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Recent Advances in Protein and Peptide Drug Delivery: A Special Emphasis on Polymeric Nanoparticles

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Abstract

Proteins and peptides are widely indicated in many diseased states. Parenteral route is the most commonly employed method of administration for therapeutic proteins and peptides. However, requirement of frequent injections due to short *in vivo* half-life results in poor patient compliance. Non-invasive drug delivery routes such as nasal, transdermal, pulmonary, and oral offer several advantages over parenteral administration. Intrinsic physicochemical properties and low permeability across biological membrane limit protein delivery via non-invasive routes. One of the strategies to improve protein and peptide absorption is by delivering through nanostructured delivery carriers. Among nanocarriers, polymeric nanoparticles (NPs) have demonstrated significant advantages over other delivery systems. This article summarizes the application of polymeric NPs for protein and peptide drug delivery following oral, nasal, pulmonary, parenteral, transdermal, and ocular administrations.

Keywords

Brain; hydrophobic ion-pairing (HIP) complex; nasal; ocular; parenteral; polymeric nanoparticles; protein and peptide drug delivery; pulmonary; transdermal

Introduction

Recent advancements in biotechnology have resulted in the development of novel therapeutically active proteins and peptides. Some of the recently approved proteins and peptides are listed in Table 1. These potent therapeutics are indicated for several chronic conditions such as cancer, hepatitis, diabetes, rheumatoid arthritis, and leukemia [1]. However, several pharmaceutical and biopharmaceutical challenges limit their clinical application. Parenteral administration is the major route for administration of therapeutic proteins and peptides [2]. However, requirement of frequent injections due to short *in vivo* half-life of proteins and peptides is a major concern. Moreover, in some chronic conditions, frequent injections are required for a longer period of time leading to poor patient

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compliance [3]. Administration of proteins and peptides by oral, pulmonary, transdermal and nasal routes are few of the potential alternatives to parenteral injections. Among non-invasive routes, oral delivery is the primary route for administration of small molecules however; it is not preferable for proteins and peptides. Oral delivery of proteins and peptides is highly challenging due to presence of proteolytic enzymes in gastrointestinal tract (GIT) and their intrinsic physicochemical and biological properties like poor stability in lower pH of gastric fluid, large molecular size and poor permeation across gastrointestinal membrane [4]. Nasal and pulmonary administration received considerable attention because of low proteolytic activity relative to oral route, highly vascularized and large absorptive surfaces especially in the lungs resulting in improved absorption. Large size and proteolytic instability are major factors for poor absorption of therapeutic proteins across nasal and pulmonary mucosal surfaces. Moreover, physiological barriers such as mucociliary clearance may further limit protein and peptide absorption [4]. Similarly, hydrophilicity and large molecular size also limit transdermal protein delivery [4].

To overcome these challenges, proteins and peptides can be delivered efficiently by encapsulating in carrier systems such as microparticles, polymeric NPs, liposomes and solid lipid NPs. In this aspect, polymeric NPs offer unique advantages over other carrier systems. Smaller size relative to microspheres renders polymeric NPs a suitable drug carrier for parenteral administration. Moreover, it has been generally observed that NPs can translocate efficiently across epithelial surfaces relative to microparticles [5, 6]. Polymeric NPs also exhibit high stability in biological fluids compared to liposomes and solid lipid NPs. In addition, versatility of formulation, sustained release, subcellular size, protection of encapsulated proteins and peptides from enzymatic degradation and tissue biocompatibility render NPs as a promising delivery system for protein and peptide delivery [6]. Moreover, physicochemical properties (e.g. hydrophobicity, surface charge), drug release profile and biological behavior (e.g. bioadhesion, targeted drug delivery, improved cellular uptake) can be modulated by application of various polymeric materials and targeting ligands [7, 8].

In this review, we have made an attempt to summarize few of the recent (five to six years) development and application of polymeric NPs for the delivery of proteins and peptides via oral, transdermal, ocular, parenteral, pulmonary and nasal routes. Barriers to proteins and peptides delivery by various routes and advantages of polymeric NPs in overcoming delivery barriers have been summarized in Table 2.

Polymeric NPs as Carriers for Proteins and Peptides

Polymeric NPs are solid colloidal carriers composed of synthetic, semi-synthetic or natural polymers with size ranging from 10 to 1000 nm [10, 11]. These carriers are usually categorized as either nanospheres or nanocapsules. In nanospheres, drug is dispersed in polymeric matrix whereas nanocapsules are reservoir system in which drug is confined within a polymeric shell. Both polymeric nanospheres and nanocapsules have been explored for the delivery of protein and peptide therapeutics. Properties of polymeric NPs are significantly affected by nature of polymers either natural or synthetic and the method of preparation. A few examples of commonly employed natural polymers include chitosan (CS), gelatin and alginate [7]. These polymers are abundantly present in nature and have

been extensively applied in oral proteins and peptides delivery. Among natural polymers, CS has shown most interesting potential, which is attributed to its better solubility at the intestinal pH, improving mucoadhesivness and permeation enhancement. In the small intestine, CS NPs can adhere to and infiltrate into mucus layer and open the tight junctions between contiguous epithelial cells [12]. Furthermore, pH-sensitive CS NPs can disintegrate and release the encapsulated drugs which then penetrate through the opened paracellular pathway. Examples of synthetic polymers investigated for proteins and peptides delivery are poly(DL-lactide co-glycolide) (PLGA), polycaprolactone (PCL), poly(lactic acid) (PLA), polyalkylcyanoacrylate, polyacrylic acid (PAA) and poly(methyl methacrylates) [7, 13, 14]. Among synthetic polymers, polyesters alone or in combination are the most relevant and most frequently studied for protein delivery. Contrary to natural polymers, synthetic polymers enable adjustable controlled drug release for a period of several days to weeks.

Proteins can be encapsulated, adsorbed or chemically linked to the surface of polymeric NPs. Protein incorporation in polymeric NPs can be achieved by various methods. Natural polymers are generally more sensitive to processing conditions. Therefore, NPs with natural polymers are generated using mild techniques including ionic gelation, polyelectrolyte complexation and coacervation [15-17]. NPs composed of synthetic polymers are normally prepared by more extensive techniques such as interfacial polymerization, emulsification–polymerization, emulsification-solvent evaporation, nanoprecipitation, salting out, supercritical fluids and emulsification solvent diffusion [18-24]. Preparation methods for NPs have been broadly reviewed in the literature and hence briefly discussed in the following section [18].

Emulsion Solvent Evaporation Method

Double emulsion solvent evaporation (w/o/w) is the most widely used method for loading biotherapeutics into polymeric NPs. In this method, an aqueous protein solution is first emulsified in polymer containing organic phase. Then, w/o/w emulsion is produced by adding w/o emulsion into an aqueous phase with emulsifier. The organic solvent is usually removed by evaporation. Emulsion solvent evaporation method provides unique advantages including control of particle size and release rate. However, major problems with this method are poor encapsulation and protein instability. Proteins are destabilized at aqueous-organic interface and shear stress which can lead to protein unfolding and aggregation [25]. Denatured and aggregated proteins are reported to produce immunogenic reactions and toxicity [26]. Addition of stabilizing excipients has been widely employed to stabilize protein at aqueous-organic interface. Excipients such as bovine serum albumin (BSA), sugars and polyethylene glycol (PEG) have demonstrated protein stabilization effect during NPs formulation [27-29]. As an alternative to w/o/w method, solid-in oil-in water (s/o/w) method is also used in order to avoid water-organic solvent interface and related protein denaturation. In the s/o/w method, the lyophilized protein powder is dispersed in an organic phase containing the dissolved polymer and this suspension is emulsified in an aqueous solution containing an emulsifying agent. Organic solvent is then evaporated and NPs are washed followed by freeze drying. Several investigators have reported that hydrophobic ion-pairing (HIP) complexation is an excellent strategy to stabilize protein during NPs fabrication and improve encapsulation efficiency. Recently, Gaudana et al. prepared BSA-

loaded NPs by combining s/o/w method with HIP complexation approach [30]. In this study, BSA was ion-paired with dextran sulfate to form HIP complex. The complex was freeze dried and loaded into PLGA NPs by s/o/w method. Significantly high entrapment of BSA in NPs was achieved with this method. Also, secondary and tertiary structures of BSA maintained following HIP complexation and NPs preparation. Similarly, higher encapsulation of human IgG-Fab fragment in PLGA NPs was achieved following HIP complexation with dextran sulfate [31]. In another study, Rastogi *et al*. have prepared insulin-loaded PCL– PEG–PCL NPs utilizing modified s/o/w method in order to achieve higher protein loading. Insulin was hydrophobically modified with sodium deoxycholate. The lipophilicity of insulin following ion-pairing with sodium deoxycholate was enhanced by 5-fold resulting in an increase in entrapment efficiency by 10–50% compared to free insulin [32].

Nanoprecipitation Method

In this method, polymers and drugs are dissolved in a polar, water-miscible solvent (DMSO, acetone, or ethanol). The solution is added in a drop by drop manner into an aqueous solution with surfactant. NPs are formed instantaneously by rapid solvent diffusion. Main advantage of the method is that the drug molecules can be encapsulated without subjecting to shearing stress and high temperatures. The method was originally developed for encapsulation of hydrophobic molecules [7, 33]. Later on, Bilati *et al*. have modified this technique to encapsulate hydrophilic proteins and peptides into PLGA NPs [24]. NPs with small size and narrow size distribution can be easily obtained by this method. Similar to s/o/w method, protein in HIP complex form can also be incorporated into NPs by this technique. Combination of HIP complexation with nanoprecipitation have demonstrated enhancement in encapsulation of proteins such as human IgG-Fab fragment and lysozyme [31, 34]. In a recent study, Gaudana *et al*. have hydophobically ion-paired lysozyme with dextran sulfate and then the complex was loaded into PLGA NPs by nanoprecipitation [34]. Higher lysozyme encapsulation was achieved and released lysozyme maintained its biological activity.

Ionic Gelation and Cocervation Techniques

Ionic gelation and cocervation techniques are mainly employed for preparing NPs composed of natural polymers (CS, gelatin and sodium alginate). In ionic gelation, CS is dissolved in acetic acid (presence/absence of stabilizer) followed by the addition of polyanion or anionic polymer under mechanical stirring at room temperature [35]. The preparation conditions are mild and protein can be encapsulated without use of organic solvents or elevated temperature. However, it is difficult to achieve long term controlled release due to solubility of polymers. Some of the parameters affecting protein encapsulation by ionic gelation method are molecular weight of polymer, initial protein concentration and polymer concentration. It is reported that BSA encapsulation efficiency was enhanced around two times with increasing molecular weight of chitosan from 10 to 210 kDa. Protein and polymer concentration have demonstrated inverse effect on protein encapsulation efficiency [36].

Supercritical Fluids

Recently, NPs are also prepared utilizing supercritical fluids. In this technique, drug and polymer are first dissolved in supercritical fluid and the solution is expanded through a nozzle. The supercritical fluid is evaporated using spraying process which eventually leads to precipitation of solute particles. Advantages of this technique include processing of biolabile pharmaceuticals under mild operating conditions, flexibility in procedures, and elimination of organic solvent in the final product. Elvassore *et al*. produced insulin-loaded PLA NPs with high product yield and maintenance of >80% of the insulin hypoglycemic activity using this method [37]. The major problems observed with this method are requirement for special equipment, poor solubility of high molecular mass (10,000) polymers and strong polar substances in supercritical CO_2 [38].

Application of Polymeric NPs in Delivery of Proteins and Peptides

Development and application of polymeric nanoparticulate carriers for the delivery of proteins and peptides via different delivery routes such as oral, pulmonary, nasal, parenteral, transdermal, and ocular have been discussed in this section.

Oral Delivery

Oral administration is the most preferred route due to its advantages such as patient convenience and compliance, avoidance of contamination and infections caused by the use of injections [39]. However, proteins and peptides exhibit poor oral bioavailability due to lower permeation across intestinal epithelium, aggregation and denaturation. Hindrance to oral administration of protein and peptide can be categorized as physical, chemical and enzymatic barriers. The physical barrier is attributed mainly to the continuous monolayer of intestinal epithelial cells which highly expresses intercellular tight junctions. Fig. 1 depicts the structure of intestinal epithelia. The intercellular tight junctions provide high physical integrity to intestinal epithelial cells [40]. It comprises of various transmembrane integral proteins, which form a continuous seal between adjacent intestinal cells. The porous structure of tight junctions contains fenestrate with the dimension of 3 to 10 Å [41]. Large molecular size and hydrophilic nature of proteins and peptides pose a challenge to passing through cellular membrane and also limit the passage through paracellular pathway [42, 43]. Physicochemical properties of polymeric NPs can be optimized to facilitate transport across intestinal epithelial cells [44]. Efflux proteins such as P-glycoprotein (P-gp) may act as a barrier and limit intestinal transport [45]. Polymeric NPs can be an excellent drug delivery systems to evade the efflux route as Pgp is incapable of recognizing NPs [46]. Moreover, rapid pH variations in GIT can induce degradation of orally administered proteins and peptides by oxidation, deamidation or hydrolysis. Proteins and peptides are vulnerable to acidic pH in the stomach [44]. Enzymatic degradation by pepsin in the stomach and/or pancreatic proteases in the intestine, and aminopeptidases existing in the brush-border membrane are other major barriers to oral protein and peptide delivery. In addition, pre-systemic degradation of peptides and proteins due to extensive first-pass metabolism may result in low dose fraction to enter the systemic circulation [47, 48]. Consequently, most proteins and peptides exhibit low absorption via oral administration. Polymeric NPs have

been thoroughly explored to overcome these barriers and improve the oral absorption of proteins and peptides.

NP Transport Mechanisms

Polymeric NPs can offer an alternative strategy to improve bioavailability of encapsulated proteins and peptides by providing protection towards degradation in gastrointestinal environment, enhancing cellular contact with intestinal membrane, and promoting absorption in the small intestine [49]. NPs could be trapped in the adherent mucus layer or translocated by intestinal epithelial cells. Possible interactions of NPs with the intestinal barrier have been depicted in (Fig. 2). There are two distinct mechanisms for NPs transport across the intestinal epithelium: paracellular pathway (between adjacent cells) and transcellular route.

Paracellular Pathway

Paracellular space represents only about 0.01-0.1% of total absorptive intestinal surface with the dimension in the order of 10 Å. Therefore, transport of macromolecules as well as NPs is severely restricted from crossing mucosal epithelia through paracellular route (Fig. 2). To improve the paracelluar transport, various enhancers such as CS, starch, thiolated polymers and calcium chelators have been successfully employed to reversibly open tight junctions.

Transcellular Transport

Transcellular route for proteins and peptides includes passive transcellular diffusion, carrier-mediated transport, transcytosis by normal enterocytes (with/without receptor-mediated) and phagocytosis by M cells (with/without receptor-mediated). Transcellular transport of NPs across intestinal epithelium generally involves the latter two pathways (Fig. 2).

Transcytosis by Normal Enterocytes

Transcytosis of NPs across the intestinal epithelial cells involves two steps: first, an endocytic process occurring at the apical membrane which is followed by transport processes which allow particles to be delivered across the basolateral membrane [50]. Intestinal epithelium is primarily composed of enterocytes. Translocation of NPs through enterocytes is severely restricted owing to low endocytic capacity of the enterocytes [51, 52].

M Cell-Mediated Phagocytosis

M cells represent only 1% of the total intestinal cells and approximately 5% of the human follicle-associated epithelium (FAE) [53]. M cells mediated phagocytosis is a major route of translocation for orally administered proteins and peptides as well as NPs due to their high transcytotic activity. Several research studies have reported that the majority of particles are translocated in the FAE [54].

Application of Polymeric NPs as a Potential Oral Delivery System for Proteins and Peptides

The application of polymeric NPs for oral protein and peptide delivery has gained significant interest in the last few decades. Recent developments in polymeric NPs based oral delivery of proteins and peptides have been described in Table 3. Several factors need to be considered and optimized to develop efficient nanocarriers for oral delivery. Uptake of NPs through intestinal epithelium is dependent on particle size, surface charge, polymer hydrophobicity, mucoadhesivity, and the presence or absence of surface ligands. Studies on the effect of particle size have demonstrated that transcytosis of NPs is improved with the decrease in particles size [55, 56]. Particles with size ranging from 50 to 500 nm are generally favorable for transport of NPs in GIT [51]. Uptake of NPs by enterocytes or M cells is highly dependent on their size [57]. Smaller particles of less than 50-100 nm in size can be transcytosed by enterocytes, while the particles with size less than 1 μm are likely to be internalized by M cells [58]. NPs surface charge is also a key factor that may alter its entry in enterocytes or M cells. The surface charge can facilitate the proximity of particles to the intestinal epithelium, and may further enhance entry through Peyer's patches. Previously, it has been observed that negatively charged poly(styrene) NPs showed a limited uptake through M cells [59]. NPs with neutral charge and size of 130 and 950 nm were taken up through Peyer's patches. It has also been reported that the NPs with negative or neutral surface charge had higher transport efficiency across Peyer's patches relative to positive charged NPs [60]. Hence, dense coating the surface of NPs with the neutral charged polymer poly(ethylene glycol) (PEG) can minimize the binding of NPs with negatively charged mucin [61]. Particle hydrophobicity is one of the major factors that may affect particle absorption by intestinal epithelium, especially for Peyer's patches. Several reports have demonstrated NPs composed of hydrophobic polymer entered efficiently through Peyer's patches [62, 63]. In addition to change in surface properties, NPs transport across oral mucosa can be improved by application of bioadhesive polymers and surface modification with targeting ligands.

The use of bioadhesive synthetic (polyacrylates, cellulose derivatives) and natural polymers (alginate, CS derivatives) can provide prolonged interaction of NPs with the intestinal barrier, which extends the residence time for permeation across the intestinal epithelium. Among bioadhesive polymers, CS is most widely investigated for oral protein and peptide delivery. In a recent study, novel nanocarrier based on alginate-dextran sulfate core, complexed with a CS-PEG-albumin shell improved oral delivery of insulin [64]. NPs entered due to high adhesion to enterocytes and especially Peyer's patch region. *In vivo* oral studies displayed significant reduction of glycaemia for 24 h. In another study, carboxylated CS graft methyl methacrylate NPs have been developed for oral insulin delivery [65]. This nanocarrier exhibited sustained release at lower pH (2.0) relative to higher pH such as 6.8 and 7.4. At 25 IU/kg, the pharmacological bioavailability was 9.7% with desirable tissue and blood compatibility.

Modifying NPs by absorption or covalent attachment of targeting ligands on the surface is an alternative strategy for enhancing NPs transport across intestinal epithelial cells. These

targeting ligands can interact with surface receptors on M cells, goblet cells, or enterocytes [66-68]. The targeting moieties studied include lectins, peptides, vitamins and wheat germ agglutinin [66, 67, 69, 70]. Targeted delivery was employed to improve bioavailability and hypoglycemic activity of insulin via oral administration. Insulin-loaded CS NPs were surface modified by conjugation with a synthetic CRTLTVRKC peptide [71]. The peptide significantly enhanced targeting to epithelium and translocation of CS NPs by interaction with a transmembrane protein stabilin-2. These interactions facilitate efficient delivery and higher transport of NPs. Yoo et al. reported the enhancement of oral drug absorption via M cell-targeting CS NPs. NPs were linked with a targeting peptide such as CKSTHPLSC (CKS9). The modified CS NPs linked with CKS9 peptide improved uptake at FAE area of Peyer's patch. In the *in vitro* transcytosis study across rate small intestine, the CKS9 peptide-modified CS NPs showed more effective transport ability and more specific accumulation into Peyer's patch regions compared with non-modified CS NPs. In another study, goblet cell-targeting NPs of insulin were developed by coupling cell targeting peptide CSKSSDYQC (CSK) with the trimethyl CS chloride surface to augment insulin oral absorption [68]. CSK peptide modification was observed to enhance transport of NPs in villi and facilitate the transition of drug molecules across the cellular membrane. CSK peptide also induced greater internalization of protein/peptides through clathrin and caveolae dependent endocytosis in HT29-MTX cells. CSK peptide modified NPs by oral administration in diabetic rats showed 1.5-fold higher bioavail-ability and greater hypoglycemic effect relative to unmodified NPs.

Recently, drug delivery through colon has emerged as an attractive strategy for delivering large molecular drugs into systemic circulation. The colon is the preferred absorption site for proteins and peptides due to following reasons: (i) less proteolytic activity of colon mucosa compared with small intestine; (ii) less endogenous metabolic enzymes; (iii) longer retention time of colon with 5 days and (iv) significant enhancement by absorption enhancers due to slow transit and stirring [72-75]. However, the colonic drug delivery system of protein and peptide is also complicated and limited due to (i) need of advanced technology, multiple formulation steps and skills of manufacturing; (ii) difficulty in accessing the colon at the distal portion of the alimentary canal; (iii) harsh conditions such as different pH and enzymes in GIT; (iv) drug loss due to binding to dietary residues, intestinal secretions, mucus in colon; (v) possible involvement of microflora in colon which could affect colonic performance via metabolic degradation of protein and peptide drugs; (vi) smaller surface area and tight junctions in the colon restrict drug transport across the mucosa into the systemic circulation [76, 77]. To overcome these challenges, polymeric NPs have been utilized for colon targeted protein and peptide delivery. Recently, Coco et al. formulated three different polymeric NPs such as pH-sensitive Eudragit® NPs, mucoadhesive trimethylchitosan (TMC) NPs, and PLGA-based NPs with targeting ligands to deliver ovalbumin (OVA) to colon for treating inflammatory bowel disease (IBD) [78]. TMC NPs exhibited highest apparent permeability for OVA in the untreated Caco-2 monolayer model, however, there was no difference among three NPs formulation in the inflamed model. Moreover, targeted PLGA NPs presented the highest accumulation of OVA in inflamed mouse colon. These results demonstrate the potential of active targeting of the colon for inflammatory bowel treatment. Laroui et al. has demonstrated the efficacy of tripeptide Lys-

Pro-Val (KPV)-loaded NPs of size 400 nm in alginate-chitosan hydrogel for the treatment of IBD via mouse model [79]. The hydrogel collapsed in the colon and NPs quickly released the drug on or within the closed area of colonocytes. KPV-loaded NPs also exhibited significant anti-inflammatory efficacy in mice. Moreover, KPV-loaded NPs generated similar therapeutic efficacy with 12,000-fold lower concentration in the formulation than that of KPV solution. These observations clearly indicate the potential of drug-loaded NPs as an effective therapeutic approach for the treatment of IBD.

Nasal Delivery

Nasal route is commonly explored for non-invasive protein and peptide delivery. Recent advancement in biotechnology, inhalation devices and targeting motifs has considerably raised research interest in protein and peptide delivery via this route. It offers advantages including large surface area, highly vascularized mucosa, porous endothelial membrane, lower enzymatic activity relative to GIT and avoidance of first-pass metabolism [101, 102]. However, pattern of deposition and size distribution through delivery device and nasal clearance mechanisms might pose a significant challenge to protein delivery [103]. Following intranasal administration, proteins can be absorbed directly into systemic circulation, central nervous system (CNS) or across GIT [104]. Miacalcin®, DDAVP®, Synarel®, Fortical® and Syntocinon® are few of the marketed proteins and peptides for nasal administration [9].

Absorption through nasal mucosa is highly dependent on nasal physiology, physicochemical properties of permeants and effectiveness of delivery devices [105]. About 1.5 to 21 mL of mucus generated by serous and seromucous glands forms a double layer of 2-5 μm in thickness [106]. Mucus acts as a first-line defense by filtering and entrapping foreign particles. Entrapped particles are expelled by cilia in the epithelial surface. This process might significantly impair protein absorption through nasal mucosa. Moreover, mucociliary apparatus poses a formidable barrier for nasal absorption. Cilia along with mucus layer generate a fluctuating movement to clear drug in approximately 12-15 min from nasal cavity [107]. In addition to physiological factors, physicochemical properties of permeants play a considerable role in altering absorption across nasal mucosa. Proteins of size greater than 1 kDa have demonstrated overall bioavailability of 0.5 to 5% [108]. Moreover, particles of size 1 μm have been reported to reach blood circulation after intranasal administration [108]. Importantly, lipophilicity and partition coefficient may alter protein and peptide absorption across nasal mucosal surface. In addition, the particle size and distribution pattern may also determine nasal protein absorption. Particles of size 10 μm or higher have been demonstrated to accumulate in the upper respiratory region. On the other hand, particles of less than 0.5 μm in size are carried by the inhaled air to the lungs. Importantly, particles of the size in the range of 5-7 μm significantly accumulate in the nasal cavity [109].

Various polymeric NPs have been examined for their efficacy in improving protein and peptide absorption across nasal mucosa. Chitosan-N-acetyl-L-cysteine (CS-NAC) NPs have been investigated for nasal delivery of insulin [110]. The particle size ranged from 140-210 nm with a zeta potential of 119.5 to 131.7 mV. Particles were spherical in shape and had loading efficiency of 13-42%. CS-NAC NPs displayed an excellent swelling behavior and

generated an initial burst followed by slow release of insulin, *in vitro*. *In vivo* studies revealed that CS-NAC NPs were more promising in reducing blood glucose levels relative to CS NPs and insulin control solution. Intranasal administration of insulin-loaded CS-NAC and CS particles diminished glucose level to 59% and 74% at 30 and 60 min post dosing, respectively. Total reduction in plasma glucose levels observed with insulin-loaded CS-NAC and CS NPs were 16.2% and 8.3% within 5 h, respectively. These results clearly signify the potential of thiolated CS in improving nasal absorption of proteins such as insulin. Hybrid poly-oligosaccharide NPs comprising of CS and cyclodextrins have been developed and investigated for nasal insulin delivery [111]. These NPs demonstrated high efficacy in reducing transepithelial resistance of nasal membrane reversibly. Moreover, intranasal administration of insulin-loaded hybrid NPs generated more than 35% reduction in glucose levels at 1 h post dosing in conscious rabbits. In contrast, insulin solution resulted in 14% reduction in plasma glucose levels after 30 min of dose administration.

CS and alginate have been employed commonly for formulating NPs due to their excellent biodegradability, biocompatibility, and mucoadhesive properties. Such high bioadhesiveness is beneficial as it will prolong the contact time of NPs with biological membrane. Moreover, CS has been known to enhance absorption across epithelium by causing transient loosening of tight junctions. Recently, chitosan/alginate blended NPs have been developed by Goycoolea *et al*. [112]. Particle size of insulin-loaded NPs ranged from 273 to 396 nm. *In vitro* release studies demonstrated nearly 80% insulin release within 20 min. The insulin-loaded NPs generated 35% reduction in plasma glucose level at 45 min post intranasal administration. PEGylated trimethyl CS nanocomplexes have demonstrated high potential in improving nasal insulin absorption [113]. These nanocomplexes have generated approximately 34-37% reduction in plasma glucose levels in rats. Thiolated CS-thioglycolic acid (CS-TGA) NPs have been developed by Shahnaz *et al*. as a promising drug carrier for leuprolide. Mean particle size of these NPs was observed to be 252 ± 82 nm. Leuprolide was released slowly over a period of 6 h. Thiolated CS NPs generated 5.2-fold higher leuprolide transport across porcine nasal mucosa relative to leuprolide solution. These NPs displayed 6.9 and 3.8- fold higher area under the curve (AUC) and maximum plasma concentration relative to leuprolide solution, respectively.

Nose to Brain Delivery

Protein and peptide brain delivery via nasal route has gained considerable attention as it can bypass blood-brain barrier (BBB) and first-pass metabolism and provides rapid brain absorption due to high blood flow and porous endothelial membrane structures [114]. BBB is considered as the most formidable barrier for drug delivery into the brain from systemic circulation [115]. Hence, direct brain delivery through intranasal route offers a more promising and viable strategy to improve absorption of therapeutic agents which have short plasma half-life. Nasal cavity can be distinguished into nasal vestibular, respiratory and olfactory regions. Olfactory region has been proposed as the major route for brain transport following intranasal administration (Fig. 3) [116-124]. Several polymeric NPs have been investigated for direct and sustained release of proteins and peptides into the brain following intranasal administration.

Cheng *et al*. demonstrated brain delivery of neurotoxin-I with polylactic acid NPs (NT-I-NPs) following intranasal administration [125]. Intranasal administration of NT-I-NPs demonstrated shorter time to reach maximum concentration (T_{max}) relative to intravenous (i.v.) administration of NT-I-NPs and NT-I solution. T_{max} values observed for intranasally and i.v. administered NT-I-NPs was 65 and 95 min, respectively. T_{max} value displayed by NT-I solution was 145 min. Moreover, AUC generated by intranasal administration of NT-I-NPs was significantly higher relative to i.v. administration of NT-I-NPs and NT-I solution. Absolute bioavailability produced by intranasal administration was about 160 and 196% higher relative to i.v. administration of NT-I-NPs and NT-I solution. These results indicate that the brain delivery of peptide loaded NPs can be significantly improved by intranasal administration relative to i.v. administration. Xia *et al*. have demonstrated the efficacy of low-molecular-weight protamine (LMWP)-modified PEG-PLA NPs to deliver therapeutic agents to CNS via olfactory and trigeminal nerve pathways [126]. Such nanoparticulate formulation could be employed for improving brain protein or peptide absorption.

PLGA NPs loaded with thyrotropin-releasing hormone (TRH) of size 108 ± 12 nm have been developed by Veronesi *et al*. [127]. Intranasal administration of TRH-loaded NPs significantly raised the stimulations required to reach stage V seizures in rats. Moreover, seizure after duration discharge was observed to be significantly reduced in rats treated with TRH-loaded NPs intranasally. These observations clearly indicate the neuroprotective potential of intranasally administered TRH-loaded NPs. A recent report discussed brain concentrations of neurotoxin-I (NT-I) peptide after intranasal administration as solution and PLA NPs coated with polysorbate-80 [128]. Investigators observed an approximately 3-fold higher accumulation of NT-I with NPs compared to solution. Concentrations of NT-I observed with NPs and solutions were 18.23 ± 3.30 ng/mL and 6.26 ± 0.23 ng/mL, respectively. Moreover, NPs generated about 2-fold higher brain concentration relative to i.v. administration (8.56 ± 0.33 ng/mL). The same group of investigators developed neurotoxin-II (NT-II)-loaded PLA NPs coated with polysorbate-80 [129]. Brain concentrations of NT-II observed after intranasal administration of solution and NPs were 0.26 ±0.03 and 8.66 ± 0.30 ng/g, respectively. NPs generated more than 30-fold higher brain concentrations of NT-II relative to solution. Moreover, NPs generated about 3-fold higher brain concentration relative to i.v. administration (2.50 ±0.21 ng/g). These observations clearly indicate that PLA NPs coated with polysorbate-80 have significant potential to enhance the antinociceptive activity of these peptides after intranasal delivery. Recently, Liu et al. demonstrated the transport of wheat germ agglutinin-modified PEG-PLA NPs into the brain following intranasal administration [130]. Ex vivo imaging analysis clearly displayed that olfactory and trigeminal nerve pathways were primarily responsible for transport of NPs to brain. Investigators also proposed that the brain transport was possibly due to extracellular transport along the nerve fibers.

Lactoferrin receptor has been reported to be overly expressed on the luminal surface of respiratory epithelium, brain capillary endothelial cells and neurons [131]. Moreover, it has been observed to be highly expressed in CNS neurodegenerative conditions such as Alzheimer's, Parkinson's and Huntington's disease. Such high expression could be useful in generating lactoferrin receptor targeted delivery of polymeric NPs to promote absorption of

proteins and peptides in these tissues. Lactoferrin functionalized PEG-co-PCL NPs exhibited significantly higher brain accumulation of coumarin-6 relative to unmodified NPs [131]. $AUC_{0\text{-}8h}$ of coumarin-6 generated by lactoferrin-modified NPs in rat cerebrum, cerebellum, olfactory tract, olfactory bulb and hippocampus were 1.36, 1.53, 1.70, 1.57 and 1.23-times higher relative to unmodified NPs. Moreover, these functionalized NPs encapsulating a neuroprotective peptide -NAPVSIPQ produced remarkable memory improvement effects at relatively lower dose than unmodified NPs. These observations demonstrate the utilization of nanoparticulate based targeted approach to improve brain delivery and efficacy of proteins and peptides. Recently, Mistry *et al.* demonstrated that polystyrene or polysorbate-coated polystyrene NPs of size 100 nm were primarily located in olfactory epithelial cells but were absent in olfactory bulb in mice [132]. Hence, investigators hypothesized that the average nanoparticulate size required for optimal axonal transport may be less than 100 nm.

Pulmonary Delivery

Pulmonary route is one of the most commonly investigated non-invasive routes to improve absorption of proteins and peptides. This route provides numerous advantages including enormous absorptive surface area (100 m2), high vascularization, thin alveolar epithelial membrane (0.1-0.2 μm) and low enzymatic activity [109]. Despite these advantages, several factors may regulate pulmonary protein and peptide absorption. Central airway epithelium is largely constituted of ciliated columnar cells which expresses tight intercellular junctions. These tight junctions may significantly limit paracellular transport of proteins and peptides. Diffusion rate in this region is directly proportional to concentration gradient and lipid solubility. However, diffusion is also observed to be influenced by molecular size and ionization capabilities of the proteins and peptides [109]. Mucus binding and subsequent clearance via mucociliary apparatus may also reduce concentrations available for absorption. Particle wettability, aggregation, crystallinity, polymorphism and susceptibility towards enzymatic degradation may also determine absorption rate of proteins and peptides. Importantly, the deposition in the respiratory tract significantly depends on the aerodynamic properties of inhaled particles and breathing patterns [133-135]. Particles with aerodynamic diameter between 5 to 9 μm are deposited by impaction in bronchial airways. Fast breathing patterns may result in deposition of 3 to 6 μm particles in similar mechanism as above. Gravitational sedimentation results in deposition of 1 to 5 μm particles in smaller airways. Importantly, particles with aerodynamic diameter less than 3 μm might be deposited in respiratory bronchioles. Slow breathing patterns can promote absorption of particles in these ranges. Particles with aerodynamic diameter of less than 500 nm can easily be deposited in alveoli through Brownian diffusion however; there are chances that small particles can be exhaled significantly. Despite these challenges, omalizumab, α1-Antitrypsin, cyclosporine A, IFN (α, β and γ), IL-2 and IL-4 mutein are some of the inhaled (pulmonary delivery) proteins that are in ongoing clinical trials [136].

Several polymeric NPs have been previously explored to improve systemic absorption of proteins and peptides following pulmonary administration. Alpha 1-antitrypsin-loaded PLGA NPs have been anticipated as a promising formulation for the treatment of respiratory diseases [137]. NPs were spherical in shape with an average size of 100-1000 nm and entrapment efficiency of around 90%. PLGA (50:50) NPs released 60% of alpha 1-

antitrypsin in 8 h however; an initial burst release of about 30% was observed. Mannitol based dry powder of insulin-loaded CS NPs has been suggested as an excellent formulation for local and systemic delivery of proteins and peptides [138]. Glycol and thiolated glycol CS NPs significantly enhanced the pharmacological availability of calcitonin following pulmonary administration [16]. Calcitonin-loaded thiolated and non-thiolated CS NPs generated availability of 40% and 27%, respectively. A marked hypocalcemic activity was observed for a period of 24 and 12 h with thiolated and unmodified CS NPs. Particles were of size 230 to 330 nm and demonstrated high calcitonin entrapment efficiency. Following intratracheal administration, thiolated CS NPs displayed 2-fold higher mucoadhesion relative to glycol CS NPs. Pulmonary delivery of modified gelatin based NPs has been proposed as an excellent delivery system to enhance insulin absorption [139]. Recently, Wanakule *et al.* suggested an enzyme responsive NPs entrapped microgel system as a promising formulation for pulmonary delivery of proteins and peptides [140]. This delivery system was proposed to overcome challenges such as alveolar macrophage uptake, non-specificity, low respirable fractions and poor deposition in deeper lung regions. Microgel system was formulated with a new Michael addition during w/o emulsion method and displayed a highly porous structure and optimal aerodynamic properties for deposition in alveolar region.

Mechanofusion™ is an excellent technique to impart remarkable inhalation properties to lyophilized nanoparticulate systems [141]. Nanocomposites were developed by loading salmon calcitonin adsorbed PLGA nanospheres on inhalable lactose (Pharmatose325M™) in Mechanofusion apparatus at 372 rpm for 30 min. This process improved the inhalation efficiency of lyophilized powder probably due to excellent powder flow ability and de-agglomeration. The respirable fraction of NP composites and nanospheres were 34.9 ± 1.5 and 12.4 ± 13.1, respectively. Importantly, nanocomposites generated superior hypocalcemic effects relative to salmon calcitonin solution and nanospheres. Following intratracheal administration of nanocomposites, approximately 50% (w/w) of nanospheres were observed to be deposited in alveoli region. However, nearly 60% of nanospheres were eliminated within 1 h of nanocomposite administration. To overcome this problem, CS-modified PLGA nanocomposites were developed by the same group of investigators [142]. Modification of PLGA with CS was performed to improve mucoadhesive properties to the nanospheres. Salmon calcitonin nanocomposites were prepared with spray drying fluidized bed granulation (Agglomaster™) and dry powder coating (Mechanofusion™) techniques. Nanocomposites developed by these processes demonstrated excellent inhalation efficiency with 50% deposition in alveoli regions following intratracheal administration in rats. Interestingly, nanocomposites developed using Agglomaster™ technique exhibited excellent redispersibility, retention time and hypocalcemic effects relative to Mechanofusion™. Moreover, the hypocalcemic effect generated by nanocomposites developed using Agglomaster™ technique were more significant relative to non-modified nanocomposites and nanocomposites developed using Mechanofusion™. These observations clearly indicate the suitability of applying nanocomposite systems developed with Agglomaster™ technique for pulmonary protein and peptide delivery.

Parenteral Delivery

Parenteral route has attracted considerable attention for nanoparticulate delivery as it can avoid first-pass metabolism, provide highest bioavailability and active targeting for drug delivery [143]. However, NPs properties such as size, charge, dissolution rate and surface coating can significantly alter the *in vivo* fate [143, 144]. Moreover, NPs with size greater than 200 nm or slow dissolution rate can be rapidly cleared by mononuclear phagocytic system [145]. This process might significantly enhance nanoparticulate uptake in liver and spleen. PEGylation has been proposed as a promising approach to evade reticuloendothelial system uptake [146-148]. In addition, the use of poly (N-vinyl-2-pyrrolidone) as a coating material to improve blood circulation time of particulate carriers has also been reported [149]. Interestingly, NPs with size between 100-300 nm can highly accumulate in tumors due to enhanced permeability and retention effect [143, 150]. For systemic to brain administration, BBB poses a formidable barrier for brain transport of proteins and peptides (Fig. 4). Brain capillary endothelial cells (BCECs) express tight intracellular junctions and occlude paracellular spaces. Moreover, BCECs are also encircled by astrocytic end feet and pericytes which further forms an implausible barrier for protein and peptide transport. Hence, there is an imperative need for the development of drug delivery systems capable of overcoming BBB following systemic administration.

In recent years, several polymeric NPs have been investigated to target poorly permeable tissues such as brain and lung following systemic administration. Lactoferrin-modified PEG-PLGA NPs have been investigated for their efficacy to treat Parkinson's disease [151]. Urocortin-loaded NPs were spherical in shape and exhibited size of 120 nm. *In vivo* studies clearly demonstrated that i.v. injection of urocortin-loaded NPs significantly reduced striatum lesions caused by 6-hydroxydopamine. Angiopep-2 peptide and EGFP-EGF1 protein functionalized PEG-PCL NPs have been developed by Huile *et al.* [152]. These functionalized NPs demonstrated remarkable potential in penetrating BBB (angiopep-2) and binding to neuroglial cells (EGFP-EGF1). Moreover, these dual functionalized NPs generated significantly higher brain accumulation relative to unmodified NPs. These targeting potential of angiopep-2 peptide and EGFP-EGF1 protein represents a novel promising formulation for the treatment of neuroglial related diseases following systemic administration. A novel CS based NPs loaded with caspase inhibitor N-Benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethyl ketone (Z-DEVD-FMK) or basic fibroblast growth factor have been observed to rapid transport across BBB following systemic circulation [153, 154].

Recently, Bao et al. developed OX26 functionalized hyperbranched polyglycerol-conjugated PLGA to improve brain delivery of endomorphins [155]. NPs were spherical in shape with an average size of 170 ± 20 nm. Drug loading and entrapment efficiency observed were 8.65 ± 1.27% and 83.96 ± 2.60 %, respectively. Nearly, 65% of endomorphin was released in first 72 h. Moreover, OX26-modified NPs generated significantly higher analgesic activity in chronic constriction injured rats relative to unmodified NPs and endomorphin itself, following i.v. injection. Xia et al. demonstrated penetratin (CPP with low cationic amino acid content) modified polyethylene glycol)-poly(lactic acid) NPs as an excellent brain drug delivery system compared to protamine (high arginine content) modified NPs [156]. Such

systems can be utilized for improving brain protein or peptide delivery following systemic administration. RVG29- and CS-conjugated pluronic-based nanocarrier has been proposed as a promising formulation for improving brain delivery of proteins such as β-galactosidase [157]. B6 peptide (CGHKAKGPRK) functionalized PEG-PLA NPs have been demonstrated as an excellent delivery system for the brain delivery of neuroprotective peptide-NAPVSIPQ (NAP) [158]. NAP-loaded B6 NPs were 118.3 ± 7.8 nm in size with entrapment and loading efficiency of 0.65 ± 0.021% and 0.57 ± 0.023%, respectively. NAP-loaded B6 NPs demonstrated remarkable improvement in learning impairment recovery, loss of hippocampal neurons, and cholinergic disruption at significantly lower doses.

The shape of NPs has been observed to affect the hydrodynamic properties and interactions with target tissues significantly [159]. Moreover, size, surface charge and modification (targeting ligand) can significantly influence the uptake mechanism at BBB [160]. This process in turn affects the endocytotic pathway mechanisms and therefore, intracellular fate of NPs. Such information might be valuable for brain specific delivery of NPs following i.v. administration.

Transdermal Delivery

Transdermal delivery offers several advantages such as enormous surface area, avoidance of hepatic first-pass, continuous and non-invasive administration and patient compliance. Moreover, skin is easily accessible and delivery can be controlled and terminated whenever required. However, transdermal protein and peptide delivery is limited by penetrative and enzymatic barriers [161]. Large size and hydrophilic nature forbid the passive diffusion of proteins across the skin. Several endopeptidases and exopeptidases are also expressed in epidermal and dermal layers of the skin which may significantly degrade proteins and peptides. Several formulation and permeation enhancing approaches have been studied for improving transdermal delivery. Among formulation approaches, encapsulation of a protein or peptide in polymeric NPs seems to be an attractive approach for enhancing transdermal delivery. However, intact polymeric NPs are only able to penetrate into the superficial layers of the stratum corneum [162]. Hence, for efficient delivery of polymeric NPs, permeation enhancers, iontophoresis or sonophoresis may be needed to facilitate permeation through stratum corneum. In a recent study, Rastogi *et al*. have studied the potential of transdermal electroporation of insulin-loaded nanocarriers for insulin delivery [163]. Insulin-loaded PCL-PEG-PCL NPs were prepared by w/o/w double emulsion solvent evaporation method. Insulin entrapment efficiency was observed to be 32.9 ± 7.6% with an average NPs diameter of about 85 ± 9.4 nm. The efficacy of electroporation of insulin as solution and NPs was compared both *in vitro* and *in vivo*. Electroporation of NPs resulted in 4-fold higher insulin deposition in rat skin relative to solution. *In vivo* efficacy was evaluated in streptozotocin-diabetic male Wistar rats. This studies demonstrated 77 ± 5% (87.2 ± 6.4 mIU/mL, t = 2 h) and 85 ± 8% (37.8 ± 10.2 mIU/mL, t = 4 h) reduction in blood glucose levels with therapeutic levels maintained for 24 and 36 h for solution and NPs, respectively. These results demonstrate the efficiency of electroporation of nanosystems as an alternative to injectable insulin administration.

Ocular Delivery

Ocular route has been utilized for both systemic and localized delivery of proteins and peptides. For systemic delivery, ocular route provides advantages such as ease of administration, avoidance of first-pass metabolism and comparatively rapid rate of absorption (over oral administration) [164]. Systemic absorption of several proteins and peptides including insulin, calcitonin, and enkephalins has been studied following ocular administration [165-167]. Currently, ocular route is mainly used for the delivery of proteins and peptides for the treatment of local ocular disorders such as age related macular degeneration, dry eye disease, or proliferative diabetic retinopathy. Lucentis® and Eylea® are the recently marketed proteins intended for the treatment of ocular diseases [9, 168]. The physiological and anatomical barriers and enzymatic degradation within the ocular environment limit the efficacy of proteins and peptides administered by ocular route [169]. Polymeric NPs have been successfully designed to overcome these barriers and improve ocular bioavailability of proteins and peptides. Polymeric NPs can improve localized ocular delivery by providing sustain release, protection from enzymatic degradation and enhancing precorneal residence time compared to aqueous eye drops. However, NPs may be eliminated rapidly from precorneal pocket. Hence, for topical administration it is highly desirable to formulate NPs with mucoadhesive properties to increase their retention time in the cul-de-sac. PEG, carbopol and hyaluronic acid have been utilized to improve NPs precorneal residence time [170-172].

The prolonged residence time allows optimal contact between the formulation and mucosa and thereby sufficient drug concentration in external ocular tissues can be achieved. In a recent study, coating of cyclosporine A (CsA)-loaded poly caprolactone (PCL)/ benzalkonium chloride (BKC) nanospheres with hyaluronic acid resulted in high concentrations of cyclosporine A into the cornea compared with non-coated NPs [172]. Cationic polymers such as Eudragit® have also been employed to increase precorneal residence time. It was observed that Eudragit® can prolong the residence time of NPs by interacting with the anionic mucins present in the mucus layer at the eye surface. Aksungur et al. evaluated efficiency of Eudragit® in improving effectiveness of CsA NPs formulations against inflammation of the eye surface [171]. The NPs were prepared using either PLGA alone or a mixture of Eudragit® RL with PLGA or were coated with Carbopol®. Tear kinetic parameters were determined following topical application of CsA-loaded NPs suspension and RestasisA® (drug-loaded emulsion) in rabbit eye (Table 4). Cellular uptake, tear film concentration of the drug and $AUC_{0\rightarrow 24h}$ were significantly higher for PLGA: Eudragit® RL (75:25)-CsA NPs (cationic NPs) and Carbopol® coated PLGA-CsA NPs (adhesive) formulations compared to Restasis A®. hese results clearly demonstrate the effect of surface characteristics of NPs in the improvement of ocular retention and bioavailability.

Even though topical administration is the major route for the anterior segment conditions, it is difficult to deliver proteins and peptides to posterior ocular tissues via topical route. Currently, intravitreal injection is the most commonly employed technique to treat posterior ocular diseases. However, short vitreal half-life of proteins and peptides and chronic nature of most of the posterior ocular diseases lead to requirement of frequent intravitreal injections. These administrations are usually associated with potential undesired side effects

such as increased risk of cataract development, vitreous hemorrhage, retinal detachment, and endophthalmitis leading to poor patient acceptance. Several research studies have demonstrated suitability of intravitreally injected NPs in the treatment of posterior segment ocular diseases. In a recent study, Kim *et al*. have developed polylactic acid/polylactic acid-polyethylene oxide NPs (PLA/PLA-PEO) for the delivery of C16Y (an integrin-antagonist peptide) to the sub-retinal space for the treatment of choroidal neovascularization (CNV) [173]. Efficacy of C16Y-NPs was evaluated in laser-induced CNV in rats. A single intravitreal administration of both C16Y peptide and C16Y-NPs inhibited CNV at 5 and 9 days post laser photocoagulation. However, CNV inhibition was significantly higher for C16Y-NPs than the C16Y peptide solution on day 5. Because of short intravitreal half-life of C16Y peptide, inhibition of the experimental CNV was less with the peptide solution as compared to C16Y-NPs. On the contrary, the prolonged C16Y peptide release from C16Y-NPs aided to more CNV inhibition following intravitreal injection. These results demonstrate the potential of intravitreally injected biodegradable polymer-based NPs for treating choroidal neovascularization related to age-related macular degeneration.

Conclusion

Proteins and peptides have attracted considerable attention in the treatment of various chronic diseases due to their high potency and specificity. However, physicochemical properties of proteins and peptides and complex physiology of the non-invasive routes pose significant challenges for site specific delivery of these macromolecules. In recent years, polymeric NPs have demonstrated considerable potential in promoting absorption of macromolecules via non-invasive routes. An ideal polymeric nanoparticulate system should be capable of generating high loading and entrapment efficiency, protecting protein integrity until it reaches the target site and releasing the encapsulated protein or peptide in a sustained manner to avoid frequent administration. Recent advancements in biotechnology and newly emerging targeting ligands have significantly boosted interest in developing novel polymeric NPs system for systemic and local delivery of proteins and peptides. Importantly, surface conjugation of polymeric NPs with targeting ligands such as antibodies and cell penetrating peptides may be further explored to improve the effectiveness of formulation. Surface modification such as coating with mucoadhesive polymers or co-administration of protease inhibitors may significantly enhance the efficacy of polymeric NPs for nasal and pulmonary delivery. Development of novel polymeric NPs to enhance bioavailability of proteins and peptides remains an active field of research. Further studies are still required to develop novel targeted NPs formulation for site specific and sustained release of proteins and peptides in a non-invasive patient complaint manner.

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Abbreviations

PASP	Poly(L-aspartic acid)
BBB	Blood-brain barrier
BCECs	Brain capillary endothelial cells
BSA	Bovine serum albumin
CKS9	CKSTHPLSC targeting peptide
CNV	Choroidal neovascularization
CPP	Cell-penetrating peptide

CS	Chitosan
CsA	Cyclosporine A
CSK	CSKSSDYQC cell targeting peptide
CS-TGA	Thiolated Chitosan -thioglycolic acid
DS	Dextran sulfate
DTPA	Diethylene triamine pentaacetic acid
FAE	Follicle-associated epithelium
GIT	Gastrointestinal tract
HIP	Hydrophobic ion-pairing
IBD	Inflammatory bowel disease
KPV	Tripeptide Lys-Pro-Val
NT-I	Neurotoxin-I
LMWP	Low-molecular-weight protamine
OVA	Ovalbumin
PAA	Polyacrylic acid
PCL	Polycaprolactone
PD	Pharmacodynamic
PK	Pharmacokinetic
PLA	Poly (lactic acid)
PLA-PEO	Polylactic acid-polyethylene oxide
PLGA	Poly (DL-lactide co-glycolide)
PMMA	Poly(methyl methacrylate)
PNIPPAm	Poly-N-isopropylacrylamide
SOD	Superoxide dismutase
TMC	Trimethylchitosan
TMC-Cys	Trimethyl chitosan-cysteine conjugate
TPP	Tripolyphosphate

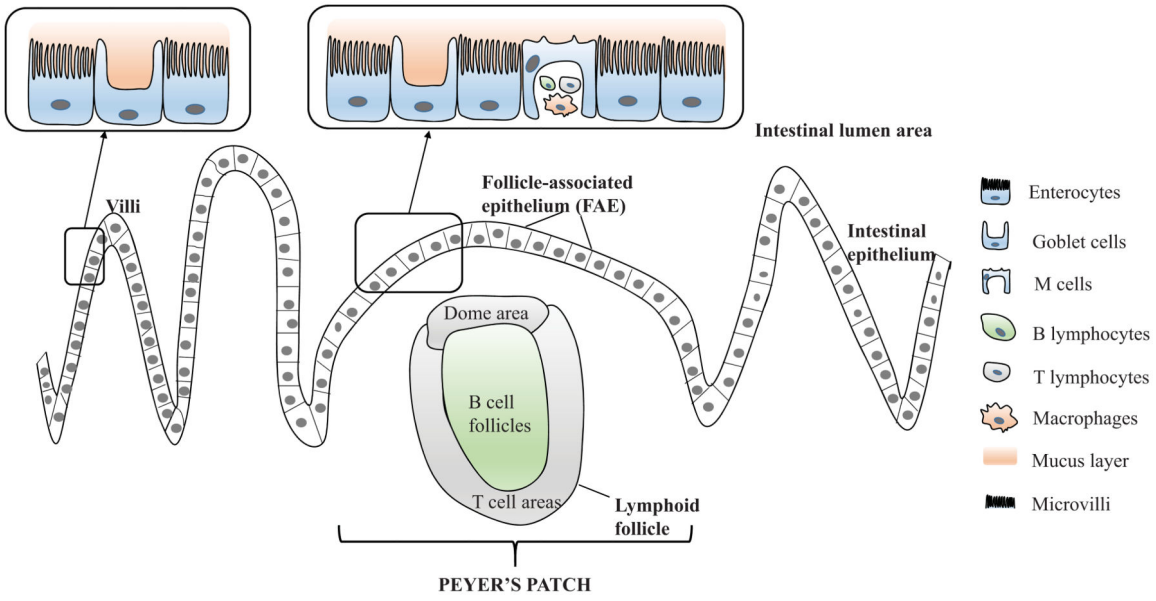

Figure 1.

Diagrammatic representation of intestinal epithelial cells with Peyer's patch, lymphoid follicle and overlying follicle-associated epithelium (FAE); Cells represented in Peyer's patch include enterocytes, goblet, lymphocytes and M cells.

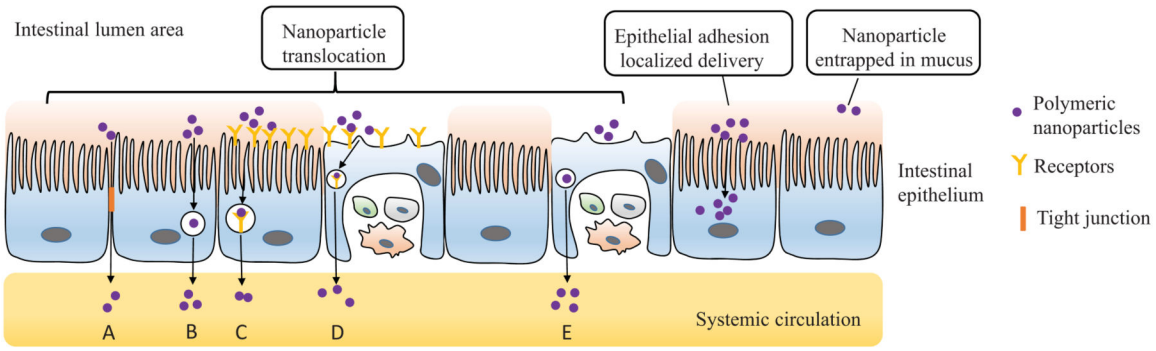

Figure 2.

Possible interactions of NPs with the intestinal barrier and the different transport mechanisms of NPs uptake: (**A**) Non-receptor mediated transcellular transport (**B**) Paracellular transport (**C**) Receptor-mediated transcellular endocytosis (**D**) Receptor and M cell medicated phagocytosis (**E**) Non-receptor M cell mediated phagocytosis.

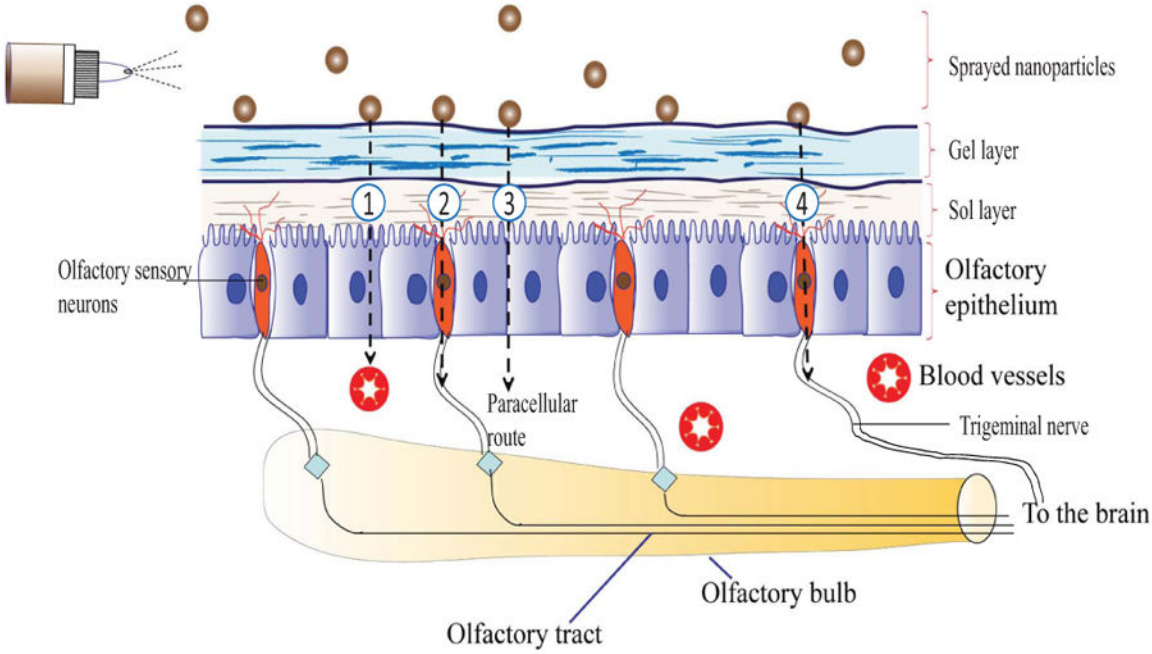

Figure 3.

Various transport mechanisms to brain following intranasal administration: (1) Nasal vasculature into the systemic circulation (2) Olfactory tract into the cerebrospinal fluid (CSF) and brain (3) Paravascular route into the brain parenchyma and (4) Trigeminal nerve pathways to the brain stem.

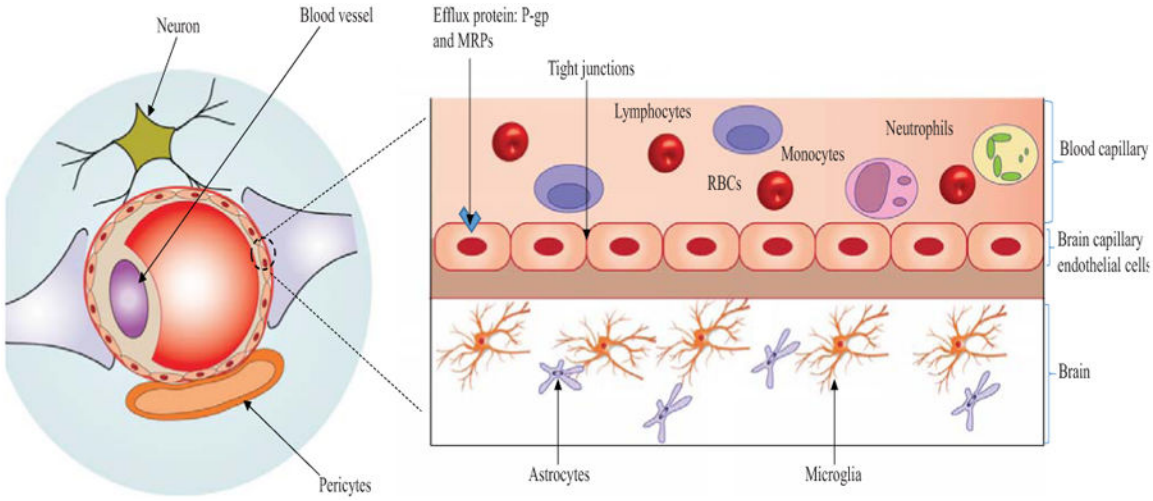

Figure 4.

A schematic representation of BBB. Note: Paracellular diffusion is highly restricted for proteins and peptides due to expression of tight junction. Carrier mediated transport and receptor/adsorptive mediated endocytosis are the major pathways for transport of proteins and peptides across BBB.

Table 1

Recently approved protein and peptide therapeutics [9]

Protein or peptide	Brand name, manufacturer	Year of FDA approval	Molecular weight	Route of administration	Half-life	Target disease
Ziv-aflibercept	Zaltrap®, Regeneron and Sanofi	2012	115 kDa	Intravenous infusion	4-7 days	Metastatic colorectal cancer
Ocriplasmin	Jetrea®, ThromboGenics Inc.	2012	27.2 kDa	Intravitreal injection	NA	Symptomatic vitreomacular adhesion
Raxibacumab	Abthrax®, GlaxoSmith Kline	2012	146 kDa	Intravenous infusion	16-19 days	Inhalational anthrax
Belimumab	Benlysta®, Human Genome Sciences, Inc.	2011	147 kDa	Intravenous infusion	19.4 days	Systemic lupus erythematosus
Ipilimumab	Yervoy ®, E.R. Squibb & Sons, L.L.C	2011	148 kDa	Intravenous infusion	15.4 days	Unresectable or metastatic melanoma
Belatacept	Nulojix®, E.R. Squibb & Sons, L.L.C	2011	90 kDa	Intravenous infusion	8-10 days	Prophylaxis of organ rejection (kidney transplant)
Brentuximab vedotin	Adcetris®, Seattle Genetics, Inc.	2011	153 kDa	Intravenous infusion	4-6 days	Hodgkin lymphoma and systemic anaplastic large cell lymphoma
Asparaginase *Erwiniachryanthemi*	Erwinaze, Jazz Pharmaceuticals, Inc.	2011	35 kDa	Intramuscular injection	16 h	Acute lymphoblastic leukemia
Aflibercept	Eylea®, Regeneron Pharmaceuticals, Inc.	2011	115 kDa	Intravitreal injection	5-6 days	Neovascular (Wet) age-related macular degeneration (AMD), Macular edema following central retinal vein occlusion (CRVO)
Velaglucerase alfa	Vpriv®, Shire US Manufacturing Inc.	2010	63 kDa	Intravenous infusion	11-12 min	Type 1 Gaucher disease
Tesamorelin	Egrifta®, EMD Serono, Inc.	2010	~5 kDa	Subcutaneous injection	26-38 min	Lipodystrophy
Tocilizumab	Actemra®, Genen-tech, Inc.	2010	148 kDa	Subcutaneous injection and Intravenous infusion	6.3 days	Rheumatoid and systemic juvenile idiopathic arthritis
Collagenase clostridium histolyti-cum	Xiaflex®, Auxilium Pharmaceuticals, Inc.	2010	~113 kDa	Intralesional injection	NA	Dupuytren's contracture
Alglucosidase alfa	Lumizyme®, Genzyme Corporation	2010	109 kDa	Intravenous infusion	2.4 h	Pompe disease
Denosumab	Prolia®, Amgen Inc.	2010	147 kDa	Subcutaneous injection	25.4 days	Postmenopausal osteoporosis
IncobotulinumtoxinA	Xeomin, Merz Aesthetics. Inc.	2010	150 kDa	Intramuscular injection	NA	Cervical dystonia
Pegloticase	Krystexxa®, Savient Pharmaceuticals, Inc.	2010	540 kDa	Intravenous infusion	NA	Chronic gout

NA: not available, Source: labels.fda.gov.

Table 2
Barriers to protein and peptide delivery and advantages of polymeric NPs

Route of Administration	Advantages	Barriers	Advantages of polymeric NPs
Oral	• High patient compliance	• Low permeability through GIT epithelia • Degradation by proteolytic enzymes • Instability at acidic pH in stomach	• Enhancement of oral absorption • Improved bioavailability • Prolonged residence time in the intestine • Sustained drug release • Enhanced stability in the GIT
Ocular	• Ease of administration • Avoidance of first-pass metabolism	• Poor permeability • Enzymatic degradation • Nasolacrimal drainage	• Protection from enzymatic degradation • Prolonged residence time in cul-desac • Lower drug loss due to tear turnover • Sustained drug release
Transdermal	• Avoidance of first-pass metabolism • Large surface area • Ease of application	• Poor permeability across stratum corneum	• Accumulation in the hair follicles creating high local concentrations of loaded drugs
Parenteral	• High bioavailability and rapid onset of action	• Rapid plasma degradation and generally poor patient compliance	• Protection from enzymatic degradation • Enhanced residence time in the plasma • Site specific delivery via targeting ligands
Pulmonary	• Large surface area • Highly vascularized mucosa • Porous endothelial membrane • Lower enzymatic activity • No first-pass metabolism	• Pattern of deposition and size distribution depending on delivery device • Mucociliary clearance mechanisms	• Improved absorption • Sustained release • Minimal enzymatic degradation of encapsulated proteins • Target specificity via surface modifications

Route of Administration	Advantages	Barriers	Advantages of polymeric NPs
Nasal	• Highly vascularized mucosa • Porous endothelial membrane • Lower enzymatic activity • Direct brain delivery • Avoidance of first-pass metabolism	• Mucociliary clearance	• Improved systemic and brain absorption • Sustained release • Minimal enzymatic degradation of encapsulated proteins

GIT: Gastrointestinal tract.

Table 3
tRecent developments in polymeric NPs based oral proteins and peptides delivery

Polymer	Protein or Peptide	Results	Ref.
PLGA	Insulin	In case of NPs, six fold higher oral bioavailability relative to plain insulin was noted in healthy rats. An equivalent blood glucose lowering effect from a 120 IU/kg oral dose of insulin NPs and 20 IU/kg subcutaneous dose of insulin solution in diabetic rats was observed.	[80]
PLGA-CS	Insulin	Positively charged (+43.1 ± 0.3 mV) CS-PLGA-NPs exhibited stronger bioadhesive potency than negatively charged PLGA-NPs and much higher relative pharmacological availability for orally delivered insulin with no increase in toxicity.	[81]
PLGA-eudragit	Salmon-calcitonin	Size ranged from 179.7 to 308.9 nm with a polydispersity index between 0.051 and 2.75, and surface charges ∼ -11 to +6 mV. Polymer type was an important factor influencing the release characteristics and the *in vivo* hypocalcemic effect.	[82]
PLA-F127-PLA	Insulin	Blood glucose concentration of oral insulin-loaded PLA-F127-29 NPs decreased from 18.5 to 5.3 mmol/L after 4.5 h; the minimum blood glucose concentration (about 4.5 mmol/L) was shown within about 5 h; the blood glucose concentration was retained at this level for additional 18.5 h.	[83]
PCL/eudragit RS	Insulin, aspart-insulin	Insulin-loaded NPs composed of PCL/Eudragit RS preserved the biological activity of aspart-insulin. The postprandial peak suppression was prolonged more than 24 h compared to regular insulin working only 6-8 h.	[84]
PAA	Leuprolide	NPs increased relative oral bioavailability of leuprolide by 4.2-fold.	[13]
	Insulin	All polymers demonstrated great insulin complexation efficiency (78 and 93%). Quaternised PAA polymer showed lower cytotoxicity than PAA.	[85]
Thiolated PAA	Insulin	Thiolated PAA improved AUC of insulin by 2.3-fold compared with unmodified PAA, which further contributed to a blood sugar reduction of 22%.	[86]
CS	Salmon calcitonin	CS nanocapsules significantly reduced the serum calcium levels, and delayed the reduction for more than 24 h, irrespective of the type and molecular weight of CS.	[87]
	Insulin	The relative bioavailability of insulin was found to be approximately 20%. These results suggest that the formulation developed in the study might be employed as a potential approach for the oral delivery of insulin.	[88]
	Insulin	In the pharmacodynamic (PD) and pharmacokinetic (PK) evaluation in a diabetic rat model, the orally administered aspart-insulin-loaded NPs produced a slower hypoglycemic response for a prolonged period of time, whereas the SC injection of aspart-insulin produced a more pronounced hypoglycemic effect for a relatively shorter duration.	[89]
CS/HPMCP	Insulin	In the pharmacodynamic (PD) and pharmacokinetic (PK) evaluation in a diabetic rat model, the orally administered aspart-insulin-loaded NPs produced a slower hypoglycemic response for a prolonged period of time, whereas the SC injection of aspart-insulin produced a more pronounced hypoglycemic effect for a relatively shorter duration.	[90]
CS/PGA-DTPA	Insulin	The oral intake of enteric-coated capsule containing CS/γPGA-DTPA NPs produced a prolonged reduction in blood glucose levels, with a maximum insulin concentration at 4 h and 20% relative oral bioavailability of insulin.	[91]

Polymer	Protein or Peptide	Results	Ref.
TMC-Cys	Insulin	TMC-Cys NPs improved insulin transport through rat intestine by 3.3-11.7 and 1.7-2.6 folds, enhanced Caco-2 cell internalization by 7.5-12.7 and 1.7-3.0 folds, and promoted uptake in Peyer's patches by 14.7-20.9 and 1.7-5.0 folds, as compared to insulin solution and TMC NPs, respectively.	[92]
Lauryl succinyl CS	Insulin	Modification of CS with both hydrophilic (succinyl) and hydrophobic (lauryl) moieties improved the release profiles, mucoadhesivity and the permeability of insulin.	[93]
Dextran-poloxamer-CS-albumin	Insulin	In the pharmacodynamic and pharmacokinetic studies with 50IU/kg nanoencapsulated insulin, 13% oral bioavailability indicated a 3- fold improvement in comparison to free insulin.	[94]
Dextran sulfate CS	rhHGF	rhHGF-loaded DS/CS NP showed potency of liver-targeting after oral administration.	[95]
Cholic acid (CA) modified dextran sulfate	Superoxide dismutase (SOD)	Higher degree of CA substitution in DS-CA can remarkably increase the cellular uptake of the loaded SOD.	[96]
Alginate/dextran sulfate/CS/albumin	Insulin	Permeation of insulin-loaded NPs was improved by 2.1-fold through Caco-2 cell monolayer, 3.7-fold through a mucus-secreting Caco-2/HT29 co-culture, and 3.9-fold through excised intestinal mucosa of Wistar rats, compared to insulin, respectively.	[97]
Alginate	Insulin	The association efficiency and loading capacities were optimized as high as 92% and 14.3%, respectively. Around 50% of the protein was partially retained by NPs in acidic gastric environment up to 24 h while around 75% of release was found under intestinal pH conditions.	[98]
Thiolated Eudragit	Insulin	The oral insulin-loaded Eul-cys NPs produced an increased and prolonged hypoglycemic action with 2.8-fold higher relative bioavailability of insulin (7.33 ± 0.33%) compared with Eul NPs (2.65 ± 0.63%).	[99]
PNIPPAm	Salmon calcitonin	All the NPs formulation (179.7 to 308.9 nm, -11 to +6 mV) showed efficient sCT encapsulation and release.	[100]

PNIPPAm: Poly-N-isopropylacrylamide; CS: Chitosan; DTPA: diethylene triamine pentaacetic acid (DTPA); TMC-Cys: Trimethyl chitosan-cysteine conjugate; PASP: poly(L-aspartic acid); PMMA: poly(methyl methacrylate); SOD: Superoxide dismutase.

Table 4

Tear pharmacokinetic parameters following topical application of CsA-loaded NPs suspension and RestasisA® (Reproduced with permission from reference [171])

Fornulation code	[a] $AUC_{0\rightarrow24h}$ (ng h/g)	[b] C max (ng/g)
P-CsA	490.42	126.12
P:E-CsA (75:25)	972.59	366.30
P:C-CsA	776.57	211.08
Restasis A®	514.24	299.02

[a] Area under the concentration-time curve between 0 and 24 h.

[b] Peak drug concentration (ng CsA/g tear).