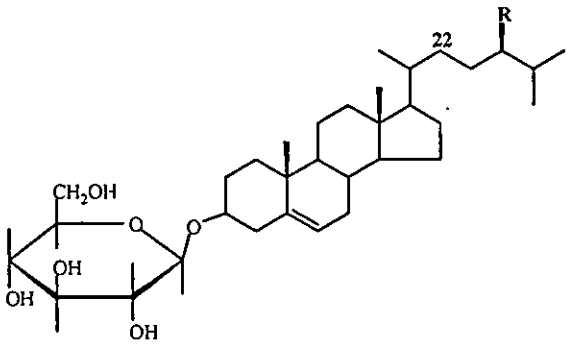

R=C_2H_5; β-Sitosterol β-D-glucoside (Sit-G, 49.9 %)
R=CH_3; β-Campesterol β-D-glucoside (29.1 %)
R=C_2H_5 and Δ^{22}; β-Stigmasterol β-D-glucoside (13.8 %)
R=CH_3 and Δ^{22}; β-Brassicasterol β-D-glucoside (7.2 %)

Fig. (1). Chemical structure of soybean-derived sterylglucoside (SG).

3. Ligand for liver-targeting: the efficacy of SG-liposomes for liver-targeting *in vivo* and the mechanism pathway of SG activity in HepG2 cells.
4. Application of SG-liposomes entrapping doxorubicin, pirarubicin and retinoic acid in cancer chemotherapy.

1. SOYBEAN-DERIVED STERYLGLUCOSIDE (SG)

Apart from functional variations at the C-24 position, phytosterols (plant sterols) exhibit structural similarities with the classical "animal sterol", cholesterol (Ch).

The type of SG used in this study was a mixture of glucoside analogues from soybean-derived sterol (SS): β-sitosterol (Sit, 49.9 %); campesterol (29.1 %); stigmasterol (13.8 %); and brassicasterol (7.2 %) (Fig. 1). Average molecular weight of SG is 570.5. Phytosterols are synthesized de novo in plants but their presence has been detected throughout the entire animal kingdom due to dietary consumption. As well as in animals, sterol concentrations in plants are high in plasma membranes and much lower in endoplasmic reticulum and mitochondoria. In fact, 160.7 mg of SS is present per 100 g of edible soybean parts and total sterol in SS-derived oil is around 7.9 mg/g. Interestingly, SG itself is produced by transition of a glucose group from UDP-glucose to SS, and it makes up approximately 10% of all sterols present.

Favorable biological properties and a lack of toxicity have prompted much interest in using SG as an emulsifier and preservative in a wide variety of pharmaceutical applications and foodstuffs. For example, Bouic *et al.* [1-3] have been researching the effects of SS and SG on lymphocytes and their impact on diseases, including HIV, hepatitis, allergies, physical stress-induced immune suppression and inflammation. Interestingly, blood Ch can be reduced by drug treatment with SS in lipemic patients since it exhibits a higher affinity for biological membranes and competitively inhibits Ch uptake [4].

1.1. Sit-G Nanoparticles

Lipid particles have gained particular importance as a non-toxic and biodegradable carrier system for solubilizing compounds that have low aqueous solubility, such as SG. Solubility of SG is 1.39 μg/mL in phosphate-buffered saline (PBS, pH7.4) at 37 °C. Nanoparticles and liposomes can be defined as colloid particles in the nanometer range of size and as phospholipid vesicles, respectively. A sample of Sit-G nanoparticles (Sit-G NP) incorporating oleic acid was prepared via modified ethanol-injection method. Dissociation of carboxylic groups on oleic acid created a negative charge on the surface of Sit-G NP. Particle size of Sit-G NP was dependent on the Sit-G amount in NP formulations. In the case of 0.5 % Sit-G NP, the size of NP was around 100 nm [5].

1.2. SG-Liposomes

Unlike polymeric and prodrug systems, nanoparticulate DDS offer the opportunity for improved absorption, as well as the capacity to transport relatively large quantities of drug. They can be effective carriers for site-specific delivery of drugs in oral and parenteral formulations. For example, liposomes incorporating sterylglucoside (SG-liposomes) can entrap labile drugs, e.g. insulin, protecting them from enzyme degradation.

When SG is incorporated into liposomes composed of dipalmitoylphosphatidylcholine (DPPC) and Ch, the glucose residue of SG migrates to the outermost bilayer. This effect can be confirmed by the increase of turbidity of SG-liposomes after incubation with lectin [6,7], IR measurement [8] and quantifying glucose released from SG-liposomes after incubation with emulsin [8]. SG and its aglycon, SS could be the component as the liposomal membrane, and their effect on the liposomal membrane have the opposite ability: SS makes the liposomal membrane rigid compared with Ch [9], but SG makes high fluidity in the hydrophobic end of the liposomal membrane around 37°C, as revealed by fluorescence polarization analysis and ESR [10].

2. SG FOR ENHANCEMENT OF NASAL AND INTESTINAL ABSORPTION

2.1. Nasal Delivery

2.1.1. General Delivery Methods

Systemic delivery of peptide-based pharmaceuticals via nasal administration is a challenging task [11]. Drugs are poorly absorbed from nasal mucosa due to the available surface area for drug absorption in nasal mucosa being smaller than that in small intestinal or pulmonary epithelium. However, nasal drug delivery offers many advantages: The avoidance of the hepatic first-pass metabolism; rapid absorption due to relatively high tissue permeability; and ease of administration since the nasal cavity has a large and vascularized surface area. In practise, nasal delivery is considered when peroral drug delivery fails, such as in the case of peptides. Nasal administration is then often ranked as less effective when compared to pulmonary delivery, and better than transdermal delivery [12]. Currently, nasally-delivered therapeutics of low molecular weight peptides such as buserelin, desmopressin and calcitonin are on the market.

2.1.2. Barriers to Mucosal Absorption

Basic mechanisms of transepithelial transport of drugs include passive transport of small molecules, active transport

of ionic compounds, and endocytosis of macromolecules. Hydrophobic drugs will be predominantly absorbed by passive transport and directly permeate into lipid bilayers (transcellular transport). Cell membranes are relatively permeable to water and small hydrophobic molecules (paracellular transport) but their tight junction proteins restrict drug permeation by exerting a barrier-effect to macromolecules and most polar compounds. An inverse relationship was established between the extent of nasal absorption and molecular weight of absorbed hydrophilic compounds [13,14]. Another obstacle for drug permeation is the mucin layer at the surface of nasal mucosa. It acts as a diffusion barrier and washes out drugs and DDS material deposited on the nasal mucosa surface [15]. Another important factor is that proteolytic enzymes may destroy the therapeutic peptide before it permeates through the nasal mucosa.

2.1.3. Mechanisms of Action of Absorption Enhancers

In the last two decades, techniques for increasing drug bioavailability and improving absorption from mucosal membrane have centered on using absorption enhancers for avoiding extensive drug degradation and overcoming poor permeation. Methods to facilitate transport of molecules across epithelial cells can be categorized into two major groups: transcellular and paracellular transport. Most of the commonly used absorption enhancers influence both the transcellular and paracellular routes of absorption, albeit in different ratios. Several of these absorption enhancers are summarized in Table 1.

Table 1. Typical Absorption Enhancers

Enhancer type	Compounds
Papracellular	Chitosan EDTA Glycyrrhetic acid derivatives Saturated fatty acid (sodium caprate)
Transcellular	Bile salts Methylated β-cyclodextrin Saponins Unsaturated fatty acid (oleic acid)

Transcellular Transport

The mechanism of transcellular transport has yet to be elucidated. Fatty acid [16], monoglycerides [17], bile salts [14,18] and surfactants [19] have all been shown to increase epithelial membrane permeability by affecting the normal function of membrane proteins or lipids. A typical example of this type of enhancer is oleic acid. It increases the mucosal fluidity by partitioning into lipid bilayers and facilitates drug transport through mucosal membrane [20]. The most likely mechanism by which detergents enhance drug absorption is by solubilizing membrane phospholipids and proteins, thus increasing membrane permeability [19]. These actions may result in not only severe membrane damage, but also toxic side-effects due to absorption enhancers interfering with cell organelles.

Paracellular Transport

Paracellular transport is the transport of molecules around or between cells. Tight junctions or similar inter-connections exist between cells. The intercellular tight junction is one of the major barriers to paracellular transport of macromolecules and polar compounds. Tight junction structure and permeability can be regulated by many potential physico-chemical factors, including the concentration of cAMP [21] and intracellular calcium concentrations [22]. Promotion of absorption by many Ca^{2+} chelators, such as EDTA, depends on their chelating activities [23]. Sodium caprate, a medium chain saturated fatty acid, induces an increase in intracellular calcium levels, resulting in the opening of tight junctions [24]. Glycyrrhizin can also trigger the opening of tight junctions and an increase in intracellular calcium levels [25].

Cyclodextrins can enhance the nasal absorption of peptide drugs, such as insulin. The mechanism of absorption enhancement of hydrophilic drugs by methylated β-cyclodextrins may be related to a temporary change in mucosal permeability (by extraction of membrane Ch) and opening of the tight junctions [26,27]. Poly-(acrylic acid) derivatives such as Carbomer 934® and chitosans have been extensively studied for their possible uses as absorption enhancers that cause the loosening of tight junctions [28,29].

2.2. Particulate Nasal and Oral Delivery System with SG

As already mentioned, the problems that make the oral route unsuitable for the systemic delivery of therapeutic peptides and proteins are the potential degradation by strong acids in the stomach and proteolytic enzymes in the intestinal tract, as well as pre-systemic elimination in the liver. Furthermore, it is known that macromolecular peptides and proteins have a very low permeability across GI mucosae. Thus, systemic bioavailability of peptide and protein pharmaceuticals by nasal and oral delivery is very low, generally less than 2%. Recently, great emphasis and study has been placed on particulate drug delivery as a novel type of DDS, that confers several advantages. First, the particle absorption process was found to be quite different from that of drug solutions transport would occur at the specialized mucosal membrane tissue, namely the mucosal associated lymphoid tissues (MALT) [30]. This transfer system may be utilized for vaccine and peptide delivery. Second, particles can migrate easily through mucous layers found on the surface of mucosae.

So far, there has only been a limited amount of information on particulate DDS incorporating absorption enhancers. The most successful examples of particulate delivery systems for nasal membranes were based on the absorption enhancers chitosan [31] and starch [32], which are bioadhesive polymers. Application of powders based on these materials increases their retention time on mucosal membranes. Liposomes have an inner aqueous phase surrounded by lipid bilayers. If peptides could be completely enveloped inside these liposomal, inner regions, they ought to be protected from attack by enzymes residing on the mucosal membrane.

2.2.1. Insulin

Systemic delivery of insulin, a protein hormone (amino acid 51, MW 5,806), by intranasal and oral administration has been studied extensively. Transmucosal permeability and mucosal absorption of insulin were found to be enhanced by co-administrating with absorption enhancers, such as bile salts (e.g., glyco- and deoxycholate), naturally occurring surfactants, or synthetic surfactants [12,15]. Numerous studies have shown that absorption enhancers can increase the bioavailability of insulin (Table 2). However, bile salts caused mucosal irritation accompanied by lipid extraction [18]. Chitosan and cyclodextrin have also been shown to enhance insulin absorption, but they suppressed the ciliary beat of the mucosae [36,37].

Recent work showed that a hypoglycemia effect appeared just after nasal administration of powdered mixture of insulin, SG and SS. This decrease in glucose levels was greater than the 2.5% bioavailability after 10 IU/kg rabbit

Table 2. Comparison of Insulin Bioavailability After Co-Administration of Absorption Enhancer Following Nasal or Oral Delivery

Route	Enhancer	Dosage form (concentration or ratio) (size)	F (%)	Dose, Animal (F)	Ref
Nasal	Sodium deoxycholate	Spray (1 %)	20	0.9 IU/kg human	[33]
	Sodium glycocholate	Spray (4 %)	67.5	0.5 IU/kg human	
	Dimethyl β-cyclodextrin	Powder	12.9 ± 4.4	4 IU/rabbit (0-3 h)	[26]
	Chitosan derivatives	Solution NP suspension (750 nm)	47.9 ± 19.4 36.1 ± 13.4	2.0 IU/kg rat (0-4 h)	[34]
	SS	SS suspension (1 %) Sit suspension (1 %)	5.3 5.5	10 IU/kg rabbit (0-6 h)	[42]
		DPPC/SS liposome (7/4) (100 nm)	5.1 ± 0.5	7.0 IU/kg rabbit (0-21 h)	[41]
	SG	SG powder (physical mixture) SG powder (lyophilized powder)	2.6 26.3 ± 0.3	10 IU/kg rabbit (0-6 h) 2 IU/kg rabbit (0-6 h)	[39]
		SG suspension (1 %) Sit-G suspension (1 %)	6.7 ± 1.4 11.3 ± 1.6	10 IU/kg rabbit (0-6 h)	[42]
		DPPC/SG liposomes (7/4) (100 nm) Freeze-dried DPPC/SG liposomes DPPC/SG liposome + insulin solution	13.3 ± 5.7 24.2 ± 5.3 15.7 ± 3.2	7.0 IU/kg rabbit (0-8 h)	[41]
Oral	Chitosan	NP suspension (120 nm) NP suspension (350 nm) NP suspension (1,000 nm)	10.2 ± 0.5 14.9 ± 1.3 7.3 ± 0.8	10 IU/kg rat	[35]
	Cholesterol	DPPC/Ch liposomes (7:2) (90 nm) DPPC/Ch liposome (7:4) (110 nm)	0.8 ± 0.6 21.9 ± 5.8	23.8 IU/kg rat (0-21 h) 15.5 IU/kg rat (0-21 h)	[45]
	SS	DPPC/SS liposome (7:2) (150 nm) DPPC/SS liposome (7:4) (110 nm)	24.1 ± 4.9 31.6 ± 5.7	28.3 IU/kg rat (0-21 h) 20.0 IU/kg rat (0-21 h)	
	SG	DPPC/SG liposome (7:2) DPPC/SG liposome (7:4)	21.1 ± 9.7 10.9 ± 3.4	21.0 IU/kg rat (0-21 h)	

F: pharmacological bioavailability (%), (F): pharmacological bioavailability from 0 h to final h, Sit: β-sitosterol, Sit-G: β-sitosterol β-D-glucoside, SS: soybean-derived sterol, SG: soybean-derived sterylglucoside, DPPC: dipalmitoylphosphatidylcholine, Ch: cholesterol, NP: nanoparticles.

achieved with a solution of insulin containing 1 w/v % sodium glycocholate [38]. The insulin powder dosage form with SG had been administered to the rabbit nasal cavity for five successive days [39]. Average bioavailability of insulin and the average pharmacological bioavailability at a dose of 2 IU/kg was approximately 25.0 and 61.6% after administration to 4 h, respectively (Fig. 2). Morphological observations revealed no severe damage to mucosal membranes [39,40]. These studies suggest that it is safe to use SG as an absorption enhancer for nasal delivery.

Nasal application of insulin-loaded liposomes triggered glucose reduction immediately after administration. Pharmacological bioavailability was greater and more prolonged in SG-liposomes than SS-liposomes; the hypoglycemia effect was persisted beyond 8 h after administration of SG-liposomes [41]. Insoluble powders and liposome dosage forms incorporating SG and SS improved bioavailability, predominantly by retarding drug elimination from the site of absorption and appeared to be suitable and effective for nasal systemic drug delivery when compared with liquid dosage forms. Comparing the insulin nasal absorption enhancement degree in main component (Sit-G) for SG and SG, Sit-G was stronger absorption ability than SG [42].

By the oral route, Gregoriadis *et al.* investigated the feasibility of systemic delivery of insulin using liposomes prepared from phosphatidylcholine and Ch [43]. A significant reduction in blood glucose levels was achieved in normal rats, with the maximum effect observed within 3 h. Patel *et al.* published the results of an extensive study on the delivery of insulin-entrapped liposomes to dogs, in the duodenal region, via a catheter [44]. Muramatsu *et al.* were able to produce a 31.6% reduction in blood glucose levels in normal rats via insulin-entrapped liposomes [45]. Oral application of insulin-loaded SS-liposomes brought glucose reduction from approximately 1 h to over 21 h after administration.

2.2.2. Erythropoietine

Erythropoietine (EPO) is a 30 kDa peptide. Oral administration of EPO-loaded liposomes incorporating SS caused a dramatic increase in blood cell proliferation [46], but EPO-loaded liposomes incorporating SG did not. These data were consistent with insulin data above [45]. In general, liposome membrane stability may be an important determining factor on the effective GI absorption of peptides from oral particulate DDS.

2.2.3. FITC-Dextran

FITC-dextrans with a varying range of molecular weights were used as a model drug to mimic the poor absorption of peptide drugs. Sit-G and Sit showed an increase in absorption enhancing effect after nasal administration of FITC-dextran 4,400 (FD-4) powder dosage form with them, and Sit-G showed higher bioavailability than Sit [47]. Comparing the degree of absorption enhancing effect of Sit-G and Sit with other absorption enhancers like cationic polymers, and bile salts, the enhancing degree of Sit-G and Sit was much weaker than cationic polymers, but in comparison of Sit and Sit-G with bile salts on bioavailability, there is no differences between them (Table 3).

Nanoparticles based on Sit-G and Sit were also tested to see how their particles would affect FD-4 absorption through nasal and intestinal mucosal membranes [47,49]. After nasal administration, the bioavailability of FD-4 with a particulate formulation was higher than powder or suspension preparations. Similarly, insulin nasal absorption was also facilitated by SG-liposomes, resulting in high hypoglycemic effect [41]. Preparations of Sit-G NP showed greater enhancement of absorption than Sit preparations [47]. These NP preparations included oleic acid in the formula, but NP based solely on oleic acid did not increase FD-4 absorption. By using NP formulations, it may be possible to ameliorate the hydrophobicity of Sit-G and Sit.

Sit-G NP enhanced FD-4 absorption predominantly in colon rather than rectum after co-administration of FD-4 with NP. The absorption-enhancing mechanism of NP will be described in the next section, but one hypothesis was that the distribution of β-D-glucosidase centred on colonic regions. Using this nature, dexamethazone β-D-glucoside has been developed as a prodrug for colon-specific delivery [50].

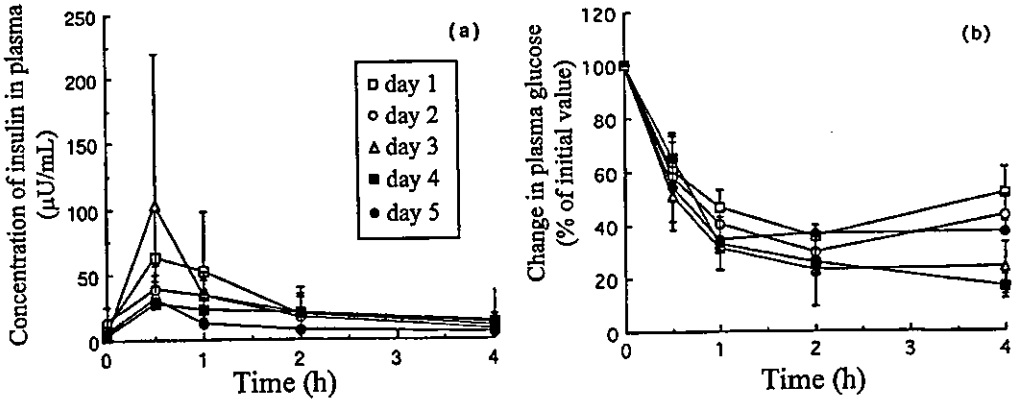

Fig. (2). Efficacy of porcine insulin powder with SG (insulin: SG= 2: 90) prepared by freeze-drying in a subchronic study.

Rabbits received drug for 5 successive days (2.0 IU/kg). Data represent blood insulin (a) and blood glucose (b) on day 1,2,3,4, or 5 (mean ± S.D.; n=3) [39].

Table 3. Influence of Absorption Enhancer on FD-4 Absorption

Route	Enhancer	Dosage form (size)	F (%)	Dose, Animal (F)	Ref
Nasal	EDTA	0.5 % solution	5.7	33.0 mg/kg rat (0-9 h)	[48]
	Glycocholate	0.5 % solution	8.8		
	Chitosan	0.5 % solution	24.4		
	Sodium caprate	1.0 % solution	8.2 ± 2.9	1 mg/kg rabbit (0-3 h)	[47] [49]
	Sit [a]	powder (< 75 μm)[b] 0.05 % suspension NP suspension (100 nm)	6.9 ± 0.4 5.8 ± 0.9* 9.2 ± 0.8**		
	Sit-G [a]	powder (< 75 μm)[b] 0.05 % suspension NP suspension (100 nm)	8.3 ± 1.0 8.9 ± 0.6** 11.7 ± 0.7**		
Ileum [c]	Sit	NP suspension	6.3 ± 2.0	1 mg/kg rat (0-3 h)	[5]
	Sit-G	0.05% suspension (< 75 μm) NP suspension (100 nm)	6.4 ± 1.0 5.4 ± 2.4		
Colon [d]	Sit	NP suspension (100 nm)	3.1 ± 0.7		
	Sit-G	0.05% suspension (< 75 μm) NP suspension (100 nm)	3.4 ± 0.2 15.4 ± 1.8 **		
Rectum [e]	Sit	NP suspension (100 nm)	2.7 ± 0.9		
	Sit-G	0.05% suspension (< 75 μm) NP suspension (100 nm)	3.1 ± 0.7 5.8 ± 1.2 *		

F: bioavailability (%), (F): bioavailability from 0 h to final h, NP: nanoparticles.
FD-4 solution in PBS was applied as control: [a] F = 2.6 ± 0.4 (%), [c] F = 5.6 ± 1.5 (%), [d] F = 2.6 ± 0.6 (%), [e] F = 2.2 ± 0.1 (%), [b] FD-4 powder as control: F = 4.1 ± 1.0 (%), **: $p<0.01$, *: $p<0.05$, compared with control.

2.3. Absorption-Enhancing Mechanism of SG

Various factors have been studied in an attempt to elucidate the mechanisms involved with absorption enhancement. Changes in transepithelial electrical resistance (TEER) after application of absorption enhancers have been linked to a relaxation of tight junctions along the paracellular route. Assessment of TEER was used to correlate intracellular calcium flux with regulation of tight junctions in these cells [22]. Typical paracellular-type enhancers, like sodium caprate, induce an increase in intracellular calcium levels irrespective of extracellular calcium ion levels, causing tight junctions to open [51]. Application of Sit-G did not change TEER values at all after 2 h timepoint. Post-administration, intracellular calcium levels were elevated only in the presence of calcium ions in the extracellular space [52]. These results suggest that the increase in intracellular calcium ion levels may be a secondary effect of membrane disruption caused by Sit-G interactions. From this perspective, SG would act as an absorption enhancer in the transcellular category.

Nevertheless, Sit-G NP caused a significant decrease in mucosal membrane resistance (TEER) immediately after application of Sit-G NP onto either nasal or intestinal mucosal membranes [5,49]. Specific inhibition of sodium-dependent glucose transporters (SGLT) suppressed this reduction (Fig. 3), but did not affect FD-4 permeation [5]. Surprisingly, treatment of mucosal membranes with endocytosis inhibitors made the absorption enhancing effect of Sit-G NP completely disappear. This effect by Sit-G NP may be due to an endocytotic process and a glucose moiety resides on Sit-G NP because Sit NP did not exhibit such behaviour.

Glucose transporters are widely distributed and can be separated into two categories: SGLT and GLUT, a non-dependent glucose transporter. Type SGLT exhibits inherent properties and can activate a SGLT-related decrease in TEER, since sodium ions become co-transported into cells when SGLT recognize glucose [53]. Recognition of glucose residues in SG by SGLT may be due to propensity of NP and liposomes to migrate into the mucus or microvillae at the surface of cell membranes.

Nasal absorption of insulin-loaded SG-liposomes caused a greater hypoglycemic effect than similar SS-liposomes [41]. Initially, it was thought that this difference might be due to liposomal release of insulin as a result of membrane destabilisation. Membranes within SG-liposomes are less rigid, making insulin easily released at partitioning junctures within SG-liposomes. This might raise the typical insulin concentration gradient higher since diffusion of insulin solution would have been restricted. On the other hand, since the membranes of SS-liposomes were stable, they would not release their drug load. When attempting oral delivery of peptides using liposomes, the rigidity of liposomal membranes may play an important factor since the relative

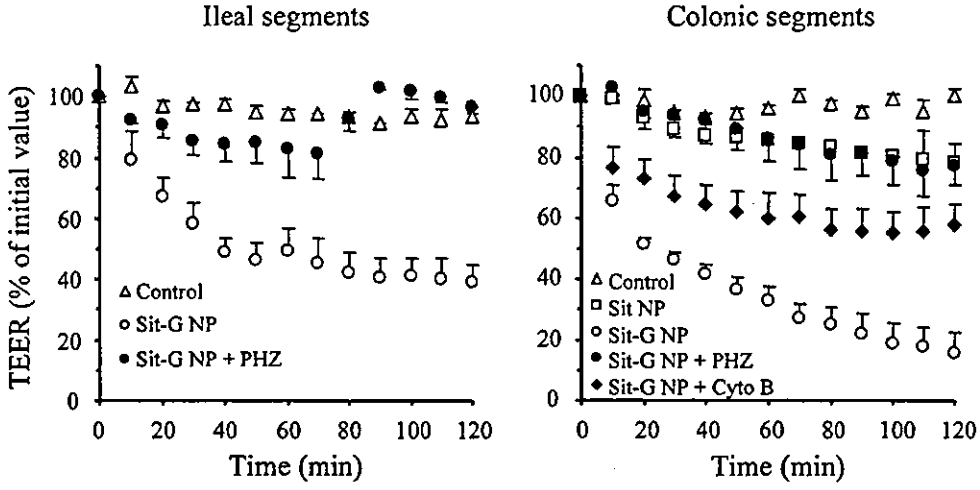

Fig. (3). Comparison of membrane resistance (TEER) after application of 0.05% Sit-G NP without or with SGLT inhibitor (PHZ) or endocytosis inhibitor (Cyto B) to the mucosal side in excised ileal and colonic mucosae in rats.

Each value was represented as the mean ± SD. (n=4). Control: Ringer's solution.

Phloridzin (PHZ) was treated on the mucsoal side before adding Sit-G NP.

Ctyochalasin B (Cyto B) at a concentration of 20 μM was added to the mucosal side.

Sit-G NP enhanced FITC-dextran 4,400 absorption in colonic segments, but not in ileal segment. [5].

distance from the administration site to the absorption site must be taken into consideration.

A second explanation for absorption enhancement of insulin by SG-liposomes is that an interaction occurs between Sit-G NP and certain bindings located at the surface of mucosal membranes. Indeed, SG-liposomes themselves facilitated absorption, as demonstrated by the faster onset of hypoglycemia from a mixture of empty SG-liposomes (insulin free liposomes) plus insulin solution compared to a preparation of insulin-loaded SG-liposomes [41]. Thus, a combination of these factors, such as the rate of insulin release and the absorption enhancement of SG-liposomes, may result in a potentiated hypoglycemic effect.

Normally, insulin at a neutral pH would form a hexamer state, but the addition of bile salts may be capable of dissociating insulin oligomers to monomers [54]. However, the trace in a circular dichroism spectra of insulin did not show any change in the conformation of insulin after an application of SG [42]. Co-administration of Sit and Sit-G with other drugs, e.g. verapamil [52] and FD-4 [47], showed the same behaviour with insulin, where their joint application resulted in an increase in insulin absorption that was substantially higher in SG than SS. Therefore, the absorption enhancement characteristics of SG and SS were not specific for just insulin and their mechanisms may be due to an interaction with mucosal membrane structures.

3. SG-LIGAND FOR LIVER-TARGETING

3.1. Liposomes for Liver Targeting

Drug targeting can be applied for therapeutic and for diagnostic purposes. The primary aim of drug targeting for therapeutic use is to manipulate the whole-body distribution of drugs. That is, to prevent distribution to non-target cells and concomitantly increase the drug concentration in target cells. Low-molecular-weight compounds have high vascular permeability, resulting in homogenous distribution in all tissues after intravenous injection. On the other hand, polymers and particles cannot efficiently cross the endothelial cell barrier in most normal tissues because the diameters of capillary pores in normal tissues are generally less than 80 nm. Therefore, after intravenous injection of polymers and particles, they distribute to specific tissue types, depending on the vascular structure. The liver and spleen possess small pores called fenestrae, which are sufficiently large to allow particles to escape the circulation. In these regions, particles smaller than 100 nm can leave the general circulation and be taken up by hepatocytes. However, capillaries in these regions are lined with active macrophages, called Kupffer cells, which remove most circulating particles. The majority of injected colloidal particles will end up in Kupffer cells rather than hepatocytes. Uptake and clearance by reticuloendothelial system (RES) is a major barrier for colloidal particles when used for intravenous targeting applications.

To defeat RES uptake and increase circulation time in blood, one of the few promising approaches is the use of long-chain hydrophilic polymers (polyethylene glycol, PEG) to form a heavily hydrated layer around the particle. The mechanism for escaping the RES is not clear, but is thought to be related to preventing adsorption of serum recognition factors, or opsonins, which mediate the uptake process.

An advantage of using particle carriers, such as liposomes, is that drugs can easily become encapsulated, either dissolved in the aqueous phase or in the lipid phase, without

the requirement of a covalent linkage between drug and carrier. Liposomal surfaces may be easily modified with specific targeted ligands, such as monoclonal antibodies, sugar residues or proteins. A summary of ligands recognized by receptors present on the sinusoidal membrane of liver is displayed in Table 4. The asialoglycoprotein receptor (ASGP-R), only found on hepatocytes [55,56], has been widely used to selectively deliver compounds to hepatocytes by exploiting the presence of galactosylated proteins. Galactose-terminated compounds, such as lactosylceramide (LC) [57,58], asialofetuin (AF) [59,60] or synthetic glycolipids [61] have been used to modify liposomes for hepatocyte-specific drug delivery. Mannose residues were used to target Kupffer or non-parenchymal cells [62-64], and can be useful for delivering immunomodulators such as muramyl peptides [65] and gamma-interferon [66]. Negatively-charged (succinylated or aconitylated) albumins have been used to target scavenger receptors [67].

Table 4. Ligands Recognized by Endocytosis Receptors on the Cell Surface of the Hepatic Cells and Used for Drug Targeting Preparations

	Ligands for endocytosis receptors
Heptatocyte	Asialoglycoprotein (ASGP) Insulin Epidermal growth factor (EGF) Low density lipoprotein (LDL) High density lipoprotein (HDL) IgA Transferrin
Kupffer cell	Mannose Acetylated LDL Fc (immunocomplex) Fiblonectin α_2-Macroglobulin
Endotherial cell	Mannose Polyanion (scavenger) Fc

3.2. *In Vivo* Efficacy of SG-Liposomes for Targeting Hepatocytes

In recent years, liposomes modified with SG (SG-liposomes) have been reported as effective, liver-targeting carriers [6,68]. Compared with bare liposomes (DPPC alone), SG-liposomes (DPPC/SG=7/2) entrapped with fluorescent dye calcein showed an increased accumulation in the liver of mice after intravenous injection (Fig. 4). The level in hepatocytes, reached a level approximately 7-fold higher than non-parenchymal cells, a figure that is comparable to liposomes modified with LC [68]. Incubation of liposomes with rat primary hepatocytes also indicated that the associated amount of SG-liposomes were almost similar to that of LC-liposomes [69]. From these results, SG-liposomes displayed relatively similar drug delivery efficiencies to galactose-mediated targeting systems.

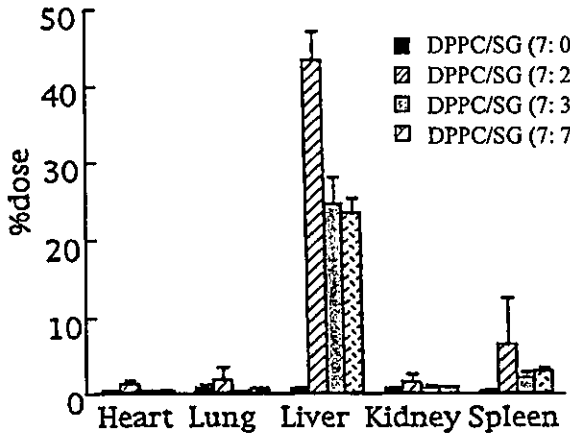

Fig. (4). Biodistribution of calcein entrapped in DPPC/SG liposomes 2 h after an intravenous administration to mice

Each value represents the mean ± S.D. of three mice [68].

Such specific biodistribution of SG-liposomes may be ascribed to the glucose residue in SG molecules. Hepatic accumulation of liposomes incorporating Ch or SS, was lower than that of SG-liposomes and the distribution SG-liposomes in the liver was mainly in parenchymal cells [6,70]. Glucose residues belonging to SG projects outward from the liposomal membrane and the maximum molar ratio of SG in DPPC membrane was 26 mol% [8]. Further incorporation of SG (DPPC/SG=7/3.5 or 7/7) decreased membrane stability in blood, resulting in less association with hepatocytes [68].

Drug carriers which are unstable in circulating blood would not be able to deliver its drug load efficiently to the targeted site. Increasing the amount of Ch in liposomes makes liposomal membrane more rigid and stable [71,72]. Among some formulations of liposomes with SG, the stability of SG-liposomes in blood was optimal at 30 mol% Ch. Approximately 80% of these SG-liposomes were accumulated in the liver 1 h after intravenous injection [6].

The fluidity of liposomal membranes is a crucial factor for preferential liver uptake. Liposomes incorporating LC and DPPC were taken up by the liver at a higher rate than similar liposomes composed of egg phosphatidylcholine or dimyristoylphsophatidylcholine [58,73]. These reports indicated that the galactose ligand is more suited to receptor-recognition in the rigid liposomal membranes. SG-liposomes having rigid membrane with Ch may augment interactions between SG and hepatocytes.

3.3. Mechanism of SG-Ligands for Liver-Targeting of HepG2 Cells

As mentioned above, SG-liposomes entrapped with calcein were seen to accumulate into the liver at a ratio comparable to LC-modified liposomes [68]. Accumulation of SG-liposomes in the liver may be related to the function of glucose residues in SG. Recognition of glucose residues at the surface of liver cells occurs as a result of glucosylated albumin in ASGP-R exposing positively-charged spacers close to glucose residues. This pathway was seen to be more

effective at uptake than other spacers when presented in murine [63] and isolated rat hepatocytes [74]. Pullulan consists of three α-1,4-linked glucose molecules that are repeatedly polymerized by α-1,6-linkages on the terminal glucose. They were also accumulated in liver parenchymal cells via ASGP-R [75]. Modification at the C-6 position of glucose, rather than C-1 or C-3 positions, allowed specific interactions between ASGP-R in primary murine hepatocytes and glucose-derivatized polyvinylbenzyl polymers [76]. Glucose residue of SG, however, does not have such a spacer and the C-1 position of glucose is connected with a sterol structure.

The liver-targeting efficacy of SG-liposomes was evaluated by means of measuring the associated amount of lipid maker DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) and microspheres with human hepatoblastoma HepG2 cells. These cells have ASGP-R on the plasma membrane [77] and the receptor-mediated association was competitively reduced by co-incubation with AF, which is a desialylated glycoprotein with terminal galactose residues. Uptake of these markers in SG-liposomes into HepG2 cells was inhibited by co-incubation with AF [78] (Fig. 5). This observation suggested that SG-liposomes trigger some interactions with hepatocytes via ASGP-R, or at least an AF-sensitive pathway.

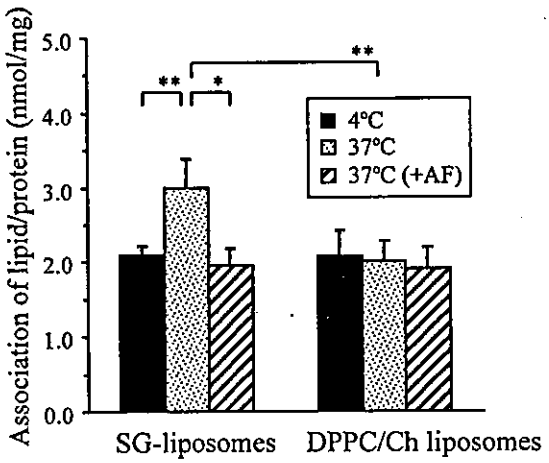

Fig. (5). Association of SG-liposomes with HepG2 cells.

HepG2 cells were incubated for 1 h with SG-liposomes (DPPC/SG/Ch=6/1/3, molar ratio, 100 μM as DPPC) or DPPC/Ch liposomes (DPPC/Ch=6/4, molar ratio, 100 μM as DPPC) labeled with 0.4 mol% lipid marker DiI at 4°C or 37°C in the absence or presence of 1 mg/mL asialofetuin (AF). The association was shown in the amount of DPPC.

Each value represents the mean ± S.D. (n=3) *: $p<0.05$, **: $p<0.01$ [78].

The ASGP-R binds glycoproteins that exhibit either β-D-galactose (Gal) or N-acetylgalactosamine (GalNAc) non-reducing terminal residues. At this point, the affinity for GalNAc is approximately 50-fold higher than for Gal, and binding hierarchy is tetraantennary > triantennary >> biantennary >> monoantennary galactosides. This effect is dependent on the structural organization of the receptor on the cell membrane, because it is not observed on the isolated receptor. In addition to this so-called "cluster effect," some researchers have shown that optimal receptor recognition of synthetic cluster glycosides is also determined by appropriate spacing (at least 15Å) of the sugar residues [79,80]. When SG is positioned onto the surface of liposomes, it may also confer multivalency and appropriate spacing to achieve quantitatively higher affinities, even if these targeting moieties held lower individual affinities.

Interaction of Sit-G with HepG2 cells was also examined by IAsys®, resonant mirror optical biosensor. Binding of Sit-G to HepG2 cell was seen to increase proportionately with the number of cells added. This reaction was higher than that of human cervical carcinoma HeLa cells, indicating that Sit-G had interacted with hepatocytes [81]. Another specific interaction of SG-liposomes with hepatocyte may be related to their affinity with glucose transporters. Hepatocytes have been found to express GLUT2, a major, facilitative, glucose transporter isoform. This affinity with glucose transporters was utilized for targeting carriers to the liver. A polystyrene-derivative containing glucose moieties was found to interact with GLUT1 from HepG2 cells [82]. Vesicles bearing glucose residues were prepared for targeting GULT to tumours [83]. Preparations of Sit-G NP were found to interact with glucose transporters (SGLT) on intestinal membranes [5]. This data supports the possibility that SG-liposomes interact with hepatic glucose transporters.

Furthermore, efficacy of SG-liposomes as drug delivery carrier was evaluated by means of incubation with FD-4 with HepG2 cells in the presence of Sit-G (Fig. 6). SG and Sit-G, a major component of SG, have been reported to be effective for enhancement of absorption of drugs across mucosal membranes [84]. The polysaccharide FD-4 represents a

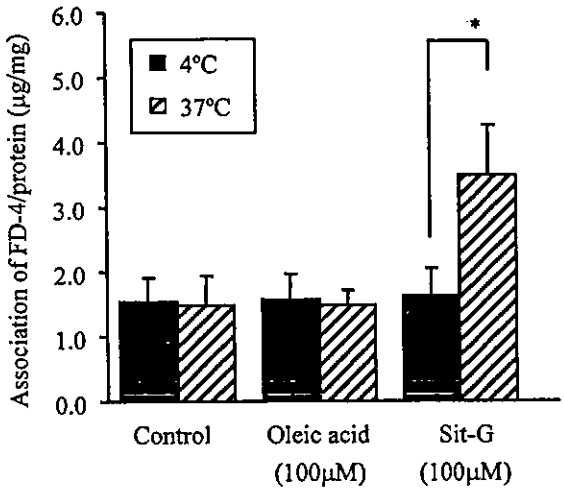

Fig. (6). Association of FD-4 with HepG2 cells enhanced by the addition of Sit-G.

HepG2 cells were incubated for 1 h in FD-4 solution (5 mg/ml) containing 1% DMSO with oleic acid or Sit-G at 4°C or 37°C. FD-4 solution containing 1 % DMSO was used as control.

Each value represents the mean ± S.D. (n=3-4) *: $p<0.05$ [81].

model of macromolecules that did not associate with HepG2 cells when incubated at 37°C. Application of oleic acid increased drug absorption of dermal [85] and mucosal [86,87] drug delivery systems, although the association of FD-4 was not enhanced by oleic acid in such conditions. Incorporation of Sit-G increased the association of FD-4 in HepG2 cells. This effect was related to the increase in Sit-G concentration and incubation time, as compared with control [81].

Bile salt and glycyrrhizin are known to act as penetration (absorption) enhancers for intestinal [25,88], nasal [12,89] and transdermal [90] absorption of drugs. They also act as ligands for drug delivery to the liver by possessing specific affinities for hepatocytes [91,92]. Bile acids are taken up by normal hepatocytes via sodium-dependent and sodium-independent processes. They act mainly through the sodium-dependent taurochorate co-transporter and covalently linked bile-acid conjugates accumulate in the liver due to their vectoriality [91]. Liposomes modified with glycyrrhizin were effectively accumulated in hepatocytes [92]. Fatty acids, such as oleic acid, possess a high affinity for hepatocytes. Their efficient cellular uptake is the result of a specific interaction with the Fatty Acid Binding Protein (h-FABP) present at the sinusoidal pole of the hepatocyte [93].

Association of FD-4 with HepG2 cells was increased by incubating with Sit-G since Sit-G perturbed lipid membranes. These findings suggested that Sit-G may exhibit a property as both a ligand for liver-targeting and a penetration enhancer at the targeted site, i.e. the liver. After intravenous injection of SG-liposomes, some of them actively transported into hepatocytes and others become passively accumulated in liver macrophages. After the degradation of liposomes by macrophages, the released drug penetrated into hepatocytes with the aid of Sit-G. It has been suggested that SG-liposomes may not only function as liver-targeting drug carrier, but they might also enhance the penetration of drugs in the vicinity of hepatocytes.

3.4. Application of SG-Liposomes in Liver Cancer Chemotherapy

Primary liver cancer, or hepatocellular carcinomas (HCC), is a rare type of cancer in Western countries, but occurs frequently in Africa and Asia. In Japan, HCC is often the sequel to chronic viral hepatitis. More common types of cancer occurring in the liver are metastatic diseases, which originate mainly from primary GI tumours. The prognosis for both HCC and metastases is poor. Treatment of either type of cancer can consist by surgery followed by chemotherapy or radiotherapy.

In cancer chemotherapy, drug targeting can be classified into passive and active targeting. The delivery of drugs to tumours can be realized by entrapping the drug in long-circulating particles. This takes advantage of physiologic factors that improve the localization of particles to target tissues relative to free drug. For example, increased capillary permeability allows passive targeting of particles to solid tumours, known as enhanced permeability and retention effect, especially in tumour or inflammatory sites.

As mentioned earlier, active liver-targeting has been reported using specific ligands, such as galactose residues, bile acid, glycyrrhyzin, and so on. For liver-directed cancer therapy, *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymers conjugated with doxorubicin (DXR) have been targeted to hepatocytes by incorporating galactosamine residues. This polymer, known as PK2, was developed as a potential treatment for primary HCC or secondary liver diseases, and is undergoing clinical testing [94,95]. Bile acid-cisplatin complexes, called Bamet, enhanced liver tumour treatment and overcame cisplatin resistance *in vitro* [96,97].

The use of liposomes for the treatment of cancer has been approached from several different directions. The longer the liposomes are able to circulate in the blood, the greater the level of accumulation to the tumour site (passive targeting). This, in fact, is the basis of several different liposomal anthracycline constructs that have given successful results in clinical trials, two of which are on the market. "Active" targeting liposomes with specific ligands, such as antibodies, glycoconjugates, or peptides, have been generally successful *in vitro*, but *in vivo* targeting has been unsuccessful. In tumours, transferrin and folate receptors (FR), and glucose transporters are expressed highly when compared to normal cells.

In a ligand-targeted application for tumours, Lee and Low demonstrated that specific binding and uptake of folate-PEG-liposomes (folate conjugated to the PEG terminus) by a FR expressed in human nasopharyngeal epidermal carcinoma (KB) or HeLa cells [98]. The therapeutic potential of folate-targeted liposomes was initially demonstrated by encapsulating the anticancer drug, DXR [98]. In the past, DNA [99] and oligonucleotides [100] have been formulated with liposomes. There are obviously many other types of macromolecular drug carriers that could be candidates for FR-mediated delivery [101].

Another advantage of particulate formulations is that they can be modified to avoid recognition by efflux pumps, such as P-glycoproteins and multidrug resistance associated protein, which are responsible for "pumping out" lipophilic drug molecules from cells and causing drug tolerance. Use of particulate systems can deliver a P-glycoprotein substrate and antigen across the plasma membrane, allowing them to by-pass P-glycoprotein and lead to intracellular accumulation. Liposomes [102], HPMA copolymer [103] and Pluronic micelles [104] incorporating DXR increased the cytotoxicity of anticancer drugs in cells that over-expressed efflux pumps.

Liver cancer treatment has been attempted with SG-liposomes entrapping DXR (Table 5), pirarubicin (THP) and all-*trans* retinoic acid (ATRA). The drug DXR is one of the most commonly used antitumour agent, but it causes severe adverse effects (e.g. cardiotoxicity, GI disorder and myelosuppression). The drug THP is a derivative of DXR that exhibits fewer adverse reactions and higher antitumour effects than DXR, but distribution in the liver is low. The drug ATRA is a retinoid that induces differentiation in cells and might be suitable for treatment of acute promyelocytic leukaemia. Preparations of SG-liposomes entrapping DXR exhibited increased the antitumour effects of DXR in rats bearing primary liver cancer induced by diethylnitrosoamine, and mice bearing M5076 metastatic liver tumours [105].

Table 5. Antitumor Effects of SG-Liposomes Entrapping Doxorubicin (DXR) Against Primary and Metastatic Liver Tumor

Liver tumor	Dosage form	Survival time (day) Mean (S.D.)	% ILS [a]
Rat primary	Saline	25.8 (17.1)	
	Free DXR	19.7 (14.1)	-49.1
	DPPC/Ch liposomes	25.6 (15.5)	-5.5
	SG-liposomes	48.2 (23.3)	92.7
Mouse metastasis (M5076)	Saline	11.4 (1.6)	
	Free DXR	13.1 (3.1)	9.1
	DPPC/Ch liposomes	15.5 (3.9)	27.3
	SG-liposomes	18.2 (10.2)	27.3

[a] Percentage increase in life span (% ILS), [(T/C-1)x100], where T and C represent the median survival time of the treated and control animals, respectively.
DPPC/Ch liposomes : DPPC/Ch=6/4 (molar ratio), SG-liposomes : DPPC/SG/Ch=6/1/3 (molar ratio) [105]

Accumulation of DXR and THP in the liver by SG-liposomes was 2.4 and 3.7-fold higher than that of free drug, causing an increase in antitumour effects in liver metastatic tumours [105,106]. Preparations of SG-liposomes entrapping ATRA have also shown efficacy against liver metastases [107-109]. These SG-liposomes enhanced the antitumour effects of these drugs owing to a change in drug distribution within the body. These features may correlate with observations of drug accumulation at tumour sites by liposomes modified with SG.

FUTURE DIRECTION

Sterylglucoside has been known for more than 30 years, but the physicochemical properties had not been made clear. We explored the novel physicochemical properties of particulate formulation incorporating Sit-G. Insoluble absorption enhancers can be used effectively in powders, liposomes and nanoparticles. Particulate SG may increase affinity for plasma membranes and reveal novel effects. Absorption enhancement effects of these enhancers have been investigated by direct application onto nasal and intestinal mucosae. However, particulate dosage forms containing enhancers that were delivered into systemic circulation were seen to circulate and passively accumulate in RES and the liver. Active accumulation was thought to have occured when an interaction was detected between enhancer and hepatocytes, such as in the case of ligand-activated receptor mediation. Thus, Sit-G has been found to fulfil many functions and exert many effects. To study the precise mechanism of absorption enhancers, it might be necessary to find an alternative ligand for targeting of tissues and cells. This appendage would also assist in predicting and quantifying the safe usage of these agents and, perhaps, uncover further novel functions in DDS.

REFERENCES

[1] Breytenbach, U., Clark, A., Lamprecht, J. and Bouic, P., (2001) *Cell. Biol. Int.*, **25**, 43-49.
[2] Bouic, P. J., (2001) *Curr. Opin. Clin. Nutr. Metab. Care*, **4**, 471-475.
[3] Bouic, P. J., (2002) *Drug Discov. Today*, **7**, 775-778.
[4] Hennessey, T. M., (1992) *Comp. Biochem. Physiol. C*, **101**, 1-8.
[5] Nakamura, K., Takayama, K., Nagai, T. and Maitani, Y., (2003) *J. Pharm. Sci.*, **92**, 311-318.
[6] Shimizu, K., Maitani, Y., Takayama, K. and Nagai, T., (1997) *Biol. Pharm. Bull.*, **20**, 881-886.
[7] Maitani, Y., Soeda, H., Junping, W. and Takayama, K., (2001) *J. Liposome Res.*, **11**, 115-125.
[8] Shimizu, K., Maitani, Y., Takayama, K. and Nagai, T., (1996) *J. Pharm. Sci.*, **85**, 741-744.
[9] Muramatsu, K., Maitani, Y., Machida, Y. and Nagai, T., (1994) *Int. J. Pharm.*, **107**, 1-8.
[10] Muramatsu, K., Masumizu, T., Maitani, Y., Hwang, S. H., Kohno, M., Takayama, K. and Nagai, T., (2000) *Chem. Pharm. Bull.*, **48**, 610-613.
[11] Chien, Y. W., Su, K. S. E. and Chang, S., (1989) in *Drugs and the pharmaceutical sciences.* (Chien, Y. W., ed.), Marcel Dekker, New York, pp. 1-26.
[12] Aungst, B. J., Rogers, N. J. and Shefter, E., (1988) *J. Pharmacol. Exp. Ther.*, **244**, 23-27.
[13] McMartin, C., Hutchinson, L. E., Hyde, R. and Peters, G. E., (1987) *J. Pharm. Sci.*, **76**, 535-540.
[14] Uchida, N., Maitani, Y., Machida, Y., Nakagaki, M. and Nagai, T., (1991) *Int. J. Pharm.*, **74**, 95-103.
[15] Hinchcliffe, M. and Illum, L., (1999) *Adv. Drug Deliv. Rev.*, **35**, 199-234.
[16] Muranishi, S., (1985) *Pharm. Res.*, **2**, 108-118.
[17] Muranushi, N., Takagi, N., Muranishi, S. and Sezaki, H., (1981) *Chem. Phys. Lipids*, **28**, 269-279.
[18] Martin, G. P., Marriott, C. and Kellaway, I. W., (1978) *Gut*, **19**, 103-107.
[19] Lichtenberg, D., Robson, R. J. and Dennis, E. A., (1983) *Biochim. Biophys. Acta*, **737**, 285-304.
[20] Turunen, T. M., Urtti, A., Paronen, P., Audus, K. L. and Rytting, J. H., (1994) *Pharm. Res.*, **11**, 288-294.
[21] Duffey, M. E., Hainau, B., Ho, S. and Bentzel, C. J., (1981) *Nature*, **294**, 451-453.
[22] Pitelka, D. R., Taggart, B. N. and Hamamoto, S. T., (1983) *J. Cell. Biol.*, **96**, 613-624.
[23] Martinez-Palomo, A., Meza, I., Beaty, G. and Cereijido, M., (1980) *J. Cell. Biol.*, **87**, 736-745.
[24] Tomita, M., Hayashi, M. and Awazu, S., (1996) *J. Pharm. Sci.*, **85**, 608-611.
[25] Imai, T., Sakai, M., Ohtake, H., Azuma, H. and Otagiri, M., (1999) *Pharm. Res.*, **16**, 80-86.
[26] Schipper, N. G., Romeijn, S. G., Verhoef, J. C. and Merkus, F. W., (1993) *Pharm. Res.*, **10**, 682-686.
[27] Merkus, F. W., Verhoef, J. C., Romeijn, S. G. and Schipper, N. G., (1991) *Pharm. Res.*, **8**, 588-592.
[28] Luessen, H. L., de Leeuw, B. J., Langemeyer, M. W., de Boer, A. B., Verhoef, J. C. and Junginger, H. E., (1996) *Pharm. Res.*, **13**, 1668-1672.
[29] Kotze, A. F., Luessen, H. L., de Leeuw, B. J., de Boer, B. G., Verhoef, J. C. and Junginger, H. E., (1997) *Pharm. Res.*, **14**, 1197-1202.
[30] Jung, T., Kamm, W., Breitenbach, A., Kaiserling, E., Xiao, J. X. and Kissel, T., (2000) *Eur. J. Pharm. Biopharm.*, **50**, 147-160.
[31] Soane, R. J., Frier, M., Perkins, A. C., Jones, N. S., Davis, S. S. and Illum, L., (1999) *Int. J. Pharm.*, **178**, 55-65.
[32] De Ascentiis, A., Bettini, R., Caponetti, G., Catellani, P. L., Peracchia, M. T., Santi, P. and Colombo, P., (1996) *Pharm. Res.*, **13**, 734-738.
[33] Pontiroli, A. E., Alberetto, M., Pajetta, E., Calderara, A. and Pozza, G., (1987) *Diabete Metab.*, **13**, 441-443.
[34] Dyer, A. M., Hinchcliffe, M., Watts, P., Castile, J., Jabbal-Gill, I., Nankervis, R., Smith, A. and Illum, L., (2002) *Pharm. Res.*, **19**, 998-1008.
[35] Pan, Y., Zheng, J. M., Zhao, H. Y., Li, Y. J., Xu, H. and Wei, G., (2002) *Acta Pharmacol. Sin.*, **23**, 1051-1056.

[36] Thanou, M. M., Verhoef, J. C., Romeijn, S. G., Nagelkerke, J. F., Merkus, F. W. and Junginger, H. E., (1999) *Int. J. Pharm.*, 185, 73-82.

[37] Uchenna Agu, R., Jorissen, M., Willems, T., Van den Mooter, G., Kinget, R., Verbeke, N. and Augustijns, P., (2000) *Int. J. Pharm.*, 193, 219-226.

[38] Yamamoto, T., Maitani, Y., Machida, Y. and Nagai, T., (1994) *S.T.P. Pharma Sci.*, 4, 133-138.

[39] Yamamoto, T., Maitani, Y., Ando, T., Isowa, K., Takayama, K. and Nagai, T., (1998) *Biol. Pharm. Bull.*, 21, 866-870.

[40] Yamamoto, T., Maitani, Y., Isowa, K. and Nagai, T., (1995) *Biol. Pharm. Bull.*, 18, 887-890.

[41] Muramatsu, K., Maitani, Y., Takayama, K. and Nagai, T., (1999) *Drug Dev. Ind. Pharm.*, 25, 1099-1105.

[42] Ando, T., Maitani, Y., Yamamoto, T., Takayama, K. and Nagai, T., (1998) *Biol. Pharm. Bull.*, 21, 862-865.

[43] Gregoriadis, G., Dapergolas, G. and Neerunjun, E. D., (1976) *Biochem. Soc. Trans.*, 4, 256-259.

[44] Patel, H. M. and Ryman, B. E., (1976) *FEBS Lett.*, 62, 60-63.

[45] Muramatsu, K., Maitani, Y. and Nagai, T., (1996) *Biol. Pharm. Bull.*, 19, 1055-1058.

[46] Maitani, Y., Hazama, M., Tojo, Y., Shimoda, N. and Nagai, T., (1996) *J. Pharm. Sci.*, 85, 440-445.

[47] Nakamura, K., Maitani, Y. and Takayama, K., (2002) *J. Control. Release*, 79, 147-155.

[48] Natsume, H., Iwata, S., Ohtake, K., Miyamoto, M., Yamaguchi, M., Hosoya, K., Kobayashi, D., Sugibayashi, K. and Morimoto, Y., (1999) *Int. J. Pharm.*, 185, 1-12.

[49] Nakamura, K., Maitani, Y. and Takayama, K., (2002) *S.T.P. Pharma Sci.*, 12, 63-68.

[50] Friend, D. R., (1992) in *Oral Colon-specific Drug Delivery*. (Friend, D.R., ed.), CRC Press, Boca Raton, pp. 153-187.

[51] Tomita, M., Hayashi, M. and Awazu, S., (1994) *Biol. Pharm. Bull.*, 17, 753-755.

[52] Maitani, Y., Nakamura, K., Suenaga, H., Kamata, K., Takayama, K. and Nagai, T., (2000) *Int. J. Pharm.*, 200, 17-26.

[53] Turner, J. R., Rill, B. K., Carlson, S. L., Carnes, D., Kerner, R., Mrsny, R. J. and Madara, J. L., (1997) *Am. J. Physiol.*, 273, C1378-1385.

[54] Li, Y., Shao, Z. and Mitra, A. K., (1992) *Pharm. Res.*, 9, 864-869.

[55] Ashwell, G. and Harford, J., (1982) *Annu. Rev. Biochem.*, 51, 531-554.

[56] Schwartz, A. L., Rup, D. and Lodish, H. F., (1980) *J. Biol. Chem.*, 255, 9033-9036.

[57] Spanjer, H. H. and Scherphof, G. L., (1983) *Biochim. Biophys. Acta*, 734, 40-47.

[58] Banno Y., Ohki K., Morita T., Yoshioka S. and Nozawa Y., (1986) *Biochem. Int.*, 12, 865-871.

[59] Ishihara, H., Hara, T., Aramaki, Y., Tsuchiya, S. and Hosoi, K., (1990) *Pharm. Res.*, 7, 542-546.

[60] Wu, J., Liu, P., Zhu, J. L., Maddukuri, S. and Zern, M. A., (1998) *Hepatology*, 27, 772-778.

[61] Murahashi, N. and Sasaki, A., (1996) *Biol. Pharm. Bull.*, 19, 418-423.

[62] Barratt, G., Tenu, J. P., Yapo, A. and Petit, J. F., (1986) *Biochim. Biophys. Acta*, 862, 153-164.

[63] Nishikawa, M., Ohtsubo, Y., Ohno, J., Fujita, T., Koyama, Y., Yamashita, F., Hashida, M. and Sezaki, H., (1992) *Int. J. Pharm.*, 85, 75-85.

[64] Nag, A. and Ghosh, P. C., (1999) *J. Drug Target.*, 6, 427-438.

[65] Daemen, T., Veninga, A., Roerdink, F. H. and Scherphof, G. L., (1986) *Cancer Res.*, 46, 4330-4335.

[66] Stukart, M. J., Rijnsent, A. and Roos, E., (1987) *Cancer Res.*, 47, 3880-3885.

[67] Kamps, J. A. A., Morselt, H. W. M., Swart, P. J., Meijer, D. K. F. and Scherphof, G. L., (1997) *Proc. Natl. Acad. Sci. USA*, 94, 11681-11685.

[68] Shimizu, K., Maitani, Y., Takayama, K. and Nagai, T., (1996) *J. Drug Target.*, 4, 245-253.

[69] Shimizu, K., Maitani, Y., Takahashi, N., Takayama, K. and Nagai, T., (1998) *Biol. Pharm. Bull.*, 21, 818-822.

[70] Muramatsu K., Maitani Y., Machida Y. and Nagai T., (1995) *Int. J. Pharm.*, 124, 19-26.

[71] Mcelhaney, R. N., de Gier, J. and van Deenen, L. L., (1970) *Biochim. Biophys. Acta*, 219, 245-247.

[72] Demel, R. A., Kinsky, S. C., Kinsky, C. B. and van Deenen, L. L., (1968) *Biochim. Biophys. Acta*, 150, 655-665.

[73] Yoshioka, S., Imaeda, N., Okano, Y., Mizukami, Y. and Katagiri, Y., (1994) *Biol. Pharm. Bull.*, 17, 640-644.

[74] Kawaguchi, K., Kuhlenschmidt, M., Roseman, S. and Lee, Y. C., (1981) *J. Biol. Chem.*, 256, 2230-2234.

[75] Kaneo, Y., Tanaka, T., Nakano, T. and Yamaguchi, Y., (2001) *J. Control. Release*, 70, 365-373.

[76] Kim, S. H., Goto, M. and Akaike, T., (2001) *J. Biol. Chem.*, 276, 35312-35319.

[77] Schwartz, A. L., Fridovich, S. E., Knowles, B. B. and Lodish, H. F., (1981) *J. Biol. Chem.*, 256, 8878-8881.

[78] Maitani, Y., Kawano, K., Yamada, K., Nagai, T. and Takayama, K., (2001) *J. Control. Release*, 75, 381-389.

[79] Lee, Y. C., Townsend, R. R., Hardy, M. R., Lonngren, J., Arnarp, J., Haraldsson, M. and Lonn, H., (1983) *J. Biol. Chem.*, 258, 199-202.

[80] Biessen, E. A., Beuting, D. M., Roelen, H. C., van de Marel, G. A., van Boom, J. H. and van Berkel, T. J., (1995) *J. Med. Chem.*, 38, 1538-1546.

[81] Kawano, K., Nakamura, K., Hayashi, K., Nagai, T., Takayama, K. and Maitani, Y., (2002) *Biol. Pharm. Bull.*, 25, 766-770.

[82] Park, K. H., Akaike, T. and Lee, K. C., (2003) *J. Biomed. Mater. Res.*, 65A, 136-143.

[83] Dufes, C., Schatzlein, A. G., Tetley, L., Gray, A. I., Watson, D. G., Olivier, J. C., Couet, W. and Uchegbu, I. F., (2000) *Pharm. Res.*, 17, 1250-1258.

[84] Yamamoto, T., Maitani, Y., Takayama, K. and Nagai, T., (1995) *Int. J. Pharm.*, 117, 129-137.

[85] Francoeur, M. L., Golden, G. M. and Potts, R. O., (1990) *Pharm. Res.*, 7, 621-627.

[86] Murakami, M., Takada, K., Fujii, T. and Muranishi, S., (1988) *Biochim. Biophys. Acta*, 939, 238-246.

[87] Wang, L. Y., Ma, J. K., Pan, W. F., Toledo-Velasquez, D., Malanga, C. J. and Rojanasakul, Y., (1994) *Pharm. Res.*, 11, 513-517.

[88] Sakai, M., Imai, T., Ohtake, H. and Otagiri, M., (1998) *J. Pharm. Pharmacol.*, 50, 1101-1108.

[89] Mishima, M., Okada, S., Wakita, Y. and Nakano, M., (1989) *J. Pharmacobiodyn.*, 12, 31-36.

[90] Ogiso, T., Iwaki, M., Yoneda, I., Horinouchi, M. and Yamashita, K., (1991) *Chem. Pharm. Bull.*, 39, 449-453.

[91] Kramer, W., Wess, G., Schubert, G., Bickel, M., Girbig, F., Gutjahr, U., Kowalewski, S., Baringhaus, K. H., Enhsen, A., Glombik, H., Mullner, S., Neckermann, G., Schulz, S. and Petzinger, E., (1992) *J. Biol. Chem.*, 267, 18598-18604.

[92] Osaka, S., Tsuji, H. and Kiwada, H., (1994) *Biol. Pharm. Bull.*, 17, 940-943.

[93] Charbon, V., Latour, I., Lambert, D. M., Buc-Calderon, P., Neuvens, L., De Keyser, J. L. and Gallez, B., (1996) *Pharm. Res.*, 13, 27-31.

[94] Seymour, L. W., Ulbrich, K., Wedge, S. R., Hume, I. C., Strohalm, J. and Duncan, R., (1991) *Br. J. Cancer*, 63, 859-866.

[95] Julyan, P. J., Seymour, L. W., Ferry, D. R., Daryani, S., Boivin, C. M., Doran, J., David, M., Anderson, D., Christodoulou, C., Young, A. M., Hesslewood, S. and Kerr, D. J., (1999) *J. Control. Release*, 57, 281-290.

[96] Briz, O., Serrano, M. A., Macias, R. I. and Marin, J. J., (2000) *Int. J. Cancer*, 88, 287-292.

[97] Dominguez, M. F., Macias, R. I., Izco-Basurko, I., de La Fuente, A., Pascual, M. J., Criado, J. M., Monte, M. J., Yajeya, J. and Marin, J. J., (2001) *J. Pharmacol. Exp. Ther.*, 297, 1106-1112.

[98] Lee, R. J. and Low, P. S., (1995) *Biochim. Biophys. Acta*, 1233, 134-144.

[99] Lee, R. J. and Huang, L., (1996) *J. Biol. Chem.*, 271, 8481-8487.

[100] Li, S., Deshmukh, H. M. and Huang, L., (1998) *Pharm. Res.*, 15, 1540-1545.

[101] Lu, Y. and Low, P. S., (2002) *Adv. Drug Deliv. Rev.*, 54, 675-693.

[102] Matsuo, H., Wakasugi, M., Takanaga, H., Ohtani, H., Naito, M., Tsuruo, T. and Sawada, Y., (2001) *J. Control. Release*, 77, 77-86.

[103] Minko, T., Kopeckova, P., Pozharov, V. and Kopecek, J., (1998) *J. Control. Release*, 54, 223-233.

[104] Rapoport, N., Marin, A., Luo, Y., Prestwich, G. D. and Muniruzzaman, M. D., (2002) *J. Pharm. Sci.*, 91, 157-170.

[105] Shimizu, K., Qi, X. R., Maitani, Y., Yoshii, M., Kawano, K., Takayama, K. and Nagai, T., (1998) *Biol. Pharm. Bull.*, **21**, 741-746.

[106] Kawano, K., Takayama, K., Nagai, T. and Maitani, Y., (2003) *Int. J. Pharm.*, **252**, 73-79.

[107] Shimizu, K., Tamagawa, K., Takahashi, N., Takayama, K. and Maitani, Y., (2003) *Int. J. Pharm.*, **258**, 45-53.

[108] Tamagawa, K., Shimizu, K., Ebine, T., Maitani, Y., Fukui, T., Kawai, K. I. and Takahashi, N., (2001) *Clin. Cancer Res.*, **7**, 3551-3558.

[109] Takahashi, N., Tamagawa, K., Shimizu, K., Fukui, T. and Maitani, Y., (2003) *Biol. Pharm. Bull.*, **26**, 1060-1063.

Sustained Release of Cisplatin from Multivesicular Liposomes: Potentiation of Antitumor Efficacy against S180 Murine Carcinoma

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ABSTRACT: Cisplatin was encapsulated into multivesicular liposomes (MVLs) and the entrapment efficiency, size distribution, and *in vitro* drug release characteristics of the cisplatin-MVLs were studied. Pharmacokinetics, tissue distribution, and therapeutic efficacy of cisplatin-MVLs were compared against injection of cisplatin solution into mice inoculated with the murine carcinoma 180 (S180) tumor. The results showed that the cisplatin-MVLs were capable of high drug loading (0.148:1 mg cisplatin/mg lipid) and high encapsulation efficiency (>80%). The mean diameter of cisplatin-MVLs was 17 μm. *In vitro* studies of cisplatin-MVLs in saline solution showed that they sustained release of encapsulated drug for >7 days. Cisplatin-MVLs showed higher drug accumulation in the liver, spleen, and tumor regions than cisplatin solution, as well as higher plasma concentrations and a longer circulation time. The therapeutic efficacy of the cisplatin-MVL preparation against S180 tumor-bearing mice is significantly higher than that of cisplatin solution. © 2004 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 93:1718–1724, 2004

Keywords: cisplatin; multivesicular liposomes; S180 tumor; sustained release; pharmacokinetics; therapeutic efficacy

INTRODUCTION

Cisplatin [*cis*-dichlorodiamine platinum (II)] is one of the most effective antitumor agents in the treatment of testicular, ovarian, head and neck, and lung cancer. However, its use is limited by significant undesirable side effects, such as nephrotoxicity, ototoxicity, neurotoxity, and myelosuppression to a lesser extent.[1] Several attempts to potentiate antitumor efficacy of cisplatin and reduce cisplatin-induced toxicities, including microspheres,[2] nanoparticles,[3] liposomes,[4] and polymer micelles[5] have been reported. Various types of liposomal formulations have been used as drug delivery vehicles to potentiate antitumor efficacy of cisplatin. However, in most cases, either unilamellar (ULVs) or multilamellar vesicles (MLVs) of cisplatin liposomes prepared by traditional methods offer low encapsulation efficacy and low drug loading.[6,7] The conventional methods of preparing liposomes, for example, reverse-phase evaporation vesicle (REV) method, are based on the passive entrapment of the drug during formation of the lipid bilayer vesicles, resulting in low encapsulation efficacy. Moreover, these processes require vigorous conditions, namely, organic solvents, sonication, and high temperature, which result in loss of drug efficiency and inactivation. Multivesicular liposomes (MVLs), prepared by a multiple emulsion method, are

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characterized by their unique structure of multiple, nonconcentric, aqueous chambers surrounded by a network of lipid membranes.[8] The structure of MVLs renders a higher aqueous volume-to-lipid ratio and much larger particle diameters compared with MLVs.[8,9] This technology facilitates loading of water-soluble drugs and improves their encapsulation efficacy. The active ingredient is encapsulated within the nonconcentric internal aqueous chambers and is released over an extended period of time. In a clinical trial in human patients with encapsulation of an antineoplastic agent, cytarabine, in MVLs maintained therapeutic cerebrospinal fluid concentrations over an extended period of time after a single intrathecal administration, which may improve therapeutic efficacy in patients with neoplastic meningitis.[10] MVLs containing cisplatin (cisplatin-MVLs) are also expected to have some degree of sustained release compared with ULVs or MLVs, and potentiate antitumor efficacy of cisplatin. Cisplatin, therefore, formulated in MVLs, was developed.

In the present study, cisplatin-MVLs were prepared. The release of cisplatin from the cisplatin-MVLs *in vitro* was studied. Pharmacokinetics, tissue distribution, and therapeutic efficacy of cisplatin-MVLs were compared with administration of cisplatin solution in mice inoculated with an S180 tumor using a single injection schedule in the tumor site. Cisplatin was entrapped into MVLs, with the aim of improving the entrapment efficiency, prolonging release time, improving drug localization in tumors, and enhancing antitumor efficacy.

MATERIALS AND METHODS

Materials and Animals

Cisplatin was purchased from Qilu Pharmaceutical Company (China). Egg phosphatidylcholine was purchased from Engel Bioengineer Company (China). Cholesterol was kindly provided by Wako Pure Chemical Industries, Ltd. (Japan). Triolein, free-base lysine, and other chemicals used were reagent grade. LACA mice were purchased from the Animal Institute of Health Science Center, Peking University.

Preparation of Cisplatin-MVLs and Cisplatin-REVs

Cisplatin-MVLs were prepared by a two-step, "water-in-oil-in-water," double emulsification process. The first step is the formation of a "water-in-oil" emulsion. A lipid combination of 4.5 μmol egg phosphatidylcholine, 4.5 μmol cholesterol, 2.0 μmol triolein, and 1.6 mg of co-membrane stabilizer was dissolved into 1.0 mL of chloroform-ether (1:1, v/v) and mixed with an equal volume of an aqueous solution containing cisplatin in 5.0% glucose (the first aqueous solution) in a 5-mL glass, screw-top vial. This mixture was sonicated to produce a water-in-oil emulsion (the first emulsion). A subsequent emulsification with 2.5 mL of a second aqueous solution in another glass vial, such as 4.5% glucose containing 40 mM lysine (the second aqueous solution), resulted in a water-in-oil-in-water double emulsion (the second emulsion). The vial was then vortexed for 10 s to decrease the size of chloroform-ether spherules. Chloroform-ether spherules suspended in the second aqueous solution were layered on the bottom of a 250-mL Erlenmeyer flask (bottom diameter, 8 cm). Chloroform and ether were removed by flushing nitrogen over the surface of the mixture at approximately 20°–37°C for 5 min. Decreasing turbidity of the suspension indicated near completion of solvent removal. The evaporation process was then allowed to proceed for a few more minutes until no odor of chloroform or ether was detectable.[8]

Cisplatin large ULV vesicles (cisplatin-REVs) were prepared by an REV method. Lipids used were the same as the cisplatin-MVLs formulation, and 3 mL of chloroform was added to the 50-mL round-bottom flask containing the lipid. The lipid solution was emulsified by sonicating for 5 min with 1 mL of aqueous solution, containing 1 mg of cisplatin in 5.0% glucose. The emulsion was transformed into a liposome suspension by removing organic solvents using a rotary evaporator at 30°C, under reduced pressure, for approximately 30 min. The size of cisplatin-REVs was in the range of 1–5 μm.

Determination of Encapsulation Efficacy

Cisplatin-MVL and cisplatin-REV preparations were centrifuged at 600*g* for 5 min to separate the free cisplatin (in the supernatant) from the liposomal cisplatin (in the pellet). The amount of cisplatin in the supernatant (C_f) and liposome pellet (C) was determined by flameless atomic absorbance spectroscopy (FAAS) (SPECTRAA 4.0; Varian, San Francisco, CA).[11] The amount of cisplatin in the initial preparation (C_t) was calculated from C and C_f. The weight of lipid in

the liposome pellet (L) was determined by enzymatic assay of phospholipids.[12] Because the total weight of lipids used in the formulation (L_t) was already known, entrapment efficiencies could be calculated using the following equation:

$$\text{Encapsulation efficacy}\,(\%) = (C/L)/(C_t/L_t) \times 100$$

In Vitro Release

Cisplatin-MVLs and cisplatin-REVs (1.39 mg lipid/mL) were centrifuged at 600*g* for 5 min and resuspended in saline solution. Aliquots of 100 μL were pipetted into 1.5-mL plastic-capped tubes containing 0.9 mL of saline solution. Each tube represented one time point, and the tubes were incubated at 37°C under dynamic conditions (rotation of 12 rpm). All *in vitro* release studies were set up three times at each time point, the tubes were centrifuged at 600*g* for 5 min, and the supernatants and pellets were separated. The samples of the supernatants were determined for cisplatin content by FAAS.

Animals and Tumor Models

Male LACA mice, each weighing about 22–30 g (4 weeks old), were used in pharmacokinetic, tissue distribution, and therapeutic efficacy studies. S180 cells were maintained by weekly transplantation of tumor cells into the peritoneal cavity. To obtain a suspension of tumor cells for transplantation, ascites fluid containing S180 cells was diluted to approximately 2×10^7 cells/mL with saline and 0.2 mL of the diluted suspension was injected subcutaneously per mouse.[13]

Pharmacokinetic and Tissue-Distribution Studies

Nine days after tumor inoculation, mice were injected at the tumor site[14] with either cisplatin solution or cisplatin-MVLs at a dose of 10 mg of cisplatin per kilogram of body weight. Each experimental time point consisted of three mice. Mice were sacrificed by cervical dislocation at indicated times after injection. Blood was collected by heart puncture and the heart, liver, spleen, lung, kidneys, and tumor were immediately removed. Tissues were carefully removed without any excess blood and weighed. Blood was collected in heparinized tubes and centrifuged to obtain the plasma fraction. Plasma and tissues were stored at −20°C until platinum analysis by FAAS.

Determination of Platinum in Plasma and Tissue Samples

Plasma and tissue samples, except the liver and tumor, were digested with HNO_3 (Newman et al., 1999), and the liver and tumor with HNO_3, H_2O_2, and a drop of *n*-octanol.[11] All samples were incubated at 85°C for 2 h, followed by centrifugation. Supernatant was diluted with double-distilled water to yield a final platinum concentration in the range of 50–400 ng/mL.

To determine the recovery of platinum, blank plasma and tissue samples were spiked with 2.0 mg of cisplatin, followed by digestion with HNO_3 or HNO_3 and H_2O_2 mixture, and then diluted with distilled water as described above. Platinum concentrations were determined by FAAS.

A calibration curve with platinum concentrations in the range of 50–400 ng/mL was run before analysis of each sample type. Values reported were the average of two separate platinum determinations for each sample. The recovery of platinum after incubation of plasma and tissues with cisplatin was 85–105%, and the RSD was 1.9–5.2%.

Therapeutic Efficacy Studies

Nine days after the mice were inoculated with the S180 tumor, they were treated by injection of cisplatin-MVLs or cisplatin solution into the tumor site at a dose of 10 mg of cisplatin per kilogram of body weight. The control group was injected with sterile saline solution instead. Each group consisted of eight mice. The tumor volume was measured daily with slide calipers every day after treatment. Each tumor volume was calculated by approximation of the solid tumor to an ellipsoid

$$V = k \times a \times b \times c$$

where V is the volume of the tumor (cm^3); a, b, and c are the length, width, and height (cm) of the tumor, respectively; and k is a constant.

Therapeutic efficacy was evaluated in terms of tumor growth rate and volume inhibited efficacy (VIE)[12]

$$\text{VIE} = (1 - V_T/V_C) \times 100\%$$

where V_T is the mean tumor volume of the cisplatin-MVLs or cisplatin solution group (treatment groups), and V_C is the mean tumor volume of the control group.

Statistical Analysis

Data from the animal experiments using mice were compared using analysis of variance and paired t test.

RESULTS AND DISCUSSION

Characterizations of Cisplatin-MVLs

Cisplatin was encapsulated at a high loading capacity of 0.148–0.444 mg of cisplatin per mg of lipid, and with good encapsulation efficacy into MVLs (>80%). Cisplatin was encapsulated into ULV liposomes, known as SPI-077, to reduce toxicity and prolong the circulating time in by intravenous injection.[6,15] The drug/lipid weight ratio of SPI-077 was 0.014:1 (mg/mg). It cannot be disregarded that the reticuloendothelial system function was saturated or damaged to some extent given that large quantities of lipid were administered.[16] Cisplatin-MVLs, with a drug/lipid weight ratio of 0.444:1, would contain a much lower amount of lipid than SPI-077, per given cisplatin dose.

Table 1 shows the stability of cisplatin-MVLs stored in 6° ± 2°C and 25° ± 2°C, respectively. The encapsulation efficacy of cisplatin at 6° ± 2°C did not decrease significantly after 3 months. There was no leakage of cisplatin from the MVLs over a 3-month period when stored at 6°C, indicating that the multivesicular matrix was stable in storage at 6°C over the indicated time period. The encapsulation efficacy of cisplatin at 25° ± 2°C was decreased from 95.7% to 83.0% after 3 months' storage, and there was a significant decrease of encapsulation efficacy at 40°C after 15 days (from 95.7% to 47.8%, data not shown), indicating that the leakage of cisplatin from the MVLs occurred when stored at 40°C.

A picture of cisplatin-MVLs under an optical microscope is shown in Figure 1. The cisplatin-MVLs were spherical. The median diameter of cisplatin-MVLs vesicle was 17 μm and 90% of vesicles were in the range of 5–25 μm.

MVLs are distinct from conventional liposomal (ULVs and MLVs) delivery systems in that each MVL encloses multiple nonconcentric internal chambers, and the average size of MVLs is about 10 times larger than the conventional liposomes.[8,17,18] Vesicle size can be modulated by the process parameters, especially by emulsification, to yield a water-in-oil-in-water emulsion. The size is not dependent on the first aqueous condition.

Figure 2 shows the *in vitro* release profiles for cisplatin-MVLs and cisplatin-REVs with the same formulation in saline at 37°C. At the time point of 168 h, there were many intact MVL spheres in solution under the light microscope. This result showed that the MVLs did not release completely in 168 h. In addition, there was some extent of absorbance between cisplatin and the lipids when the system reached equilibrium. The percentage of drug retained by the vesicles was plotted as a function of time release of the encapsulated cisplatin at the time points of 0, 0.17, 4, 8, 24, 72, and 168 h. The release profiles of cisplatin-MVLs and cisplatin-REVs were fitted with the Weibull Distribution. Using this equation, it was possible to calculate the time period required for the release of 50% of the solute load from the vesicular preparations ($t_{0.5}$).[19] The results showed that the $t_{0.5}$ for cisplatin-MVLs was 30.7 times greater than that of cisplatin-REVs.

MVL preparations can provide sustained release of entrapped drug without the "burst" effect or rapid initial release that is seen with the REVs. Because MVLs are different from ULV liposomes, such as REVs, a single breach in the external membrane of an MVL particle should not result in a total emptying of the solute load.

Pharmacokinetic and Tissue-Distribution Experiments

In the S180 tumor model mice, platinum concentrations were determined in the plasma and wet tissues of the heart, liver, spleen, lung, kidneys,

Table 1. Encapsulation Efficacy (%) of Cisplatin-MVLs Stored at Different Temperatures and Times *In Vitro*

	Time (Months)			
Temperature (°C)	0	1	2	3
6 ± 2	95.7 ± 1.2	96.3 ± 1.0	98.3 ± 1.4	98.6 ± 0.5
25 ± 2	95.7 ± 1.2	93.5 ± 0.4	92.1 ± 0.8	83.0 ± 0.5

Data are mean ± SD, $n = 3$.

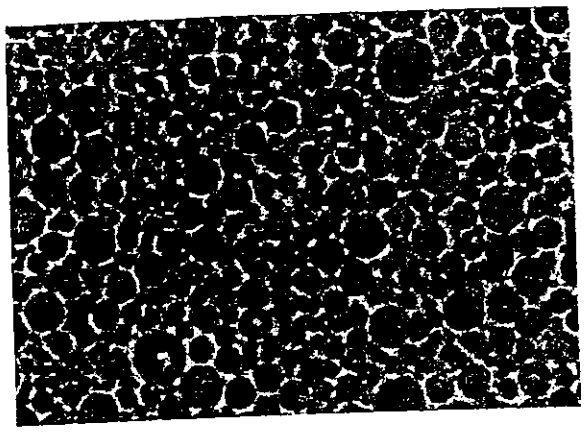

Figure 1. Optical micrograph of the cisplatin-MVLs suspended in saline solution at ×200 magnification, showing a spherical, honeycomb-like structure of tiny chambers and multivesicular nature.

and tumor at different time points after treatment. Pharmacokinetic and tissue-distribution analysis of drug disposition were based on the concentration of platinum in each tissue at the experimental time points 0.5, 8, and 48 h, respectively, after injection (as shown in Fig. 3).

The results indicated that Cisplatin-MVLs remained in the blood circulation for a significantly longer duration than an injection of cisplatin solution. After 8 h, the plasma concentration of cisplatin solution degraded to zero, but the concentration of cisplatin-MVLs was still high.

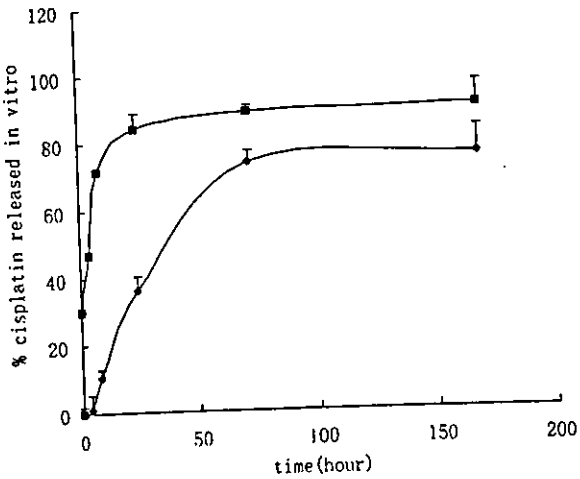

Figure 2. *In vitro* release characteristics of liposomes in saline solution under gentle rotating conditions (12 rpm) at 37°C. The data represent the mean ± SD, $n = 3$. ◆, cisplatin-MVLs; ■, cisplatin-REVs.

The most remarkable effect in this tissue-distribution study was the elevated concentration of platinum in the tumor site due to cisplatin-MVLs, as compared with cisplatin solution, reached to 2.5-, 4.0-, and 8.1-fold at 0.5, 8 and 48 h, respectively. This feature may be related to the capacity of cisplatin-MVLs to sustain release and accumulate at the tumor site. The figures indicated that cisplatin-MVLs in the organs relating to the mononuclear phagocytic system (liver and spleen) were higher than the cisplatin solution. These results suggest that a substantial amount of liposomes were transported and accumulated into the mononuclear phagocytic system. This effect may be related to the lipophilic properties of liposomes. The platinum concentration of cisplatin-MVLs in the heart was higher than that of cisplatin solution at each experimental point. This was because some liposomes were targeted to the cardiac muscle.[20] Cisplatin toxicity primarily affects renal function and the platinum of cisplatin-MVLs in this region was in the proximity of that for cisplatin solution at the three time points. This finding suggests that cisplatin-associated toxicity from cisplatin-MVLs might not be exacerbated. There were no significant differences between the groups treated with cisplatin-MVLs and cisplatin solution in the lung region. Whether cisplatin-MVLs are associated with increases in hepatic or splenic toxicity will require further investigation.

Therapeutic Efficacy

Four days after the mice were inoculated with the S180 tumor, the swells were found. Therapeutic efficacy was studied subsequent to a single injection on day 9 after S180 inoculation. Figure 4 charts the tumor growth rate in terms of mean tumor volume (cm^3) compared with the day before treatment for each day after treatment with cisplatin-MVLs or cisplatin solution. The results of paired t test for mean tumor volume, measured daily posttreatment for both preparations, showed significant difference between them ($p < 0.05$) after 9 days posttreatment. After 9 days posttreatment, the mean tumor volume in the cisplatin-MVLs, cisplatin solution, and the control group were 2.12, 12.00, and 31.68 cm^3, respectively. According to the equation above, the VIE of the cisplatin-MVLs group (93.3%) and cisplatin solution group (62.1%) was calculated. The results showed that the VIE of the cisplatin-MVLs was 31.2% higher than that of the cisplatin solution group.

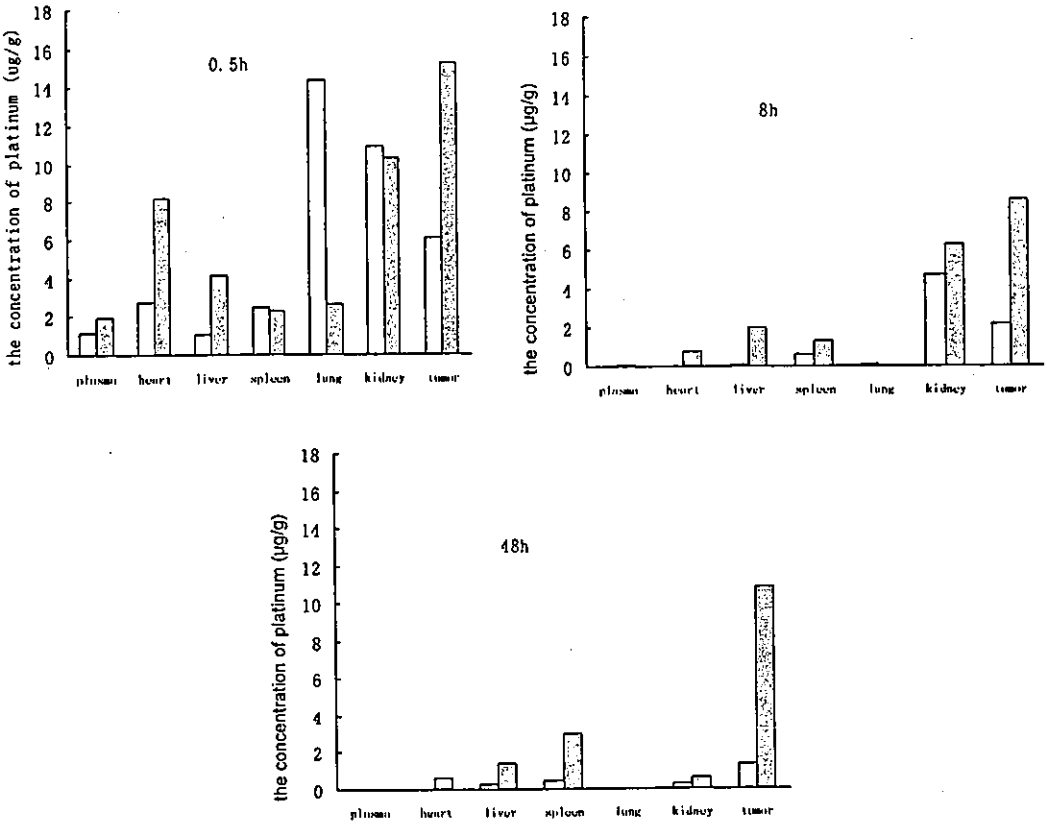

Figure 3. The platinum mean concentrations of different tissues at the experimental time points 0.5, 8, and 48 h, respectively. □, Cisplatim solution; ▣, cisplatin-MVLs; $n = 3$.

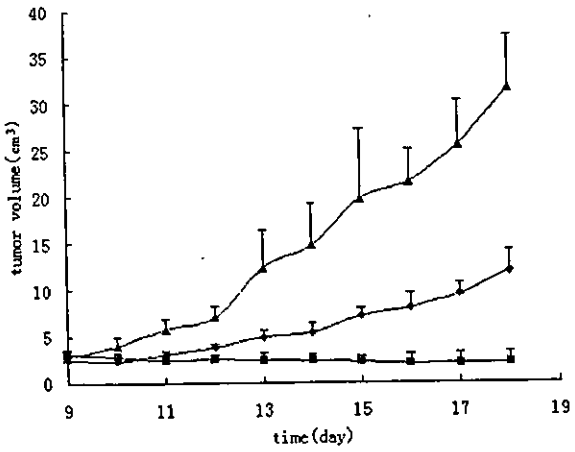

Figure 4. The tumor volume (cm^3) of mice inoculated with S180 tumor after treatment with the cisplatin solution (●), the cisplatin-MVLs (■), or the sterile saline for control (▲). Cisplatin solution or the cisplatin-MVLs was injected in the tumor site at a dose of 10 mg cisplatin/kg body weight. The data represent the mean ± SD, $n = 8$.

Studies of therapeutic efficacy indicate that cisplatin-MVLs had significant antitumor activity and were effective in reducing mean tumor volume of mice inoculated with S180 tumor, compared with the cisplatin solution group. When cisplatin-MVLs were injected in the tumor site, cisplatin solute was released from the MVL delivery system and exposed to tumor cells. Furthermore, as a result of the unique structure of cisplatin-MVLs, the rate of drug release was slow and the concentration of free cisplatin remained high at the tumor site—a combination that augmented tumoricidal efficiency.

CONCLUSIONS

The present findings indicate that the drug entrapment capability of cisplatin-MVLs is higher than cisplatin-REVs and their *in vitro* sustained-release profile of free cisplatin more suitable because of higher plasma concentrations and

prolonged circulation. The accumulation of drug in the liver, spleen, thymus, and tumor areas was higher for the cisplatin-MVLs preparation than that of cisplatin solution. The therapeutic efficacy of cisplatin-MVLs against S180 tumor-bearing mice is significantly higher than that of the cisplatin solution.

REFERENCES

1. Durant JR. 1980. Cisplatin: A clinical overview. In: Prestayko AW, Crooke ST, Carter SK, editors. Cisplatin: Current status and new developments. New York: Academic Press, pp 317–321.
2. Garcia-Contreras L, Abu-Izza K, Lu DR. 1997. Biodegradable cisplatin microspheres for brain implantation: Preparation and characterization. Pharm Dev Technol 2(1):53–65.
3. Avgoustakis K, Beletsi A, Panagi Z, Klepetsanis PG, Karydas AG, Ithakissios DS. 2002. PLGA-mPEG nanoparticles of cisplatin: *In vitro* nanoparticle degradation, *in vitro* drug release and *in vivo* drug residence in blood properties. J Control Release 79:123–135.
4. Steerenberg PA, Storm G, de Groot G, Claessen A, Bergers JJ, Franken MA, van Hoesel QG, Wubs KL, de Jong WH. 1988. Liposomes as a drug carrier system for *cis*-diamminedichloroplatinum(II). II. Antitumor resistance *in vivo*, induction of drug resistance, nephrotoxicity and Pt distribution. Cancer Chemother Pharmacol 21:299–307.
5. Mizumura Y, Matsumura Y, Hamaguchi T, Nishiyama N, Kataoka K, Kawaguchi T, Kawaguchi M, Hrushesky WJ, Moriyasu F, Kakizoe T. 2001. Cisplatin-incorporated polymeric micelles eliminate nephrotoxicity, while maintaining antitumor activity. Jpn J Cancer Res 92(3):328–336.
6. Newman MS, Colbern GT, Working PK, Engbers C, Amantea MA. 1999. Comparative pharmacokinetics, tissue distribution, and therapeutic effectiveness of cisplatin encapsulated in long-circulating, pegylated liposomes (SPI-077) in tumor-bearing mice. Cancer Chemother Pharmacol 43:1–7.
7. Freise J, Mueller WH, Magerstedt P, Schmoll HJ. 1982. Pharmacokinetics of liposome encapsulated cisplatin in rats. Arch Int Pharmacodyn Ther 258: 180–192.
8. Kim S, Turker MS, Chi EY, Sela S, Martin GM. 1983. Preparation of multivesicular liposomes. Biochim Biophys Acta 728:339–348.
9. Ye Q, Asherman J, Stevenson M, Brownson E, Katre NV. 2000. DepoFoam™ technology: A vehicle for controlled delivery of protein and peptide drugs. J Control Release 64:155–166.
10. Murry DJ, Blaney SM. 2000. Clinical pharmacology of encapsulated sustained-release cytarabine. Ann Pharmacother 34:1173–1178.
11. Li WJ. 1989. Determination of cisplatin in biological tissues and fluids by graphite furnace atomic absorption spectrometry. Chin J Pharm Anal 9: 82–85.
12. Qi XR, Maitani Y, Nagai T. 1995. Effect of soybean-derived sterols on the *in vitro* stability and the blood circulation of liposomes in mice. Int J Pharm 114:33–41.
13. Qi XR, Maitani Y, Nagai T, Wei SL. 1997. Comparative pharmacokinetics and antitumor efficacy of doxorubicin encapsulated in soybean-derived sterols and poly(ethylene glycol) liposomes in mice. Int J Pharm 146:31–39.
14. Anonymous. 2000. First phase II results of cisplatin/epinephrine in primary liver cancer. Oncology 14(1):89.
15. Colbern GT, Hiller AJ, Musterer RS, Working PK, Henderson IC. 1999. Antitumor activity of Herceptin® in combination with STEALTH® liposomal cisplatin or nonliposomal cisplatin in a HER2 positive human breast cancer model. J Inorg Biochem 77:117–120.
16. Bandak S, Goren D, Horowitz A, Tzemach D, Gabizon A. 1999. Pharmacological studies of cisplatin encapsulated in long-circulating liposomes in mouse tumor models. Anticancer Drugs 10:911–920.
17. Kim T, Murdane S, Gruber A, Kim S. 1996. Sustained-release morphine for epidural analgesia in rats. Anesthesiology 85(2):331–338.
18. Katre NV, Asherman J, Schaefer H, Hora M. 1998. Multivesicular liposome (DepoFoam™) technology for the sustained delivery of insulin-like growth factor-I (IGF-I). J Pharm Sci 87(11):1341–1346.
19. Xi NZ. 1996. Pharmaceutical science, 3rd ed. Beijing: People's Health Press, p 455.
20. Tang CS, Su JY, Li ZP, Zhang LZ, Yang J, Qi M, Liu FA, Tang J. 1992. The feasibility study of treating myocardial ischemia disease by the liposome-directed drug delivery system. Science in China (Series B) 2:165–171.

Pharmaceutical Research, Vol. 21, No. 4, April 2004 (© 2004) *Research Paper*

Novel Chitosan Particles and Chitosan-Coated Emulsions Inducing Immune Response via Intranasal Vaccine Delivery

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Purpose. The aim of this study was to prepare a novel vaccine carrier particulate system (nanoparticles and emulsions) with chitosan and to evaluate the effect of this system on the immune response for intranasal delivery.

Methods. Chitosan nanoparticles (NP) and chitosan-coated emulsions (CC-Emul) were prepared by improvement of the method previously reported and by modified ethanol injection methods, respectively. The rats were immunized with the particles adsorbed with ovalbumin (OVA) and cholera toxin (CT) by intranasal (i.n.) and intraperitoneal (i.p.) administration.

Results. NP and CC-Emul could be prepared with particle diameter from about 0.4 μm to 3 μm. IgG induced by i.n. of NP was comparable with that by i.p., and IgA induced by i.n. of 0.4-μm- and 1-μm-size NP was significantly higher than control (OVA and CT). IgG and IgA induced by i.n. of 2-μm-size CC-Emul were significantly higher than those with control.

Conclusions. The novel chitosan particles used simple preparation methods showed high OVA adsorption. When administered intranasally, NP and CC-Emul induced systemic immune response in rats. These findings suggested that CC-Emul and the smaller-size (0.4 μm) NP are effective for targeting to nasal-associated lymphoid tissues (NALTs) in nasal vaccine delivery.

KEY WORDS: chitosan; emulsion; immune response; intranasal; nanoparticle.

INTRODUCTION

Mucosal vaccine delivery is very attractive for inducing a protective immune response because many pathogens invade the body through mucosal surfaces. The main function of mucosa-associated lymphoid tissue is the selective uptake of antigens and the induction of local immune responses (1). In nasal inoculation, particle antigens are mainly taken by the M-cell connected to the nasal-associated lymphoid tissues (NALTs), whereas soluble antigens are mainly absorbed at the nasal epithelium (2,3). Particle antigens will be processed at the NALT and preferentially drain to the antigen-presenting cells (APCs). Because emulsions are directed to lymph, the development of antigen particulate carrier systems (nanoparticle and emulsion) that allow mucosal vaccine delivery is of considerable interest.

Chitosan derived by the deacetylation of chitin, which is a polymer of D-glucosamine and *N*-acetyl-D-glucosamine, has high biodegradability and low toxicity. Chitosan particle delivery system can reduce the clearance rate from the nasal cavity, thereby increasing the contact time of the delivery system with the nasal mucosa (4). Chitosan suspensions or micro- and nanoparticles have been reported to have immune stimulating activity such as increasing accumulation and activation of macrophage and polymorphonuclear cell, promoting resistance to infections by microorganisms, and inducing cytokines (5). Among the various particle properties, the effective particle size on immune responses appears to be a key factor but has not been intensively investigated. The uptake of chitosan micro- (>1 μm) and nanoparticles from the nasal cavity was reported in the past decade (5,6). However, the optimal size of particles remains unclear for intranasal vaccine delivery. Although numerous nanoparticle preparation methods are known, sufficient antigen loading of nanoparticles and emulsions remains a challenge. Therefore, various-size novel chitosan particles and chitosan-coated emulsions loaded with antigen were prepared.

The aim of this study was to prepare and characterize chitosan nanoparticles and chitosan-coated emulsions for adsorptive loading of ovalbumin (OVA) and to evaluate the effect of particle size of chitosan nanoparticles (NP) and chitosan-coated emulsion (CC-Emul) loaded with OVA and cholera toxin (CT) on the immune response for intranasal vaccine delivery.

MATERIALS AND METHODS

Chemicals

Soybean oil and three kinds of chitosan (chitosan 10, chitosan 100, and chitosan 500 with a deacetylation degree of about 80 mol% with molecular weights of 10 kDa, 100 kDa, and 500 kDa, respectively) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). OVA was obtained from Worthington Biochemical Corporation (Lakewood, NJ, USA), and CT obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). Eight-week-old male Wistar Kyoto Rats (WKY rat) were purchased from Oriental Yeast, Co. Ltd. (Tokyo, Japan). Tween 80 and oleic acid (OA) were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Egg phosphatidylcholine (EPC) was from QP Co. Ltd. (Tokyo, Japan). All other reagents were of analytical grade.

Preparation of NP

Three different size NP (average diameters of about 700 nm, 1300 nm, and 3000 nm for individual batches) were prepared using chitosan 10, chitosan 100, and chitosan 500, respectively, by improvement of methods previously reported by Lubben *et al.* (7). Briefly, 0.25% (v/v) of chitosan 10, chitosan 100, and chitosan 500 solutions were prepared in 2% acetic acid aqueous solution. Then, 1 ml of 10% (w/v) sodium sulfate was added to 100 ml of each chitosan solution. Moreover, to obtain smaller NP, the NP sample prepared by chi-

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ABBREVIATIONS: APC, antigen presenting cell; CC-Emul, chitosan-coated emulsions; CT, cholera toxin; EPC, egg phosphatidylcholin; NALTs, nasal-associated lymphoid tissues; NP, chitosan nanoparticles; OA, oleic acid; OVA, ovalbumin.

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