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New strategies to improve the intranasal absorption of insulin: the application of new safe absorption enhancers and new drug delivery systems with increased retention time, nanocarriers, and so on.

New strategies to improve the intranasal absorption of insulin

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Recently, intranasal delivery of insulin as an alternative route of parenteral administration has been widely studied because it bears close resemblance to the 'pulsatile' pattern of endogenous insulin secretion during meal time. However, insulin is not well absorbed through nasal mucosa because of its large molecular size, hydrophilicity and low permeability through the membrane. This review describes the main barriers preventing nasal insulin absorption, and special attention is given to new approaches to improve the intranasal absorption of insulin, including the application of new safe absorption enhancers and the use of appropriate delivery systems. It seems that bioadhesive delivery systems or water-insoluble powders with absorption enhancers are the most promising methods for intranasal delivery of insulin.

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Introduction

Diabetes has become the third most common disease that heavily threatens human health in the world, following cardiovascular diseases and cancers. According to the World Health Organization, more than 180 million people worldwide suffer from various forms of diabetes, and it is estimated that the number of people with diabetes will double by 2030. Left uncontrolled, diabetes can lead to coronary heart disease, kidney failure, blindness, limb amputations and premature death.

Diabetes mellitus is a chronic metabolic disorder that results from a failure of the body to produce the hormone insulin and/or an inability of the body to respond adequately to circulating insulin. It can be grouped into two types – type 1 and type 2. Type 1 diabetes, previously referred to as 'insulin-dependent diabetes mellitus' (IDDM), is characterized by an absolute loss of insulin secretion, mainly owing to the selective autoimmune destruction of pancreatic β-cells [1]. Patients suffering from type 1 diabetes need to inject insulin daily and self-monitor blood glucose. Type 2 diabetes, previously known as 'non-insulin-dependent diabetes mellitus' (NIDDM), ranges from predominantly insulin resistance with relative insulin deficiency to predominantly an insulin secretory defect with insulin resistance. Patients with type 2 diabetes mellitus are usually treated initially with oral antidiabetic agents but, as the disease progresses, most patients eventually require insulin to maintain glucose control. Therefore, insulin is not only vital in the treatment of IDDM but also widely used in the treatment of NIDDM.

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It is clear that optimal insulin therapy should mimic the normal physiological secretion of insulin and minimize the risk of hypoglycemia. However, because of insulin's large molecular size, hydrophilicity and low permeability through the membrane, subcutaneous injection has been the only method of delivering it to patients with diabetes mellitus for the past 80 years. Subcutaneous insulin administration does not lead to optimal pharmacodynamic properties of the applied insulin, absorption into the blood stream (even with rapid-acting insulin analogues) is not that rapid, and a precise mimicking of the prandial physiological insulin secretion pattern is not possible. In addition, subcutaneous injection is associated with tissue invasion, infection and poor patient compliance, especially in the cases of chronic diseases when frequent dosing is required.

Therefore, much attention has been paid to the development of non-parenteral routes. The main alternatives studied for insulin delivery include nasal, pulmonary, dermal, rectal and oral routes [2], as shown in Fig. 1. Pulmonary insulin application led to a rapid absorption of insulin across the mucosa. In 2006, the Federal Drug Administration approved the first commercially available pulmonary inhaled insulin, Exubera. The relative bioavailability was low (approximately 10%), however, the dose of inhaled insulin must be ten times higher than the dose applied subcutaneously to induce a comparable metabolic effect. Moreover, the long-term consequences of the inhalation of insulin (i.e. the development of insulin antibodies, changes in lung function and lung safety) were raised during clinical development. Because of these problems, Exubera failed to gain acceptance from both patients and physicians and was withdrawn from the market in October 2007.

Dermal insulin application does not result in a reproducible and sufficient transfer of insulin across the highly efficient skin barrier. The dream of an 'insulin tablet' has not become reality because of the low permeability of insulin through the gastrointestinal mucosa and susceptibility to chemical and enzymatic degradation in the gastrointestinal tract. By contrast, intranasal insulin therapy has considerable potential for controlling post-prandial hyperglycemia in the treatment of both IDDM and NIDDM, based on its advantages (described in the following section), and is attracting more and more attention. However, effective insulin absorption via the nasal route is improbable without the help of absorption enhancers and/or prolonging the residence time of the drug formulation in the nasal cavity. This article discusses the barriers that prevent nasal insulin absorption and new strategies to improve its intranasal absorption.

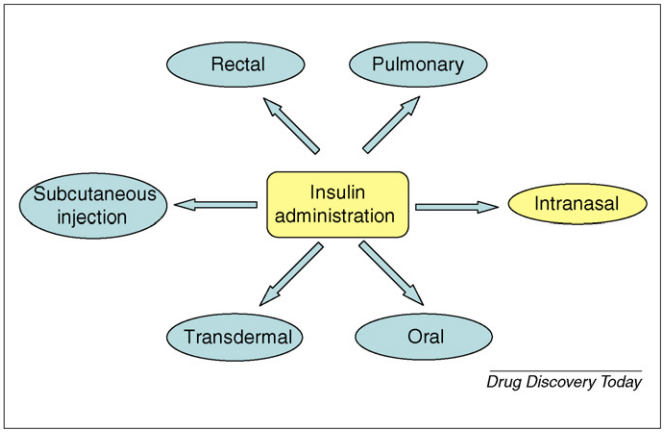

FIGURE 1

The main administration routes for insulin delivery.

Intranasal delivery of insulin

Advantages of insulin intranasal delivery

Nasal administration has attracted a lot of interest as an alternative route for the systemic delivery of insulin [3]. Several potential advantages contribute to the attainment of adequate bioavailability of insulin and rapid onset of action comparable to injections, including the large surface area of nasal mucosa available for insulin absorption (approximately 150 cm^2, nasal epithelial surface covered with numerous microvilli), a porous endothelial membrane, the relatively high permeability of the nasal epithelial membrane, lower enzymatic activity than the gastrointestinal tract and a highly vascularized subepithelial layer that passes directly into the systemic circulation, thereby avoiding the first-pass metabolism in the liver. The ready accessibility of nasal administration also makes it possible for patients on long-term therapy to self-medicate. In addition, the possibility of obtaining pharmacokinetic profiles that mimic the 'pulsatile' endogenous secretion of insulin in healthy volunteers provides opportunities for the design of an optimal replacement therapy. Furthermore, after nasal administration, part of the drug can enter directly into the brain tissue or cerebrospinal fluid through olfactory neurons. With these advantages, the nasal administration of insulin for systemic medication has been widely investigated in recent years, and many projects are now under clinical development [4], as shown in Table 1.

Disadvantages of insulin intranasal delivery

Although nasal delivery of insulin has many advantages, there are also some barriers that hamper the absorption of insulin across the nasal mucosa [5], and the bioavailability of insulin is generally less than 1%.

The low permeability of the nasal mucosa is one of the major limitations for attaining sufficient nasal bioavailability of therapeutic proteins and peptides [6]. Another important factor for low membrane transport is the general rapid clearance of the administered formulation from the nasal cavity owing to the mucociliary clearance mechanism. For non-mucoadhesive formulations, the half-life of clearance is approximately 15–20 min [7], which shortened nasal drug absorption time. It has also been suggested that the deposition of a formulation in the anterior part of the nasal cavity can decrease clearance and promote absorption because the anterior part of nasal cavity has no or very few ciliated cells that provide a faster clearance of particles [8]. The third contributing factor to the low bioavailability of insulin is the possibility of enzymatic degradation. The nasal cavity contains many different kinds of enzyme, such as the monooxygenase, reductase, transferase, esterase and proteolytic enzymes. Aminopeptidases are the most important proteolytic enzymes, including exopeptidases (such as mono- and diaminopeptidases) that can cleave peptides at their N and C termini and endopeptidases (such as serine and cysteine) that can attack internal peptide bonds [9].

TABLE 1

Insulin formulations for non-parenteral administration under clinical development

Administration route	Formulation	Company	Clinical trial	Date
Intranasal	Spray	Nastech Pharmaceutical Company, Inc.	Phase II	June 2008
Intranasal	Nasulin™	CPEX Pharmaceuticals, Inc.	Phase IIa	December 2009
Intranasal	Spray	MDRNA, Inc.	Received European patent	January 2010
Pulmonary	Microsphere	Baxter Healthcare	Phase I	April 2007
Pulmonary	Dry powder	MicroDose Technologies, Inc.	Phase I	August 2007
Pulmonary	Technospheres/Insulin (AFRESA)	MannKind	Submitted a New Drug Application	March 2009
Transdermal	PassPort™ patch	Altea Therapeutics	Phase I	January 2007
Transdermal	TPM-02/Insulin gel	Phosphagenics Limited	Phase Ib	May 2007
Oral	Nanoparticle	BioSante Pharmaceuticals, Inc.	Preclinical study	June 2004
Oral	Tablet	Emisphere Technologies, Inc.	Phase II	April 2006
Oral	Bioadhesive nanoparticle (Nodlin)	Shanghai Biolaxy	Phase I	November 2009

Strategies and perspectives to enhance the intranasal absorption of insulin

To overcome the various absorption barriers and achieve effective insulin absorption via the nasal route, researchers have studied many methods. Several approaches seem to be promising.

Chemical modification

Chemical modification of the primary peptide structure or the formation of prodrugs can protect the drug from proteolytic degradation or improve its permeation characteristic across the nasal mucosa.

Protecting insulin from proteolytic degradation. Chemical modification of peptides and proteins has been reported with several macromolecules such as polyethyleneglycol (PEG), poly(styrene maleic acid) copolymer, albumin and dextrans [10]. The steric protection conferred by pegylation protects conjugates from proteolysis, shields them from the immune system and decreases their rate of clearance from the circulatory system via intracellular uptake and kidney filtration. Shechter *et al.* [11] designed and prepared a spontaneously hydrolyzable prodrug by conjugating insulin through its amino side chains to a 40-kDa PEG containing sulfhydryl moiety. A single subcutaneous administration of PEG40–Fmoc–insulin to healthy and diabetic rodents facilitates prolonged glucose-lowering effects 4–7-fold greater than similar doses of the native hormone [11]. This might be successful for injection but not for transmucosal delivery, however, because pegylation of insulin increased hydrophilicity and molecular weight (MW) and size, which decreased the permeability of insulin across the mucous membrane.

Improving the permeation characteristics of insulin. It is well known that a major barrier for the passage of peptides and proteins through biological membranes is hydrophilicity. A potentially useful approach is the chemical modification of peptides and proteins to produce prodrugs and analogues that are more lipophilic. Many studies on insulin acylation or alkylation have been carried out concerning intestinal route [12,13]. Muranishi *et al.* [12] chemically modified three peptides (thyrotropin-releasing hormone, tetragastrin and insulin) by attaching fatty acid moieties (acyl chains) to their amino termini to increase peptide lipophilicity, thus facilitating absorption. These analogues retained more than 64% of the pharmacological activities of the parent peptides, as assessed after intravenous injection in rats. The lipophilic derivatives were more suitable for intestinal absorption, and the stability of some derivatives against intestinal enzymatic degradation was also improved. Asada *et al.* [13] acylated bovine insulin with one or two of the short fatty acids (six carbons), caproic acid, medium-length (12 carbons) lauric acid and/or long-chain (16 carbons) palmitic acid. The observed enhancement was attributed to the increased lipophilicity and also to the inhibition of insulin self-association. Although the usefulness of chemical modification of insulin has been clearly established for gastrointestinal tract absorption, to date, there are no examples for the nasal route. In addition, chemical modification might be successful for small peptides but seems to be much less useful for larger peptides because of the complexity of their structure.

Using enzyme inhibitors

Proteolytic enzyme inhibitors could prevent the hydrolysis of peptide and protein drugs in the nasal cavity and, thus, improve the stability of drugs at the absorption site. For example, camostat mesilate, an aminopeptidase and trypsin inhibitor, improved the nasal delivery of vasopressin and desmopressin [14]. Inhibitors with a trypsin-inhibiting activity were useful for enhancing the nasal absorption of salmon calcitonin [15]. Yamamoto *et al.* [16] demonstrated that 0.01% aprotinin, a serine protease inhibitor, reduced the metabolism of insulin and proinsulin by approximately 70–80% within 2.5 hours in homogenates of an albino rabbit buccal mucosa, which would otherwise have degraded at a rapid rate. However, proteolytic enzyme inhibitors themselves cannot facilitate the penetration of drugs across the epithelia membrane and, therefore, are generally unable to considerably improve bioavailability in the absence of other absorption-enhancing measures. For example, Aungst [17] observed no improvement of buccal insulin bioefficacy in rats upon coadministration with either aprotinin or a peptidase-inhibiting pentapeptide (Glx-Gly-Pro-Leu-Gly-Pro). Furthermore, the enzyme inhibitors will

affect the normal metabolism of the body, resulting in serious side-effects. Although there were no reports about enzyme inhibitors used for the nasal absorption of insulin, the above studies have demonstrated that using enzyme inhibitors does not seem to be an effective and safe method of improving the nasal absorption of insulin.

Using absorption enhancers

The use of absorption enhancers or promoters is the most common approach to improving the nasal absorption of insulin. Ideally, absorption promoters should be rapid-acting, resulting in transient and reversible modulation of the absorptive properties or physiology of the nasal mucosa, and not be absorbed systemically. They should be devoid of any toxic, irritating or allergic activity. The degree of absorption enhancement should be predictable and reproducible. They should also not permit entry of potentially dangerous environmental materials and should be compatible with drugs and adjuvants in the preparation. Ultimately, for the treatment of diabetes, the compound must be safe for chronic nasal administration [18]. So far, many compounds – such as surfactants, bile salt and its derivatives, and fatty acid and its derivatives – have been investigated as absorption enhancers for nasal delivery of insulin, as shown in Table 2. They are capable of improving the transport or absorption of insulin by different mechanisms, they might help solubilize or stabilize the drug or inhibit directly the enzymatic activity, they might extract the

TABLE 2

The absorption enhancers used for intranasal delivery of insulin

Absorption enhancers	Insulin dose	Formulation	Species	BA[a] (%)	C_{min}[b] (% of baseline)	C_{max} (mIU/L)	Safety[c]	Refs
Bile salt and its derivatives								
1% sodium deoxycholate	0.5 IU/kg	Aerosol	Rat		50		–	[89]
1% sodium taurodihydrofusidate (STDHF)	0.4 IU/rabbit	Solution	Rabbit	5.2			–	[90]
	8 IU/rat	Solution	Rat	18.0				
Surfactants								
0.5% lysophosphatidylcholine (LPC)	16.7 IU/kg	Solution	Rat		35		–	[91]
0.125% dodecylmaltoside	2 IU/rat	Solution	Rat			750	+	[92]
0.5% Laureth-9	10 IU/kg	Solution	Rat	28.7			–	[93]
0.5% sucrose cocoate	0.5 IU/rat	Solution	Rat	12.5		200	+	[52]
3.5% soybean-derived sterol	10 IU/kg	Peanut oil suspension	Rabbit	11.8			+	[94]
1.0% sterol glucoside	10 IU/kg	Peanut oil suspension	Rabbit	11.6			+	
Cyclodextrins								
5% DM-βCD	2 IU/kg	Solution	Rat	100			+	[18]
5% DM-βCD	2 IU/rabbit	Solution	Rabbit	0.8				[44]
30% DM-βCD	2 IU/rabbit	Solution	Rabbit	3.2				
1.9 μmol/dose DM-βCD	4 IU/rabbit	Powder	Rabbit	12.9		640		
5% DM-βCD	0.4 IU/rat	Solution	Rat	108.9		737		[45]
5% α-CD	0.4 IU/rat	Solution	Rat	27.7		115	–	
Cell-penetrating peptides								
0.5 mM L-R8	10 IU/kg	Solution	Rat	5.2		38.9	+	[41]
0.5 mM D-R8	10 IU/kg	Solution	Rat	9.7		95.8	+	
0.5 mM D-penetratin	10 IU/kg	Solution	Rat	15.8		140.6	+	
0.5 mM L-penetratin	10 IU/kg	Solution	Rat	33.3		444.6	+	
0.5 mM L-penetratin	1 IU/kg	Solution	Rat			80		[95]
0.5 mM shuffle (R,K fix) 2	1 IU/kg	Solution	Rat			245	+	
Cationized polymers								
0.5% chitosan	2 IU/kg	Solution	Sheep		43	191	+	[20]
0.5% chitosan	2 IU/kg	Solution	Rat	47.9	40.1			
	2 IU/kg	Nanoparticle	Rat	37.7	59.7			[84]
0.5% chitosan	100 IU/sheep	Solution	Sheep	3.6	53.0	179.1		
	100 IU/sheep	Nanoparticle	Sheep	1.3	72.6	106.2		
85.7% chitosan	128 IU/sheep	Powder	Sheep	17.0	38.1	743.1		
0.2% sperminated gelatin	10 IU/kg	Solution	Rat		61.7	315	+	[36]
0.2% aminated H-gelatin	10 IU/kg	Solution	Rat		66.9		+	[34]
0.4% aminated H-gelatin	10 IU/kg	Solution	Rat		62.3			
0.2% aminated H-gelatin	10 IU/kg	Solution	Rat		Approx. 63			[35]
0.2% aminated L-gelatin	10 IU/kg	Solution	Rat		Approx. 82		+	
Chelators								
0.5% EDTA-2Na	10 IU/kg	Solution	Rat	3.5			+	[93]

[a] Relative bioavailability compared with subcutaneous.
[b] C_{min} represents the minimum blood glucose level.
[c] '–' means unsafe and '+' means increased safety.

membrane protein or remove the outer layer of mucous membrane by interacting with the lipid bilayer, and they might alter properties of the mucus layer by opening tight junctions between the cells [5]. All of these lead to the improvement of the permeability of the epithelial cell layer.

However, many of the enhancers that are effective in improving the nasal absorption of insulin caused severe irritation and damage to the nasal mucosa at the concentrations required to effectively promote nasal absorption [5]. Thus, researchers strive to discover novel materials or better uses of existing materials to improve the safety of nasal delivery systems. Here, we list some promising absorption enhancers for intranasal insulin delivery.

Cationic polymers. Among these new absorption enhancers, the cationic polymers seem to be the most effective and safest materials for improving the transport of insulin. They could interact with the luminal surface of mucus membranes directly by an ion–ion interaction and then induce signals that would open tight junctions, resulting in intercellular permeation [19].

Chitosan and its derivatives. Chitosan and its derivatives are the most well-known absorption enhancers with good safety profile. Chitosan is a positively charged linear polysaccharide produced from chitin present in the shells of crustaceans by a process of deacetylation and is available in a range of MWs, viscosity grades and degrees of deacetylation. Illum *et al.* [20] described that chitosan solutions at 0.5% (w/v) concentration were highly effective in increasing the absorption of insulin across nasal mucosa in rats and sheep. It has been shown that chitosan is bioadhesive and able to interact strongly with the negatively charged components of nasal epithelial cells and the overlaying mucus layer, thereby providing a longer contact time for insulin transportation across the nasal membrane before the formulation is cleared by the mucociliary clearance mechanism. In addition, chitosan (in Caco-2 cell culture studies) increases the paracellular transport of polar drugs by transiently opening the tight junctions between the epithelial cells [21]. The enhancing effect of chitosans was higher if they had a higher MW and a lower degree of acetylation (higher positive charge) [22]. In addition, a single and chronic application (28 days) of chitosan to nasal epithelia did not produce any obvious tissue damage [23]. Further studies in human volunteers showed that application of chitosan solutions for seven days did not have harmful effects [24].

In spite of its superior properties, chitosan has a major drawback: its solubility is poor above pH 6. At physiological pH, chitosan will precipitate from solution and lose its capacity to enhance drug permeability and absorption, which can only be achieved in its protonated form in acidic environments [25]. By contrast, N-trimethyl chitosan chloride (TMC), a partially quaternized chitosan derivative, shows perfect solubility in water over a wide pH and concentration range. The reason for this improved solubility is the substitution of the primary amine with methyl groups and the prevention of hydrogen bond formation between the amine and the hydroxylic groups of the chitosan backbone. TMC also has bioadhesive properties and enhancement of permeability in neutral and basic pH environments [26]. The degree of quaternization (DQ) of TMC plays an important part in the solubility, the absorption-enhancing ability and the mucoadhesive properties of this polymer, especially in neutral and basic pH environments. This might be explained by the charge density on the TMC molecules, determined by the degree of quaternization, it influences the interaction of this polymer with the negatively charged sites on the cell membranes and/or within the tight junctions. At pH 7.2, TMC with a DQ of 60% (TMC60) showed higher mannitol transport enhancement ratio across Caco-2 cells than TMC40 [27]. However, Boonyo *et al.* [28] demonstrated that TMC40 was the most potent nasal antigen delivery platform compared with TMC20 and TMC60 in mice model. Hamman *et al.* [29] indicated that TMC48 showed the strongest absorption-enhancing property. These contradictions can probably be explained by the different experimental conditions, model drugs, and so on.

Recent studies have demonstrated that thiolated chitosan, another chitosan derivative, has higher mucoadhesive and permeation-enhancing properties than unmodified chitosan. When thiolated chitosan was used, in particular chitosan–TBA (chitosan-4–thiobutylamidine conjugate), the nasal absolute bioavailability of insulin ($7.24 \pm 0.76\%$) was obviously higher than unmodified chitosan formulation ($2.04 \pm 1.33\%$) in conscious rats [30]. The thiol functions on the chitosan backbone enable thiolated chitosans not only to form disulfide bonds with mucus glycoproteins but also to form inter- and intra-molecular disulfide bonds, which endowed thiolated chitosans with higher mucoadhesiveness, *in situ* gelling properties and excellent cohesive properties that guarantee a prolonged controlled release of embedded therapeutic ingredients. Moreover, the thiol functions could inhibit protein tyrosine phosphatase, which seems to be involved in the opening and closing process of the tight junctions, thereby strongly improving the permeation-enhancing effect of chitosan [31].

Because of their low toxicity, biocompatibility, biodegradability and mucoadhesive properties and their tight junction modulator effect, chitosan and its derivatives are promising candidates to enhance drug delivery in a clinical setting.

Poly-L-arginine. Natsume *et al.* [32] found that poly-L-arginines of different MWs (8.9, 45.5 and 92.0 kDa) can markedly enhance the nasal absorption of fluorescein isothiocyanate (FITC) labeled dextran (MW 4.4 kDa; FD4) in an *in vivo* rat model when applied with FD4 (33 mg/kg) at a concentration of 0.5%. Subsequently, Miyamoto *et al.* [33] studied the effects of concentration and MW of poly-L-arginine on the nasal absorption of FD4 in rats. The bioavailability ($F_{0\text{–}9\ h}$) of FD4 increased with the increasing concentration of poly-L-arginine. For each applied concentration, the poly-L-arginine exhibited a MW dependence as far as the enhancement of FD4 absorption was concerned. After nasal coadministration of FD4 with poly-L-arginines, the amount of protein, phospholipids and lactate dehydrogenase (LDH) leached from *in vitro* isolated rabbit nasal epithelia was similar to that found after the application of physiological saline. These results indicate that poly-L-arginine is a promising candidate exhibiting a suitable balance between enhancing activity and being a safe means of delivering peptides and proteins by the nasal route [32].

Cationized gelatin. Gelatin itself is bioadhesive and able to provide a long contact time for insulin transportation across the nasal membrane. Cationization enables gelatin to interact with the luminal surface of mucus membranes directly through an ion–ion interaction and then induce signals that would open routes for intercellular permeation.

Wang *et al.* [34] reacted ethylenediamine with gelatin to form aminated gelatin, which significantly increased the nasal

absorption of insulin without any marked leaching of LDH into the nasal cavity. The enhancing effect of aminated gelatin with different numbers of amino groups on the nasal absorption of insulin in rats depended on the MW and the number of amino groups [35]. Aminated gelatins with a higher MW and larger amino group content were more effective in enhancing the nasal absorption of insulin.

Sperminated gelatin, another cationized gelatin, is also able to improve the absorption of peptide and protein drugs through mucosal membranes while causing negligible mucosal damage. The AUC of immunoreactive insulin levels in the plasma after nasal administration of insulin were increased 5.3-fold by the addition of 0.2% sperminated gelatin, and the plasma glucose levels fell in a manner dependent on the insulin levels. In Calu-3 cell monolayer permeation experiments, sperminated gelatin showed significant enhancing effects on 5(6)-carboxyfluorescein, FD4 and insulin [36].

Cell-penetrating peptides. Cell-penetrating peptides (CPPs, also known as 'protein transduction domains'), a new class of peptides that was discovered in 1994, have been demonstrated to facilitate the delivery of hydrophilic macromolecules over the plasma membrane without altering their activities, both *in vitro* and *in vivo* [37,38]. When covalently linked with a cargo, including polypeptides and oligonucleotides with many times their own molecular mass, these peptides are still able to translocate.

Insulin transport across Caco-2 cells was dramatically increased by the conjugation of insulin with Tat peptide (CGGGYGRKKRR-QRRR). The transporting efficiency of insulin–TAT conjugate was six to eight times higher than that of free insulin, as demonstrated by calculating effective permeability [39]. Morishita *et al.* [40] demonstrated that coadministration of oligoarginine markedly increased intestinal insulin absorption without causing detectable damage in cellular integrity, and an *in situ* intestinal perfusion experiment showed that D-R_8 (D-form arginine octamer) had the strongest enhancing effects on insulin intestinal absorption. The same group studied the effect of penetratin and octa-arginine on the nasal absorption of insulin for the first time, showing that L-penetratin was the most effective promoter of insulin absorption without causing detectable damage to the integrity of cells in the nasal respiratory mucosa. A dose-dependent relationship of L-penetratin and insulin bioavailability was established. The pharmacological availability and bioavailability of nasally administered insulin was up to 76.7% and 50.7% relative to the subcutaneous route, respectively [41].

The entry mechanism of CPPs into cells is still extensively debated. There are many hypotheses to explain how these peptides could possibly deliver various kinds of molecules and much larger macromolecular structures into cells, but none of them has been justified. The toxic effects of CPPs were also controversial. These problems have been reviewed by Vivès *et al.* [42]. Notwithstanding that the detailed mechanism of CPPs entry into cells is poorly understood, their ability to traverse the membrane into the cytoplasm has provided a new and powerful biological tool for overcoming the low permeability of peptide and protein drugs through the epithelial cell membrane, which is the greatest barrier to the nasal delivery of macromolecular drugs.

Cyclodextrins. In recent years, research about cyclodextrin (CD) and its derivatives as an absorption enhancer for insulin nasal delivery has been very active. CD and its derivatives are deemed to be safe and efficacious. Shao *et al.* [43] reported that the relative effectiveness of the CDs in enhancing insulin nasal absorption was found to be in the following descending order of dimethyl-β-CD (DM-βCD) (5% w/v) > α-CD (5% w/v) > β-CD (1.8% w/v), hydroxypropyl-β-CD (HP-βCD) (5% w/v) > γ-CD (5% w/v). Among the CDs investigated, DM-βCD is the strongest absorption promoter, and powder formulations are more effective than liquid formulations. The absolute bioavailability of the nasally administered insulin-DM-βCD powder was $13 \pm 4\%$ in rabbits [44]. However, large interspecies differences existed in the absorption of insulin with DM-βCD. Coadministration of 5% (w/v) DM-βCD with insulin (0.4 IU) solution highly improved the nasal absorption of insulin in anesthetized rats, resulting in a bioavailability of $108.9 \pm 36.4\%$ compared with intravenous administration (0.05 IU) and a strong decrease in blood glucose level (to 25% of the initial value) [45]. By contrast, the nasal administration of insulin (2 IU)-DM-βCD (5%, w/v) liquid formulations in anesthetized rabbits did not result in a significant change in serum insulin and blood glucose concentrations [44]. Similarly, DM-βCD in solution did not affect insulin nasal absorption in human [4]. The effect of DM-βCD on ciliary movement was concentration dependent and reversible, and the ciliotoxicity was particularly mild. Irreversible cilio-inhibition occurred only at concentrations (10%, w/v) exceeding those used in pharmaceutical formulations (1.0–5.0%, w/v) and/or at an unusual exposure time (45 min), and in an *in vivo* situation, dilution and mucociliary clearance contribute to a further decrease in local concentrations of the applied compound. Thus, DM-βCD is a safe absorption enhancer for nasal delivery of drugs [46]. HP-βCD and randomly methylated-β-CD (RM-βCD) have higher water solubility and lower toxicity than DM-βCD. Less than 20% (w/v) solutions of HP-βCD and 10% (w/v) RM-βCD did not induce gross tissue damage and could keep the histological integrity of the nasal mucosa, and in an *in vivo* situation, repeated RM-βCD doses did not cause irritation to the nasal mucosa [47]. CDs, therefore, seem to be safe and effective enhancers for use in nasal insulin therapy.

The absorption enhancement afforded by CDs can be attributed primarily to their ability to reduce the physical and/or metabolic barriers to these peptides and proteins [48]. (i) CDs can protect peptides and proteins against enzymatic, as well as chemical, degradation by including the hydrophobic side chains of the peptide within the CD cavity or directly inhibiting the proteolytic enzymes. (ii) The hydrophilic CDs can remove some specific lipids from biological membranes through the rapid and reversible formation of inclusion complexes, leading to an increase in membrane permeability. (iii) CDs can interact directly with the hydrophobic chain of peptide and protein drug molecules, altering its intrinsic aggregation or permeability through the phospholipid bilayer. The relative effects of various CDs in causing insulin hexamer dissociation were found to follow the descending order of DM-βCD (15%, w/v) > DM-βCD (10%, w/v) > DM-βCD (5%, w/v) > DM-βCD (1.8%, w/v) > α-CD (5%, w/v) > HP-βCD (5%, w/v) > γ-CD (5%, w/v) > β-CD (1.8%) [43]. (iv) CDs can change the distribution of tight junction proteins, thereby opening the tight junction between the epithelial cells and increasing permeability [43].

Tight junction modulators. New approaches for enhancing intranasal drug delivery based on recent discoveries on the molecular

biology of tight junctions are obviously improving the bioavailability of 'non-Lipinski' small molecules, peptides, proteins and oligonucleotide drugs. Tight junction modulators (TJMs) can open tight junctions safely and reversibly to enhance tissue permeability and drug transport. The novel TJM excipients must be chemically stable and retain permeation enhancement under conditions relevant for therapeutic storage and use [49].

Tight junction modulating lipids. As tight junction proteins associated with (and in some cases to affect) lipid raft structure, a screen was developed to identify lipids that alter tight junction properties. Glycosylated sphingosines, alkylglucosides, oxidized lipids and ether lipids were identified as TJMs, and several active lipid treatments could increase the permeability of the barrier tissue for FITC-labeled 3 kDa dextran and peptide YY3-36 [50]. Pillion *et al.* [51] demonstrated that alkylglycosides containing maltose or sucrose linked to alkyl chains between ten and 14 carbons in length could increase the bioavailability of insulin. Administration of a nasal insulin formulation containing 0.5% sucrose cocoate, a mixture of sucrose esters of coconut fatty acids, caused a rapid and significant increase in plasma insulin levels, with a concomitant decrease in blood glucose levels [52]. However, Chen *et al.* demonstrated that cells treated with alkylglucosides showed very high cytotoxicity and low viability at concentrations of 0.2–0.4% [50].

Tight junction modulating peptides. PN159, a peptide sequence, was identified as a novel TJM peptide capable of reducing transepithelial electrical resistance across a tissue barrier with a rapid onset and increasing paracellular transport of low MW drug and larger molecules with low cytotoxicity and high retention of cell viability [53]. In addition, a formulation containing peptide YY3-36 and PN159 was dosed intranasally in rabbits, resulting in a dramatic increase in bioavailability [53]. PN159 would be a safe and potent TJM to enhance drug delivery by nasal and gastrointestinal routes of administration.

Zonula occludens toxin (Zot), a 44.8-kDa protein located in the cell envelope of the bacterial strain *Vibrio cholerae*, is capable of reversibly opening the tight junction between cells and increasing the paracellular transport of drug candidates of varying MWs or low bioavailability in a non-toxic and dose-dependent manner [54]. *In vitro* experiments in rabbit ileum demonstrated that Zot reversibly increased intestinal absorption of insulin by 72% in a time-dependent manner [55]. In another study, insulin bioavailability increased from 5.4% in control to 10.7% and 18% when intragastrically coadministered with 2 μg/kg and 4 μg/kg Zot, respectively [56].

Recently, a hexamer peptide, H-FCIGRL-OH (AT1002), which retains the Zot biological activity, has been identified and synthesized. It has been demonstrated that AT1002 could enhance the nasal absorption of large hydrophilic markers (PEG4000 and inulin) *in vivo* [57]. AT1002-induced tight junction opening was accompanied by activating src and mitogen-activated protein kinase pathways, increasing ZO-1 tyrosine phosphorylation reversibly, which induced ZO-1 redistribution. AT1002 might also affect serine/threonine phosphorylation of ZO-1. In addition to its effects on tight junctions, AT1002 also caused rearrangement of actin stress fibers that have been involved in the regulation of tight junction assembly and function in Caco-2 and IEC6 cells. Functionally, AT1002 caused a reversible reduction in transepithelial electrical resistance and an increase in lucifer yellow permeability in Caco-2 cell monolayers [58].

The studies demonstrated that TJMs showed potential utility for transmucosal drug delivery, they provide a new possibility of high bioavailability for the intranasal delivery of peptide and protein drugs.

Nitric oxide donors. Nitric oxide (NO), a major secretory product of mammalian cells, has been identified as an important regulator and/or effector of many phenomena in cardiovascular, nervous and immune systems. Moreover, NO is a regulator of epithelial tight junctions and can enhance the permeability and absorption of drugs across various mucosal membranes [59], however, the exact mechanism of action is unknown. Salzman *et al.* [59] reported that NO donors increased the permeability of water-soluble compounds across Caco-2 cell monolayers with neither loss of cell viability nor LDH release. In the presence of NO donors, the rectal [60] and intestinal [61] absorption of insulin increased significantly. Among the NO donors investigated, S-nitroso-N-acetyl-DL-penicillamine was the most effective enhancer and could improve the nasal absorption of human granulocyte colony-stimulating factor [62]. Recently, Baker *et al.* [63] reported that sodium nitroprusside, an NO donor, caused a profound fall in intracellular potential and membrane resistance in nasal epithelial cells from healthy subjects. These results suggested that NO donors would be effective intranasal absorption enhancers of peptide and protein drugs.

N-acetyl-L-cysteine. N-acetyl-L-cysteine (NAC) is an effective mucolytic agent that has been widely used clinically in bronchopulmonary diseases to reduce both the viscosity and the tenacity of mucus and to facilitate its removal. The long history of clinical use of this compound suggests that it is likely to have low toxicity and no local irritation. NAC has been reported to have a mild absorption-enhancing effect at a high concentration (20%) [64], probably owing to the increased accessibility of the drug to the epithelial membrane. The combination of NAC and non-ionic surfactant could strongly increase the nasal absorption of peptides and proteins, and its promotion in dry powder formulations was more effective than that in liquid [65]. As a speculative mechanism, NAC can reduce mucus viscosity, which enables the target drug and the surfactant molecules to diffuse more efficiently through the epithelial membrane. This enhanced accessibility of both the target drug and the surfactant molecules led to synergistic absorption enhancement [65]. In addition, toxicology studies have shown that even in the absence of surfactant, NAC could also effectively promote nasal absorption of peptides and proteins without irritation and damage to the nasal epithelial cell membrane [66]. All these results suggest that the combination strategy of a mucolytic agent and a non-ionic surfactant might be widely applicable to various mucosal deliveries of poorly absorbed hydrophilic compounds.

Using delivery systems with increased retention time

The mucoadhesive approach has been developed to improve intranasal drug absorption because it could prolong the intimate contact time of the formulation on the nasal mucosa by adhering to the surface of the mucus layer. Different methods can be used to achieve mucoadhesion, thereby enhancing bioavailability.

Bioadhesive microsphere delivery system. To avoid rapid clearance owing to ciliary beating and prolong the residence time in the

TABLE 3

Bioadhesive microsphere delivery system for intranasal delivery of insulin

Microspheres	Enhancers (mg/kg)	Insulin dose (IU/kg)	Species	RB[a] (%)	AB[b] (%)	C_{min}[c] (%)	C_{max}[d] (mIU/L)	Refs
Hyaluronic acid ester microspheres		2	Sheep	11				[72]
Chitosan microspheres		4	Rat		44	33		[96]
Dextran microspheres		1	Rat			48		[67]
		1	Rat			25		[68]
Starch microspheres		0.75	Rat		30	40		[69]
		1.70	Rat		33	64		
		2	Sheep	10.7	4.5			[70]
	LPC (0.02)	2	Sheep	31.5	13.1			
		2	Sheep	3.6			97	[97]
	LPC (0.02)	2	Sheep	6.4		89.6	94	
	LPC (0.05)	2	Sheep	25.3		46.4	380	
	LPC (0.10)	2	Sheep	19.6		41.6	335	
	SGDC (0.08)	2	Sheep	31.9		55	776	
	STDHF(0.08)	2	Sheep	16.5		43.5	409	
Unfractionated sodium polystyrene sulfonate microparticles		28/rabbit	Rabbit	6.13		66.2	300.3	[98]
Fractionated sodium polystyrene sulfonate microparticles		28/rabbit	Rabbit	6.50		55.2	413.0	
Styrene–divinylbenzene copolymer microparticles		28/rabbit	Rabbit	2.71		57.3	267.8	
Gelatin microspheres		5	Rat			77.2		[71]
Aminated gelatin microspheres		5	Rat			69.3		

[a] Relative bioavailability to subcutaneous administration.
[b] Absolute bioavailability.
[c] Minimum plasma glucose levels (percentage of base glucose levels).
[d] Maximum plasma insulin levels.

nasal mucosa, different kinds of microspheres have been used in the intranasal delivery of insulin (Table 3), such as crosslinked dextran microspheres [67,68], starch microspheres [69,70], aminated gelatin microspheres [71] and hyaluronic acid ester microspheres [72]. All types of microspheres that have been used as nasal drug delivery systems are water-insoluble but absorb water into the sphere's matrix, resulting in swelling of the spheres and the formation of gel. These bioadhesive microsphere systems had low toxicity and were safe.

Because of the bioadhesive nature of microspheres, the formulation is retained in the nasal cavity for an extended time period, thereby improving systemic bioavailability even without the use of enhancer systems. As reported recently, the half-life of clearance for starch microspheres was found to be in the order of 240 min, compared with 15 min for the liquid and powder control formulations [7]. Microspheres have been suggested to exert several mechanisms for absorption-enhancement effects on the nasal delivery of peptides and/or proteins. Microspheres deposit in the less- or non-ciliated anterior part of the nasal cavity with slower nasal clearance. The bioadhesive effect of the microspheres decreases the rate of clearance of the drug from the nasal cavity and thereby enables a longer contact time with the absorptive epithelium [73]. The gelled system provides a local high drug concentration in close contact with the epithelial absorptive surface. Furthermore, it has been shown in a study employing monolayers of Caco-2 cells that the absorption of water by the microspheres from the mucus layer can induce reversible and transient shrinking of the epithelial cells and widening of the tight junctions and, therefore, the transport of hydrophilic compounds could be increased [73]. In addition, aminated gelatin microspheres could also open the tight junctions through the interaction of positively charged materials with the negatively charged epithelium membrane [71].

Bioadhesive powders. Besides designing bioadhesive microspheres, mucoadhesion can simply be achieved by using bioadhesive excipients, such as chitosan, hydroxypropyl cellulose, hydroxypropyl methylcellulose, microcrystalline cellulose, starch and carbopol. Bioadhesive powders are interesting because they can increase the residence time of drugs in the nasal cavity, improve the contact between the mucosa and the drug, increase the drug concentration at the site of deposition, and facilitate drug permeation through the mucosa by opening the tight junctions between the epithelial cells.

Nagai *et al*. [74] studied the effect of different bioadhesive polymers in powder form on enhancing the nasal bioavailability of insulin in beagle dogs. The absorption of insulin from nasal mucosa was the fastest in the preparation with microcrystalline cellulose, the plasma glucose level decreased to 49% after 30 min with a corresponding increase in serum insulin levels to 455 mIU/L. With the addition of hydroxypropyl cellulose and neutralized Carbopol 934, the powder formulation resulted in the reduction of glucose levels to 39% and 42%, respectively. The hypoglycemic effect was sustained with the formulation containing neutralized carbopol and the total effect was one-third the extent of that produced by intravenous injection. Dondeti (P. Dondeti, PhD thesis, University of Rhode Island, 1994) evaluated different bioadhesive polymers for nasal delivery of insulin in rabbits. The bioadhesive polymers evaluated were listed in the decreasing order of efficiency of polyacrylic acid > crosslinked polyacrylic acid > polyethylene oxide > chitosan > alginate.

Callens *et al.* [75] demonstrated that the bioavailabilities of insulin obtained with the powder formulations containing drum-dried waxy maize starch and Carbopol® 974P were significantly higher than those containing maltodextrins and Carbopol® 974P mixtures. The bioavailability of insulin increased as the ratio of Carbopol® increased. The bioavailability in the powder formulation containing drum-dried waxy maize starch and 10% Carbopol® 974P was as high as 14.4%.

Gel preparations. The use of bioadhesive nasal gel containing insulin not only promotes the prolonged contact between the drug and the absorptive sites in the nasal cavity but also facilitates direct absorption of medicament through the nasal mucosa.

Morimoto *et al.* [76] reported that the nasal administration of insulin in 0.1% and 1% (w/v) polyacrylic acid gels showed maximum hypoglycemic effects at 30 min and one hour after administration, respectively. In another study [77], nasal administration of insulin-loaded polyacrylic acid microparticles suspended in 1% (w/v) polyacrylic acid gel resulted in a noticeable and sustained hypoglycemic effect for seven hours in normal rabbits. Recently, a group [78] indicated that after administration of insulin in gel form using the combination of carbopol and hydroxypropyl methylcellulose as gelling agents, the absorption of the drug through the nasal mucosa was high in the first 0.5 to 1.5 hours with a sharp decline in blood sugar and rise in insulin plasma concentration.

Thermosensitive hydrogel can be dropped or sprayed easily into the nasal cavity and spread on the nasal mucosa in solution state. After being administered into the nasal cavity, the solution can be transformed into viscous hydrogel at body temperature, leading to decreased nasal mucociliary clearance rate and slow drug release. A thermosensitive hydrogel containing insulin, designed and prepared by simply mixing N-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride and PEG with a small amount of α–β-glycerophosphate, apparently decreased the blood glucose concentration (40–50% of initial blood glucose concentration) for at least four to five hours after administration, and no apparent cytotoxicity was found after application [79].

These studies have demonstrated that the administration of insulin intranasally in gel form is a pleasant and painless alternative to injectable insulin.

Insoluble powder formulations. A powder formulation using a water-insoluble compound was demonstrated to be effective as a drug carrier to improve nasal bioavailability. A powder formulation using $CaCO_3$ improved the nasal absorption of elcatonin and drugs with a wide range of MWs by increasing the residence time and topical concentration of the drugs by attaching to the powder particles [80].

The nasal absorbability of drugs can be influenced by the filler species used in the dry powder formulation, and the selection of filler species is important in formulation design. For instance, the hygroscopic properties of the filler might influence the drug dissolution and diffusion processes in the nasal cavity; the particle size distribution of the filler might influence the spread of the drug particles and, thus, affect the absorption area; and some fillers might strongly adsorb drug molecules on their surfaces, thereby influencing the release of drugs [81]. Matsuyama *et al.* [81] examined the influence of filler species on the nasal absorbability of peptide drugs via a powdery formulation system, revealing that the use of less wettable powders provided better nasal absorbability. Using ethylcellulose as a water-insoluble filler and NAC as an absorption enhancer, the nasal bioavailability of insulin reached $23.4 \pm 10.6\%$.

However, it is generally difficult to achieve a satisfactory effect for macromolecular drugs by increasing retention time in the nasal cavity alone because it has to simultaneously overcome the physical barrier of the epithelium for a drug to permeate into the body circulation. Therefore, the combined use of penetration enhancers and mucoadhesion should be an effective means of promoting the nasal absorption of macromolecular drugs such as insulin.

Using nanoparticles

Because peptides and proteins were poorly absorbed and highly susceptible to the harmful environment of the nasal cavity, the use of nanoparticulate systems has the advantage of protecting the peptide drugs from the harsh environment of the nasal cavity, enhancing their intranasal absorption and controlling the release of the encapsulated or adsorbed drugs. In addition, nanoparticles have a higher surface area to cover the highly vascularized nasal absorptive area, providing a greater concentration gradient.

Chitosan is deemed to be the safest and most effective absorption enhancer and has bioadhesive properties, hence, chitosan nanoparticles as a carrier for the nasal delivery of insulin have been widely studied. For example, blood glucose level fell to 60% of basal level one-hour post-intranasal instillation of insulin-loaded chitosan nanoparticles to conscious rabbits [82]. This absorption enhancement can be explained by the fact that chitosan nanoparticles with positive charge would intensify the contact between insulin and the nasal absorptive mucosa, thus leading to an increased insulin concentration at the absorption site. Further improvement of the properties of chitosan nanoparticles can be achieved by surface modification. For instance, PEG-g-chitosan copolymers can increase the solubility and improve the biocompatibility of chitosan. Moreover, modification of chitosan with PEG can resist adsorption of plasma proteins when in contact with blood through the steric repulsion mechanism. Intranasal administration of insulin-loaded PEG-g-chitosan nanoparticles in rabbits enhanced the absorption of insulin to a greater extent than a suspension of insulin–PEG-g-chitosan and control insulin solution [83].

However, a recent study showed that the insulin–chitosan solution formulation was significantly more effective than the complex and nanoparticle formulations. As shown in the sheep model, the most effective chitosan formulation for nasal insulin absorption was a chitosan powder delivery system with a bioavailability of 17.0%, as compared to 1.3% and 3.6% for the chitosan nanoparticles and chitosan solution formulations, respectively [84]. This might be explained by the reduced amount of positive charge available at the surface of the nanoparticles, the nanoparticles had basically only a low effect on the opening of the tight junction, most of the insulin-loaded chitosan nanoparticles were entrapped into the Caco-2 monolayer or attached to the cell surface, leading to the failure of the nanoparticles to enhance the permeation of insulin across the Caco-2 cell monolayer [85]. Our work also demonstrated that PEGylated trimethyl chitosan copolymers significantly enhanced the uptake of insulin in Caco-2 cells by adsorptive endocytosis. However, nanocomplexation did

TABLE 4

Comparison of different strategies for improving intranasal absorption of insulin

Strategies	Advantages	Disadvantages	Effectiveness
Chemical modification	Protects the drug against proteolytic degradation Improves the drug's permeation across the nasal mucosa owing to increased lipophilicity	Decreased pharmacological activities of the parent peptides Not very useful for larger peptides such as insulin	Limited effect
Use of enzyme inhibitors	Improves the stability of drugs at the absorption site	Unable to dramatically improve bioavailability in the absence of other absorption-enhancing measures Might affect the normal metabolism of the body and cause side-effects	Not very effective
Use of absorption enhancers	Improves the permeability of the epithelial cell layer based on different mechanisms	Some of the mechanisms can cause irritation and damage to the nasal mucosa	The most common and effective approach
Use of delivery systems with increased retention time (especially insoluble powder formulations)	Prolongs the intimate contact time of the formulation on the nasal mucosa by adhering to the surface of the mucus layer		The most promising, especially when combined with absorption enhancers
Use of nanoparticles	Protects insulin from degradation in the nasal cavity Enhances insulin intranasal absorption and controls the release	Complicated preparation process Many negative reports	With paradoxical reports

not seem to enhance transcellular insulin transport across cell monolayers, which is in line with animal data in rats, and the decrease in plasma glucose levels induced by PEG(5k)40-g-TMC(100) insulin nanocomplexes during the four-hour investigation was not significantly different from that induced by the insulin control solution [86].

Besides, chitosan-reduced gold nanoparticles could improve its surface properties for binding of biomolecules and improve its uptake into cells effectively. After intranasal administration of insulin-loaded gold nanoparticles to diabetic rats, blood glucose level was decreased by 20.27% [87].

Moreover, Jain *et al.* [88] investigated starch nanoparticles as a mucoadhesive carrier for transnasal insulin delivery. Insulin loaded starch nanoparticles containing sodium glycocholate demonstrated a rapid and sustained hypoglycemic action (70% reduction of plasma glucose) for six hours, and the plasma insulin level reached 258 mIU/L one hour after intranasal administration.

Concluding remarks

Although researchers have made great efforts to improve the nasal bioavailability of insulin (Table 4), formulations that meet clinical needs have not yet been reported. The two most important factors that hamper the absorption of insulin across the nasal mucosa are low permeability of the nasal mucosa to large molecules and rapid mucociliary clearance of formulations from the nasal cavity. Consequently, formulation design must try to overcome the two barriers – for example, by using a mucoadhesive drug delivery system or coadministration with absorption enhancers. According to previous research work, it seems that bioadhesive microsphere delivery systems or water-insoluble powders with absorption enhancers are the most promising for nasal absorption of insulin. Future studies should focus on the screening of safe and effective absorption enhancers, and the search for appropriate bioadhesive and water-insoluble excipients, with a view to achieve higher nasal bioavailability of insulin.

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