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In vitro cellular interaction and absorption of dispersed cubic particles

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Abstract

A precursor type oily liquid formulation comprising monoolein, Pluronic F-127 and ethanol has been prepared as a carrier for lipophilic drugs. When dispersed in water, the liquid precursor formulation produces sub-micron (200–500 nm) sized lipid particles, named 'nanocubicles'. The interaction between nanocubicles and Caco-2 cell was studied, and the absorption of nanocubicles by cells was observed by various microscopic techniques. Lipid droplets were observed in cytosol after incubation with nanocubicles with time. The degree of pyrene absorption encapsulated in nanocubicles was dependent on particle size and incubation time. The amount of pyrene absorbed by Caco-2 cells was ca. 20% of total at 37 °C after an 8-h incubation. When nanocubicles with a bigger average particle size (ca. 600 nm) were applied, the uptake rate was reduced to 10% under identical experimental conditions. The nanocubicles were easily solubilized by bile salts to produce mixed micelles. As bile salt concentration increased, pyrene absorption into the jejunum of rat everted sac in vitro increased.
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1. Introduction

To increase the bioavailability of hydrophobic drugs, drugs must be fully solubilized in carriers without precipitation and delivered into the gastrointestinal tract, where they are absorbed by intestinal absorptive cells. Since most of the small hydrophobic drug molecules are absorbed by simple diffusion, solubilization of the drug is one of the most important factors. In the lipid-based drug delivery systems, the hydrophobic drugs are solubilized in lipid carriers including liposomes, microemulsions or solid lipid nanoparticles (Constantinides, 1995; Al-Meshal et al., 1998; Mehnert and Mäder, 2001).

About a decade ago, Swedish scientists have succeeded in developing a dispersion of liquid crystalline cubic phase of hydrated lipids (Gustafsson et al., 1996). The dispersed particles were named Cubosome®. Since the internal structure of the Cubosome® consists of lipid domain, water channel and interfacial region, Cubosome® has been used to encapsulate amphiphilic, lipophilic and protein drugs (Bojrup et al., 1996; Engström et al., 1996; Landh and Larsson, 1996; Sheikh et al., 1999).

Administration routes of Cubosome® have also been diverse. Cubosome® prepared with glycolipids

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was used as a vaccine adjuvant via subcutaneous injection (Sheikh et al., 1999). Cubosome® also helped maintain the plasma concentration of somatostatin for up to 6 h after intravenous injection (Engström et al., 1996). Cyclosporin A loaded in Cubosome® was administered into stomach and exhibited similar bioavailability to a commercial formulation (Bojrup et al., 1996). Even though Cubosome® has some of the outstanding features to become successful delivery systems for protein and lipophilic drugs, there are also some of the shortcomings. To prepare nano-sized Cubosome®, the mixture of monoolein, emulsifier and water needs to be microfluidized at ca. 80 °C and cooled slowly to room temperature. Many drugs cannot endure the high temperature and pressure, and therefore are destabilized during this harsh process. To overcome problems associated with the preparation process, we have prepared a precursor type formulation that can be dispersed in water by mere shaking or vortexing at ambient temperatures. The liquid precursor formulation comprises monoolein, emulsifier, organic solvent and drug and produces nano-sized particles termed 'nanocubicles' encapsulating drugs at a high efficiency (Kim et al., 2000). Our nanocubicle may be similar to, but can also be different from Cubosome® in some respects. It is well-known that the preparation procedure determines the type of produced particles even the identical mixture was used as a starting material (Almgren et al., 2000). For instance, if the cubic phase consisting monoolein, emulsifier and water is sonicated instead of being microfluidized, small 'folded vesicles' are formed initially, and only slowly some of these particles evolve into well-shaped Cubosome® (Gustafsson et al., 1996). The characteristics of our nanocubicle are under investigation. In the mean time, we will call the particles produced by dispersing our liquid precursor formulation 'nanocubicles' to differentiate them from Cubosome®.

Previous study has shown that nanocubicles encapsulating insulin effectively lowered the serum glucose level of rat when delivered via peroral route (Chung et al., 2002). In this paper, we prepared nanocubicles encapsulating a hydrophobic probe, pyrene and performed in vitro uptake experiment using Caco-2 cells and rat jejunum everted sac to study the absorption mechanism of nanocubicles by the digestive tract and to evaluate nanocubicles as an oral drug delivery carrier for hydrophobic drugs.

2. Materials and methods

2.1. Materials

Distilled monoglyceride (RYLO MG 19, Danisco Ingredients, Brabrand, Denmark) with the following fatty acid composition was used: oleic acid 88%, linoleic acid 10%, other fatty acid 2%. This monoglyceride will be referred to as monoolein in this paper. Pluronic F-127 (Poloxamer 407, $PEO_{98}PPO_{67}PEO_{98}$) was obtained from BASF (Parsippany, NJ). Pyrene, paraformaldehyde, Triton X-100, propidium iodide (PI), ribonuclease A (RNase A), fluorescein isothiocyanate labeled phalloidin (phalloidin-FITC), sodium taurodeoxycholate (NA-TDC) and Krebs–Ringer bicarbonate buffer (KBR; pH 7.4) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium cacodylate buffer, propylene oxide, osmium tetroxide, lead citrate and Spurr's Kit were purchased from Electron Microscopy Sciences (Fort Washington, PA). Uranyl acetate was purchased from Merck (Darmstadt, Germany). All other chemicals used were of analytical grade.

2.2. Preparation of liquid formulation and nanocubicles

To prepare a liquid precursor formulation, 100 mg monoolein and 20 mg Pluronic F-127 were completely dissolved in 2 ml absolute ethanol. Ethanol in the mixture was evaporated completely under vacuum to obtain a viscous mixture. The mixture was mixed again in 120 mg ethanol to form a liquid precursor formulation. The liquid formulation was stored at room temperature or at 4 °C for further experiments. For the pyrene-loaded liquid formulation, pyrene dissolved in absolute ethanol was used instead of 2 ml absolute ethanol at the first step of the preparation process. The weight ratio between monoolein and pyrene was 100:6 in the final liquid formulation.

Nanocubicles were prepared by dispersing the liquid precursor formulation in 5–2000 times excess water by vortexing at 3200 rpm for 30 s.

2.3. Characterization of nanocubicles

The average particle size and the surface potential of the nanocubicles were determined by quasielastic laser

light scattering with Malvern Zetasizer® (Malvern Instruments Ltd., UK) as described previously (Chung et al., 2001) after 300- and 3×10^4-folds dilution in water, respectively. The particle size distribution will be represented by the average size (diameter) and the variance (polydispersity) of the Gaussian distribution function in logarithmic axis mode.

The nanocubicles were observed by using low temperature scanning electron microscopy (JSM 5410 LV, JEOL, Japan) with an Oxford CT1500 cryotransfer system.

The internal liquid crystalline phase was investigated by small angle X-ray diffraction. Nanocubicle dispersions were transferred to 1 mm diameter quartz capillary and sealed with epoxy. Copper Kα X-rays (1.542 Å) were produced using an X-ray generator (FL CU 4KE, Bruker, Germany) and operated at 40 kV and 45 mA. The exposure time was 2.5 h, and the sample-to-detector distance was 30 cm. X-ray diffraction (XRD) data was obtained by General Area Detector Diffraction System (GADDS, Bruker, Germany) at 25 °C.

Encapsulation efficiency of pyrene in nanocubicle was measured with Centricon® (YM-100, Millipore Corp., Bedford, MA) as described previously (Chung et al., 2002) and the fluorescence intensity of the free pyrene in the filtrate was compared to the total intensity in the dispersion before filtration. For in vitro release test, liquid formulation was dispersed in PBS solution and was shaken at a frequency of 10^3 min^{-1} at 37 °C with an isothermal shaker (Thermomixer 5436, Eppendorf, Germany). At various time points, the concentration of pyrene in bulk water was determined by using Centricon®.

2.4. Caco-2 cell culture and incubation with nanocubicles

Caco-2 cells were obtained from the American Type Culture Collection and cells from passages 40–75 were used. Caco-2 cells were grown at 37 °C/5% CO_2 in culture medium consisting of Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids and 1% penicillin-streptomycin (GIBCO BRL, Grand Island, NY). For microscopy observation and cellular association study, cells were plated at a density of 5×10^4 cells/1.8 cm^2 on the Lab-Tek™ 4-well chamber slide (Nunc, Roskilde, Denmark) and plated at a density of 3×10^5 cells/9.5 cm^2 on a Falcon® 6-well plate, respectively. For transmission electron microscopy (TEM), cells were incubated on the Thermanox™ cover slip (Nunc) at a density of 5×10^4 cells/1.8 cm^2. Cells were cultured for 3 days prior to use and the confluency was ca. 95%.

To incubate the pyrene-loaded and empty nanocubicles with cells, the liquid formulation was dispersed in culture medium without serum to form nanocubicles and the fetal bovine serum corresponding to 10% of total volume was added to each well. The final concentration of nanocubicles dispersion corresponded to 0.5 mg of the liquid formulation in 1 ml cell culture media. For microscopic observations, the cells were incubated with 1 ml of the above nanocubicle dispersion for 3 h at 37 or 4 °C. To measure the pyrene concentration associated with the cells as a function of time and particle size, 3 ml of the nanocubicle dispersions were added to each well and incubated with cells up to 8 h at 37 °C.

2.5. Microscopy

The morphology change of live cells was observed by using phase contrast microscope (CK40, Olympus, Japan) fitted with 20× objective after incubation of Caco-2 cells with nanocubicles at 37 or 4 °C.

For fluorescence microscopy, cells were incubated with 4% paraformaldehyde in PBS for 30 min at 4 °C after removing the nanocubicle dispersions. Cells were permeabilized with 1% Triton X-100 for 5 min at RT, incubated with 10 μg/ml RNase A for 1 h at 37 °C and with 5 μg/ml PI for 20 min. The slides were mounted in fluorescent mounting medium (DAKO, Denmark) and cells were visualized by using a fluorescence microscope (BX51, Olympus JAPAN) fitted with 20× objective.

For confocal microscopy, cells were incubated with 1 μg/ml phalloidin-FITC for 1 h at room temperature after PI staining and observed by confocal laser scanning microscope (Multiphoton Imaging System, Radiance 2000 MP Bio-Rad, Hercules, CA) with IR-Laser (Tsunami, Spectra-Physics Inc., Mountain View, CA). Pyrene fluorescence was excited using a 2-photon excitation system. The fluorescence of PI and FITC was excited with Ar/Kr laser.

For TEM, cells on cover slip were washed and fixed in 2% paraformaldehyde–2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2). Cells were post fixed with 1% osmium tetroxide for 2 h and stained overnight with 0.5% uranyl acetate at 4 °C. Cells were dehydrated, infiltrated and polymerized with 100% Spurr's resin. Ultra thin sections were stained with 2% uranyl acetate and lead citrate and examined with transmission electron microscope (JEM-1010, JEOL, Japan).

2.6. Cellular association of nanocubicles encapsulating pyrene

After incubation with pyrene-loaded nanocubicles, the cells were washed and harvested. The pellets were dried overnight in a speed vacuum evaporator (Modulspin 4808, Hanil R&D, South Korea) and pyrene was extracted with ethyl acetate by shaking (Thermomixer 5436, Eppendorf, Germany) for 24 h. Concentration of pyrene was determined by using ISS K2 multifrequency phase and modulation fluorometer (ISS, Champaign, IL). Pyrene was excited using a 300-W xenon arc lamp (ILC Technology, Sunnyvale, CA). To obtain the emission fluorescence intensity at 390 nm, the slit openings for both excitation and emission lights were 0.5 mm. The excitation wavelength (λ_{Ex}) was 336 nm.

2.7. Solubilization of nanocubicles by Na-TDC and absorption of monoolein-taurodeoxycholate mixed micelles by everted sac

Nanocubicle dispersions were prepared and mixed with various concentrations of taurodeoxycholate. The final concentration of nanocubicles was 20 mg of the liquid formulation in 1 ml of the 0–15 mM taurodeoxycholate solutions. And the average particle diameter of each solution determined with dynamic light scattering using Brookhaven BI-200SM goniometer and BI-9000AT autocorrelator (Lee et al., 1999).

Male Sprague–Dawley, 6 weeks old, weighing 150 g were fasted for 24 h and killed by a cervical dislocation. The peritoneal cavity was opened and proximal jejunum (10–30 cm distally from the ligament of Treitz) was stripped from the mesentery and placed in a Petri dish containing KBR solution (pH 7.4, 4 °C). The jejunum was everted with a stainless steel rod and washed for 1 min in three dishes each containing KBR solution. After cutting the jejunum by 2 cm in length, the terminal sites were ligated with suture. The experiment was finished in 1 h after the jejunum was taken out of the rat since the tissue was viable only for a few hours (Barthe et al., 1998). Sacs were incubated with above mixed solutions of nanocubicles and taurodeoxycholate in a 6-well plate. After an 1-h incubation at 37 °C, the sacs were removed from the solution and washed three times. After it was weighed and homogenized, pyrene was extracted with ethyl acetate (Bouchard et al., 1998). The concentration of pyrene was analyzed using fluorometer as described previously.

3. Results and discussion

3.1. Characterization of nanocubicles

Nanocubicle in the aqueous solution is a particulate system as imaged by low temperature scanning electron microscope (Fig. 1). The deformation of nanocubicle by high temperature and high electron beam produced during the sample preparation could be prevented by freezing the sample. The average size of nanocubicles was ca. 250 nm (polydispersity: 0.203) when observed by photon correlation spectroscopy (300-fold dilution, Melvern Zetasizer®) and zeta potential was ca. −30 mV (3×10^4-fold dilution). The negative zeta potential resulted from the fatty acid, which is a minor component in monoolein. The size

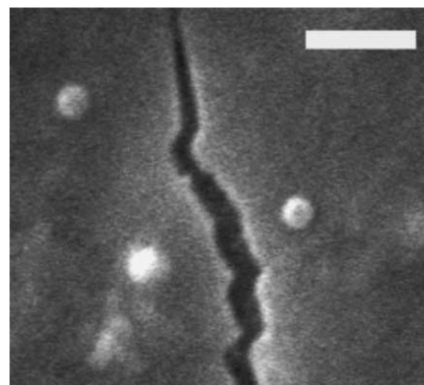

Fig. 1. Low temperature scanning electron microscope image of the dispersion of nanocubicles. The scale bar corresponds to 1 μm.

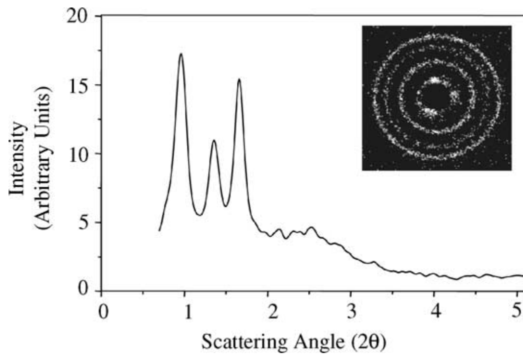

Fig. 2. Small angle X-ray diffraction pattern of nanocubicle.

and zeta potential of nanocubicles containing pyrene were 258.5 nm (polydispersity: 0.390) and −28.6 mV, respectively, which were virtually identical to those without pyrene.

The internal structure of the nanocubicles was investigated by small angle X-ray diffraction. The particles in the dispersion had to be concentrated by centrifugation to obtain clear XRD pattern. The concentrated particles were easily redispersed in water with a simple pipetting and retained original size distribution. It indicates strongly that the centrifugation process did not cause a gross rearrangement of the structure of nanocubicles. The small angle X-ray diffraction in Fig. 2 shows the repeat spacing ratio of $\sqrt{2}:\sqrt{4}:\sqrt{6}$. Therefore, it is highly probable that the internal structure of the nanocubicles is a body-centered cubic, *Im*3*m* phase (Macgillavry and Rieck, 1962). Lattice parameter of the unit cell was calculated to be 131.4 ± 1 Å. The phase and the lattice parameter are very similar to those of cubic phase consisting of monoolein, Pluronic F-127 and water (Landh, 1994) and Cubosome® (*Im*3*m* cubic liquid crystalline phase, Gustafsson et al., 1996, 1997), but further experiments are necessary to figure out the similarity and difference between the structures of the nanocubicle and Cubosome®. This result suggests that the lipid particles with the internal structure of cubic phase can be constructed by dispersing a precursor containing monoolein and emulsifier in water without the aid of mechanical devices. Due to this internal structure, nanocubicles can encapsulate lipophilic as well as hydrophilic protein drugs.

To visualize the interaction of nanocubicles with cells and the location of lipid absorption, pyrene was encapsulated in nanocubicles (Plant et al., 1985, 1987). Pyrene can also be regarded as a hydrophobic model drug to evaluate nanocubicle as a lipid-based drug delivery system. The encapsulation efficiency of pyrene in the nanocubicles was ca. 99.8%. 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. The in vitro release study of pyrene was performed in PBS at 37 °C, but pyrene was not released at all over 24 h, probably due to the extremely hydrophobic nature of the probe ($\log P$ of pyrene = 5.2, Miller et al., 1985) and the stability of nanocubicles in release medium.

3.2. Microscopy

In vitro cell experiment was performed to predict the uptake mechanism of nanocubicles by cells. When Caco-2 cells were incubated with nanocubicles, morphology change of the cells was observed (Fig. 3B–D). Punctual structure appeared in and on the cells at 37 °C, when observed with phase contrast microscope. The number of these structures increased with time at 37 °C (Fig. 3B and C) whereas they were hardly visible at 4 °C (Fig. 3D) upon incubation. We suspected the cell death, because the cell membranes resembled those undergoing apoptosis or necrosis. MTT assay showed, however, that cell viability did not decrease at this nanocubicle concentration (0.5 mg of the liquid formulation in 1 ml media) when compared to the control experiment for at least 8 h.

To determine the exact location of theses structures, cells were observed further at a higher resolution by transmission electron microscope (Fig. 3E and F). The droplets with low-electron density (<2 μm) were observed inside the cell after incubation with nanocubicles. There was no droplet-like structure on the cell surface suggesting that punctual structures were resulted from the lipid droplets formed in cell cytosol. Some lipid droplets were membraneous structures and some were not.

The localization of the fluorescence probe, pyrene, was visualized by fluorescence microscope (Fig. 4B). The cells that were not treated with the nanocubicles are shown in Fig. 4A. The nuclei of the cells were stained with propidium iodide (red color). We did not observe any blue fluorescence of pyrene in the cells without nanocubicles. When cells were incubated with

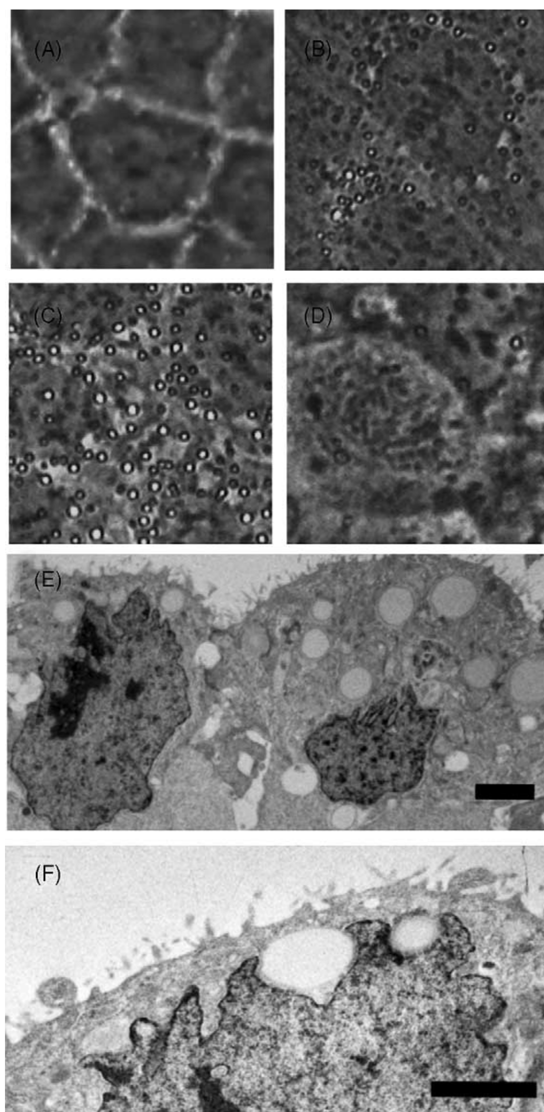

Fig. 3. Phase contrast and transmission electron microscopy. Caco-2 cells were incubated with nanocubicles at 37 °C for 1 h (B) or 3 h (C) and 4 °C for 3 h (D), and morphology change of live cell was observed with phase contrast microscopy. Untreated cells were used as a control (A). Caco-2 cells were incubated with nanocubicles at 37 °C for 3 h and observed with transmission electron microscopy (E and F). The bars correspond to 2 μm.

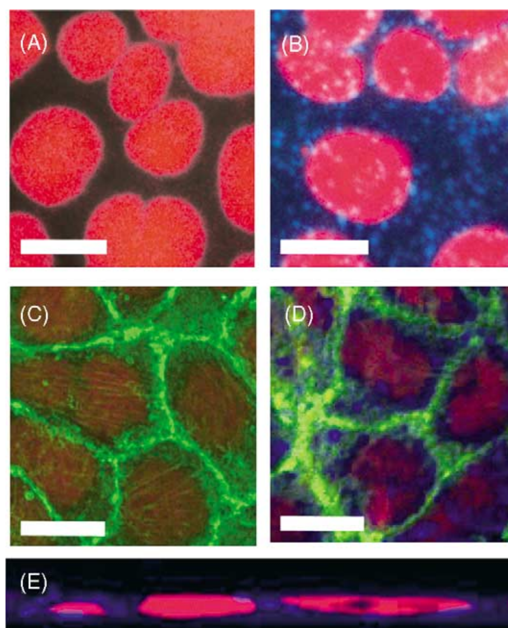

Fig. 4. Fluorescence and confocal microscopy. Caco-2 cells were incubated at 37 °C for 3 h without nanocubicles (A and C). Caco-2 cells were incubated at 37 °C for 3 h with nanocubicle encapsulating pyrene (B and D). The localization of lipid droplets were visualized using fluorescence (A and B) and confocal microscopy (C–E). The vertical (E) as well as horizontal (D) section is shown. Top and bottom of (E) are apical and basal sides, respectively, of Caco-2 cells. The bars correspond to 10 μm.

nanocubicles containing pyrene for 3 h at 37 °C, the blue fluorescence was observed in the cells (Fig. 4B). Intense punctual fluorescence as well as diffused blue fluorescence was observed in most of the field. The number of dotted structures and the intensity of the diffused fluorescence increased with time (data not shown).

Inside of the cell was observed by confocal microscopy (Fig. 4C–E). Caco-2 monolayer was horizontally as well as vertically sectioned at intervals of 0.5 μm. Control experiment was performed without nanocubicles (Fig. 4C). F-actin and nuclei were stained with phalloidin-FITC (green color) and propidium iodide (red color), respectively (Fig. 4C and D). Since the cells were focused approximately at the mid-plane, nuclei were very clear, but the actin filaments were not clear except at the periphery of the cells. When cells were incubated with nanocubicles, many large droplets (ca. <2 μm) with blue fluorescence of pyrene were detected inside the entire cells in the mid-plane (at a depth of 3.5 μm from the cell surface) (Fig. 4D) whereas the blue fluorescence was not observed in the control experiment (Fig. 4C). When the cell monolayer was vertically sectioned,

large droplets emitting blue fluorescence was observed clearly in the cytosol and around the nuclei (Fig. 4E). Some of the blue droplets were overlapped with the red color of nuclei. For clearance's shake, the green fluorescence of the actin filaments is not shown for the vertical section.

Even though we do not know the exact nature of these droplets, the size and position of the punctual structure observed by phase contrast microscopy and the droplets observed by TEM (Fig. 3) seem to coincide. Also the blue punctual fluorescence of pyrene observed by fluorescence and confocal microscope (Fig. 4B and D) has similar size, position and frequency of appearance to the droplets seen by TEM. This result suggests that hydrophobic model drug, pyrene, was accumulated in the lipid droplets after penetration into the cell membrane.

Further experiments are needed to elucidate the exact nature of the droplets. These droplets appear to be similar to the large lipid body formed during lipid digestion and absorption in intestinal absorptive cells (Shen et al., 2001). Since monoolein is bioadhesive and can enter the enterocyte by simple diffusion with or without the aid of bile salts, these structures could be resulted from triglyceride synthesized from the absorbed monoolein (Nielsen et al., 1998). Strauss has observed intestinal fat absorption in vitro from mixed micelles containing linolenic acid, monoolein, and bile salt (Strauss, 1966). The uptake of fatty acids and monoglycerides into the cell was not associated with detectable morphological change. Once within the cell, these lipids were converted into dense osmiophilic droplets, clearly visible within the endoplasmic reticulum and in the matrix of the cytoplasm. Since enzyme related reactions were needed to synthesize triglyceride of chylomicron in cell, this process was temperature dependent. In our experiment, the droplet-like structures were visible at 37 °C, but not seen at 4 °C. Therefore, the droplets formed in our experiments may be similar in nature to the large lipid body formed after digestion of fats and oils (Dobbins, 1983).

3.3. Cellular association of nanocubicles

We have shown qualitatively that the component of nanocubicle was absorbed by cells. As a next experiment, we have quantified the cellular uptake

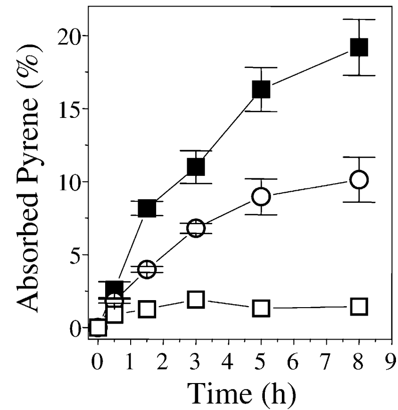

Fig. 5. Absorption of nanocubicles by 1×10^6 Caco-2 cells as a function of incubation time and the size of nanocubicles. Caco-2 cells were incubated at 37 °C for 8 h with the nanocubicles of an average size of 267.8 nm and polydispersity of 0.261 (■, $n = 3$), with the nanocubicles of an average size of 567.7 nm and polydispersity of 1 (○, $n = 3$) and egg PC emulsion of an average size of 250 nm and polydispersity of 0.207 (□, $n = 2$). Data are represented as mean ± S.E.M.

efficiency. Caco-2 cells were incubated for up to 8 h with the nanocubicles encapsulating pyrene at a concentration of 0.5 mg of the liquid formulation in 1 ml DMEM containing 10% fetal bovine serum at 37 °C (Fig. 5). To examine how the uptake efficiency of nanocubicles correlates with the particle size, the cells were incubated with the dispersions of normal (size: 267.8 nm, polydispersity: 0.261) and large (size: 567.7 nm, polydispersity: 1.000) sized nanocubicles. Emulsion comprising egg phosphatidylcholine and soybean oil was also prepared and compared with nanocubicles. The emulsion size was ca. 250 nm and zeta potential was ca. −45 mV. Large particles were prepared by shaking the mixture manually a few times instead of vortexing after adding the liquid formulation in water. The amount of cellular-associated pyrene increased smoothly with time at 37 °C upon incubation for both dispersion systems. After an 8-h incubation at 37 °C, the cellular-associated pyrene was ca. 20 and 10%, for the dispersions of the normal and large sized particles, respectively. The absorption ratio of emulsion formulation was 2%. The number of associated nanocubicles was approximately two times higher for small sized particles than that of the large one, and 10 times higher at 37 °C than that of emulsion formulation. When Caco-2 cells were

incubated with nanocubicles (ca. 250 nm) at 4 °C, the absorption ratio was only 2% (data not shown). This result coincides well with that by phase contrast microscopy, where the interaction between nanocubicles and cells at 4 °C seems to be much weaker than at 37 °C. Since it is highly probable that nanocubicle do not retain its internal structure at 4 °C, the phase behavior of the particles must be studied further.

3.4. Solubilization of nanocubicles by Na-TDC and absorption of the mixed micelles by rat intestinal jejunum everted sac

In the gastrointestinal tract, the lipid particles can be digested by many enzymes and solubilized by bile salts. Since the main ingredient of lipid nanocubicle is monoolein, we studied the changes in the structure of nanocubicles and in the absorption of nanocubicle dispersion by intestinal absorptive cells in the presence of various concentrations of Na-TDC. We have used nanocubicles with the average size of ca. 495 nm (prepared by dispersing 20 mg of the liquid formulation in 1 ml KBR buffer). Due to the high concentration, the average size was bigger than those used for earlier experiments. Since the concentration of bile salt in human is ca. 5 mM at fasted and 10–20 mM at fed state, 0–15 mM Na-TDC was prepared to encompass the physiological range (Lindahl et al., 1997). The accurate size of mixed micelles was measured using dynamic light scattering (Fig. 6). As taurodeoxycholate solution was added to cubic particles, the size of particles decreased from 495 to 114 and 34 nm when the final concentration of taurodeoxycholate was 10 mM (Fig. 6A and B). When the final concentration of the taurodeoxycholate was 15 mM, all of cubic particles were fully solubilized and produced mixed micelles with the size of 8 nm (Fig. 6C). The size of taurocholate micelle (15 mM) was ca. 3 nm in the absence of nanocubicles (Fig. 6D).

Since bile salt destroyed the nanocubicle structure completely, the absorption of the loaded drug, pyrene, by the cells can be altered greatly. To study the effect of bile salt solubilization on the absorption by the intestinal absorptive cells, the mixtures of nanocubicle encapsulating pyrene and bile salt at different concentrations were added to the rat jejunum everted sac (Fig. 7). After an 1-h incubation, pyrene concentration in the jejunum was quantified by extraction. There

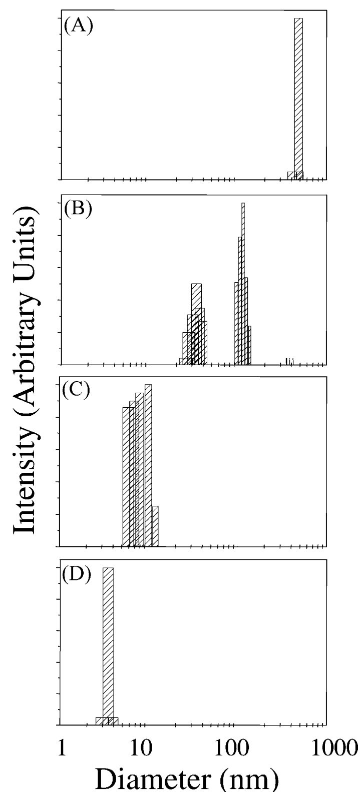

Fig. 6. The diameter change of nanocubicle upon mixing with sodium taurodeoxycholate (Na-TDC) measured with dynamic light scattering. Nanocubicles were mixed with 0 mM (A), 10 mM (B) and 15 mM (C) taurodeoxycholate solution. The final concentration of nanocubicles was 20 mg of the liquid formulation in 1 ml bile salt solution. The diameter of bile salt micelle at 15 mM was measured in the absence of nanocubicles (D).

was a small increase in the pyrene absorption when bile concentration was low (2–5 mM). As the bile salt concentration increased, the concentration of pyrene inside the cells increased. When nanocubicles are administered orally, they can be solubilized by the bile salts in the small intestine into mixed micelles and absorbed into the intestinal absorptive cells. Nanocubicles are likely to be taken up by the intestinal absorptive cells very slowly by the diffusion of monoolein when the bile salt concentration is low or very fast by the aid of bile salt as the form of bile salt mixed micelles when the bile salt concentration is high. It is possible that ethanol and bile salt used in the experiment can enhance the absorption. Ethanol has been known to enhance cellular drug absorption by para-cellular

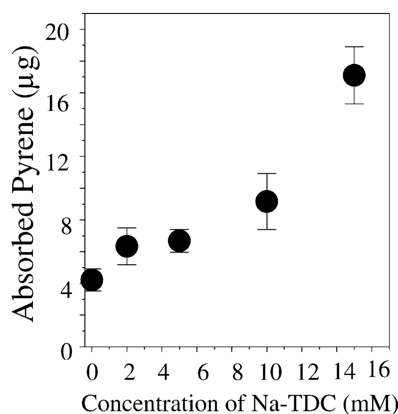

Fig. 7. The amount of pyrene absorbed by 100 mg everted rat jejunum as a function of sodium taurodeoxycholate concentration. One milliliter of the medium contains 0.5 mg pyrene and 20 mg of the dispersed liquid formulation, nanocubicles. Vertical bars denote ±S.E. of the mean value for three separate determinations.

permeability increase, but the working concentration was very high compared to that under our experimental conditions (Ma et al., 1999) since the concentration of ethanol in cell culture media and KBR solution were 0.025% in Caco-2 cell and 1% in everted sac experiment, respectively. Negative control experiments carried out in 0.025 and 1% ethanol show that ethanol in this concentration range did not enhance the absorption of pyrene. It has been known that the penetration enhancing effect of monoolein–bile salt mixed micelle is higher than that of bile salt micelle alone (Muranishi, 1985). Therefore, it is possible that the absorption of monoolein is enhanced by bile salt and vice versa.

Balandraud-Pieri et al. (1997) reported that bioavailability of cyclosporin A in the bile salt-monoolein mixed micelle was higher than that by coadministration of bile salt and emulsion. Emulsion containing monoolein was used as a lipophilic drug carrier to deliver hydrophobic steroid, danazol, more effectively. Since monoolein is a digested form of triglyceride as described previously, it can easily diffuse through cell membrane without further digestion (Charman et al., 1993).

It is clear from this study that nanocubicles have unique features to become an effective oral drug delivery system because it can be solubilized by bile salt without help of lipase. Probably a better system could be a mixed micelle between bile salt and monoolein in terms of the oral bioavailability only. But in TEM observation, high concentration of bile salt together with monoolein, however, was toxic to enterocyte (data not shown).

Another advantage of nanocubicles is stabilizing and penetration enhancing effect on protein drugs. We have shown previously that the nanocubicles increased the uptake efficiency of orally administered insulin (Chung et al., 2002). And monoolein has known to increase the penetration of macromolecules through the cell membrane because of its fusogenic property (Muranishi, 1985).

As a next project, we are currently working on in vitro uptake mechanism and in vivo uptake study of the nanocubicle encapsulating protein drugs. We expect that the nanocubicles can serve as peroral drug delivery system for protein and lipophilic drugs.

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