Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmaceutics and Biopharmaceutics 69 (2008) 426–435
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Effective insulin delivery using starch nanoparticles as a potential trans-nasal mucoadhesive carrier

Akhlesh Kumar Jain [a,1], Roop K. Khar [b], Farhan J. Ahmed [b], Prakash V. Diwan [a,*]

[a] Pharmacology Division, Indian Institute of Chemical Technology, Hyderabad, India
[b] Department of Pharmaceutics, Jamia Hamdard (Deemed) University, New Delhi, India

Received 1 December 2006; accepted in revised form 3 December 2007
Available online 8 December 2007

Abstract

Mucoadhesive nanoparticles (NPs) could be an exciting prospect for trans-nasal insulin delivery as they have higher surface area to cover highly vascularised nasal absorptive area providing a greater concentration gradient; hence the present study makes an attempt in this regard. Starch NPs were prepared by different crosslinkers using various methodologies and were loaded with insulin. Emulsion crosslinked particles were smaller in size compared to gel method (351 vs 997 nm), and size is further reduced when epichlorohydrin is used as crosslinking agent compared to $POCl_3$ (194 vs 810 nm). NPs of epichlorohydrin emulsion were further optimized with variable crosslinking to evaluate the effect of degree of crosslinking on *in vivo* performance. *In vitro*, a size dependent first order diffusion controlled insulin release with an initial burst effect was found, which is higher with NPs of small size and least crosslinking. Formulation of EE–NPs with Na glycocholate showed a superior hypoglycemic action compared to other NPs formulations containing the former and lysophosphatidylcholine as permeation enhancers. The hypoglycemic effects were more pronounced with medium crosslinked NPs (EE–L2–NPs), which showed a nadir of 70% reduction of plasma glucose and significant effects untill 6 h. The peak plasma insulin level (C_{max}) of medium crosslinked EE–L2–NPs (258 μIU/ml at 1 h) vindicates the pharmacodynamic effect, which was found to be superior compared to all other formulations. The release rate and higher associated surface area might work in tandem, and could be greatly amplified when combined with permeation enhancers to make starch NPs an efficient trans-nasal mucoadhesive carrier of insulin.

Keywords: Nanoparticles; Insulin; Nasal delivery; Permeation enhancers; Mucoadhesion

1. Introduction

In the recent past, systemic delivery of protein bioactives using trans-nasal administration is gaining considerable attention. Major factors limiting the bioavailability of nasally administered protein drugs include: (a) poor permeability across lipophilic membranes due to hydrophilicity of these agents, (b) poor permeability via the paracellular route due to large size, and/or (c) the mucociliary clearance mechanism that rapidly removes the non-mucoadhesive formulations from the absorption site [1,2]. In general, the residence time of a protein delivered through the nasal mucosa is only 15–30 min. Various strategies such as co-administration of absorption enhancers and enzyme inhibitors, microspheres, nanoparticles and bioadhesives have been attempted in order to improve the bioavailability of nasally administered protein drugs [3–7]. Absorption enhancers such as bile acids, fat salts and cyclodextrins have been extensively studied to facilitate drug absorption, however they have the tendency towards irritation or damage to mucosa [8–10]. On the other hand, bioadhesive controlled-release carriers were found to be an exciting prospect as they decrease the effect of mucociliary clearance [11–16]. In view of this, bioadhesive delivery systems are being designed to adhere to various tissue surfaces, mainly

* Corresponding author. Pharmacology Division, Indian Institute of Chemical Technology, Uppal Road, Tarnaka, Hyderabad 500 007, India. Tel./fax: +91 40 27193753.
E-mail address: diwan@iict.res.in (P.V. Diwan).
[1] Present address: Pharmaceutics Division, Central Drug Research Institute, Chattar Manzil Palace, Lucknow, UP 226 001, India.

0939-6411/$ - see front matter © 2008 Published by Elsevier B.V.
doi:10.1016/j.ejpb.2007.12.001

the mucosal epithelium. As a more fitting example, the nasal mucosa is characterized by a highly vascularised and relatively large absorptive surface area (150 cm^2) [17]. In addition, drug delivery via trans-nasal route has an advantage of bypassing first pass metabolism by the liver as blood is drained directly from the nose into the systemic circulation. Irrespective of mechanisms involved, the main advantages of bioadhesive systems are higher residence time at the site of action, local delivery to a selected site, and intimate contact with mucosal epithelium [18,19].

Polysaccharides such as starch, chitosan, alginate, cellulose and cellulose derivatives have been widely used as mucoadhesive nasal drug delivery systems due to their biocompatibility and hydrophilicity [20–23]. Such bioadhesive systems based on microspheres were found to significantly enhance systemic absorption of conventional drugs and polypeptides across the nasal mucosa, even, devoid of absorption enhancing agents [24,25]. In this regard, Illum et al. and other research groups have investigated the use of microspheres as a nasal delivery carrier for the improved nasal absorption of insulin [5,26]. Moreover, extensive studies have been carried out to examine the use of starch microspheres as a biocompatible nasal delivery system [6,18,24,27]. The particulate carriers such as crosslinked polysaccharide are capable of absorbing moisture and become a bioadhesive gelled system. The absorption of water from the mucus layer by such carriers dehydrates the mucosal surface and facilitates passage of the drug through paracellular tight junctions [18,28]. Increase in drug absorption via this pathway is found to occur, mainly, during short periods when the tight junctions are separated [19,29]. In spite of great potential as a bioadhesive carrier, starch microspheres are not normally sufficient to provide clinically relevant plasma levels of large polypeptides. To address above limitations, extensive studies were carried out on microsphere formulations comprising permeation enhancers [6,24,30] and few studies dealt with polysaccharide nanoparticles (NPs). The chitosan NPs were found to be effective vehicles for the trans-nasal insulin delivery, however, were shown to be inferior insulin transport vehicles compared to physical mixture powder formulation or simple solution when the glutamate salt of the polymer was used to make formulations [31–33]. It was reported that absorption of intact NPs is about 1% [34] and the absolute transported amount is same between NPs of 10–200 nm in size [35]. In view of low fraction of particle translocation, it could be presumed that the predominant mechanism of trans-nasal absorption of drugs is bioadhesive and shows permeation enhancing effects of particulate carriers. We envisage that NPs in contrast to microspheres could be an exciting prospect as they have higher surface area, and would cover highly vascularised nasal absorptive area. The present study investigates starch nanoparticles as a mucoadhesive carrier for trans-nasal insulin delivery. Starch nanoparticles were prepared by different methods using epichlorohydrin and $POCl_3$ as crosslinkers to obtain NPs of various sizes suitable for insulin loading and evaluated for *in vitro* release and *in vivo* performance. The aim for making different types of NPs was to develop particles of different sizes, and study whether there is a size dependency for insulin delivery. Considering size-dependent drug loading and release, we aimed to design the NPs of 150–300 nm size which will have significant loading, and cover reasonable area of nasal mucosa. NPs of smaller size were evaluated to study the effect of crosslinking. Trans-nasal delivery of insulin loaded NPs with and without permeation enhancers such as sodium glycocholate and lysophosphatidylcholine was evaluated, in the STZ (streptozotocin) induced diabetic rats by measuring blood glucose and plasma insulin levels, to develop an efficient carrier.

2. Materials and methods

2.1. Materials

Soluble starch (potato), sodium glycocholate (Na Glyco), lysophosphatidylcholine (Lyso), streptozotocin (STZ) and bicinchoninic acid (BCA) protein assay kit were purchased from Sigma, St. Louis, USA. Human recombinant insulin USP (28.9 IU/mg) was purchased from Serological Corporation, Oxford, UK. Triethylamine (TEA), epichlorohydrin (Epi), $POCl_3$ and trifluoroacetic acid (TFA) were obtained from E-Merck Ltd., Mumbai, India. Insulin ELISA kits were procured from Mercodia, Sweden. All the other solvents and reagents used were of analytical grade.

2.2. Methods

2.2.1. Preparation of crosslinked starch nanoparticles (NPs)

2.2.1.1. Polysaccharides crosslinking by gel method

2.2.1.1.1. Crosslinking with epichlorohydrin. Starch nanoparticles were prepared according to the method described elsewhere with slight modifications [36]. Two grams of soluble starch was dispersed homogeneously in 1 M NaOH. Crosslinking was carried out by adding 0.18 ml of epichlorohydrin under constant stirring. Stirring was stopped and the reaction was left to proceed at 70–80 °C until (6 h) a soft gel was obtained. The resulting gel was diluted with water, mechanically ground and subjected to sonication for 15 min (Sonics, Vibra cell™, USA). The gel like crosslinked particulates so obtained were fractionated by centrifugation at 6000 rpm for 10 min and the precipitate (larger particulates) was discarded. The NPs were spray-dried (Mini spray dryer, Buchi, Switzerland) in the following manner. Spray drying conditions used were: Inlet temperature 200 °C, outlet temperature 140 °C; pump control 3; aspirator control 19; heating control 12; flow indicator 700; to obtain a fine powder.

2.2.1.1.2. Crosslinking with $POCl_3$. This was carried out according to previous report with some modifications [37]. Five grams of starch was solubilized by homogenization in 2 M NaOH (12 ml). The temperature was adjusted to 4 °C by ice bath immersion, and $POCl_3$ (2.0 g) was added drop-

wise together with a solution of 7.5 ml of 10 M NaOH under stirring. It was subjected to homogenization at 14,000 for 15 min (Ultra Turrax, T195, Germany) and pH was adjusted to 7.0 with 0.1 N HCl, resulting gel was diluted with water, sonicated, centrifuged and spray-dried as described previously.

2.2.1.2. Polysaccharides crosslinking by emulsion method

2.2.1.2.1. Crosslinking with epichlorohydrin. The procedure of a previous report was used with some modifications [38]. One gram of soluble starch was solubilized in 1 M NaOH containing 2% Tween 80 (8 ml). The aqueous polymer phase was emulsified in liquid paraffin (40 ml) containing 2% Span 80 by stirring at 22,000 rpm for 10 min. Crosslinking was carried out by adding epichlorohydrin (0.1–0.3 ml) dropwise under agitation at 2000 rpm with constant stirring at 70–80 °C for 6–8 h. The resultant particles were washed thrice with equal volumes of petroleum ether/hexane and precipitated with *n*-propanol/petroleum ether mixture (1:1). The organic phase was evaporated (Rotavapor) and the dried particles were re-dispersed in de-ionized water, subjected to centrifugation at 10,000 rpm for 15 min and spray-dried as described previously.

2.2.1.2.2. Crosslinking with $POCl_3$. The procedure of a previous report was used with some modifications [39]. Starch (5 g) was solubilized in 2 M NaOH (12 ml) and emulsified in liquid paraffin (40 ml) containing 2% span 80 by stirring at 22,000 rpm for 10 min. The temperature of dispersed aqueous polymer was brought to 4 °C using ice bath immersion and crosslinking was carried out by adding $POCl_3$ (2.5 g) dropwise together with a solution of 10 M NaOH (7.5 ml). This step was carried out while stirring at 2000 rpm. The oil phase was removed by washing thrice with equal volumes of hexane/petroleum ether. The aqueous phase was diluted sufficiently with de-ionized water, pH was adjusted to 7.0, and the NPs were spray-dried as described previously.

2.2.2. Drug loading in nanoparticles

Insulin was loaded in nanoparticles by a post loading method [39]. Accurately weighed quantity of NPs (50 mg) was incubated with 100 μl of insulin solution (15 mg/ml dissolved in 0.01 N HCl) to obtain 3% w/w insulin entrapment in the particles. Permeation enhancers were also dissolved in the insulin solution at a concentration of 4.5 mg/ml to obtain a dose level of 0.1 mg/kg and added in the respective formulations. The resulting gel was allowed to saturate for 24 h and lyophilized and stored at 4 °C until further use.

2.2.3. Size and size distribution

Nanoparticles were characterized for the particle size and size distribution by photon correlation spectroscopy (PCS) technique using zeta sizer 3000HS (Malvern, UK). The NPs powder was re-dispersed in de-ionized water, diluted suitably and particle size was measured at an angle of 90° at 20 °C. The measurements were performed in triplicate.

2.2.4. In-vitro drug release study

Accurately weighed quantity of nanoparticles (≈10 mg) was suspended in PBS 7.4 (1 ml), sealed in dialysis bags (M.W. 12,000 cutoff, Sigma, USA) and immersed in 25 ml of PBS 7.4 at 37 °C and kept for magnetic stirring. The samples were withdrawn at scheduled intervals (replaced with equivalent amount of diffusion media) and the insulin content was estimated by reverse phase HPLC as described by Chalasani et al. [38]. Briefly, mobile phase of 33.5% acetonitrile and water containing 1.5% triethylamine (TEA), pH adjusted to 2.5 with trifluoroacetic acid (TFA), was delivered at a flow rate of 1.3 ml/min to estimate insulin on C8 column. Release study of each NPs formulation was performed in triplicate.

2.2.5. In vivo studies

Male Wistar rats (200 ± 20 g) used in the present study were procured from National Institute for Nutrition, Hyderabad, India, and were acclimatized in our animal facility for one week before study. The animals were housed at 22 ± 2 °C temperature with 12 h light/dark cycle and 50–70% relative humidity. The *in vivo* efficacy of the formulations was tested in diabetic rats (STZ induced) after administering the formulations by nasal route. The protocol for animal studies was approved by the Institutional Animal Ethical Committee of IICT, Hyderabad, India, and study was carried out in accordance with principles of laboratory animal care and approved protocol. Diabetes was induced by i.p. administration of streptozotocin (45 mg/kg in citrate buffer, pH 4.0). Blood samples (200 μl) were withdrawn from the tail vein into Eppendorf tube containing 5 μl of 5% EDTA. The plasma was separated by centrifugation (Mini centrifuge, Spinwin) at 6000 rpm for 10 min. The blood glucose levels were assessed by estimating plasma glucose content using glucose assay kit (Autopak, Bayer's Diagnostics) on a Blood Analyzer (Technicorn RA-50, Italy). Animals showing more than 250% of fasting blood glucose levels were considered as a diabetic. Diabetic rats having 450 ± 30 mg/dl of blood glucose levels were chosen and divided into groups of five animals each. The animals had *ad libitum* access to a standard chow diet (Nutrilab, Bangalore) and water.

2.2.5.1. Pharmacological evaluation. In all the experiments of the present study, the nasal formulations were administered at a dose equivalent to 10 IU/kg insulin. In the 1st experiment, six groups of five animals each were used to evaluate delivery potential of NPs with Na glycocholate as permeation enhancer while 2nd experiment was conducted in five groups of five animals each to know the effect of lysophosphatidylcholine. In both the experiments a control group of plain insulin solution administered nasally was included. In the 3rd experiment, six groups of five ani-

mals each were used to evaluate the effect of crosslinking and compared with plain insulin and plain NPs control without permeation enhancers. Control group includes nasal administration of insulin solution and 2 IU/kg SC administration as a positive control.

The formulations were administered to conscious diabetic rats according to the procedure of a previous report [40]. Briefly, the dose was weighed into 200 μl pipette tip connected to a syringe via polyethylene tube that has a cotton filter to prevent back flow. Dose was administered to the nostril of the conscious rats by blowing air through the device while holding them in upright position.

At scheduled intervals as expressed in the figures the blood samples (200 μl) were collected and estimated for plasma glucose levels as described previously. The percentage change in blood glucose levels was calculated in comparison to the blood glucose level at zero time point (measured just prior to dosing) of each animal and was graphically expressed as percentage of initial glucose content. The portions of remaining plasma samples were stored at −80 °C to estimate plasma insulin level.

The stored plasma samples were thawed and analyzed for the insulin content using the insulin ELISA kits according to manufacturer's kit procedure. Briefly, each concentration (25 μl) in triplicate was added into microplate wells followed by Enzyme conjugate (100 μl) and incubated for 1 h at 25 °C. Plate was washed and TMB substrate (200 μl) was added and incubated for 15 min. The reaction was stopped and absorbance was recorded at 450 nm.

The pharmacokinetic and pharmacodynamic parameters were calculated using plasma drug/glucose concentration vs time profile with non-compartment based pharmacokinetic software 'Ramkin'. The relative pharmacological efficacy in terms of hypoglycemic action and relative bioavailability of insulin were calculated according to the following equations [40]:

$$\text{Relative pharmacological efficacy } (\%) = \frac{\text{AUC nasal} \times \text{Dose sc}}{\text{AOC sc} \times \text{Dose nasal}} \times 100$$

$$\text{Relative bioavailability } (\%) = \frac{\text{AUC nasal} \times \text{Dose sc}}{\text{AOC sc} \times \text{Dose nasal}} \times 100$$

The area over the curve (AOC) is the area defined as the area over the blood glucose level/time curve and below the baseline. AUC is area under the curve of insulin level vs time profile.

2.2.6. Statistical analysis

The results were expressed as means ± SD. Statistical data analysis was performed using the Student *t*-test with $P < 0.05$ as the minimal level of significance. For group comparison one-way ANOVA followed by Bonferroni multiple comparison tests was applied using 4.0, Prism software.

3. Results and discussion

3.1. Size and size distribution

The average particle size (Z_{avg}) and the polydispersity index (PDI) of these NPs are expressed in Table 1. Despite high polydispersity, the volume distribution of more than 80% particles is in the average size range. Thus, the present study investigates the potential of polydisperse NPs of various sizes. The mean size of particles prepared by different methodologies is significantly different ($P < 0.01$), except statistically insignificant differences in the mean size of various epichlorohydrin crosslinked particles of emulsion method ($P > 0.01$). Particles prepared by emulsion method were more uniform in size distribution as evident from relatively narrow PDI and have smaller mean size compared to NPs obtained from gel method ($P < 0.01$). Crosslinking with epichlorohydrin in place of $POCl_3$ has further reduced the mean size ($P < 0.01$). Hence, EE–NPs (Table 1) were further optimized to evaluate the effect of degree of crosslinking on the *in vivo* performance of these carriers. Of the various NPs prepared in this regard, significant difference ($P < 0.01$) was observed only between the mean size of EE–L1–NPs and EE–L3–NPs. The mean size of NPs obtained from emulsion method (EE–L1–NPs, EE–L2–NPs, and EE–L3–NPs) was significantly less ($P < 0.01$) compared to gel crosslinked NPs (194–229 vs 810 nm). There was slight increase in size (more change in PDI) after insulin entrapment but increase was insignificant when the particles were washed to remove surface insulin (data not shown). The surface associated insulin was not washed after the post loading process as this would be cost prohibitive. Also, it is presumed that surface insulin would provide a loading dose and high concentration gradient for rapid onset, and might help in the diffusion process for absorption.

3.2. In-vitro drug release study

Insulin release from starch NPs followed first order diffusion controlled profile with a burst effect. *In vitro* release studies indicate that surface insulin is rapidly dissociated as

Table 1
Starch nanoparticles prepared by various methodologies and their size analysis

Crosslinker/method	Delivery system	Size[a] Z_{ave} (nm) mean ± SD	Polydispersity index
$POCl_3$/gel	POG–NPs	997.2 ± 15.4	0.64
$POCl_3$/emulsion	POE–NPs	351.3 ± 8.7	0.46
Epi/gel	EG–NPs	810.4 ± 13.1	0.61
Epi/emulsion	EE–NPs	194.2 ± 6.3	0.41
Epi (0.1 ml)/emulsion	EE–L1–NPs	229.5 ± 7.2	0.44
Epi (0.2 ml)/emulsion	EE–L2–NPs	203.1 ± 8.1	0.43
Epi (0.3 ml)/emulsion	EE–L3–NPs	194.2 ± 6.3	0.40

[a] Size analysis was carried out before insulin entrapment in the NPs; SD ($n = 3$) is deviation from the mean size.

indicated by burst release (19–28%). Therefore, the slight increase in the size due to aggregation during insulin loading process is expected to be a temporary effect, and release rate is characteristic of each NPs carrier. The NPs of gel method released 81% of insulin in 12 h, whereas emulsion crosslinked particles showed a faster release (85–90% in 12 h; Fig. 1). Difference in the release was significant between NPs obtained by emulsion and gel methods ($P < 0.01$), although the difference was insignificant at 12 h. Also, the difference was insignificant between different NPs of either gel or emulsion crosslinked particles ($P > 0.01$). Thus, particles produced by the gel method and with an apparently larger size showed a slower release and it is presumed to be a result of relatively higher diffusional path length and less specific surface area of large NPs compared to small size NPs. Conversely, NPs produced by the emulsion method were small in size and showed a faster release that is ascribed mainly to the higher surface area and also to the relative decrease in diffusional path length vis-à-vis size. As expected, burst release is higher with NPs of small size. Moreover, the burst release was higher with emulsion crosslinked particles, irrespective of the type of crosslinking agent, which is presumed to be due to decrease in size. The degree of crosslinking was found to have an effect on the insulin release. At three investigated crosslinking concentrations, as expected the release was higher with NPs of least crosslinking (EE–L1–NPs; 95% compared to EE–L3–NPs; 90% release in 12 h; Fig. 2). Release from least crosslinked EE–L1–NPs was significantly different compared to other more crosslinked NPs ($P < 0.01$) untill 8 h and insignificant thereafter. Overall, the data indicate a size dependent insulin release from polydisperse starch NPs. As opposed to microspheres, the greater drug release rate from the NPs that is associated to higher nasal surface is expected to provide a high local drug concentration at the absorptive surface.

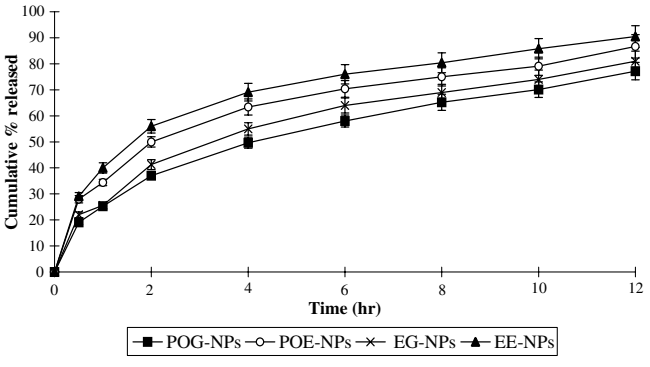

Fig. 1. *In vitro* insulin release profile of various crosslinked starch nanoparticles in PBS pH 7.4 at 37 °C (mean ± SD, $n = 3$). Difference was significant between POG–NPs and EG–NPs vs POE–NPs and EE–NPs ($P < 0.01$). Difference was insignificant between POG–NPs vs POE–NPs and EG–NPs vs EE–NPs ($P > 0.01$). POG–NPs = NPs prepared with $POCl_3$ crosslinking by gel method; POE–NPs = $POCl_3$ crosslinking by emulsion method. Similarly, EG–NPs = epichlorohydrin gel method NPs and EE–NPs = epichlorohydrin emulsion method NPs.

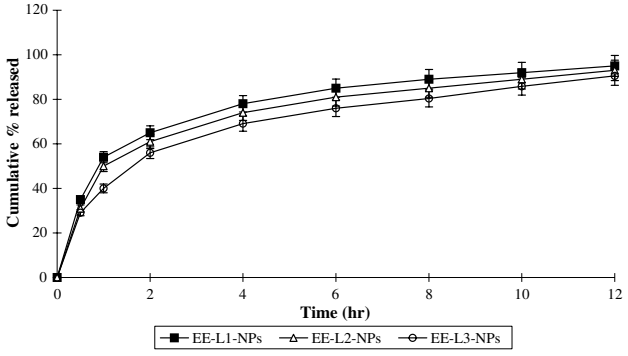

Fig. 2. Effect of crosslinking on *in vitro* insulin release profile of epichlorohydrin crosslinked starch NPs in PBS pH 7.4 at 37 °C (mean ± SD, $n = 3$). Release was significantly different between EE–L1–NPs vs other crosslinked NPs ($P < 0.01$) till 8 h and difference was insignificant thereafter. EE–L1–NPs = low crosslinked epichlorohydrin emulsion method NPs; EE–L2–NPs = medium crosslinked epichlorohydrin emulsion method NPs; EE–L3–NPs = highly crosslinked epichlorohydrin emulsion method NPs.

The higher local concentration as a result of higher release rate nullifies effects of pre-systemic clearance of protein drugs. The greater release rate of NPs is particularly useful with molecules of short *in vivo* half-life such as insulin. The pre-systemic clearance of insulin, which comprises mucociliary drainage, sneezing and enzymatic degradation, would be more with simple higher concentration solution. On the other hand, bioadhesive NPs stay for prolonged period of time at nasal mucosa and also open the tight junction to enhance the transmucosal permeation vis-à-vis bioavailability, which is not the case with simple higher concentration solution.

3.3. Trans-nasal insulin delivery potential of the NPs carriers in STZ induced diabetic rats

3.3.1. Effect of particle size and co-administration of sodium glycocholate

The effect of sodium glycocholate (0.1 mg/kg) on transnasal insulin using bioadhesive starch NPs in STZ diabetic rats is shown in Fig. 3. Among various NPs tested, EE–NPs showed a better and prolonged hypoglycemic action. The hypoglycemic action of EE–NPs was found to be superior with a maximum reduction of 65% basal plasma glucose at 1 h compared to 50% reduction of POE–NPs formulation. With both the formulations, there is a rapid onset followed by significant reductions for 6 h ($P < 0.05$) compared to plain insulin control formulation. Overall, the effects of hypoglycemic action are significantly higher with EE–NPs ($P < 0.01$) and all other NPs formulations ($P < 0.05$) compared to control group. However, the hypoglycemic effects are insignificant ($P > 0.05$) between different NPs formulations, except EE–NPs showing significantly higher effects ($P < 0.05$) until 4 h. In a nutshell, the formulations comprising NPs made from emulsion method showed slightly higher, but statistically insignificant, action compared to gel method NPs, irrespec-

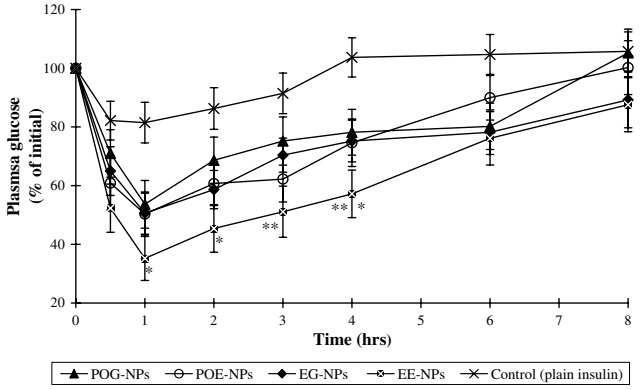

Fig. 3. Hypoglycemic effect of starch NPs of different methodologies in the presence of Na glycocholate after nasal administration to STZ induced diabetic rats (mean ± SE, $n = 5$). *Significantly higher action of EE–NPs compared to POE–NPs at 1, 2 and 4 h ($P < 0.05$) and insignificant at 6 h ($P > 0.05$). **Significantly higher action of EE–NPs compared to EG–NPs at 3 and 4 h ($P < 0.05$) and insignificant at 1 and 2 h ($P > 0.05$).

tive of crosslinker. These results are presumed to be due to the small size of emulsion particles, which is in line with *in vitro* faster insulin release of the present study, and previous reports [26]. Furthermore, NPs obtained from epichlorohydrin crosslinking, as a result of smaller size, showed better result compared to NPs prepared by $POCl_3$ crosslinking. The estimated plasma insulin levels are in accordance with the pharmacodynamic effects obtained with these formulations (Fig. 4). Plasma insulin levels of small sized NPs formulations obtained from emulsion method (EE–NPs and POE–NPs) are significantly higher ($P < 0.05$) compared to NPs made of gel method. Further, the plasma insulin levels of EE–NPs are significantly higher ($P < 0.05$) compared to all other groups at 1st ($P < 0.01$), 2nd and 3rd hours ($P < 0.05$). The peak plasma insulin level (C_{max}) at 1 h was found to be in order of EE–NPs (263 μIU/ml) > POE–NPs (136 μIU/ml) > EG–NPs (75 μIU/ml) > POG–NPs (71 μIU/ml). The effect with EE–NPs was sustained up to 8 h.

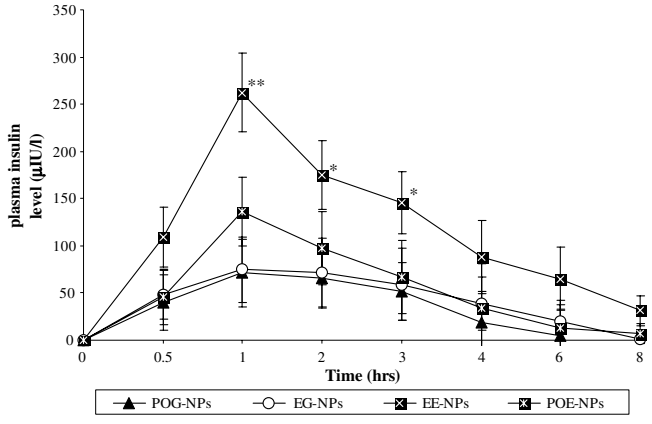

Fig. 4. Plasma insulin levels after nasal administration of various NPs formulations in the presence of Na glycocholate to STZ diabetic rats (mean ± SD, $n = 5$). Plasma insulin levels of EE–NPs are significantly higher compared to all other NPs ** at 1 h ($P < 0.01$); * 2nd and 3rd hours ($P < 0.05$).

3.3.2. Effect of co-administration of lysophosphatidylcholine

A similar trend of nasal insulin delivery was found with lysophosphatidylcholine as a permeation enhancer at a dose of 0.1 mg/kg (Fig. 5). The hypoglycemic action of EE–NPs was found to be superior with a maximum reduction of 55% basal plasma glucose at 1 h compared to 39% of POE–NPs formulation. Significant reduction of plasma glucose level was found until 4 h ($P < 0.05$) with EE–NPs, which was superior compared to all other formulations. The difference in hypoglycemic action is insignificant between other NPs formulations ($P > 0.05$). Similar to Na glycocholate co-administration, EE–NPs showed higher plasma insulin levels (208 μIU/ml) compared to all other formulations (data not shown). This is in line with general trend observed with higher efficacy as the size decreases. The trans-nasal potential delivery of EE–NPs with and without permeation enhancer is depicted in Fig. 6. The data indicate that NPs plus permeation enhancers showed

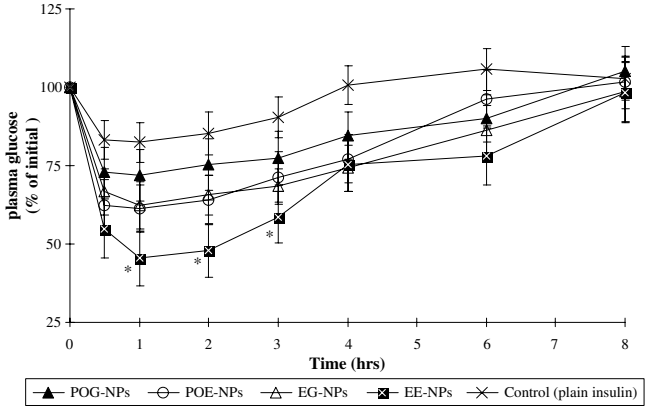

Fig. 5. Hypoglycemic effect of starch NPs of different methodologies in the presence of lysophosphatidylcholine after nasal administration to STZ induced diabetic rats (mean ± SE, $n = 5$). *Significantly higher action of EE–NPs compared to POG–NPs at 1–3 h ($P < 0.05$) and insignificant at 6 h ($P > 0.05$).

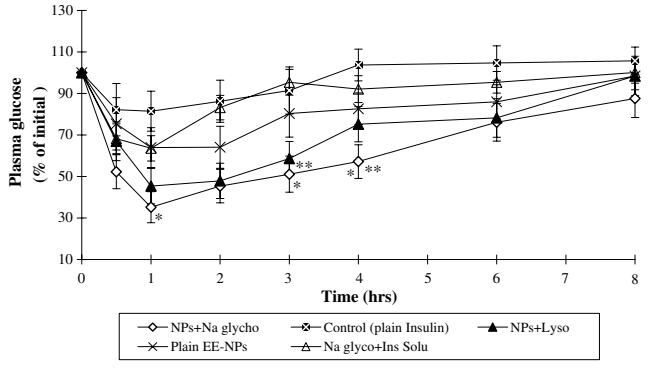

Fig. 6. Comparative hypoglycemic effects of EE–NPs in the presence of Na glycocholate and lysophosphatidylcholine after nasal administration to STZ diabetic rats (mean ± SE, $n = 5$). *Significantly higher effect ($P < 0.05$) when Na glycocholate was co-administered compared to lysophosphatidylcholine and plain NPs without permeation enhancers. **$P < 0.05$ compared to plain NPs without permeation enhancers.

a higher efficacy compared to insulin NPs without these agents. The hypoglycemic action of EE–NPs is significantly higher ($P < 0.05$) when Na glycocholate was co-administered compared to lysophosphatidylcholine, NPs without permeation enhancers and plain insulin co-administered with Na glycocholate (Fig. 6). On the other hand, lysophosphatidylcholine as permeation enhancer showed significantly higher efficacy only at 3 h ($P < 0.05$) compared to NPs without permeation enhancers.

3.3.3. Effect of crosslinking on trans-nasal insulin

In order to optimize the effective EE–NPs formulation, NPs of three different arbitrary concentrations of epichlorohydrin crosslinking were prepared and the trans-nasal insulin delivery potential of EE–L1–NPs (0.1 ml), EE–L2–NPs (0.2 ml) and EE–L3–NPs (0.3 ml) formulations was assessed (Fig. 7). The hypoglycemic action was studied with more effective Na glycocholate as permeation enhancer. All the formulations showed rapid and maximum reductions at one hour. Predictably, least crosslinked EE–L1–NPs showed a rapid and short duration effect compared to slightly less but sustained action of highly crosslinked EE–L3–NPs. While the hypoglycemic effects of different crosslinked products are significantly higher compared to control group ($P < 0.05$), the difference in the hypoglycemic action is insignificant ($P > 0.05$) between these NPs formulations. However, the hypoglycemic effect of medium crosslinked EE–L2–NPs is significantly higher compared to EE–L1–NPs (least crosslinked) at 2, 6 and 8 h ($P < 0.05$). Although hypoglycemic effects are statistically insignificant, EE–L2–NPs showed a better and prolonged pharmacological effect compared to EE–L1–NPs that is in contrast to *in vitro* release. Overall, EE–L2–NPs showed a better and prolonged hypoglycemic effect compared to EE–L1–NPs and EE–L3–NPs. The exact reason for this behaviour is not clear, it is likely, despite minor differences, that EE–L2–NPs have less surface insulin (as evident from the burst effect at 1st hour) and release rate is more regulated. A portion of free insulin is prone to presystemic effects, and the minor differences in the effective absorption could translate to higher efficacy for drugs with narrow therapeutic index drugs such as insulin. Whereas, the release rate might be too slow with EE–L3–NPs to maintain effective concentrations as insulin is short acting. Nonetheless, the difference in *in vitro* insulin release between closely related crosslinked products is insignificant, except 2nd hour for EE–L3–NPs. The formulation showed a nadir of 70% reduction of plasma glucose with significant effects untill 8 h ($P < 0.05$) compared to control group. Peak plasma insulin levels (C_{max} at 1 h) of EE–L2–NPs (351 μIU/ml) are higher compared to other NPs, EE–L1–NPs (304 μIU/ml) and EE–L3–NPs (263 μIU/ml; Fig. 8). The plasma insulin levels between three formulations are not significantly different at 1 h, however, EE–L2–NPs showed significantly higher ($P < 0.05$) levels up to 6 h compared to EE–L1–NPs formulation. It is worth mentioning here that ELISA method estimates insulin in its active conformation towards antibodies compared with RIA methods, which measures higher levels due to total radioactivity of fragments and metabolites.

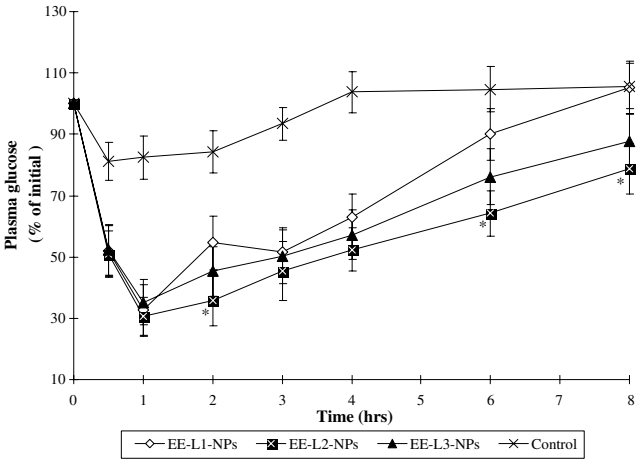

Fig. 7. Effect of crosslinking on hypoglycemic effect of EE–NPs in the presence of Na glycocholate after nasal administration to STZ induced diabetic rats (mean ± SE, $n = 5$). *Hypoglycemic effect of EE–L2–NPs is significantly higher ($P < 0.05$) compared to EE–L1–NPs (highly crosslinked).

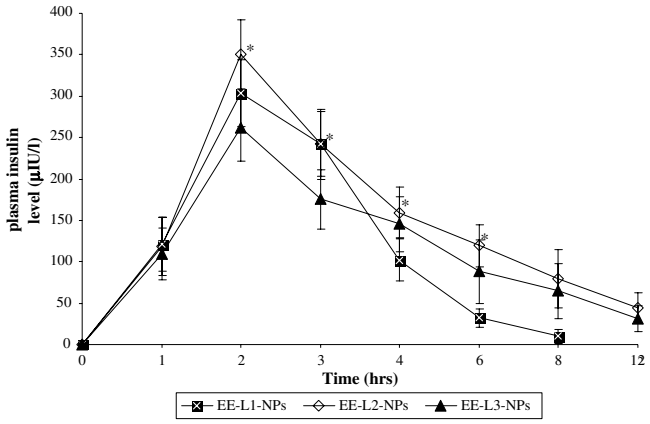

Fig. 8. Effect of crosslinking on plasma insulin levels after nasal administration of EE–NPs in the presence of Na glycocholate to STZ diabetic rat (mean ± SD, $n = 5$). *Plasma insulin levels of EE–L2–NPs are significantly higher ($P < 0.05$) compared to EE–L1–NPs formulation.

3.3.4. Assessment of pharmacological efficacy of insulin via mucoadhesive NPs

Different research groups reported successful trans-nasal insulin delivery, perhaps the best among the non-invasive routes, with microspheres formulations containing permeation enhancers. In this regard, starch microspheres were shown to greatly enhance the absorption of insulin [29], gentamicin [41], and human growth hormone [42]. It was reported that, starch microspheres were shown to have a high degree of swelling in contact with aqueous media, which prolongs residence time of the formulation. For an optimum effect, the drug has to be available for absorption, when the particles swell resulting in the temporary widening of the

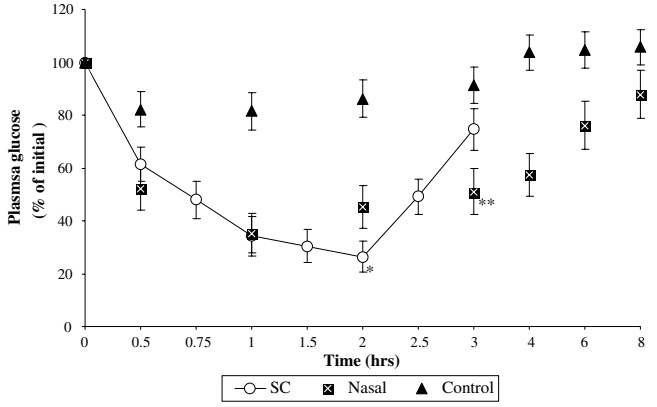

Fig. 9. Hypoglycemic effect of EE–NPs in presence of Na glycocholate after nasal administration and plain SC insulin to STZ induced diabetic rats (mean ± SE, $n = 5$). *Hypoglycemic action of sc insulin is higher ($P < 0.05$) at 2 h compared to nasal NPs. **Hypoglycemic action of nasal insulin is significantly higher compared to SC and control.

tight junctions [29]. Previously reported formulations showed consistent and reliable reductions of blood glucose level for 3–4 h with an absolute bioavailability of approx. 30% [29]. As an extension, the present study investigates usefulness of starch nanoparticles as they have shown size dependent *in vitro* insulin release and also due to the fact that nanoparticles are expected to cover a greater surface area of nasal mucosa. Since drug loading of microspheres is many fold higher compared to NPs, the mucociliary clearance of particles would result in higher dosage loss with the microspheres. The hypoglycemic effect of SC insulin (2 IU/kg) was compared with 10 IU/kg of nasal EE–NPs (Fig. 9). The hypoglycemic effects were not significantly different at 0.5 and 1 h between nasal and SC insulin administration. The hypoglycemic action of SC insulin is higher ($P < 0.05$) at 2 h compared to nasal NPs and effects are almost close to initial levels (T_0, glucose levels before treatment) within 3 h. On the other hand, hypoglycemic effect of nasal insulin is higher ($P < 0.05$) compared to SC insulin at 3rd hour and significant effects were found until 6 h compared to control formulation of nasal insulin solution. The pharmacological efficacy of insulin loaded starch NPs in terms of blood glucose reductions and plasma insulin levels is shown in Table 2. The data indicate a size dependent pharmacological availability as noticeable from blood glucose reductions vis-à-vis relative bioavailability in terms of plasma insulin levels. Maximum effects were found with medium crosslinked epichlorohydrin NPs obtained by emulsion method (EE–L2–NPs) that has shown 48.6% of relative pharmacological efficacy, which is significantly higher ($P < 0.05$) compared to highly crosslinked EE–L3–NPs, plain NPs without Na glycocholate and Na glycocholate solution (Table 2). The data of plasma insulin levels of EE–L2–NPs indicate a similar trend with a relative bioavailability of 44.3%, which is significantly higher ($P < 0.05$) compared to other NPs formulation and plain NPs.

NPs might have been retained in the nasal cavity during entire period of the period of pharmacological effect (6–8 h). The diminished activity thereafter may be due to inadequate therapeutic concentrations or complete release from NPs. According to a previous report [11], micron size mucoadhesive microspheres were retained for a period of 4 days. On the other hand, chitosan NPs were found to have a retention of 5–6 h. Fernandez-Urrusuna et al. [32,33] reported improved trans-nasal insulin delivery with chitosan

Table 2
Pharmacodynamic parameters and pharmacokinetic parameters of various starch nanoparticles

Formulation	Pharmacological parameters			Pharmacokinetic parameters		
	C_{max}* ± SE (% activity)	AOC* ± SE (mg h/dl)	Relative pharmacological efficacy** (%)	C_{max}* ± SE (μIU/ml)	AUC* ± SE (μIU h/ml)	Relative** bioavailability (%)
POG–NPs + Na Glyco [a,b]	46.4 ± 8.1	280.4 ± 78.5	31.1 ± 8.7	71.7 ± 35.1	632.6 ± 72.9	23.4 ± 2.7
POE–NPs + Na Glyco[a,b]	49.7 ± 7.2	218.8 ± 79.1	24.2 ± 8.8	136.1 ± 38.4	588.5 ± 94.2	21.7 ± 3.5
EG- NPs + Na Glyco[a,b]	49.4 ± 7.4	270.8 ± 72.8	30.1 ± 8.1	74.7 ± 38.5	612.6 ± 111.4	22.6 ± 4.1
EE–NPs + Na Glyco[a,b,***]	64.8 ± 7.5	353.6 ± 88.4	39.3 ± 9.8	262.4 ± 42.7	972.1 ± 156.7	35.9 ± 5.8
POG–NPs + Lyso	28.1 ± 8.3	165.8 ± 58.1	18.4 ± 6.5	79.7 ± 25.8	558.8 ± 89.7	20.6 ± 3.3
POE–NPs + Lyso	38.2 ± 7.5	159.4 ± 59.7	17.7 ± 6.6	156.1 ± 36.3	467.1 ± 73.2	17.3 ± 2.7
EG–NPs + Lyso	33.7 ± 7.6	181.7 ± 62.4	20.2 ± 6.9	81.7 ± 31.7	573.7 ± 94.9	21.2 ± 3.5
EE–NPs + Lyso[a,b]	54.6 ± 8.6	242.8 ± 76.8	27.0 ± 8.5	208.4 ± 41.4	681.8 ± 113.7	25.2 ± 4.2
EE–L1–NPs + Na Glyco[a,b]	67.4 ± 8.3	281.3 ± 93.9	31.2 ± 10.5	304.2 ± 44.7	704.2 ± 135.6	26.1 ± 5.0
EE–L2–NPs + Na Glyco[a,c]	69.5 ± 8.7	436.9 ± 101.2	48.6 ± 11.3	351 ± 46.3	1196.7 ± 205.4	44.3 ± 7.6
EE–L3–NPs + Na Glyco[a,b,***]	64.8 ± 7.1	353.6 ± 88.4	39.3 ± 9.8	262.4 ± 42.7	972.1 ± 156.7	35.9 ± 5.8
Plain EE–L3–NPs	36.1 ± 9.6	118.2 ± 41.5	13.1 ± 4.6	63.4 ± 28.6	348.5 ± 47.6	12.9 ± 1.7
Na Glyco + Ins solu	64.7 ± 5.3	98.9 ± 52.3	11.1 ± 3.7	82.4 ± 22.3	283.7 ± 31.2	10.5 ± 1.5
Insulin solution SC	73.6 ± 5.9	179.8 ± 59.6	100	363.7 ± 52.7	540.4 ± 111.3	100

Na Glyco = sodium glycocholate; Lyso = lysophosphatidylcholine.

[a] AOC and relative pharmacological efficacy are significantly higher ($P < 0.05$) compared to plain NPs formulation.

[b] AUC and relative bioavailability are significantly higher ($P < 0.05$) compared to plain NPs formulation.

[c] AUC and relative bioavailability are highly significant ($P < 0.01$) compared to plain NPs formulation and significantly higher ($P < 0.05$) compared to other NPs formulation.

* Parameters were calculated from individual rat data.

** Relative bioavailability was calculated relative to SC insulin solution.

*** Both the formulation made of emulsion method and crosslinked with equal quantity of epichlorohydrin.

nanoparticles compared to control formulation of plain chitosan hydrochloride polymer solution. On the other hand, Dyer et al. [31] reported a pharmacological efficacy of 48% with plain chitosan glutamate solution compared to 38% of preloaded chitosan NPs and 37% of postloaded chitosan NPs formulations. This report concludes that chitosan solution and chitosan insulin physical mixture are superior compared to nanoparticles. The results of our study show a comparative bioavailability at a dose of 10 IU/kg. These results are in line with our observations that very fast release in the case of nanoparticles of the present study, solution and physical mixture of previous report provides a greater drug concentration at the absorptive surface that might enhance permeability of trans-nasal insulin via paracellular pathway as described previously.

4. Conclusions

In the present study, starch NPs formulations of insulin containing permeation enhancer after nasal administration demonstrate a rapid and sustained hypoglycemic action until 6 h. These effects are presumed to be rapid due to release of insulin from the NPs, which are associated to a higher mucosal surface leading to greater concentration gradient at the absorptive area. The trend is consistent with a size dependent faster release of insulin *in vitro*. Such a fast release is useful for molecules of short *in vivo* half lives, hence provides therapeutic concentrations for a long time, and results in a better therapeutic effect. The degree of crosslinking was found to modulate the release of insulin from such carriers and has an effect on *in vivo* performance. NPs obtained with a particular crosslinking of epichlorohydrin (medium crosslinking of the present study) demonstrated superior effects in the presence of sodium glycocholate as permeation enhancer compared to lysophosphatidylcholine. The release rate and higher associated surface area might work in tandem for the effective insulin delivery, which would be greatly amplified when combined with permeation enhancers to make mucoadhesive NPs as an efficient trans-nasal carrier. Nonetheless, results of this study necessitate further optimization to develop a viable trans-nasal carrier.

Acknowledgements

The authors are thankful to Dr. Kishore B. Chalasani for valuable discussion. The authors are grateful to the Director, Indian Institute of Chemical Technology, Hyderabad, India for extending the facilities to carry out the work. One of the authors, Akhlesh K. Jain, would like to thank CSIR, New Delhi, for awarding Senior Research Fellowship.

References

[1] D. O'Hagan, L. Illum, Absorption of peptides and proteins from the respiratory tract and the potential development of locally administered vaccine, Crit. Rev. Ther. Drug Carrier Syst. 7 (1990) 35–97.
[2] R.J. Soane, M. Frier, A.C. Perkins, N.S. Jones, S.S. Davis, L. Illum, Evaluation of the clearance characteristics of bioadhesive systems in humans, Int. J. Pharm. 178 (1999) 55–65.
[3] G.S. Tirucherai, I. Pezron, A.K. Mitra, Novel approaches to nasal delivery of peptides and proteins, STP Pharma Sci. 12 (2002) 3–12.
[4] C. Witschi, R.J. Mrsny, In vitro evaluation of microparticles and polymer gels for use as nasal platforms for protein delivery, Pharm. Res. 16 (1999) 382–390.
[5] L. Illum, N.F. Farraj, A.N. Fisher, I. Gill, M. Miglietta, L. Benedetti, Hyaluronic acid ester microspheres as a nasal delivery system for insulin, J. Control. Release 29 (1994) 133–141.
[6] N.F. Farraj, B.R. Johansen, S.S. Davis, L. Illum, Nasal administration of insulin using bioadhesive microspheres as a delivery system, J. Control. Release 13 (1990) 253–261.
[7] C. Callens, E. Adriaens, K. Dierckens, J.P. Remon, Toxicological evaluation of a bioadhesive nasal powder containing a starch and Carbopol† 974 P on rabbit nasal mucosa and slug mucosal, J. Control. Release 76 (2001) 81–91.
[8] P. Tengamnuay, A.K. Mitra, Bile salt-fatty acid mixed micelles as a nasal absorption promoter of peptides. In vivo nasal absorption of insulin in rats and effects of mixed micelles on the morphological integrity of the nasal mucosal, Pharm. Res. 7 (1990) 370–375.
[9] P.A. Baldwin, C.K. Klingbeil, C.J. Grimm, J.P. Longeneker, The effect of sodium taurodihydrofusidate on the nasal absorption of human growth hormone in three animal models, Pharm. Res. 7 (1990) 547–552.
[10] M.E. Sangalli, L. Zema, A. Maroni, A. Foppoli, F. Giordano, A. Gazzaniga, Influence of betacyclodextrin on the release of poorly soluble drugs from inert and hydrophilic heterogenous polymeric matrices, Biomaterials 22 (2001) 2647–2651.
[11] R.J. Soane, M. Hinchcliffe, S.S. Davis, L. Illum, Clearance characteristics of chitosan based formulations in the sheep nasal cavity, Int. J. Pharm. 217 (2001) 183–191.
[12] S.E. Leucuta, G. Ponchel, D. Duchene, Oxprenelol release from bioadhesive gelatin/poly(acrylic) acid microspheres, J. Microencapsul. 14 (1997) 511–522.
[13] H.G. Choi, J.H. Jung, C.S. Yong, C.D. Rhee, M.K. Lee, J.H. Park, K.M. Park, C.K. Kim, Formulation and in vivo evaluation of omerprazole buccal adhesive tablet, J. Control. Release 68 (2000) 405–412.
[14] C.M. Lehr, J.A. Bouwstra, E.H. Schacht, H.E. Junginger, Design and testing of a bioadhesive drug delivery system for oral application, Int. J. Pharm. 78 (1992) 43–48.
[15] B.S. Lebe, A.S. Hoffman, Mucoadhesive drug carriers based on complexes of poly(acrylic acid) and PEGylated drugs having hydrolysable PEG-anhydride-drug linkages, J. Control. Release 69 (2000) 237–248.
[16] C.H. Lee, Y.W. Chien, Development and evaluation of mucoadhesive drug delivery system for dual-controlled delivery of nonoxynol-9, J. Control. Release 39 (1996) 93–103.
[17] T. Nagai, Y. Machida, Bioadhesive dosage forms for nasal administration, in: V. Lenaerts, R. Gurny (Eds.), Bioadhesive Drug Delivery Systems, CRC Press, Boca Raton, FL, 1992, pp. 69–78.
[18] E. Bjork, U. Isaksson, P. Edman, P. Artursson, Starch microspheres induce pulsatile delivery of drugs and peptides across the epithelial barrier by reversible separation of the tight junctions, J. Drug Target. 2 (1995) 501–507.
[19] E. Bjork, P. Edman, Characterization of degradable starch microspheres as a nasal delivery system for drugs, Int. J. Pharm. 62 (1990) 187–192.
[20] V. Lenaerts, Y. Dumoulin, MA. Mateescu, Controlled release of theophylline from cross-linked amylose tablets, J. Control. Release 15 (1991) 39–46.
[21] M.C. Bonferoni, S. Rossi, M. Tamayo, J.L. Pedras, G.A. Dominguoz, C. Caramella, On the employment of E-carrageenan and hydroxypropyl-methylcellulose mixtures, J. Control. Release 30 (1994) 175–182.
[22] S. Miyazaki, A. Nakayama, M. Oda, M. Takada, D. Attwood, Chitosan and sodium alginate based bioadhesive tablets for intraoral drug delivery, Biol. Pharm. Bull. 17 (1994) 745–747.

[23] D.K. Yao, T. Peng, H.B. Feng, Y.Y. He, Swelling kinetics and release characteristics of crosslinked chitosan: polyether polymer network (Semi-IPM) hydrogels, J. Polym. Sci. A: Polym. Chem. 32 (1994) 1213–1223.
[24] L. Illum, Bioadhesive formulations for nasal peptide delivery, in: E. Mathiowitz, C.M. Lehr, D. Chickering (Eds.), Bioadhesion in Drug Delivery Issues in Fundamentals, Novel Approaches and Development, Marcel Dekker, 1998, pp. 507–539.
[25] L. Ryden, P. Edman, Effect of polymers and microspheres on the nasal absorption of insulin in rats, Int. J. Pharm. 83 (1992) 1–5.
[26] L. Pereswetoff-Morath, P. Edman, Dextran microspheres as a potential nasal drug delivery system for insulin in vitro and in vivo properties, Int. J. Pharm. 124 (1995) 37–44.
[27] E. Bjork, S. Bjurstron, P. Edman, Morphologic examination of rabbit nasal mucosa after nasal administration of degradable starch microspheres, Int. J. Pharm. 75 (1991) 73–80.
[28] L. Illum, The nasal delivery of peptides and proteins, Trends Biotech. 9 (1991) 284–289.
[29] E. Bjork, P. Edman, Degradable starch microspheres as a nasal delivery system for insulin, Int. J. Pharm. 47 (1988) 233–238.
[30] L. Illum, A.N. Fisher, I. Jabbal–Gill, S.S. Daviss, Bioadhesive starch microspheres and absorption enhancing agents act synergistically to enhance nasal absorption of polypeptides, Int. J. Pharm. 222 (2001) 109–119.
[31] A.M. Dyer, M. Hinchcliffe, P. Watts, J. Castile, I. Jabbal-Gill, R. Nankervis, A. Smith, L. Illum, Nasal delivery of insulin using novel chitosan based formulations: a comparative study in two animal models between simple chitosan formulations and chitosan nanoparticles, Pharm. Res. 19 (2002) 998–1008.
[32] R. Fernandez-Urrusuna, D. Romani, P. Calvo, J.L. Vila-Jato, M.J. Alonso, Development of a freeze dried formulation of insulin-loaded chitosan nanoparticles intended for nasal administration, STP Pharma Sci. 5 (1999) 429–436.
[33] R. Fernandez-Urrusuna, P. Calvo, C. Remunan-Lopez, J.L. Vila-Jato, M.J. Alonso, Enhancement of nasal absorption of insulin using chitosan nanoparticles, Pharm. Res. 16 (1999) 1576–1581.
[34] A.J. Almeida, H.O. Alpar, M.R.W. Brown, Immune response to nasal delivery of antigenically intact tetanus toxoid associated with poly (L-lactic acid) microspheres in rats, rabbits, and guinea-pigs, J. Pharm. Pharmacol. 45 (1993) 198–203.
[35] Y. Huang, M.D. Donovan, Microsphere transport pathways in the rabbit nasal mucosal, Int. J. Pharm. Adv. 1 (1996) 298–309.
[36] A.M. Peyrot, J.M. Sautereau, F. Rabnel, J.F. Tocanne, D. Samain, Supramolecular biovectors (SMBV):a new family of nano particulate drug delivery systems, Synthesis and characterization, Int. J. Pharm. 102 (1994) 25–33.
[37] I. DeMiguel, K. Ioualalen, M. Bonnefous, M. Peyrot, F. Nguyen, M. Cervilla, N. Soulet, R. Dirson, V. Reiumajou, L. Imbertie, Synthesis and characterization of supramolecular biovector (SMBV) specifically designed for the entrapment of ionic molecules, Biochim. Biophys. Acta 237 (1995) 49–58.
[38] K.B. Chalasani, J.G. Russell-Jones, S.K. Yandarpu, P.V. Diwan, S.K. Jain, A novel vitamin B12-nanosphere conjugate carrier system for peroral delivery of insulin, J. Control. Release 117 (2007) 421–429.
[39] K.B. Chalasani, Novel ligand coupled nano-constructs for the peroral delivery of proteins, Ph.D. Thesis, Dr. H.S. Gour Univeristy, Sagar, India, 2004.
[40] A.H. Krauland, V.M. Leitner, V. Grabovac, A. Bernkop-schnurch, In vivo evaluation of a nasal insulin delivery system based on thiolated chitosan, J. Pharm. Sci. 95 (2006) 1–10.
[41] L. Illum, N.F. Farraj, H. Critchley, S.S. Daviss, Nasal administration of gentamicin using a novel microsphere delivery system, Int. J. Pharm. 46 (1988) 261–265.
[42] L. Illum, N.F. Farraj, H. Critchley, S.S. Daviss, B.R. Johansen, D.T. O'Hagan, Investigation of the nasal absorption of biosynthetic human growth hormone in sheep-use of a bioadhesive microsphere delivery system, Int. J. Pharm. 63 (1990) 207–211.