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Dextran microspheres as a potential nasal drug delivery system for insulin – in vitro and in vivo properties

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Abstract

The influence of the particle size of dextran microspheres on nasal absorption and the localisation of insulin in the spheres have been investigated in rats. Using scanning electron microscopy, it was shown that the freeze-drying process used to load insulin into the microspheres had a significant impact on the integrity and surface properties of the spheres. It was confirmed via confocal laser scanning microscopy (CLSM) that the distribution of insulin in the dextran microspheres was governed by the cut-off limit of the spheres. A cut-off of 5000 Da excludes insulin from the dextran matrix, leaving insulin on the surface of the spheres. A cut-off of 30 000 Da, on the other hand, allows insulin to penetrate into the swollen spheres and be deposited inside the spheres after the lyophilisation process. Spheres with insulin on the surface were more effective in promoting insulin absorption than those with insulin distributed within the dextran matrix. There seems to be a tendency for the particle size to influence the kinetics of the insulin effect curve.

Keywords: Nasal administration; Microsphere; Insulin; Confocal laser scanning microscopy

1. Introduction

The nasal administration of peptides and proteins has attracted great interest during the past 10 years. A major drawback is the low systemic bioavailability of large molecules (< 10%) and consequently much effort has been directed at enhancing drug absorption by different means. A significant step was taken by Nagai et al. (1984) when they introduced the use of dry powder, e.g., water-insoluble cellulose derivatives, as an absorption enhancer of insulin given nasally. A similar concept but using well characterised microspheres was introduced by Illum et al. (1987). The microspheres form a gel-like layer which is cleared slowly from the nasal cavity, resulting in a prolonged residence time of the drug formulation. It was speculated that an increased contact time could possibly increase the absorption efficiency of the drug. In later works it has been shown that starch microspheres increase the absorption of insulin (Björk and Edman, 1988), gentamicin (Illum et al., 1988) and human growth hormone (Illum et al., 1990).

The mechanism for the increased absorption of drugs administered with degradable starch microspheres (DSM) has been shown to be due to

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an effect on tight junctions between the epithelial cells and not only on the increased contact time. The rapid absorption of insulin seen in animals when given together with microspheres could be reproduced in a Caco-2 cell line in vitro, demonstrating a pulsatile effect of the starch spheres on the tight junctions (Björk et al., 1994). In a transmission electron microscope the epithelial monolayers were investigated prior to, immediately after, 15 min after and 180 min after administration. It was clearly shown that the tight junctions started to separate already after 3 min and that separation continued up to 15 min after administration. 3 h later, the tight junctions were comparable with the controls, thus indicating that the enhancing effect of DSM is rapid and reversible.

Experiments with insulin administered with dextran microspheres (Rydén and Edman, 1992) and hyaluronic acid ester microspheres (Illum et al., 1994) in rats show a rapid decrease in plasma glucose with a normalisation of the glucose level within a couple of hours. Although microspheres of different materials appear to act in the same manner and give the same type of absorption kinetics, there are some differences.

The starch microspheres used by Björk and Edman (1988) seem to be more effective in promoting insulin absorption than the dextran spheres tested by Rydén and Edman (1992). This might be due to differences in the characteristics of the starch and dextran microspheres. The starch spheres had a narrow size distribution with a mean particle size of 45 μm in the swollen state, whereas the dextran spheres were of commercial grade with a particle size distribution in the dried state of 50–180 μm. There was also a difference in cut-off, i.e., molecules that could be included in the DSM had to have a molecular mass less than 30 000–50 000 Da and the corresponding molecular mass for the dextran spheres was less than 5000 Da. Insulin with a molecular mass of approx. 5700 Da could therefore be included in DSM but not in the dextran spheres. However, during the lyophilisation process used to load insulin into the microspheres, the migration of water out of the spheres could also result in the migration of insulin from the inner part of the starch microspheres to the surface. It is therefore difficult to predict whether there are differences in the localisation of insulin in these two types of microspheres. Insulin could be situated on the surface of the spheres irrespective of the type of sphere used.

To investigate whether the particle size and localisation of insulin in the spheres have any significant impact on the nasal absorption in rats, different sieve fractions and two qualities of dextran microspheres were used. One quality had a high cut-off, theoretically allowing insulin to be incorporated into the spheres, and the other quality had a low cut-off which should prevent insulin from penetrating into the spheres. These microspheres were characterised with reference to particle size, surface characteristics, localisation and release rate of insulin. The absorption-enhancing effect of the spheres was studied in rats.

2. Materials and methods

2.1. Materials

Sephadex® G-25 fine, Sephadex® G-50 fine and coarse were obtained as gifts from Pharmacia, Sweden. Human crystalline monocomponent insulin was obtained as a gift from Novo Nordisk, Denmark. Insulin-FITC, with 1 mol FITC per mol insulin, was purchased from Sigma, USA. All other chemicals were of analytical grade.

2.2. Preparation of spheres

Dextran microspheres (Sephadex® G-25 fine, G-50 fine and coarse, Pharmacia, Sweden) with cut-off limits of 5000 and 30 000 Da, respectively, were sieved. The fractions < 45 and 90–150 μm were mixed with a 100 IU/ml solution of human monocomponent insulin (Novo Nordisk, Denmark) in the ratio of 100 mg spheres per ml insulin solution. The suspensions were lyophilised and passed through sieves of 63 and 180 μm, respectively. The amount of immunoreactive insulin in the spheres was determined by RIA. In the preparation of spheres for confocal laser scanning microscopy (CLSM), the human mono-

component insulin was replaced with an equal amount of insulin-FITC.

2.3. Characterisation of microspheres

2.3.1. Particle size

An optical light microscope (Vanox, Olympus) was used to determine the size of the microspheres according to BS 3406 (British Standard, 1963).

2.3.2. Surface characteristics

The impact lyophilisation has on the integrity of the spheres was studied with a scanning electron microscope (JEOL, JSM 820). Microspheres before and after lyophilisation were coated with gold/palladium and photomicrographs were taken at a magnification of 700 ×.

2.3.3. Localisation of insulin in the spheres

Localisation of insulin, i.e., on the surface and/or inside the sphere, was examined in a confocal laser scanning microscope (CLSM; Molecular Dynamics, UK). Confocal scanning records the signal from a fluorochrome at a specified single focal plane within the spheres. Rejection of out-of-focus light allows optical sectioning of the intact spheres. The instrument consists of a Nikon inverted microscope, a MultiProbe 2001, an argon/krypton laser and a Silicon Graphics computer with Image Space software. The laser set to 30% of full power at a wavelength of 488 nm was used as the excitation source. Light emitted from the fluorescein coupled to insulin passed through a 510 nm filter and was detected by a photomultiplier. The spheres were viewed in a 100 × 1.4 NA objective at a horizontal optical plane 8 μm into the sphere.

2.3.4. In vitro release

10 mg of the spheres were placed in a diffusion chamber according to the method outlined by Björk and Edman (1990). The membrane used was a coarse nylon net with a pore size of 10 μm to keep the spheres on the donor side and allow free diffusion of insulin to the receiving compartment that contained physiological saline. The chamber was immersed in a water bath at 37° C, and samples of 100 μl were withdrawn at intervals up to 2 h. Each sample was replaced with an equal volume of physiological saline. The amount of insulin in the samples was determined according to Smith et al. (1985). The water-soluble sodium salt of bicinchoninic acid (BCA) is a sensitive, stable and specific reagent for Cu^{+} produced in the reaction of protein with alkaline Cu^{2+} (biuret reaction). Since BCA is stable under alkaline conditions, a one-step procedure can be accomplished instead of the two-step Folin-Lowry protein assay. A micro method that can detect dilute protein solutions (0.5–10 μg/ml) was used. To 100 μl of sample was added 100 μl of a reagent that was a mixture of 1 vol. of 4% $CuSO4 \cdot 5H_2O$, 25 vol. of 4% BCA and 26 vol. of a carbonate buffer at pH 11.25. The analysis was performed in a microwell plate that was incubated in a moist chamber for 60 min at 60° C and subsequently cooled to room temperature. Absorbance was then measured in a microplate reader at 560 nm. The protein concentration in the samples was determined from a standard curve. The release was calculated as percent of the amount released after 3 h.

2.4. Animal experiments

Male Lewis × DA F1-hybrid rats weighing 250–300 g were divided into four groups. The groups were given insulin 1 IU/kg in (1) Sephadex® G-25 sieve fraction < 45 μm; (2) Sephadex® G-50 sieve fraction < 45 μm; (3) Sephadex® G-50 sieve fraction 90–150 μm and empty Sephadex® G-25 sieve fraction < 45 μm without insulin was given as control. The groups contained six, five, six and four animals, respectively.

The animals were fasted for 15–17 h prior to the experiments. They were anaesthetized with intraperitoneal injection of thiobutabarbital sodium (Inactin, BYK Gulden) and placed in a supine position on heated plates to maintain body temperature. The trachea and carotid artery were cannulated (Rydén and Edman, 1992).

The spheres were weighed into a polyethylene tube to give a dose of 1 IU/kg and were subsequently administered through the nostril 30 min after surgery by blowing air from a syringe through

Table 1
Mean particle size of dextran microspheres before and after the lyophilisation procedure to load insulin into the microspheres

	Mean particle size (μm)		Number of particles counted	
	Before loading of insulin	After loading of insulin	Before loading of insulin	After loading of insulin
Sephadex G-25	24	32	819	829
Sephadex G-50	26	34	636	824
Sephadex G-50	103	104	752	802

the tube. Blood samples were withdrawn from the carotid artery at intervals during a 4 h period.

After centrifugation, the plasma was withdrawn and frozen for glucose analysis by an enzymatic method using catalysis by hexokinase and glucose-6-phosphate dehydrogenase in a Beckman Clinical System 700. Statistical significance was tested by using the Student-Newman-Keul test.

3. Results

3.1. Characterisation of microspheres

Examination of the microspheres in a light microscope and a scanning electron microscope revealed changes in both size and structure after the lyophilisation process. The smaller sieve fraction (< 45 μm) for both qualities of the dextran spheres showed an increase in particle size after freeze-drying, whereas the size of the larger sieve fraction was not influenced (Table 1). Also, the structure of the spheres was altered, which could be seen in the scanning electron microscope. Before lyophilisation, both qualities of the dextran microspheres showed smooth and practically spherical particles (Fig. 1A). For the spheres with a low cut-off (Sephadex® G-25) the surface was altered from smooth to rough after the lyophilisation process but was still intact without cracks or holes (Fig. 1B). For the microspheres with the higher cut-off (Sephadex® G-50), the change was greater. The smooth surface was altered and a significant number of large holes could be seen on the surface. The spherical form of G-50 was also dramatically changed (Fig. 1C). The changes observed for both Sephadex® G-25 and G-50 occurred even when the spheres were lyophilised without insulin.

A comparison between the two different qualities of dextran microspheres in CLSM revealed a significant difference between the two. The fluorescein signal from insulin-FITC appears as a

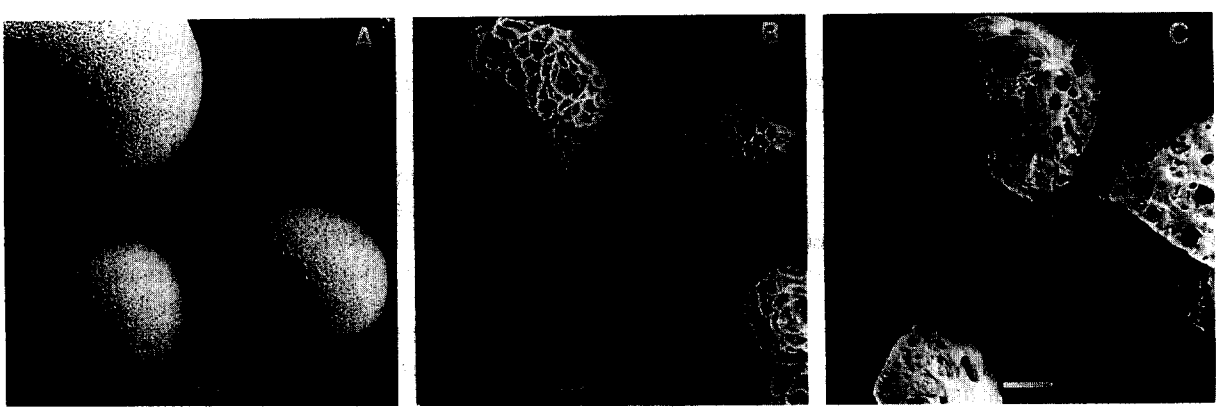

Fig. 1. SEM photomicrographs of dextran microspheres, sieve fraction < 45 μm. (A) Sephadex G-25 and Sephadex G-50 before lyophilisation, (B) Sephadex G-25 after lyophilisation and (C) Sephadex G-50 after lyophilisation. Bar denotes 10 μm.

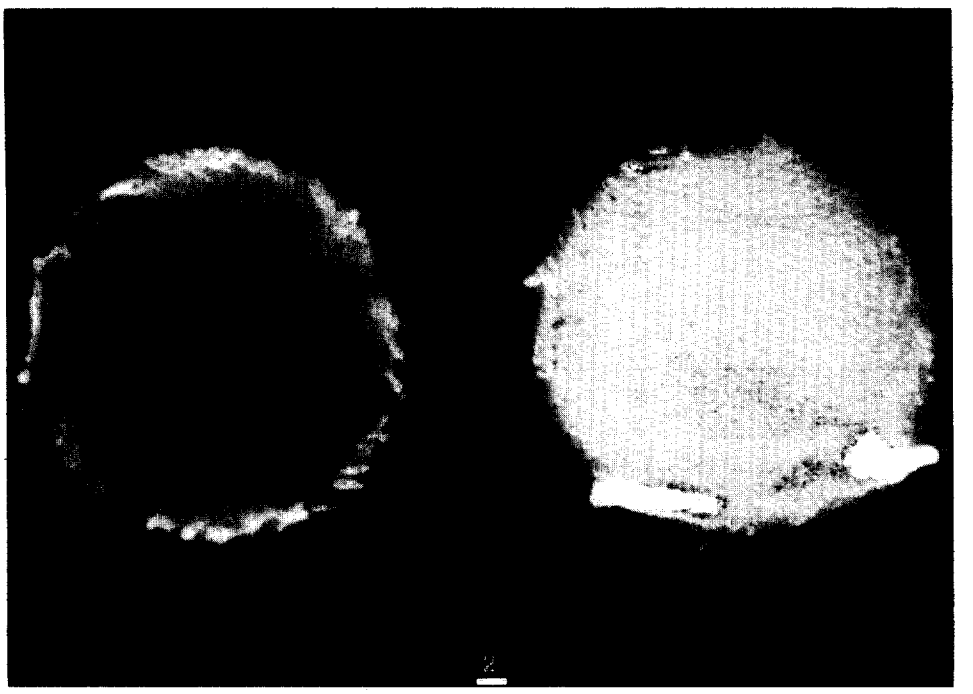

Fig. 2. Confocal fluorescence images at a depth of 8 μm into the dextran microspheres of sieve fraction < 45 μm. To the left, Sephadex G-25 with insulin on the surface, and to the right, Sephadex G-50 with insulin incorporated into the sphere. Bar = 2 μm.

light area in the image of the spheres. The brighter the image the stronger is the signal, i.e., the more the insulin-FITC at that location. As can be seen to the left in Fig. 2, the spheres with a low cut-off (Sephadex® G-25) have bright spots of insulin-FITC only on the surface of the spheres. Looking to the right in Fig. 2, the spheres with the higher cut-off (Sephadex® G-50) show insulin-FITC at both the surface and inside the spheres. An even spread of insulin-FITC throughout the whole interior of the spheres indicates that the insulin is entrapped inside the matrix of the spheres.

The difference in localisation of insulin had no impact on the release rate of insulin from the spheres studied. For both qualities, irrespective of particle size, 80% of the insulin was released within 15 min (Fig. 3).

3.2. Animal experiments

Even though the difference in localisation of insulin had no effect on the in vitro release of insulin, it had a considerable impact on the results obtained in the in vivo study. Sephadex® G-25 with insulin on the surface of the spheres

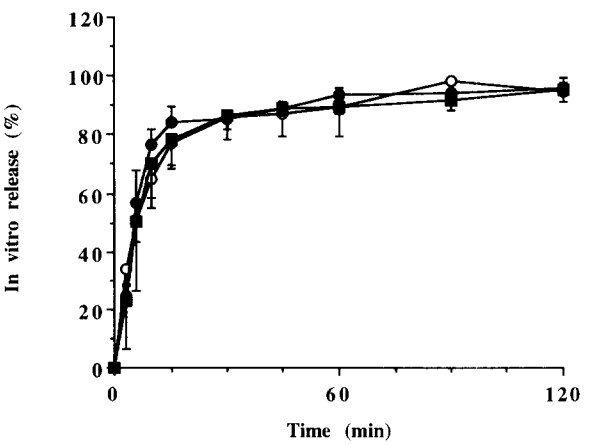

Fig. 3. In vitro release of insulin (mean ± SD) from three different dextran microspheres. (○) Dextran microspheres (Sephadex G-25), particle size 32 μm; (●) dextran microspheres (Sephadex G-50), particle size 34 μm; and (■) dextran microspheres (Sephadex G-50), particle size 104 μm.

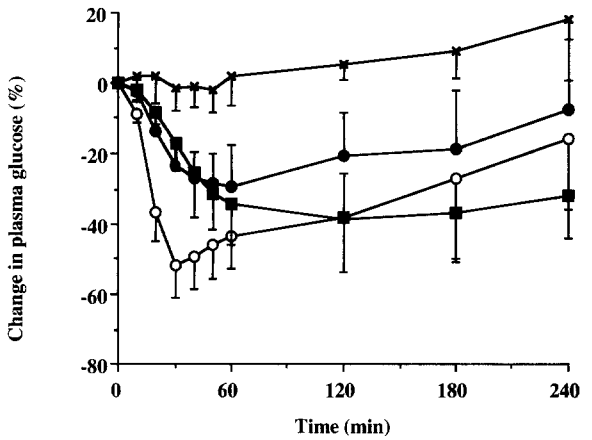

Fig. 4. Change in plasma glucose (mean ± SD) after intranasal administration of insulin 1 IU/kg with three different dextran microsphere systems and a control group that received empty dextran microspheres. (○) Dextran microspheres (Sephadex G-25), particle size 32 μm; (●) dextran microspheres (Sephadex G-50), particle size 34 μm; (■) dextran microspheres (Sephadex G-50), particle size 104 μm; and (×) control.

was more effective in promoting insulin absorption than Sephadex® G-50 with insulin distributed into the sphere matrix (Fig. 4). Insulin 1 IU/kg administered with the 32 μm Sephadex® G-25 microspheres induced a 52% decrease in plasma glucose within 30 min after administration. Sephadex® G-50 spheres with equivalent insulin dose showed a different pattern. During the first 50 min after administration there was a significant difference ($p < 0.05$) between the G-25 and G-50 microspheres. For the G-50 spheres, the maximum decrease in plasma glucose was lower and the particle size seemed to influence the kinetic profile of the effect curve. G-50 spheres with a particle size similar to that of G-25 showed a 30% lowering of plasma glucose within 60 min, whereas for the larger spheres the maximum decrease (39%) was reached after 120 min. However, there was no significant difference between the two different sizes of G-50 dextran microspheres. Empty dextran spheres given as controls had no influence on the plasma glucose level. All three dextran systems differed significantly ($p < 0.05$) from the control group throughout the whole experimental period.

4. Discussion

The freeze-drying process induces considerable changes in the structure of the dextran microspheres. Depending on the type of dextran spheres, G-25 or G-50, the size and the surface structure are significantly altered. Especially G-50 dextran spheres are dramatically affected; large pores are created in the spheres, with changes in size and shape as a result. A plausible explanation could be that lyophilisation causes the partial destruction of the internal matrix of the spheres. Depending on the degree of cross-linking, the resistance against physical stress varies between the spheres used in this study. G-25 dextran spheres have a higher degree of cross-linking than the G-50 spheres and consequently G-25 is more robust and retains its structure better than G-50. As the cross-linking degree governs the cut-off limit, G-25 should have the major part of the insulin on the surface, which was also confirmed on examination in the confocal laser scanning microscope. Sephadex® G-50, on the other hand, is more loosely cross-linked, having a greater cut-off limit, thereby allowing insulin to be incorporated into the sphere matrix. In the CLSM, it is shown that the insulin remains inside the G-50 spheres during the freeze-drying process (Fig. 2).

In the in vitro release experiment there was an excess of water available for the swelling of the spheres. The spheres absorb water rapidly and completely, irrespective of the different swelling capacity of the spheres, and the dissolution and diffusion of insulin out of the spheres are not hindered. Due to this experimental set-up, the release is not dependent on the localisation of insulin in the spheres. In the in vivo experiment, on the other hand, there was probably a limited amount of moisture in the nasal cavity that could be absorbed by the spheres. An incomplete or slower release of insulin from Sephadex® G-50 can be expected, since water must penetrate into the spheres for complete swelling of the dextran matrix prior to dissolution and diffusion of insulin out of the spheres. Sephadex® G-25, with insulin on the surface, will be less affected by the limited amount of moisture, since the insulin is immedi-

ately available for dissolution. In this situation, more insulin will be released initially from Sephadex® G-25 than from G-50. The G-25 spheres have a swelling capacity of 4–6, i.e., the spheres will hold 4–6-times their own weight of water, whereas the swelling factor of G-50 spheres is 9–11. This indicates that the G-50 spheres need more water to be completely swollen and that could decrease the release rate of insulin compared to the G-25 spheres in situations where there is a limited amount of water.

According to earlier findings, it is the amount of insulin available initially that will determine the maximum decrease in plasma glucose (Rydén and Edman, 1992). When microspheres absorb water and swell, they will cause the tight junction to separate (Björk et al., 1994). Since this is a reversible process, an increase in absorption will only take place during the short period that tight junctions are separated. The amount of insulin available for absorption during that period is the insulin that has dissolved and diffused out of the spheres. This will be governed by the localisation of insulin in dextran microspheres and the swelling factor. Degradable starch microspheres with a similar swelling capacity and matrix structure to the dextran spheres used in these experiments showed small differences in vitro release rate but no differences in vivo behaviour for starch microspheres with swelling factors of 5, 8 and 17 (Björk and Edman, 1990). It is therefore more likely that the localisation of insulin is of greater importance to the in vivo behaviour of dextran spheres than the swelling factor.

When comparing this study with results obtained with DSM (Björk and Edman, 1988) it is obvious that the G-50 dextran spheres are not as efficient as DSM in lowering the plasma glucose level, although the size, cut-off limit, and swelling factor of the spheres and the insulin dose used are comparable. An explanation for the difference seen between G-50 spheres and DSM might be that the insulin in the dextran spheres is not as available for dissolution and diffusion as in the DSM, despite the similar characteristics. The changes in the surface structure caused by the lyophilisation process observed in scanning electron microscopy might also have induced physical changes in the internal structure of the spheres, thus affecting the availability of insulin. G-25 dextran spheres, on the other hand, with a smaller cut-off limit and swelling factor than DSM, had an in vivo effect similar to that of DSM. G-25 microspheres with insulin on the surface are not susceptible to changes of the internal structure of the spheres. The insulin dose will be available for dissolution and, consequently, absorption is not dependent on changes in size and shape of the spheres. In view of the different in vivo behaviour of dextran microspheres and starch microspheres it is not possible to generalise the importance of the localisation of insulin in different types of microspheres.

Another parameter that might influence the in vivo performance of the microspheres is the size of the spheres. A reduction of the surface area of the spheres should, theoretically, reduce the contact area between the nasal mucosa and the spheres. Furthermore, an even distribution of the spheres on the mucosa might also be restricted by using large spheres. However, in this study no significant difference in the in vivo study due to particle size could be detected, possibly because the difference in mean particle size of the spheres used, 34 and 104 μm, is still too small to have any impact on the in vivo behaviour of the spheres. Additionally, it was noted that the kinetics of the effect curve seems to be influenced by particle size. However, the observed difference in plasma glucose between the two groups in the latter phase of the experiment is not significant. Part of the explanation for this might be the large variations obtained in plasma glucose in the latter phase of the experiments.

The results of this study clearly show that an optimised nasal microsphere system that mimics the physiological behaviour of insulin should have the insulin available for dissolution immediately after administration. When the spheres swell by taking water from the mucus layer and the underlying epithelial cells, resulting in a temporary widening of the tight junctions, the insulin should be available for diffusion and absorption. It might be possible to obtain different effect profiles of insulin by choosing different sizes of microspheres.

References

Björk, E. and Edman, P., Characterisation of degradable starch microspheres as a nasal delivery system for drugs. *Int J. Pharm.*, 62 (1990) 187–192.

Björk, E. and Edman, P., Degradable starch microspheres as a nasal delivery system for insulin. *Int. J. Pharm.*, 47 (1988) 233–238.

Björk, E., Isaksson, U., Edman, P. and Artursson, P., Starch microspheres induce pulsatile delivery of drugs and peptides across the epithelial barrier by reversible separation of the tight junctions. *J. Drug Targeting*, (1994) in press.

British Standard 3406: Part 4, 1963.

Illum, L., Farraj, N.F., Crithley, H. and Davis, S.S., Nasal administration of gentamicin using a novel microsphere delivery system. *Int. J. Pharm.*, 46 (1988) 261–265.

Illum, L., Farraj, N.F., Davis, S.S., Johansen, B.R. and O'Hagan, D.T., 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.

Illum, L., Farraj, N.F., Fisher, A.N., Gill, I., Miglietta, M. and Benedetti, L.M., Hyaluronic acid ester microspheres as a nasal delivery system for insulin. *J. Controlled Release*, 29 (1994) 133–141.

Illum, L., Jørgensen, H., Bisgaard, H., Krogsgaard, O. and Rossing, N., Bioadhesive microspheres as a potential nasal drug delivery system. *Int. J. Pharm.*, 39 (1987) 189–199.

Nagai, T., Nishimoto, Y., Nambu, N., Suzuki, Y. and Sekine, K., Powder dosage form of insulin for nasal administration. *J Controlled Release*, 1 (1984) 15–22.

Rydén, L. and Edman, P., Effect of polymers and microspheres on the nasal absorption of insulin in rats. *Int. J. Pharm.*, 83 (1992) 1–10.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C., Measurement of protein using bicinchoninic acid. *Anal. Biochem.*, 150 (1985) 76–85.