Phospholipid Deformable Vesicles for Buccal Delivery of Insulin

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To investigate the possibility of the enhancing effect of deformable vesicles on buccal delivery of insulin, two kinds of vesicles with and without the presence of sodium deoxycholate (deformable vesicles and conventional vesicles) were prepared by reverse phase evaporation methods. The liposomal entrapment efficiency was determined by column chromatography. The particle size and morphology of the vesicles were also evaluated. The hypoglycemic effects, insulin concentrations, and residual amounts of insulin deposited in the buccal membrane after buccal administration of insulin vesicles to rabbits were investigated. Compared with subcutaneous administration of insulin solution, the relative pharmacological bioavailability and the relative bioavailability of buccal administration of insulin vesicles were determined. The results showed that the entrapment efficiencies of the deformable and conventional vesicles were 18.87±1.78% (n=3) and 22.07±2.16% (n=3), respectively. The particle sizes of the deformable and conventional vesicles were 42.5±20.5 nm and 59.7±33.8 nm, respectively. There were no significant differences in appearance between the two types of vesicle. Compared with subcutaneous administration of insulin solution, the relative pharmacological bioavailability and the relative bioavailability in the insulin-deformable vesicles group were 15.59% and 19.78%, respectively, which were higher than in the conventional insulin vesicles ($p<0.05$), blank deformable vesicles and insulin mixture groups ($p<0.05$). Deformable vesicles have an enhancing effect on buccal delivery of insulin and may be a better carrier than conventional vesicles for buccal delivery of protein drugs.

Key words insulin; deformable vesicle; buccal delivery; relative pharmacological bioavailability; relative bioavailability

To date, a wide variety of polypeptidic drugs have been evaluated for buccal absorption.[1] Buccal delivery of peptides and proteins has potential advantages over other available routes.[2] It avoids degradation by gastrointestinal enzymes and first-pass hepatic metabolism. Buccal delivery has high patient compliance and excellent accessibility, and self-placement of a dosage form is possible. Because of the natural function (*i.e.*, to line and protect the inner surface of the cheek) of the buccal mucosa, it is less sensitive to irritation and damage than the other absorptive mucosae. Furthermore, there are fewer proteolytic enzymes at work as compared with oral administration[3] and in addition, the buccal mucosa is highly vascularized.[4]

Liposomal formulations with encapsulated drugs have been investigated for buccal administration.[5,6] Peptide entrapment within liposomes is also possible.[7,8] Applications of liposomal formulations in buccal delivery always result in an increase in local, and a decrease in systemic drug concentration.[5] Buccal administration of human insulin in streptozocin-diabetic rats was investigated and no significant difference in the blood glucose level profile was observed after administration of liposome vesicles containing insulin (LEVINS).[7]

In recent years, a novel type of highly deformable lipid vesicle (transfersome) has been developed to enhance the transdermal delivery of water-soluble drugs when applied onto the skin nonocclusively.[9,10] Transfersomes have been defined as specially designed vesicular particles, consisting of at least one inner aqueous compartment surrounded by a lipid bilayer with appropriately tailored properties. Accordingly, transfersomes resemble lipid vesicles (liposomes) in morphology but not in function.[11] Some authors suggest that transfersomes can respond to external stresses by rapid shape transformations requiring low energy.[12] This high deformability allows them to deliver drugs across barriers, including the skin. To prepare these vesicles, surfactants have been incorporated into the vesicular membrane and sodium cholate or sodium deoxycholate has been used for this purpose.[13,14]

The oral mucosa is morphologically similar to the skin, and in particular the epithelium has the same composition and physiological roles as the skin.[15,16] Therefore we assumed that the deformable vesicles could enhance the buccal delivery of water-soluble polypeptidic drugs as a carrier. Little known about the influence of deformable vesicles on the buccal drug delivery, however.

The aim of the present study was to investigate the effects of conventional and deformable vesicles on the buccal delivery of insulin, which was chosen as a model drug. We prepared insulin-deformable vesicles using sodium deoxycholate in the present investigation. The hypoglycemic effects and insulin concentrations after buccal administration of the conventional and deformable insulin vesicles to rabbits were determined. The entrapment efficiencies, vesicle size, and morphology were also investigated.

Experimental

Materials Crystalline porcine zinc insulin (26.3 IU/mg) was purchased from Xuzhou Biochemical (P.R. China), and soybean phosphatidylcholine (SPC) from Lucas Meyer GmbH (purity >95%, Germany). Sodium deoxycholate and cholesterol were supplied by Sigma (St. Louis, MO, U.S.A.). Blood glucose assay kits were obtained from Zhongsheng High-tech Bioengineering Company (P.R. China) and insulin radioimmunoassay kits from the China Institute of Atomic Energy (P.R. China). Buccal sprayers were kindly provided by Pfeiffer Company (Germany). Sephadex G-75 was purchased from Pharmacia (Sweden). Acetonitrile was the product of Fisher (chromatography grade, U.S.A.). All other chemicals and solvents were of analytical grade.

Preparation of Lipid Vesicles Two kinds of lipid vesicle containing SPC were prepared by a conventional rotary evaparation-sonification method. Briefly, appropriate amounts of SPC (5.5%, w/v) and cholesterol (0.5%, w/v) were dissolved in a co-solvent of chloroform and *n*-hexane (5 : 7). Insulin was dissolved in phosphate-buffered saline (PBS, pH 7.4) and then added to the mixture of SPC and cholesterol. Bath sonification (2 min) was carried out (CQ 250 ultrasonic processor, China) to form an emulsion. The organic solvent in emulsion was evaporated to a jelly film under vacuum rotary evaporation (23 °C). The film was then hydrated with sodium deoxy-

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cholate-PBS (pH 7.4) solution, shaken, and then placed in an ultrasonic bath for 1 min (23 °C) to obtain small vesicles. When sodium deoxycholate in PBS (pH 7.4) was added, the weight ratio of SPC and surfactant was 2.2 so that the total weight of the compositions remained equal. When sodium deoxycholate in PBS was added, deformable vesicles were obtained, and when PBS alone was added, conventional vesicles were obtained. The final concentration of insulin in vesicles was about 50 IU/ml.

Characterization of Vesicles The diameters of the two types of vesicle were determined using a Nicomp 370 Submicron Particle Sizer (Brookhaven Instrument Corp., U.S.A.). Samples for transmission electron microscopy (TEM) were prepared at room temperature by conventional double-staining methods using 2% phosphotungstic acid buffer (pH 6.0) and then viewed on a Hitachi-500 model transmission electron microscope (Hitachi, Japan).

Content and Entrapment Efficiency Determination Concentrations of insulin in the two types of vesicle were determined by high-pressure liquid chromatography (HPLC). The HPLC system consisted of a pump (Model HP 1100, Hewlett-Packard, U.S.A.), a UV detector (Model HP 1100, Hewlett-Packard, U.S.A.) at 220 nm, a data station (Model HP3395, Hewlett-Packard, U.S.A.) and a Hypersil C_{18} ODS column (250×4.6 mm, Dalian Elite Scientific Instruments Co. Ltd.) maintained at 30 °C. The mobile phase was composed of 0.013 M sodium dihydrogen phosphate, 0.05 M anhydrous sodium sulfate, and acetonitrile (35 : 35 : 30, v/v/v) and delivered at a flow rate of 1.0 ml/min. The injection volume was 50 μl and the relative retention time of insulin was about 5 min.

Content determination was carried out by dissolving both vesicles in 80% (v/v) ethanol and measuring with HPLC.

Separation of nonentrapped drug and vesicles was carried out by passage through a Sephadex G-75 column (45×2 cm). One milliliter of vesicle sample was added dropwise to the center of the column and eluted by PBS (pH 7.4) at the speed of 1.0 ml/min. The content of free drug was determined by the HPLC method. The entrapment efficiency was calculated as the ratio of drug weight within vesicles to that in the vesicular suspension.

Blood Sample Collection and *in Vivo* Buccal Delivery Studies Male rabbits were randomly divided into six groups (4 in each group): group 1, buccal administration of insulin-deformable vesicles (10 IU/kg); group 2, buccal administration of conventional insulin vesicles (10 IU/kg); group 3, buccal administration of a physical mixture, which contained blank deformable vesicles and insulin (10 IU/kg); group 4, buccal administration of insulin solution (10 IU/kg, pH 7.4); group 5, buccal administration of PBS (pH 7.4); and group 6, subcutaneous administration of insulin solution (1IU/kg, pH 7.4).

After overnight fasting before the experiment, the rabbits in groups 1—5 were intravenously anaesthetized with sodium pentobarbital 120 mg/kg and secured on their backs on boards. The esophaguses were surgically ligated to prevent swallowing of the dosing solution. Predose blood samples were collected and drug solutions (groups 1 and 4, insulin buccal spray; group 5, PBS) were administered using a buccal sprayer (0.14 ml/spray). The esophagus was untied 10 min after administration of the drug solution. Insulin solution (1 IU/kg) was subcutaneously administered to group 6 to determine bioavailability. Blood samples (0.5 ml) were collected from the ear veins at predetermined time intervals (0, 20, 30, 40, 50, 60, 90, 120, 150, 180, 240, 300, 360, 420, 480, 540 and 600 min) and plasma was separated. Blood glucose levels were measured immediately with blood glucose assay kits using the glucose oxidase methods and plasma insulin concentrations were assayed using radioimmunoassay kits.

Relative Pharmacological Bioavailability Calculation The area above the blood glucose curve (*AAC*)[17] was calculated by the trapezoidal method from the blood glucose data. The relative pharmacological bioavailability (Fp) was calculated using the following equation:

$$Fp=(AAC_{bu}/AAC_{sc})/(dose_{bu}/dose_{sc})$$

Where the subscripts bu and sc refer to buccal and subcutaneous administration, respectively. Student's *t*-test was used to determine statistical significance.

Relative Bioavailability Calculation The area under the curve (*AUC*) was calculated by the conventional trapezoidal method from the plasma insulin level. The relative bioavailability (Fr) was calculated using the following equation:

$$Fr=(AUC_{bu}/AUC_{sc})/(dose_{bu}/dose_{sc})$$

Where the subscripts bu and sc refer to buccal and subcutaneous administration, respectively. Student's *t*-test was used to determine statistical significance.

Determination of Insulin Deposited in the Buccal Membrane of *in Vivo* Experiments At the end of *in vivo* experiments (10 h later), the rabbits were anesthetized with an overdose of sodium pentobarbital (150 mg/kg) by intravenous injection. Similar to the method employed by Hayakawa *et al.*,[18] mucosae were removed immediately with a #11 blade. To remove excess drug on the surface, the buccal membrane tissues were rinsed three times with ice-cold Tris–HCl buffer (pH 7.4, 0.1 M), 80% ethanol, and then ice-cold Tris–HCl buffer (pH 7.4, 0.1 M). The tissues were cut into small pieces and homogenized in Tris–HCl buffer 3 ml at 40 °C. The resulting mixture was centrifuged for 40 min at 124000×***g*** at 40 °C in a Beckman L8-70M ultracentrifuge, and the supernatant was collected. Supernatant 50 μl was used in the determination, and the insulin content in the buccal membrane was assayed using radioimmunoassay kits.

To validate the buccal membrane washing procedure, 50 μl of vesicles containing insulin were administered onto the buccal membrane surface and washed 30 min later as a control. The insulin content in the buccal membrane was subsequently determined.

Results

Physical Properties of Vesicles Both conventional and deformable vesicles appeared as transparent colloidal dispersion with bluish emulsifying light. The average diameters were 57.7±33.8 nm and 42.5±20.5 nm, respectively.

Figure 1 shows the transmission electron photomicrographs of two types of small vesicles. Both types were spherical or oval liposomes with obvious whorls, although the whorls of deformable vesicles were slightly more distinct than those of the conventional vesicles. There were no significant differences in appearance between the two small vesicles. TEM photomicrographs validated the results of particle size measurements.

Determination of Content and Entrapment Efficien-

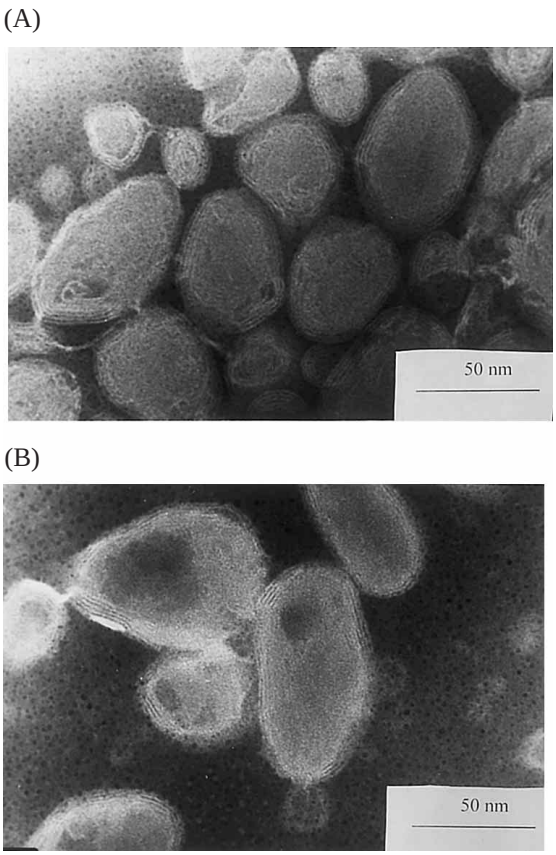

Fig. 1. Transmission Electron Photomicrographs of Conventional and Deformable Insulin Vesicles with Magnification of 150000

(A) Insulin-deformable vesicles; (B) conventional insulin vesicles.

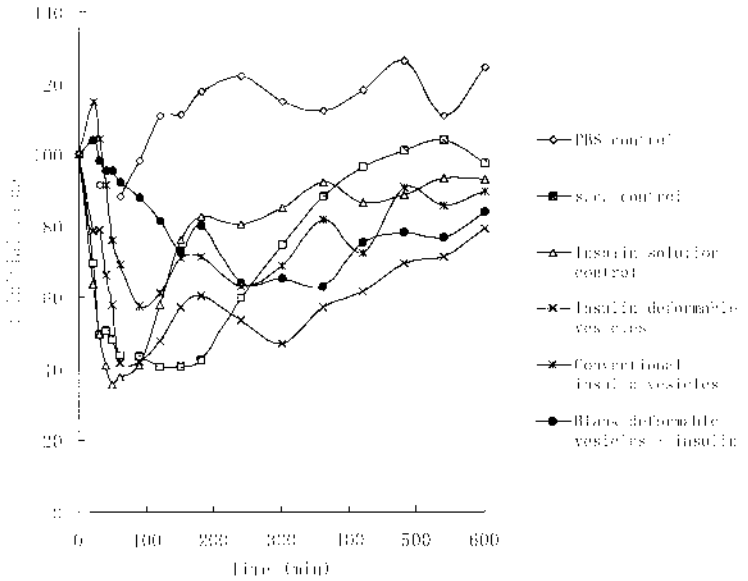

Fig. 2. Plasma Glucose Levels after Subcutaneous and Buccal Insulin Administration to Rabbits (n=4)

Table 1. Relative Pharmacological Bioavailabilities (Fp) after Buccal Administration of Insulin Vesicles to Rabbits

	AAC (%·min, mean±S.D.)	Fp (%)
Subcutaneous control	15277.33±4213.80	—
Insulin solution control	12751.59±6563.94	8.35
Insulin-deformable vesicles	23824.02±6926.01	15.59
Conventional insulin vesicles	13885.51±6756.37	9.09
Blank deformable vesicles+ insulin solution	14020.81±4892.10	9.18

*, $p<0.05$; △, $p>0.05$; n=4.

cy The regression equation for insulin content (μg/ml) in 80% (v/v) ethanol ranging from 50 to 1000 μg/ml was: $C=6.9319\times10^{-8}A-1.008\times10^{-3}$ ($r^2=0.9994$), where C (μg/ml) and A represented the concentration and peak area of insulin, respectively. The mean recovery was 97.3±2.4% (n=3). The precision assay showed that the relative standard deviations within 1 day and among every other day were all below 10%.

The contents of insulin-deformable vesicles and conventional vesicles determined by the HPLC method were 1.96± 0.07 mg/ml and 1.80±0.14 mg/ml, respectively.

Column recovery of nontrapped drug was 97.40%. The elution volume of blank vesicles was 32—38 ml and that of insulin was 48—85 ml. Vesicles and insulin were separated at 38 ml. The entrapment efficiencies of deformable and conventional vesicles were 18.87±1.78% (n=3) and 22.07± 2.16% (n=3), respectively.

Blood Glucose Levels and Hypoglycemic Effects Figure 2 shows the hypoglycemic effects after buccal or subcutaneous administration of insulin formulations (six groups). The maximal hypoglycemic effect with insulin-deformable vesicles was seen at about 1.0 h, and the maximal decrease in blood glucose reached 41.83%. Ten hours after buccal administration, the blood glucose levels were still lower than the value at 0 h. Thus the deformable vesicles had long-term hypoglycemic effects.

Table 1 summarizs the relative pharmacological bioavailabilities in each group. Compared with subcutaneous administration of insulin solution, the relative pharmacological bioavailability of deformable vesicles was 15.59%, which was greater than that of the conventional preparation ($p<0.05$). It was also greater than that of the physical mixture of blank vesicles and insulin ($p<0.05$), which indicated that insulin entrapment in flexible vesicles played an important role in the hypoglycemic effect. The Fp values in the conventional insulin vesicles group and the physical mixture group were all greater than in the control group 5 (buccal administration of insulin solution), but the difference did not reach significance ($p>0.05$).

Plasma Insulin Concentrations Figure 3 shows the plasma insulin concentrations after buccal delivery of insulin vesicles, which also confirmed the long-term effects of the insulin-deformable vesicles.

Table 2 summarizes the relative bioavailabilities in each group. Compared with subcutaneous administration of insulin solution, the relative bioavailability of deformable vesicles was 19.78%, which was also greater than that of the conventional vesicles ($p<0.05$). The Fp values in the conventional insulin vesicles group and the physical mixture group were all greater than the control group that received buccal administration of insulin solution, but the difference did not

Table 2. Relative Bioavailabilities (Fr) after Buccal Delivery of Insulin Vesicles to Rabbits

	AUC (mIU/L·min, mean±S.D.)	Fr (%)
Subcutaneous control	16837.11±2653.70	—
Insulin solution control	17799.95±1662.50	10.57
Insulin-deformable vesicles	33308.64±2787.62	19.78
Conventional insulin vesicles	19092.02±2348.34	11.34
Blank deformable vesicles+ insulin solution	20549.46±2877.67	12.20

*, $p<0.05$; △, $p>0.05$; $n=4$.

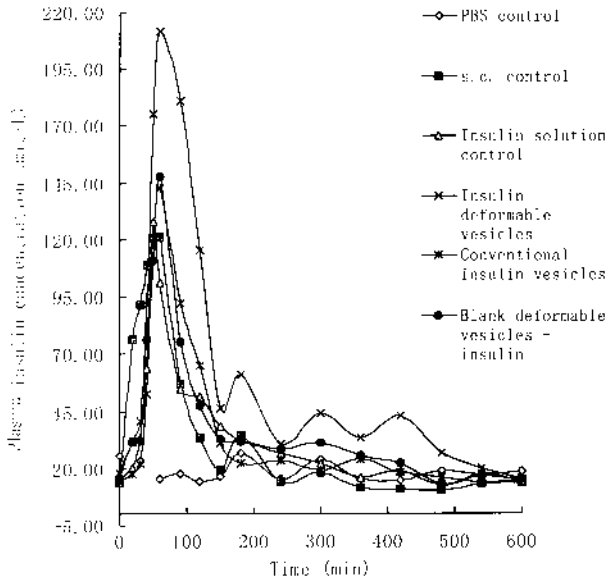

Fig. 3. Plasma Insulin Concentrations after Subcutaneous and Buccal Insulin Administration to Rabbits ($n=4$)

reach statistical significance ($p>0.05$). These results were in agreement with those from hypoglycemic experiments. Insulin entrapment in the deformable vesicles was essential to promote much more insulin penetration into the buccal membrane.

Residual Insulin in the Buccal Membrane Table 3 shows the residual amount of insulin in the buccal membrane. Compared with the controls, both deformable and conventional vesicles transported fairly large amounts of insulin into the rabbit buccal membrane, totaling 67.1±16.1 mIU/cm^2 and 32.2±11.3 mIU/cm^2, respectively. It also demonstrated that the possibility that more insulin in deformable vesicles penetrated the buccal membrane and entered the blood.

Discussion

Buccal membrane characteristics lead to better absorption of insulin *via* buccal delivery. In this study, the relative pharmacological bioavailability of insulin solution in rabbits was 8.35% and the relative bioavailability was 10.57%. Compared with the bioavailability of oral insulin delivery (<1%),[19] the absorption of buccal insulin delivery is relatively good.

For the choice of experimental animal species in buccal drug delivery studies, one important factor influencing permeation is the keratinized state of buccal tissue.[20] The

Table 3. Residual Amount of Insulin in the Buccal Mucosa after Administration of Different Preparations

Group	Residual insulin (mIU/cm^2)
Control	13.4±4.1
Insulin-deformable vesicles	67.1±16.1*
Conventional insulin vesicles	32.2±11.3*
Insulin solution control	15.3±4.7
Blank deformable vesicles+insulin	35.4±10.1*

*, $p<0.05$ compared with insulin solution control group ($n=4$).

human oral mucosa can be subdivided into a nonkeratinized area consisting of the floor of the mouth (sublingual), the buccal mucosa (cheeks), and a keratinized area consisting of the gums (gingiva), the palatal mucosa, and the inner lips. Generally, the permeability of nonkeratinized tissue is greater than that of the keratinized. Most of the buccal mucosa in rabbits is nonkeratinized mucosal lining tissues, which are similar to those in humans. Therefore rabbits were widely used in previous investigations of buccal delivery.[21—23]

In this paper, blood glucose levels were measured using blood glucose assay kits with the glucose oxidase method, and plasma insulin concentrations were assayed using radioimmunoassay kits. These two methods are common in the studies on insulin delivery. These two types of kit have been used widely in hospitals and laboratories. The precision and accuracy validations were carried out before the pharmacodynamic and pharmacokinetic studies and these methods proved feasible in our experiments.

Rabbits in the blank control group were administered PBS, and there were no changes in blood glucose levels and plasma insulin concentrations, which still fluctuated around the normal values. This confirmed that fasting procedure before the experiments might not cause suppression of blood glucose and elevation of plasma insulin levels, which was also observed by Liu *et al*.[24]

The relative pharmacological bioavailabilities (from pharmacodynamic results) and the relative bioavailabilties (from pharmacokinetic results) were determined after buccal administration of insulin-deformable vesicles. The characteristics of the pharmacodynamic data and the pharmacokinetic data are different. Therefore these two methods could be used to determine the absorption of insulin-deformable vesicles *via* the buccal route from two viewpoints.

When we examined the residual insulin in the buccal membrane, Large amounts of drug were deposited in the membrane after buccal administration of conventional and

deformable vesicles. It appeared that lipid vesicles were capable of drug adsorption and accumulation similar to transdermal drug delivery.[25,26]

Two main factors may be involved in the enhancing effect of deformable vesicles on buccal insulin delivery.[27] First, the deformable vesicles containing sodium deoxycholate are capable of penetrating the interstices of the buccal membrane under the influence of transbuccal hydration force caused by the difference in water concentration between the buccal surface and buccal interior, as found in the transdermal delivery of deformable vesicles.[9—14] When deformable vesicles were administered, the transdermal amount of drug was usually about 10 times greater than when conventional vesicles were administered.[28] In our experiments, the increase in the transbuccal amount of insulin was not as great when the deformable vesicles were administered. This might be because the drug-deformable vesicles could be administered nonoccclusively in buccal drug delivery, but the buccal mucosa might remain moist. Therefore the transbuccal hydration force was not great as that in transdermal drug delivery. On the other hand, hydration might loosen the interstices, which would be beneficial for the accumulation of insulin. Second, fusion of vesicles with the buccal membrane also contributed to the enhancement effect, which was similar to transdermal drug delivery.[29] Bile salts have been demonstrated to influence the physicochemical properties of phospholipid bilayers, strongly perturbing the phospholipid alkyl chain order and resulting in a large decrease in the order parameter.[30] The increase in the fluidity of bilayers might lead to an increase in fusion. Furthermore, it has been reported membrane ripples were found after the addition of cholate to phospholipid vesicles, although the surfaces previously appeared smooth under freeze-fracture electron microscopy. These ripples may serve as intermembrane attachment sites for membrane fusion.[31] Therefore the fusion effect of vesicles containing cholate with buccal membrane lipid was greater than that of vesicles without cholate.

The most effective enhancers for buccal drug delivery are surfactants, such as bile salts. They have often been used to enhance the absorption of insulin across the mucous membranes from various sites.[16,32,33] As bile salts can serve as enhancers, sodium deoxycholate not entrapped into the vesicles may enhance the absorption of insulin across the buccal membrane. On the other hand, bile salts not only act as enhancers, but also they play an important role in preparing the deformable vesicles with special characteristics. In addition, a series of Tweens and Spans have been also used for this purpose.

Although the ability of the buccal mucosae to recover rapidly means that the toxicity of absorption enhancers may not be as significant a factor as for other mucosal sites,[34—36] it is essential to select the appropriate type and concentration of enhancer to minimize irritation of the mucosal membranes. Phospholipids are considered nontoxic vehicles and appropriate surfactants may be used in deformable vesicle formulations, resulting in good bioavailability of drugs. Therefore deformable vesicles may be promising carriers for the buccal delivery of polypeptidic drugs.

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