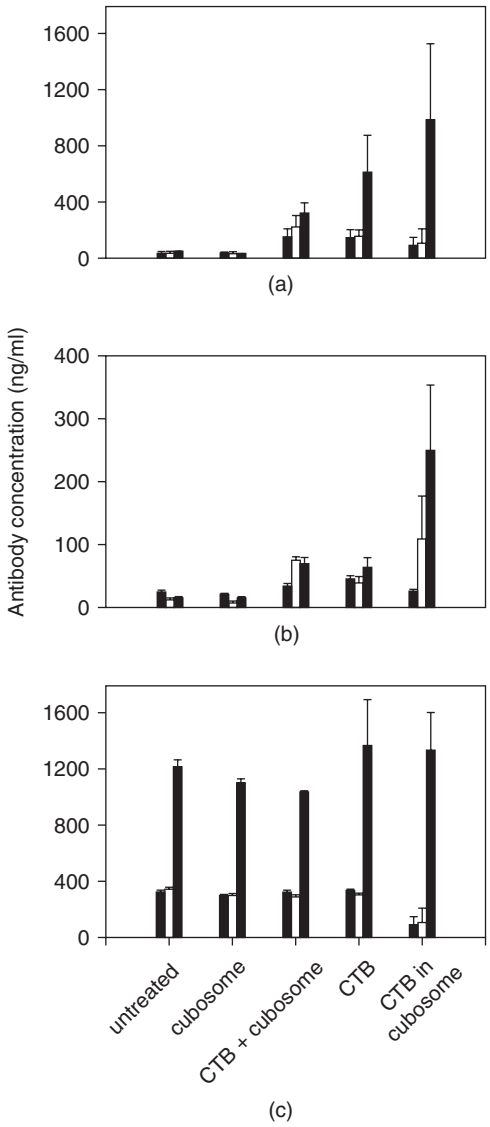


Figure 13.13 CTB-specific IgG (A), IgA (B) and IgM (C) antibody response in serum 2, 4, and 6 weeks after first immunization. Groups of 5- to 6-week-old Balb/c mice were orally immunized three times at intervals of 2 weeks. Values represent geometric mean ± S.E. (n = 5 to 6).

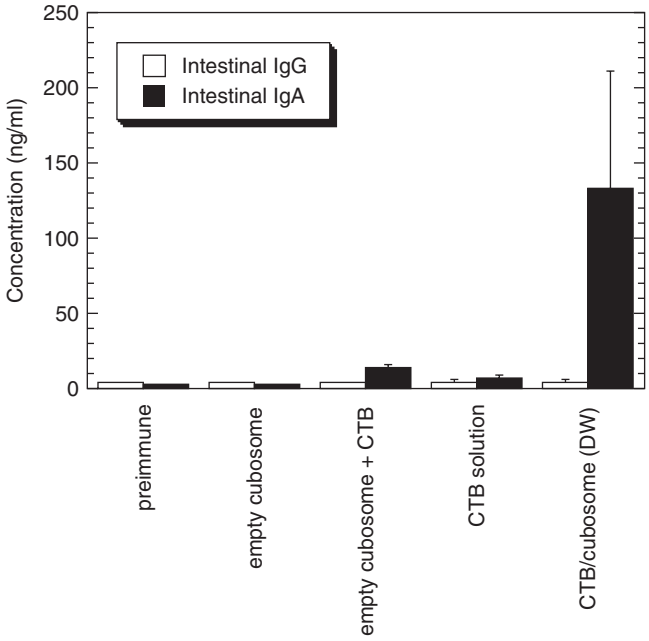


Figure 13.14 CTB-specific IgG (A) and IgA (B) responses in intestinal lavage samples 2, 4, and 6 weeks after first immunization. Groups of 5- to 6-week-old Balb/c mice were orally immunized three times at intervals of 2 weeks. Values represent geometric mean ± S.E. (n = 5 to 6).

levels of the mice fed CTB loaded inside Cubosomes were the highest among the experimental groups, showing that the oral vaccination was more efficient when the antigen was encapsulated inside Cubosome. Local immune reaction, intestinal IgA, was even higher for the group of mice orally administered CTB inside Cubosomes (Figure 13.14). The results imply that CTB inside the Cubosome was indeed taken up by the immune cells and elicited immune reactions efficiently.

13.17 CONCLUSIONS

Self-dispersing homogeneous liquid formulations containing monoolein, emulsifiers, and organic solvents have been prepared. The liquid formulation was a single anhydrous liquid and was stable for more than 6 months at room temperature and at 4°C. The homogeneous

liquid formulation can be dispersed easily in an excess amount of water by mere shaking or vortexing to form Cubosomes. Our liquid formulation overcame some of the stability problems associated with the Cubosomes especially when protein drugs were used. Since our formulation is an anhydrous homogeneous mixture, it does not undergo oxidation or hydrolysis, which can potentially destabilize the system. The liquid formulation can be used as a drug delivery system since many drugs can be mixed easily in the formulation. The interaction between Cubosomes and Caco-2 cells was studied by various microscopic techniques. Lipid droplets were observed in the cytosol of enterocytes after incubation with Cubosomes. The amount of pyrene absorbed by Caco-2 cells was ca. 20% of the total at 37°C after an 8-h incubation. Cubosomes were easily solubilized by bile salts to produce mixed micelles. As the bile salt concentration increased, pyrene absorption into the jejunum of rat everted sac *ex vivo* increased.

Cubosomes were used as a peroral insulin delivery system. Serum glucose levels could be controlled for more than 6 h after oral insulin administration for normal and diabetic rats. Cubosome can also deliver antigens efficiently into the immune cells in the intestine resulting in prominent systemic and local immunity. Even though we have limited the delivery route of Cubosome to the oral tract in this chapter, other routes including those with mucosal tissues can be excellent targets for drug delivery using Cubosomes.

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