J. Pharm. Pharmacol. 1994, 46: 547–552
Received September 17, 1993
Accepted January 17, 1994

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Calcitonin and Insulin in Isobutylcyanoacrylate Nanocapsules: Protection Against Proteases and Effect on Intestinal Absorption in Rats

PHILIP J. LOWE AND CATHY S. TEMPLE

Drug Preformulation and Delivery, Ciba Pharmaceuticals, Wimblehurst Road, Horsham RH12 4AB, UK

Abstract—One of the major limiting steps for the absorption of peptide drugs from the intestine is proteolytic degradation. To slow this degradation, human calcitonin was trapped in polyacrylamide nanoparticles, and human calcitonin and insulin were encapsulated with polyisobutylcyanoacrylate. Human calcitonin trapped in polyacrylamide nanoparticles showed no delayed release characteristics and thus would not provide protection from proteases. Proteolytic degradation of human calcitonin and insulin in polyisobutylcyanoacrylate nanocapsules was slower than the free peptides in solution. The plasma pharmacokinetic profiles were consistent with increased survival time of the peptides in the intestine, with higher plasma concentrations of the peptides in the later time samples compared with the controls. However, the nanocapsules gave no significant overall enhancement of peptide absorption. This led to the conclusion that the nanocapsules released the peptides into the intestinal lumen, with small amounts then being absorbed but the rest largely degraded.

One of the major barriers to the successful delivery of protein and peptide drugs via the intestinal route is degradation by intestinal proteases. One system which should protect peptides from proteases is encapsulation with polymers. The hypothesis for this study was that if we could encapsulate peptides and achieve absorption of the encapsulated system from the intestine, we should enhance the delivery of the drug to the systemic circulation.

The use of particulates can be exemplified by the work of Jani et al (1990), who showed that polystyrene microspheres could be taken up by the gastrointestinal tract of rats. The extent of uptake was dependent on the size of the particles, with the smaller sizes showing greater uptake. Uptake was present in both the small intestine and the colon, including Peyer's patches and mesentery. Although most of the microspheres remained with the intestinal tissue, small amounts were found in the liver and spleen indicating that particles were not simply adsorbed to intestinal tissue, but were by some mechanism transported into the body.

There are many examples of the use of particulate systems which attempt to achieve systemic delivery of peptide drugs via the intestinal tract. These include proteinoid microspheres, which use thermally condensed amino acids to encapsulate drug compounds in nanoparticles which dissolve in specific pH ranges (Steiner & Rosen 1988). Microparticles with a mucosal binding entity such as a lectin or vitamin B_{12} have been claimed to effect oral peptide delivery (Russell-Jones et al 1987). Aprahamian et al (1987), Damgé et al (1988, 1990) and Michel et al (1991) have used polycyanoacrylate to encapsulate insulin to achieve a systemic effect following oral delivery. However, few of these studies determine the extent of absorption of the encapsulated peptides into the systemic circulation.

Correspondence: P. J. Lowe, Drug Preformulation and Delivery, Ciba Pharmaceuticals, Wimblehurst Road, Horsham RH12 4AB, UK.

We used two systems. The first was to trap calcitonin within polyacrylamide nanoparticles (Speiser 1972; Birrenbach & Speiser 1976). The second was encapsulation with polyisobutylcyanoacrylate (PIBCA). The former was chosen as this method produces smaller particles. Using the water-in-oil emulsion technology of Speiser (1972), particles of 40–50 nm could easily be produced.

Materials and Methods

Preparation of polyacrylamide nanoparticles

The polyacrylamide nanoparticles were produced using a water-in-oil emulsion system. The procedure was similar to that detailed by Birrenbach & Speiser (1976). Two and a half millilitres acrylamide/N,N'-methylenebisacrylamide (Sigma, Poole) (40%/5% aq.) plus 2·5 mL 20 mg mL^{-1} calcitonin (human calcitonin, Ciba, Basel) was added to 175 mL 5% bis(2-ethylhexyl) sodium sulphosuccinate (DOS, Sigma), 2·5% polyoxyethylene 4 lauryl ether (Brij 30, Sigma) in n-hexane (BDH, Lutterworth). This optically clear micro-emulsion was degassed under vacuum and kept under nitrogen, loaded into an irradiation reactor and polymerized for 5 min with a 125 W UV lamp (Fotodyne Model 3-4400). The extent of polymerization was evaluated by bromine addition test. The particles were isolated and purified by adding 200 mL 80% ethanol, which caused the polyacrylamide to precipitate. The precipitate was collected, washed with 50 mL n-hexane then 100 mL absolute ethanol and lyophilized. The preparation was also carried out on a small scale using calcitonin labelled with ^{125}I (Amersham, Aylesbury).

Release of ^{125}I-calcitonin from nanoparticles

The release of ^{125}I-calcitonin from the polyacrylamide nanoparticles was measured by adding phosphate-buffered saline to the lyophilized polyacrylamide particles. Samples were taken at intervals into ice-cold ethanol, precipitating the polyacrylamide particles. The samples were allowed to

precipitate for at least 30 min and centrifuged at 14 000 g for 2 min. The ethanol pellets and supernatants were counted for ^{125}I radioactivity. The ethanol pellet plus supernatant counts gave the amount of particles which were dispersed from the lyophilized preparation. Since calcitonin is soluble in 80% ethanol, whereas polyacrylamide is not, the supernatant as a percentage of the total gave the amount of ^{125}I-calcitonin which was released from the particles.

Preparation of PIBCA nanocapsules

PIBCA nanocapsules were prepared in a manner similar to that described by Al Khouri Fallouh et al (1986). To 20 vol rapidly stirred 0·25% Poloxamer 188 pH 6 was added a mixture of 0·05 vol Miglyol 812, 0·05 vol isobutylcyanoacrylate (IBCA) and 0·2 vol 10 mg mL^{-1} calcitonin in 0·1% (aq.) acetic acid, in 10 vol ethanol, dropwise from a separating funnel. IBCA polymerized on the surface of the Miglyol 812 droplets as soon as it came into contact with water, forming nanocapsules.

The nanocapsules were purified by one of two methods. Following Al Khouri Fallouh et al (1986), the ethanolic mixture was rotary evaporated to remove ethanol and achieve the desired calcitonin concentration. The second method used diafiltration through YM30 Amicon membranes. In this method ethanol and all non-encapsulated calcitonin and Poloxamer 188 was removed and the nanocapsules were transferred to 0·1% acetic acid, in which the formulation was more stable and did not aggregate at high concentrations. After concentration, PIBCA debris was removed by centrifugation at 2000 g for 15 min. The concentration of calcitonin was determined by immunoassay as for the plasma samples, after disruption of the nanocapsules by adding ethanol to a concentration of 80%. The percent encapsulation of calcitonin in nanocapsules was determined by Airfuge centrifugation at 167 000 g for 60 min, using ^{125}I-calcitonin as tracer. Samples of supernatant and uncentrifuged control were compared to calculate percent encapsulation.

Degradation of nanoencapsulated ^{125}I-proteins

^{125}I-Calcitonin or ^{125}I-insulin was incorporated into PIBCA nanocapsules as for calcitonin. The degradation of 0·4 mg mL^{-1} protein and encapsulated protein was assessed at 37°C in phosphate-buffered media at pH 7·4 by precipitation with trichloroacetic acid (TCA). The degradation was assessed with reference to a zero time control and non-centrifuged 100%. To simulate the intestinal tract, 1 mg mL^{-1} pancreatin (USP, Sigma) with or without 5 mg mL^{-1} bile extract (Sigma B 8631) was used.

Measurement of particle size

Particle size was measured by dynamic light scattering (DLS), or photon correlation spectroscopy (PCS), using a Malvern 4700 laser spectrometer. The samples were examined at 25°C using a 632·8 nm laser at an angle of 90°. The samples were diluted with a relevant solution, either water or 0·1% acetic acid as appropriate. To minimize dust, all diluents were filtered through 0·2 μm filters before use.

Pharmacokinetic experiments

These were carried out as detailed by Hastewell et al (1992). Anaesthetized, fasted, male Wistar rats, 240–270 g, were fitted with a carotid artery cannula and the abdomen was opened to allow access to the gastrointestinal tract. The intestinal doses of the formulations were administered through a small incision using a Gilson positive-displacement pipette for volumes up to 50 μL, or by syringe and needle for larger volumes. The injection site was isolated from the remaining intestinal tract by ligation after dosing to prevent absorption from damaged tissue. Two hundred and fifty microlitre samples of blood were withdrawn at appropriate time intervals. These were prevented from clotting by the addition of 3 μL (15 units) heparin solution. The volume withdrawn from the animal was replaced with heparinized (50 units mL^{-1}) saline. The samples were centrifuged and plasma stored at −80°C. For the reference pharmacokinetics, the peptides were administered via the tail vein and the blood collected as above; no operative procedure was conducted on the gastrointestinal tract.

Plasma peptide assays

Calcitonin levels were determined using a commercial immunoassay (ELSA human calcitonin, CIS Bioindustries International) as described by Hastewell et al (1992). The plasma level of immunoreactive insulin was determined using a commercial immunoassay (INSI-PR, CIS Bioindustries International). The samples were diluted 4-fold with 4·5% human serum albumin before analysis. This minimized the interference between rat plasma and the antibody to insulin and gave the best signal-to-noise ratio. The antiserum was not specific to human insulin. Since there was a 30% cross-reactivity, the assay also measured endogenous rat insulin. Because of this high endogenous insulin the pharmacokinetic profiles were corrected such that the time zero sample was defined as having zero concentration of insulin. Thus, the results are expressed as change in plasma insulin, not absolute concentration.

Calculation of absorption and expression of results

To determine the absorption, the area under the plasma concentration time curve (AUC) was measured using the trapezoidal rule. The AUC for the test formulation was referenced to the intravenous AUC where the absorption was defined as 100%. The absorption was calculated for the period zero to 120 min without extrapolation, as the nature of the pharmacokinetics beyond 120 min was not known. In the plasma pharmacokinetic profiles, the means of the data are presented with error bars representing the standard error of the mean (s.e.m.).

To compare formulations statistically, medians were used rather than means as much of the data showed a skewed rather than a normal distribution. The data were compared using the COMPARE procedure in RS/1 software (BBN, Cambridge, MA, USA) on a DEC VAX-based computer system. Due to the non-normal nature of the data, this procedure used the non-parametric or robust analysis proposed by Hoaglin et al (1983) and DuMouchel (1987). Significant differences were deemed to have occurred if $P \leq 0.05$.

Table 1. Physical parameters for calcitonin polyacrylamide nanoparticles.

Composition of reactants			Incorporation into pellet (%)	Particle size (nm)	Polydispersity
Monomer (%)	Calcitonin				
	(%)	($mg\,mL^{-1}$)			
22·5	4·5	11·3	4·5	54	0·227
43·7	2·5	11·3	23·1	2362	0·328
22·5	20·0	56·4	16·0	258	0·513
43·7	11·0	56·4	16·6	1145	0·359

Results

Polyacrylamide nanoparticles

The large-scale preparation of calcitonin polyacrylamide nanoparticles, when measured by PCS, gave a size of 39 nm and a polydispersity of 0·146. To measure the entrapment of calcitonin in the nanoparticles we used ^{125}I-calcitonin as a tracer, using small scale preparations. Nanoparticles were synthesized with different proportions of calcitonin and different weight concentrations of polyacrylamide (total acrylamide plus bisacrylamide, %T). In all cases the majority of the ^{125}I-calcitonin radioactivity that was not trapped in the pelleted nanoparticles was lost into the hexane surfactant ethanol phase (Table 1). A maximum of 23% calcitonin was incorporated into polyacrylamide particles. The particle size was not the same for the different preparations. As the %T was increased the size grew such that the particles could no longer be considered to be nanoparticles. Increasing the proportion of calcitonin also increased the particle size, although not to the same extent. The smallest particle size was correlated with the lowest incorporation of calcitonin.

When phosphate-buffered saline was added to the dry polyacrylamide particles, greater than 80% dispersed into the aqueous phase within 15 min. The dispersed particles released calcitonin within 5 min. However, not all calcitonin was released. With the 23% T, 20% calcitonin nanoparticles, over 60% of the ^{125}I-calcitonin remained with the particles, whereas for the other preparations less than 20% of the ^{125}I-calcitonin remained in the particles.

Table 2. Effect of calcitonin and Miglyol 812 concentrations on size of nanocapsules and yield of encapsulated calcitonin.

Miglyol 812 (%)	Calcitonin ($mg\,mL^{-1}$)				
	0·0067	0·021	0·067	0·21	0·67
	Size of nanocapsules (nm)				
0·017			115	88	Flocculated
0·053			142	117	112
0·17	173	190	145	139	135
0·53			185		
1·7			340		
	Yield (%)				
0·017			78	66	Flocculated
0·053			79	59	33
0·17	73	51	63	38	31
0·53			38		
1·7			35		

Physical characterization of calcitonin PIBCA nanocapsules

When synthesized with increasing amounts of calcitonin, the amount of peptide pelleted by the Airfuge increased. However, the yield of peptide in the final pelleted nanocapsules was maximal at 0·067 $mg\,mL^{-1}$ (Table 2). When the concentration of Miglyol 812 was lowered, the size of the nanocapsules decreased and the percentage yield and encapsulation increased. As the concentration of Miglyol 812 was increased the percentage yield decreased due to particles floating rather than pelleting, suggesting the density was inversely proportional to the concentration of oil. Without Miglyol 812 the calcitonin-PIBCA formed flocculant material in addition to small nanocapsules. Therefore, Miglyol 812 was considered necessary for stability.

The final conditions chosen were 0·21 $mg\,mL^{-1}$ calcitonin and 0·017% Miglyol 812 to produce small nanocapsules. For the comparison with insulin, larger nanocapsules were synthesized using 0·067 $mg\,mL^{-1}$ peptides and 0·17% Miglyol 812. The insulin nanocapsules measured 305 nm with a polydispersity of 0·187. The encapsulation of insulin was 95 ± 1% compared with 90% for calcitonin.

Protease degradation of PIBCA nanocapsules

When calcitonin or insulin was incubated at 37°C with 1 $mg\,mL^{-1}$ pancreatin, the peptides lost their ability to be precipitated by TCA. This indicated that the peptides were being degraded to smaller mol. wt products (Fig. 1). Calcitonin appeared to be more sensitive to protease degradation than insulin; however, this may have been an artefact of the TCA-precipitation system, as the mol. wt of insulin (5750), unlike calcitonin (3418), could probably be reduced by half without losing the ability to be TCA-precipitable. Thus, one should not compare calcitonin with insulin directly, but only relative to their respective nanocapsule formulations. Identical nanocapsule formulations of both insulin and calcitonin were less sensitive to protease degradation than the free peptides (Fig. 1). Mono-exponential decay curves fitted to the data showed that the degradation rate of insulin was reduced 90-fold, whilst that for calcitonin was reduced 135-fold.

When 5 $mg\,mL^{-1}$ bile extract was added to 1 $mg\,mL^{-1}$ pancreatin, the degradation rate was increased for insulin nanocapsules and reduced for insulin (Fig. 1A). From mono-exponential curve fits, the difference in rate of degradation between free insulin and insulin nanocapsules in this situation was only 4-fold. As with insulin, bile extract slowed the degradation of calcitonin (Fig. 1B). With both small and large calcitonin nanocapsules there was no

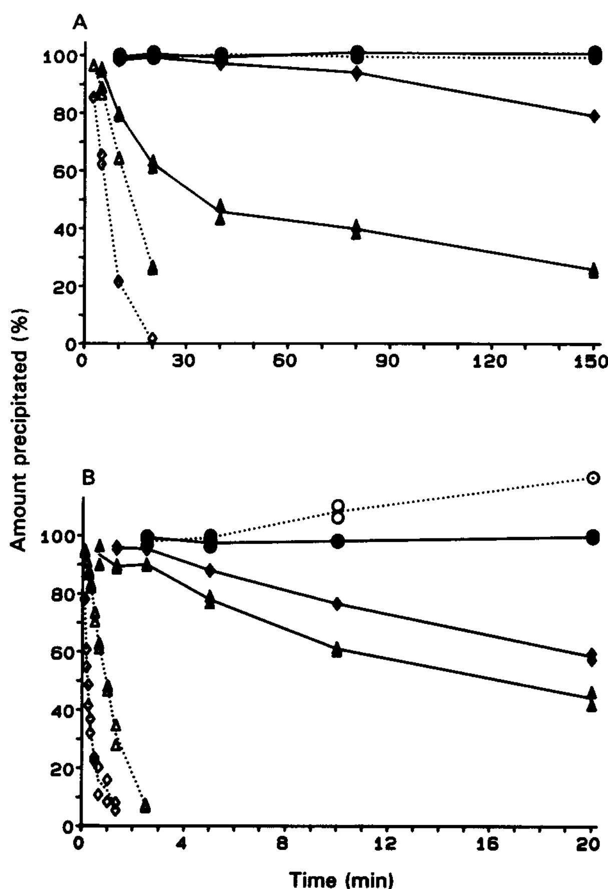

FIG. 1. Effect of $1\,\text{mg}\,\text{mL}^{-1}$ pancreatin and $5\,\text{mg}\,\text{mL}^{-1}$ porcine bile extract on the TCA-precipitability of (A) insulin and (B) calcitonin. The open symbols are peptide solutions and the closed symbols are peptide nanocapsules. The circles are the controls without pancreatin or bile extract, the diamonds pancreatin and the triangles pancreatin plus bile extract. For clarity, early time data points have been omitted from the nanocapsule and control peptide curves.

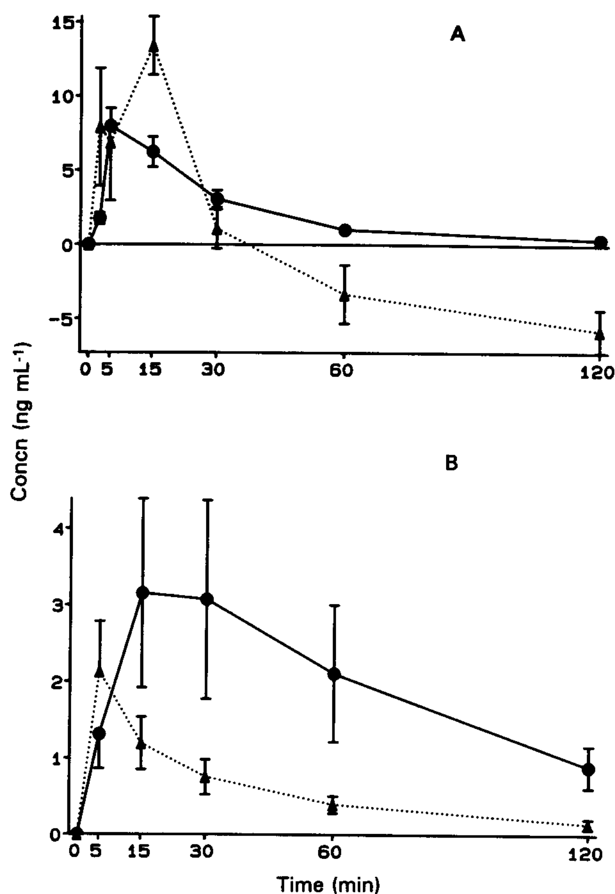

FIG. 2. Effect of nanocapsule formulation on the pharmacokinetics of duodenally administered (A) insulin and (B) calcitonin. The triangles are the control solutions and the circles the nanocapsules. For calcitonin $0{\cdot}2\,\text{mg}\,\text{kg}^{-1}$ was dosed, for insulin $2{\cdot}07\,\text{mg}\,\text{kg}^{-1}$. For the calcitonin nanocapsules n = 11; n = 6 for the calcitonin solutions. For the insulin nanocapsules n = 5; n = 3 for the insulin solution.

significant effect of bile extract and no significant difference between the two sizes of nanocapsule (results not shown). Mono-exponential curve fits showed that with pancreatin and bile extract there was a 17- and 21-fold reduction in degradation rate for the small and large calcitonin nanocapsules, respectively, compared with free calcitonin under the same conditions. It was also noted that ^{125}I-calcitonin solution was not 100% TCA-precipitable at time zero; hence the results were normalized. The increase in TCA-precipitability of the control calcitonin solution beyond 10 min most probably indicated some change in the structure of the molecules, possibly aggregation to fibrils (Arvinte et al 1993).

Pharmacokinetics of PIBCA nanocapsules

Insulin solution in 1% acetic acid at $2{\cdot}07\,\text{mg}\,\text{kg}^{-1}$ gave a pharmacokinetic profile which from a defined zero at time zero reached t_{max} at 15 min and went below zero after 30 min (Fig. 2A). Unlike insulin solution, the same dose of nanocapsule-encapsulated insulin gave a pharmacokinetic profile with a t_{max} at 5 min and positive plasma insulin levels for the experimental period. The median absorption of insulin was calculated to be −0·23% for the control (less than zero due to the negative levels) and 1·27% for the insulin nanocapsules. There was no significant difference in the AUC for the two sets of data.

Small calcitonin nanocapsules were dosed duodenally to rats at $0{\cdot}2\,\text{mg}\,\text{kg}^{-1}$ and compared with a control of the same dose of calcitonin dissolved in 0·1% acetic acid (Fig. 2B). The nanocapsule formulation gave a later t_{max} at 15–30 min compared with the control which had a t_{max} of 5 min. The C_{max} was not significantly different for the two formulations. When the individuals' absorption values were examined, there was no significant difference between the control or nanocapsule formulation of calcitonin. The data did not give normal distributions; rather the data was skewed. In this case the median gave a better indication of the absorption for each formulation than the mean. The median absorption for the nanocapsule formulation was 3·68% compared with 1·69% for the control.

Discussion

The hypothesis for particulate delivery of calcitonin was to protect the peptide from degradation by proteases in the

small intestine, delivering the compound to the systemic circulation after absorption of the intact particles. The ideal particle should be small, preferably 50 nm or less so that there should be significant uptake by the intestinal tissues; polyacrylamide nanoparticles gave the required small size. Although calcitonin could be incorporated into polyacrylamide nanoparticles, the loading efficiency was low (< 5%) for the required size of particle and the peptide came out of the nanoparticles immediately after rehydration such that they would not be able to protect it from protease degradation.

Calcitonin and insulin were successfully incorporated into PIBCA nanocapsules. How the peptides incorporate into the nanocapsules is not known, but calcitonins have marked hydrophobic regions in their structure which enable them to interact with lipidic structures (Epand et al 1983, 1986; Viani et al 1992). We suggest that calcitonin molecules are at the interface between the Miglyol oil droplet and the aqueous phase, together with PIBCA and poloxamer 188. Insulin, which also has hydrophobic characteristics, most likely behaves similarly. The notable point is that to be used in the preparation, the peptides must dissolve in high percentages (95–98%) of a solvent such as ethanol.

When incorporated into PIBCA nanocapsules, both insulin and calcitonin were significantly more resistant to protease degradation than the free peptides. However, the small intestinal lumen does not contain proteases alone, but also bile, generating approximately 10 mM bile salts. Although still significant, the protection offered by nanoencapsulation was reduced by the introduction of bile extract. The surfactant bile acids are likely to aid in the disruption of the oily core of the nanocapsules and allow lipases to hydrolyse the triglyceride oil.

The pharmacokinetics reflected the degradation resistance of the nanocapsules, generating profiles characteristic of sustained delivery (Grass & Robinson 1990) since the pharmacokinetic profile showed relatively higher plasma concentrations at the later time points. However, as these increases at the later time points were balanced by lower initial concentrations, together with variability in the individuals' results, we concluded that there was no significant overall enhancement of absorption. Thus, the hypothesis that the nanocapsules should be absorbed and enhance delivery was likely to be incorrect, since the peptide seemed to be released into a protease-containing environment and largely degraded.

In their original experiments, Damgé et al (1988) and Michel et al (1991) gave single doses of insulin to various regions of the diabetic rat intestine and observed significant hypoglycaemia for up to three weeks, suggesting that the nanocapsules were slowly releasing the insulin from some site in the body. However, they did not demonstrate systemic delivery of insulin, only an extended hypoglycaemia in diabetic rats. In an additional experiment we also collected blood samples for 14 days from rats dosed with calcitonin nanocapsules, but we failed to detect any increase in immunoreactive calcitonin in the plasma over this period of time (results not shown). Insulin has local effects on intestinal tissue (Wollen & Kellet 1988) which may explain how insulin nanocapsules function to lower blood glucose not through systemic action, but by affecting the intestinal cells directly. Any local effects of insulin on the intestinal tissue only complicate any interpretation of the effects of delivery systems using this particular drug. This should not, however, have been the case for calcitonin.

There are many barriers to the oral delivery of protein or peptide drugs. The stomach has a low pH and is a protease-containing environment. In the small intestine the pH is approximately neutral, but the total concentration of proteases is of the order of 1 mg mL^{-1}. The small intestine also contains detergent bile acids. At the cellular level there is the mucus barrier, brush-border peptidases, tight junctions of limited permeability, epithelial and endothelial cells. The mucus barrier should not be underestimated. It restricts the diffusion of proteases such as trypsin and chymotrypsin which would otherwise damage the epithelial cells. Thus, if these proteins of mol. wt 24 and 25 kDa, respectively, do not penetrate mucus, the probability of nanocapsules of 100–200 nm penetrating and reaching the enterocytes is very low. Free calcitonin and insulin, being much smaller at 3418 and 5750 Da, respectively, can diffuse through the mucus layer as long as they survive the protease environment.

Although nanocapsules would act as depot formulations for parenteral use as suggested by Couvreur et al (1979), they are not likely to be feasible systems for the oral delivery of substantial quantities of proteins and peptides (milligram range). The current hypothesis to explain the uptake of small amounts of particles is that there is selective absorption via M-cells, especially in Peyer's patches. The capacity of this system is, however, likely to be very low and thus only useful for protein or peptide drugs which have very high activities and thus small quantities (less than 1 μg) have to be delivered. Moreover, from M-cells and Peyer's patches, substances are directed to the lymphatics and then the immune system, from which there is no proven connection for systemic delivery of the intact compound.

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