Expert Opinion

Commercial challenges of protein drug delivery

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The discovery of insulin in 1922 marked the beginning of research and development to improve the means of delivering protein therapeutics to patients. From that period forward, investigators have contemplated every possible route of delivery. Their research efforts have followed two basic pathways: one path has focused on non-invasive means of delivering proteins to the body; and the second path has been primarily aimed at increasing the biological half-life of the therapeutic molecules. Thus far, the commercial successes of protein delivery by the nasal, oral and pulmonary routes have been more opportunistic rather than the application of platform technologies applicable to every protein or peptide. In several limited cases, sustained delivery of peptides and proteins has employed the use of polymeric carriers. More successes have been achieved by chemical modification using amino acid substitutions, protein pegylation or glycosylation to improve the pharmacodynamic properties of certain macromolecules. Today, commercial successes for protein and peptide delivery systems remain limited. The needle and syringe remain the primary means of protein delivery. Major hurdles remain in order to overcome the combined natural barriers of drug permeability, drug stability, pharmacokinetics and pharmacodynamics of protein therapeutics.

1. Introduction

The biotechnology boom has created many therapeutic proteins that are being used to treat diseases that were incurable 10 years ago. Formulating protein delivery systems so that they maintain their stability and remain within their efficacious and safe target doses remains a challenge. Two general approaches have been used in order to address the delivery of protein therapeutics. One technology set has focused on non-invasive means of transporting therapeutic proteins to the body. Two oral peptide products and three nasal products are approved in the US. Several pulmonary insulin products are in clinical trials. The second technology set is aimed at increasing the half-life of the molecules once administered. Several extended-release poly(lactide-co-glycolide) (PLGA) low-molecular-weight peptide products are marketed today. There are also several chemically altered protein products that have reached the market using technologies such as amino acid substitution and pegylation. The limited successes in protein drug delivery reflect the difficulty of these research and development efforts. Thus, the hypodermic needle and syringe still remains the most practical route for protein delivery despite many attempts to develop non-invasive systems or reduced frequency administration. Why has protein drug delivery been so difficult? This review focuses on those products that have indeed reached the market and examines the properties of those drugs and technologies that have allowed product approval and commercial marketing.

2. Early history of protein delivery

2.1 Blood transfusions

The history of protein drug delivery can probably first be traced to attempts to perform blood transfusions. The first successful blood transfusions on record took place in 1665 by Dr Richard Lower, an Oxford physician. He conducted his initial experiments with dog-to-dog transfusions. He kept an exsanguinated dog alive by connecting it to the carotid artery of a donor dog using a quill [1].

This was followed by additional attempts to conduct animal-to-human transfusions by various investigators in Europe in the late 1600s. The Paris Society of Physicians ultimately outlawed animal-to-human transfusions in 1678 because of numerous adverse reactions, many resulting in death. James Blundell, a British obstetrician, performed the first successful transfusion of human blood to a patient for the treatment of postpartum haemorrhage. He extracted a small amount of blood from a husband's arm and he successfully transfused the man's wife. Between 1825 and 1830 he performed 10 documented transfusions, 5 of which proved beneficial to his patients, and published these results. He also devised various instruments for performing blood transfusions [2]. Samuel Armstrong Lane in London conducted the first delivery of a clotting factor protein drug to treat a disease in 1840 when he performed a whole blood transfusion specifically to treat haemophilia [3].

2.2 Vaccination

Another early example of protein delivery was the development of the smallpox vaccine. Edward Jenner, in 1796, noticed that milkmaids developed blisters when milking cows that had cowpox, but these young women never seemed to get smallpox, even when their families and villages were dying of smallpox. Jenner eventually discovered that inoculation with this related cow virus offered protection against smallpox [4].

2.3 Insulin: the first pure therapeutic protein

Many of the most significant lessons in protein drug delivery systems have occurred using insulin as a model. There are many reasons for this, not the least being that insulin was the first pure protein therapeutic molecule discovered and that diabetes affects an increasing percentage of the world's population. The discovery of insulin by Frederick Banting and Charles Best in 1922 is naturally associated with the beginning of research aimed at finding optimal means to administer an exogenous protein to patients [5].

2.3.1 Early insulin delivery research

Soon after insulin's discovery, investigators attempted many different modes and routes of insulin administration. Aside from the subcutaneous route of injection, early investigators examined rectal, intestinal, intratracheal, peritoneal, vaginal, scrotal sac, oral, dermal, pulmonary and nasal routes with various degrees of limited success and mostly failure [6]. Other efforts focused on ways to increase the short half-life of insulin that necessitated multiple daily injections. Lewis reported unsuccessful attempts to sustain insulin release by injecting the hormone in oily suspensions, acacia solutions and lecithin solutions [7]. Other investigators attempted to administer insulin in the presence of adrenaline, posterior pituitary extracts and astringent metals. These attempts also proved to be unsuccessful. Hagedorn eventually showed that insulin combined with basic protein protamine resulted in continuous insulin action for 3 – 12 h [8]. This protamine–insulin combination had an altered isoelectric point of ~ 7.3 and dissolved slowly following subcutaneous injection. This was a clear improvement over the 7 – 15 min circulating half-life of insulin. Scott and Fisher later combined 1.5 mg of protamine and 0.20 mg of zinc per 100 units of insulin to form protamine zinc insulin [9]. It had an onset of action ~ 6 – 8 h after injection and lasted 24 h. A sustained daily requirement of insulin with a single injection was achieved when combined with regular, rapidly acting insulin. Hallas-Moller and others showed the unique interaction of proteins with metal ions in 1952 when he developed the Lente insulin by adding zinc to insulin in an acetate buffer. By using this technique, different particle sizes of insulin could be fabricated as having various periods of action in the range of 18 – 30 h [10]. The protein chemistry manipulations initiated for insulin for the purpose of improving pharmacodynamics were about to be impacted with the dawn of the biotechnology age.

3. Recombinant proteins

In 1974, the laboratories of Stanford geneticist Stanley Cohen and University of California San Francisco biochemist Herbert Boyer reported the expression of a foreign gene implanted in bacteria by recombinant DNA methods [11]. Cohen and Boyer showed that DNA could be sliced with restriction enzymes and reproduced by inserting the recombinant DNA into an *Escherichia coli* bacterium. The application of this technology to human therapeutics became feasible in 1977 when Genentech produced the first recombinant human protein: somatostatin [12].

A year later, Boyer's group inserted a synthetic version of the human insulin gene into the bacterium *E. coli*. In 1982 another milestone was reached when Genentech received approval from the FDA to market the first recombinant DNA drug: a genetically engineered human insulin. This early work seeded the development of today's growing biopharmaceutical industry.

4. The growth of the biotechnology industry

This pioneering work of recombinant DNA technology ultimately enabled the production of protein therapeutics at commercial scale. The biotechnology industry has been very productive ever since, as worldwide sales volume of the 10 major therapeutic protein classes amounted to 33.3 billion

Table 1. Top protein-based prescription drug brand by 2003 worldwide sales [14,15].

Drug name	2003 sales ($ MM)	Indication
Erythropoeitin & analogues	9451	Anaemia
Insulin	5264	Diabetes
Interferons	4709	Hepatitis C
LHRH analogues	2533	Prostate cancer
GCSF	2522	Neutropenia
Rituximab	2063	Non-Hodgkin
Infliximab	1729	Crohn's disease and rheumatoid arthritis
Glatiramer acetate	1418	Multiple sclerosis

GCSF: Granulocyte colony stimulating factor; LHRH: Luteinizing hormone releasing hormone.

dollars in 2002. This is a 24% increase over the 2001 annual sales of 26.9 billion dollars for protein and peptide drugs [13]. **Table 1** lists the largest protein-based drugs by 2003 sales volume. The significant growth of the protein and peptide drug market is projected to continue, as novel biopharmaceutical agents are targeting previously untreatable diseases. All these drugs are marketed in the injectable form and many of these drugs require daily or weekly injections. By the end of 2005, the patents for many of these protein therapeutics begin to expire, and life cycle management has become an inducement for biopharmaceutical companies to further improve these protein drug dosage forms.

5. Formulation challenges of protein drugs

Soon after the beginning of the biotechnology age it became apparent that protein biopharmaceutical development was significantly more difficult than traditional pharmaceutical drug development. The development of even small peptide-based therapeutics involves elaborate organic and synthetic chemistry. Molecular sizes of proteins are orders of magnitude larger than traditional pharmaceuticals, and they have secondary and tertiary structures which make them very susceptible to physical and chemical degradation. Proteins are easily denatured by heat or by agitation, and often go through structural changes when exposed to water and organic solvents. Consequently, they are frequently maintained at refrigerated temperatures along with stabilising additives for long-term storage. Proteins also need to be packaged in a sterile manner.

The physical size of protein drugs and their susceptibility to degradation are key determinants of their delivery route. Non-invasive delivery of proteins by the oral route would be very desirable, and there have been interesting efforts to develop oral protein formulations similar to the ones cited earlier with regard to insulin [6]. Unfortunately, these efforts have been hampered by low bioavailability [16].

5.1 Non-invasive routes

The oral, nasal and pulmonary routes have been the primary non-invasive routes of protein delivery investigated so far. This field of research remains active despite the observations that the bioavailability of peptides and proteins has proven to be very low in most of the non-invasive routes tested. The high cost of many of these complex molecules also may limit the number of protein drugs that would be economically feasible to deliver via these non-invasive routes.

5.2 Oral delivery of peptides and proteins

There have been numerous efforts to deliver protein molecules via the oral route. Enteric coatings and capsules can be used to protect the drug from the acidic environment of the stomach [17]. However, avoidance of proteolytic enzymes and absorption of these relatively large molecules through a membrane designed to actively uptake only single amino acids, di- or tripeptides presents many more challenges. Indeed, Joslin, Gray and Root and others unsuccessfully attempted to deliver insulin via the oral route immediately after the discovery of insulin [18].

Nobex Corporation and Emisphere Technologies have conducted interesting preclinical and clinical trials administering insulin via the oral route using proprietary enhancer molecules. In one study, postprandial glucose levels were controlled with a proprietary oral insulin formulation [19]. One little appreciated potential advantage of orally administered insulin is that it is delivered through the portal circulation. Those investigators involved in oral insulin delivery studies indicate that the first-pass metabolism of insulin by the liver is actually more physiological than subcutaneously delivered insulin. It is argued that lower peripheral insulin concentrations are observed with oral insulin compared with subcutaneous insulin. This may reduce the risk of hypoglycaemia, which is a common side effect of subcutaneous insulin administration.

In this study, the authors state that their proprietary oral insulin formulation was administered at doses of 0.5 and 1.0 mg/kg [19]. These oral insulin doses provided postprandial glucose control similar to 8 Units of subcutaneous injected regular insulin. One can calculate that at the lower dose of 0.5 mg/kg of oral insulin, 980 Units of insulin would be administered to a 70 kg subject (0.5 mg/kg x 70 kg x 28 Units/mg = 980 Units of insulin). The bioavailability of the oral insulin relative to subcutaneous insulin is thus ~ 0.81% (8 Subcutaneous International Units versus 980 Oral International Units). Therefore, an outstanding question remains as to whether the healthcare system or the pharmaceutical industry will be able to justify the significantly added cost of oral insulin at such low bioavailabilities.

5.2.1 Oral peptide products today

There are indeed several marketed oral peptide products. However, these molecules are significantly smaller than insulin, which has a molecular weight of 5808. Insulin is considered a relatively small protein. **Table 2** compares the

Table 2. Properties of oral peptide drugs.

Drug	Number of amino acids	Molecular weight (Daltons)	Daily oral dose (mg)	Bioavail-ability (%)
DDAVP	9	1183	0.050 – 0.80	0.16
Cyclo-sporin	11	1203	1500	30
Insulin (experi-mental)	51	5808	35 (estimated)	1 – 5(?)

DDAVP: Desmopressin acetate.

Table 3. Properties of nasal peptide drugs.

Drug	Number of amino acids	Molecular weight (Daltons)	Daily oral dose (µg)	Bioavail-ability (%)
DDAVP	9	1183	10 – 40	3.2
Synarel®	9	1322	400	2.8
Miacalcin®	32	3432	0.2	3

DDAVP: Desmopressin acetate.

properties of the two marketed oral peptide products and experimental insulin, their doses, molecular weights and oral bioavailabilities.

Desmopressin acetate (DDAVP) is a synthetic cyclic analogue of the natural pituitary hormone 8-arginine vasopressin: an antidiuretic hormone affecting renal water conservation. It is marketed by Aventis Pharmaceuticals, and is approved for the treatment of diabetes insipidus, in which the patient needs antidiuretic replacement therapy and primary nocturnal enuresis. The oral bioavailability of DDAVP is quite variable and ranges between 0.08 and 0.16%, but the dose ranges from 50 to 800 µg [20,21]. This low dosage combined with the relative ease of manufacturing this relatively small peptide compared with insulin helps to justify the marketing of the oral dosage form of this peptide drug, even with an oral bioavailability significantly < 1%.

Novartis and Roche Pharmaceuticals market an oral peptide drug that has some quite unusual properties. Cyclosporin is a small lipophilic cyclic polypeptide of 11 amino acids. Cyclosporin is indicated for the prophylaxis of organ rejection in kidney, liver and heart allergenic transplants. It is also approved for the treatment of psoriasis and rheumatoid arthritis. This peptide has a very unique set of chemical properties such that its oral bioavailability is 30% compared with intravenous injection [22]. The cyclic structure of the peptide helps protect it from proteolytic endopeptidases. The lipophilic properties of the compound favour its uptake from the intestinal mucosa. The oral formulation of cyclosporin immediately forms a microemulsion in an aqueous environment [23].

Thus, it is apparent that opportunities to succeed with the oral delivery of protein drugs are very limited. Only two small peptides with very unique properties allow them to be delivered by the oral route. In the case of DDAVP, the drug dosage is small enough to allow its 0.16% oral bioavailability to be a viable pharmaceutical product. In addition, it is a cyclic peptide whose structural features may inhibit intestinal degradation. The other case is cyclosporin, whose unique chemical and physical structure allows it to be delivered by the oral route with an unusually high bioavailability. It too is a cyclic peptide composed of hydrophobic amino acids. Therefore, one may conclude that the efforts to develop an oral formulation for a drug as large as insulin remain formidable if we compare that challenge with what is already known about the properties of cyclosporin and DDAVP.

5.3 Nasal delivery of peptides and proteins

Table 3 summarises the nasal-delivered commercial products available today. There are three peptide drugs currently marketed for systemic distribution via the nasal route. Novartis' Miacalcin® nasal spray is a calcitonin analogue with a molecular weight of 3432 Da. It is used in the treatment of osteoporosis. In clinical trials, it has been shown to reduce the incidence of vertebral fractures by > 50% in elderly women [24-26]. The bioavailability of the Miacalcin nasal spray is 3% compared with the injectable form, but the administered nasal dose is only 0.2 µg. It is clear that the small Miacalcin dosage is a key reason that allows it to be marketed despite its low nasal bioavailability.

Synarel® is the nasal form of the lipophilic luteinizing hormone releasing hormone (LHRH) agonist, nafarelin, marketed by Hoffmann-La Roche, Inc. and used to treat endometreosis. Like the orally delivered peptides, it has a relatively low molecular weight of 1322 Da. In one clinical study the bioavailability of a single dose of intranasal nafarelin was evaluated in 15 healthy female volunteers [27]. Each subject received a 400 µg intranasal and a 25 µg intravenous dose of nafarelin, separated by at least 7 days. Systemic bioavailability of nafarelin was in the range of 1.15 – 5.62% and averaged 2.82 ± 1.23%. Nafarelin was readily absorbed by the nasal mucosa, and therapeutic blood levels were rapidly achieved and maintained for a prolonged period of time. Nafarelin also contains hydrophobic amino acids that may enhance its nasal absorption. Nafarelin's bioavailability combined with its relatively low dose proved adequate enough to achieve the desired therapeutic effect because of its inherent high biological potency and its pharmacodynamic properties.

As described in the oral delivery section, DDAVP is a synthetic analogue of the natural pituitary hormone 8-arginine vasopressin: an antidiuretic hormone affecting renal water conservation. It has also been developed in a nasal dosage form. Its bioavailability is about 20-times higher via the nasal route compared with the oral route as seen in **Table 3** [28]. Like its oral form, the structure and relatively low dosage permit the commercialisation of the DDAVP nasal dosage form.
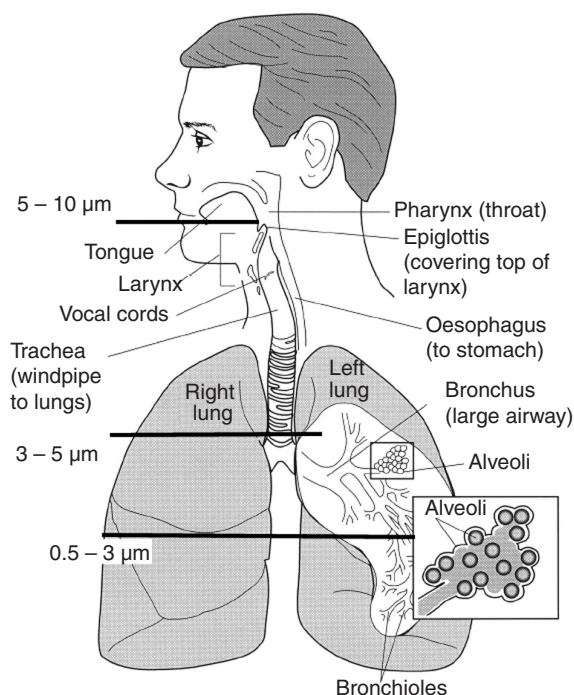
Figure 1. The respiratory tract and areas targeted by different sized particles.

There have also been experimental studies with nasal delivery of human growth hormone and insulin. In one study, the bioavailability of growth hormone was concluded to be low (3.8 – 8.9%) compared with intravenous administration [29]. In this study, the administered nasal doses were only 150 – 600 µg of human growth hormone using didecanoyl-L-α-phosphatidylcholine as a penetration enhancer.

Another clinical study determined the nasal route bioavailability of insulin to be about 5% using 1% (w/v) 9 lauryl ether as an enhancer [30]. Despite these measurable bioavailabilities, another study of nasal insulin in 31 diabetic patients concluded 'that due to low bioavailability and to a high rate of therapeutic failure, intranasal insulin treatment is not a realistic alternative to subcutaneous insulin injections at the present time' [31].

Thus, these studies and the summary of marketed nasally delivered peptides in **Table 3** support the limited capacity of the nasal mucosa to be a non-invasive route for peptide or protein delivery. All successfully delivered peptides through the nasal route are relatively low-molecular-weight moieties. The relatively low administered dosages range from 0.2 to 400 µg. Finally, chronic use of penetration enhancers to assist drug transport across the nasal mucosa also represents a potential safety concern for drugs that must be administered chronically.

5.4 Pulmonary delivery of proteins

Successful pulmonary insulin delivery was demonstrated soon after the discovery of insulin in 1925 [32]. Recent research has shown that the key to obtaining effective systemic delivery via

the lungs is to have the drug reach the alveoli or deep lung. In order to achieve this goal it has been determined that the aerodynamic particle size of the insulin must be in the 0.5 – 3 µm range [33]. **Figure 1** shows a diagram of the respiratory tract and indicates the targeted area of the lung for systemic absorption of proteins. Several companies, including Nektar, Alkermes and Aradigm, have shown have shown that aerosolised insulin can effectively be delivered to the alveolar region of the lung using a variety of devices and formulations in diabetic patients [34,35]. The bioavailability of pulmonary insulin in clinical trials has been estimated to be ~ 10% compared with subcutaneous injection [34]. More than 2000 patients have so far received inhaled insulin in clinical trials worldwide; some for as long as 5 years. Results from the Phase III clinical trials suggest that Nektar's Exubera® product may be as effective as injected insulin and superior to oral agents in lowering blood glucose in patients with diabetes. Clinical trials have shown that inhaled insulin also effectively controls postprandial glucose levels [34,35,37]. Many clinicians believe that the benefits of inhaled insulin for the treatment of diabetes will provide an effective alternative means for controlling plasma glucose. However, concerns have been raised about the safety of inhaled preparations and whether inhaled insulin will compromise lung capacity or damage lung tissue in long-term use. The Exubera product dossier was submitted to the European Medicines Evaluation Agency in March 2004 for marketing approval in Europe. In August 2004, the European regulatory authorities determined that Exubera was not licensable at this time and raised further major objections. The extended path to regulatory approval of inhaled insulin clearly illustrates the hurdles that innovative companies must endure in order to bring these novel products to market. Although no pulmonary insulin products have reached the market, several second-generation technologies are under development; for example, Epic Therapeutics, a Baxter Healthcare subsidiary, has developed a remarkably monodispersed, 1 – 3 µm formulation of insulin microspheres suitable for deep lung delivery. These microspheres are unique because they are virtually all insulin and they show excellent chemical stability over time. **Figure 2** shows the light scattering and time of flight particle size distribution of these insulin microspheres [36]. This same technology is also serving as a testing tool for the delivery of other systemic proteins via the lung, such as human growth hormone and α_1-antitrypsin for the treatment of chronic emphysema.

5.5 Additional early-stage pulmonary and oral technologies

Syntonix has linked the FcRn region of an antibody to large protein molecules. This apparently enables receptor-dependent uptake of these drugs. Further development of this approach may allow the possibility of more efficient transport of protein drugs across the epithelial cell barrier than diffusion-based technologies. A recent publication showed that FcRn-dependent absorption was more efficient in the upper

Light scattering: 95% particle volume between 0.95 and 1.2 µm

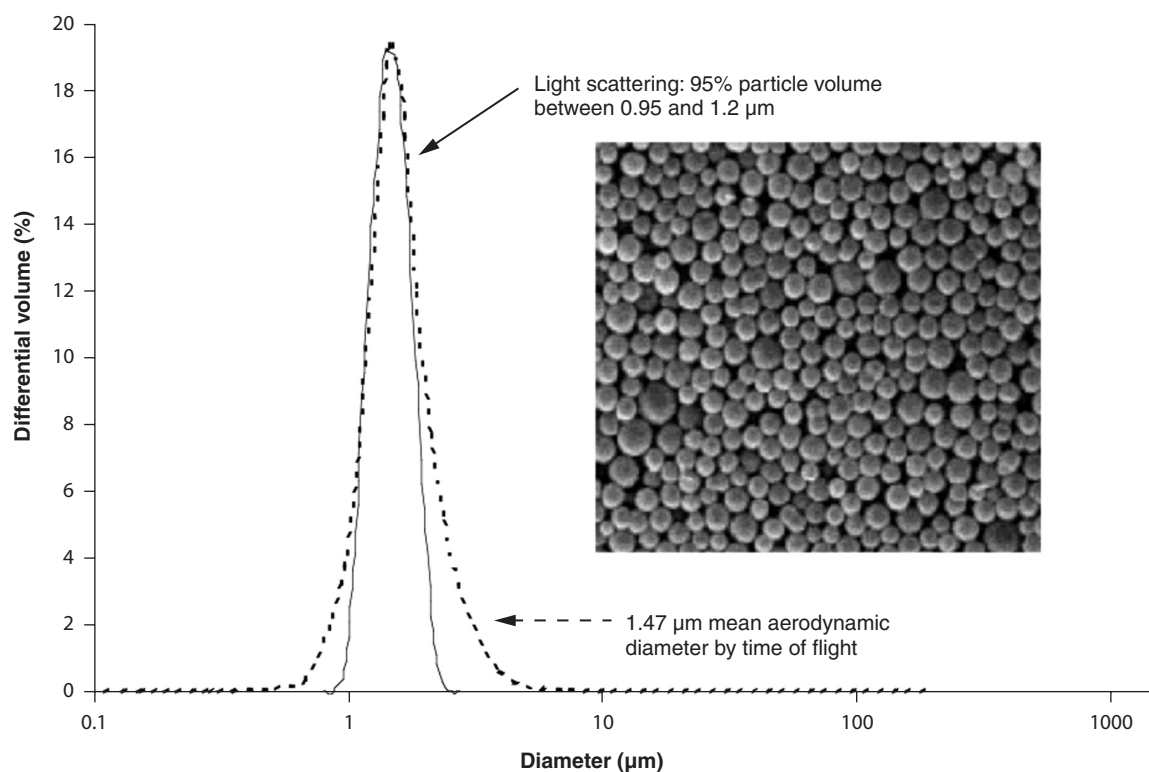1.47 µm mean aerodynamic diameter by time of flight

Figure 2. Scanning electron microscopy, light scattering and time of flight determination of particle size of insulin microspheres engineered for deep lung distribution.

and central airways of the lung where epithelial expression of FcRn is detected [38]. A recombinant 'monomeric-Epo' Fc fusion protein comprising a single molecule of Epo was conjugated to a dimeric Fc fusion protein. This fusion protein exhibited enhanced pharmacokinetic and pharmacodynamic properties. The bioavailability of the EpoFc monomer when delivered through the lung was approximately equal to that reported for unconjugated Epo delivered subcutaneously in humans. Buccal delivery of insulin has been the focus of Generex Biotechnology. They claim to have been able to obtain significant insulin transport through the buccal membrane in the mouth in early studies [39].

6. Polymeric protein delivery to increase half-life

Much of the initial research in the polymer-based protein drug delivery field was pioneered in the laboratory of Robert Langer at the Massachusetts Institute of Technology. The first problems addressed were the short biological half-lifes of these protein molecules. Techniques were developed to incorporate solid protein particles into a non-degradable polymer matrix that would slowly release the protein over an extended time. This was accomplished by low-temperature casting of solid protein particles into a polymer solution. Evaporation of the

polymer solvent left a matrix consisted of tortuous channels of protein that allowed extended release of protein drugs over time. **Figure 3** shows the long-term control of blood glucose concentrations in streptozotocin-induced diabetic rats using a single subcutaneous insulin matrix implant [40]. These non-erodible ethylene vinyl acetate copolymer insulin implants contained ~ 80 mg of insulin. These early studies demonstrated the feasibility of developing efficacious sustained-release therapies using polymeric delivery systems.

6.1 Clinical applications of sustained-release peptide delivery systems

A key consideration for all polymer matrix-based delivery systems is the selection of compounds with wide therapeutic indices. This is a direct result of the difficulty in producing commercially viable, injectable delivery systems that did not result in an initial 'burst' of drug release within the first few hours after administration [40]. Consequently, drugs were chosen for incorporation into polymeric injectable systems that were both effective at very low doses, and which had minimal or no untoward side effects at very high doses. These constraints limited the choice of peptide and protein compounds considerably. Furthermore, the maintenance of the peptide or protein drug's chemical stability while releasing at physiological temperature and pH is a major concern for the pharmaceutical
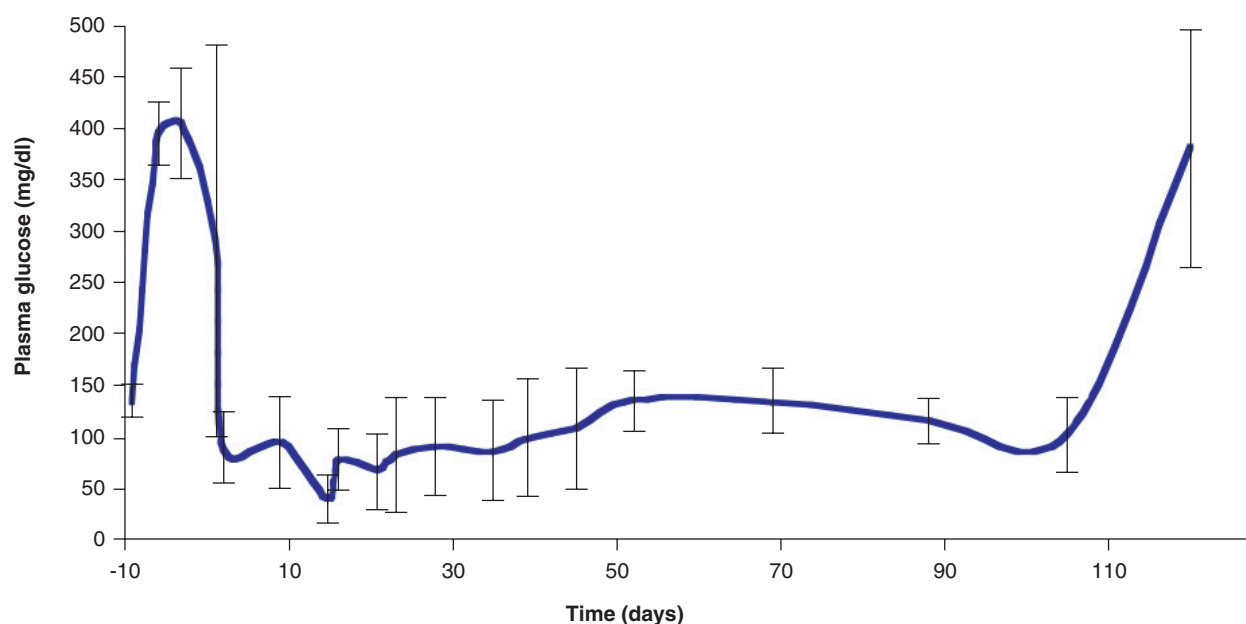
Figure 3. Long-term treatment of eight streptozotocin-induced diabetic rats by insulin polymer implant. Each implant contained ~ 80 mg of insulin.

industry who are responsible for bringing stable, reliable and reproducible products to market. The peptide and protein compounds that did indeed reach commercialisation include several LHRH agonists, octreotide and human growth hormone.

6.1.1 LHRH agonists

The first marketed sustained-released products utilised a biodegradable copolymer of lactic and glycolic acid (PLGA) to release LHRH agonists for the treatment of prostate cancer. LHRH is normally secreted in pulses; however, sustained release of LHRH results in the inhibition of steroid hormone release [41]. Prostate cancer is a steroid-dependent tumour; therefore, blocking release of testosterone results in shrinkage of the prostate tumour. The first such product was a relatively large 1.5 mm subcutaneous PLGA implant containing a LHRH analogue called goserelin. AstraZeneca markets this implant. The drug is delivered subcutaneously through a 14- or 16-gauge needle. Pain occurring at the injection site has been associated with this relatively large needle used to inject the implant [42]. In an attempt to improve patient comfort, Takeda Abbott Pharmaceuticals formulated a sustained-release PLGA microsphere that could be injected through 20-gauge or smaller bore needles.

There are several methods of incorporating protein or peptide molecules into PLGA microspheres. Solvent evaporation techniques are widely used [43]. The goal of most of these delivery systems is to sustain the drug's release effectively over several days, weeks or months. Such manufacturing processes usually require the use of organic solvents such as

dichloromethane. These sustained-release depot products have been used to treat prostate cancer effectively in man for more than a decade [44]. The LHRH peptide analogues are also used to treat endometriosis, fibroid tumours and precocious puberty. Epic Therapeutics and Baxter Healthcare Corporation have developed an interesting, new totally aqueous microsphere fabrication process that also displays several months extended release of LHRH analogues [45].

6.1.2 Octreotide

Another PLGA peptide sustained-release delivery system is octreotide LAR (long-acting release) (Novartis Pharmaceuticals). The molecular of octreotide acetate is 1019. It is a long-acting with pharmacological actions mimicking those of the somatostatin. Octreotide is indicated for the relief of symptoms associated with gastroenteropancreatic endocrine tumours including carcinoid tumours with features of carcinoid syndrome, VIPomas and glucagonomas. It is also approved for symptomatic control and reduction of growth hormone and somatomedin C plasma levels in patients with acromegaly. Octreotide LAR is administered by injection into the gluteal muscle every 28 days. Local side effects of octreotide include pain, stinging or burning at the injection site and occur in ~ 28% patients treated with octreotide LAR [46].

There are several reasons unique to the LHRH analogues and to octreotide as to why they have succeeded as sustained-release delivery systems. One key feature of these two sustained-release delivery systems is that the LHRH agonists and octreotide are both relatively small peptide molecules with molecular weights of ~ 1000 Da. Unlike much larger
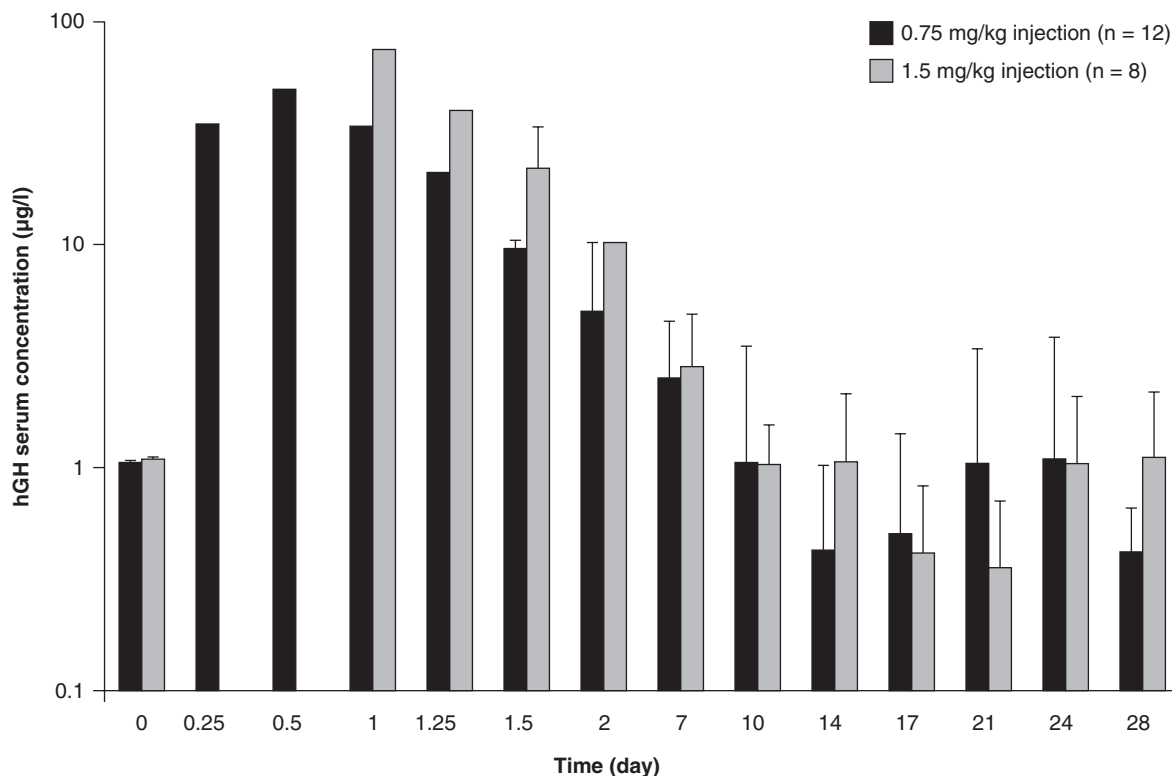
Figure 4. Mean single dose hGH concentrations in GH-deficient pediatric patients. Data from [47].
GH: Growth hormone; hGH: Human growth hormone.

therapeutic proteins, these peptides lack a three-dimensional structure. Therefore, under physiological release conditions at 37°C, and the local acidic pH due to PLGA polymer degradation, these peptides are not very susceptible to denaturation or chemical degradation.

Another important consideration is that octreotide and the LHRH analogues have fairly wide therapeutic indices. They have low toxicity potential even during the initial burst of drug release after drug administration [47,48].

A recent report indicated that the use of the PLGA polymer poses potential risk of chemical reactions via an acylation mechanism between the incorporated peptide and the lactide and glycolide monomer units. The study showed that the degradation of PLGA microspheres resulted in the production of acylation products with salmon calcitonin and parathyroid hormone analogue$_{1-34}$. No acylation products were observed in leuprolide microspheres; even after 28 days' release. This shows the different stabilities among various peptides according to the primary structure. These results also highlight the potential chemical reactions when working with more complex protein molecules in the development of extended-release delivery systems [49].

Therefore, from an extended-release drug delivery point of view, we see that the polymeric-based sustained-release technology has been very much constrained to low-molecular-weight peptides rather than large therapeutic protein molecules.

6.1.3 Human growth hormone

One exception to the restriction of sustained-release delivery systems to small peptides was the Nutropin Depot® product developed by Alkermes and Genentech. This PLGA-based microsphere product provided sustained release of human growth hormone over a 2- or 4-week period from a single injection. Human growth hormone (hGH) is a 191 amino acid protein with a molecular weight of 22,125 Da. Before the depot product, up to seven injections/week were required in order to treat growth hormone deficiency effectively in paediatric patients. **Figure 4** shows the sustained release hGH concentrations from a single injection over a 1-month period in growth hormone-deficient children. The depot product was approved in 1999 by the US FDA as a treatment for growth hormone deficiency in paediatric patients. In June 2004, Genentech and Alkermes announced their decision to discontinue commercialisation of Nutropin Depot. The companies stated that their decision was based on the significant resources required by both companies to continue manufacturing and commercialising the product.

What could have impacted the manufacturing and commercialisation of this novel sustained-release product? Among the possible reasons are that Nutropin Depot was developed using a complex, low-temperature spraying method aimed specifically at maintaining the biochemical activity of the protein during manufacture [101,50]. One of the difficulties in manufacturing these PLGA microsphere products is the final

Table 4. PEG molecular mass and half-life. Three PEG chains were bound to the SOD molecule [54].

Protein	Half-life (h)
SOD	0.08*
SOD-PEG 1900 Da	1.5*
SOD-PEG 5000 Da	11.0*
SOD-PEG 72,000 Da	36.0‡

*Rats; ‡Mice.

PEG: Polyethylene glycol; SOD: Superoxide dismutase.

product yield. Commercial-scale PLGA-based microsphere products may have a wide particle size distribution after manufacturing. It is necessary to sieve the microspheres in order to remove microspheres or aggregates that are too large to inject through reasonable sized syringes and needles. Sieving these microspheres can significantly reduce yields and increase manufacturing costs, especially with high-cost proteins such as hGH. Techniques have been reported in the literature that result in the fabrication of PLGA microspheres with somewhat narrow particle size ranges [51]. However, such techniques have yet to be applied to commercial manufacture or to water-soluble protein molecules.

The adverse event profile of the Nutropin Depot product also showed the potential side effects when administering the PLGA dosage form to children. In studies involving 138 paediatric patients treated with Nutropin Depot, the most frequent adverse reactions were injection-site reactions, which occurred in nearly all patients [52]. On average, two to three injection-site adverse reactions were reported per injection. These reactions included nodules (61% of injections), erythema (53%), pain post-injection (47%), pain during injection (43%), bruising (20%), itching (13%), lipoatrophy (13%) and swelling or puffiness (8%). The intensity of these reactions was generally rated mild-to-moderate, with pain during injection occasionally rated as severe (7%). The discontinuation of this novel product is further evidence of the complexity of developing viable sustained-release delivery systems for high-molecular-weight protein molecules.

The Nutropin Depot was also plagued with a dosage form that used a comparatively large 21-gauge needle for injecting paediatric patients and had a volume of injection that could be as large as 1.2 ml. This competed with a relatively pain-free daily dosage form that uses a very small 30-gauge needle.

Thus, PLGA-based systems for protein-based therapeutics still remain limited and suboptimal. A single sustained-release delivery platform for all proteins remains elusive despite many novel approaches in this field.

7. Chemical means of altering protein half-life

Other approaches that have been used with success include the chemical alterations of protein molecules in order to extend their activity or perhaps hasten their onset of action.

Pegylation, glycosylation and amino acid alterations of proteins have resulted in several successful marketed products.

7.1 Pegylation

Companies such as Nektar and Enzon pegylate proteins by covalently attaching a flexible strand of polyethylene glycol (PEG) to a protein. Pegylation of a protein generally masks the protein's surface, effectively increases the protein's molecular size, reduces renal ultrafiltration, inhibits antibodies or antigen processing cells, and reduces degradation by proteolytic enzymes. Therefore, the protein's distribution is significantly altered.

There are numerous studies showing that extended serum half-lifes can be obtained by chemically adding the PEG molecule to the therapeutic protein. To couple PEG to a protein, it is first necessary to activate the polymer by converting the hydroxyl terminus to some functional group capable of reacting with the functional groups found on the surface of proteins. The most common route has been to activate the PEG with functional groups suitable for reaction with lysine and N-terminal amino groups [53]. In one study the effect of pegylation was shown in animals. **Table** 4 shows the effect of increasing the molecular weight of PEG on the superoxide dismutase (SOD) molecule [54]. The data clearly shows that the half-life of SOD is increased 450-fold when 72,000 molecular weight PEG is compared with the native protein.

There are several approved pegylated proteins currently in clinical use. The first FDA-approved pegylated product was Enzon's Adagen®. It is a PEG-modified version of the bovine enzyme adenosine deaminase (ADA). It is used to treat ADA-deficient severe combined immunodeficiency disease, commonly known as the 'bubble boy disease.' Another Enzon product called Oncaspar® is a PEG-modified version of the enzyme L-asparaginase used as a chemotherapeutic agent for acute lymphoblastic leukaemia. Clinical studies examining the efficacy and safety of Hoffmann-La Roche, Inc.'s peginterferon-α_{2a} (Pegasys) in patients with hepatitis C-related cirrhosis or bridging fibrosis showed that 180 μg of peginterferon-α_{2a} administered once weekly was significantly more effective than 3 million units of standard interferon-α_{2a} administered three times weekly [55]. Like Pegasys, Schering-Plough's peginterferon-α_{2b} (PEG–Intron®) is also indicated for the treatment of chronic hepatitis C. Pfizer's pegvisomant (Somavert®) is a pegylated hGH receptor antagonist used in the treatment of acromegaly. Amgen's pegfilgrastim (Neulasta®) is used to decrease the incidence of infection, manifested by febrile neutropenia.

Pegylating a protein is not a simple chemical reaction. There can be other problems encountered when conjugating the protein of interest to PEG such that the activity of the protein can be significantly decreased. There can be variations in the number of PEG chains bound to the protein, which leads to polydispersity of the newly formed molecules. There can also be difficulties in determining the exact sites of conjugation in polypeptides [54].

Table 5. Insulin amino acid substitutions and pharmacodynamics [58].

Insulin type	Onset of action (h)	Duration (h)	Position A-21	Position B-28	Position B-29	Position B31-32
Regular	0.5	2-4	Asparagine	Proline	Lysine	-
LysPro	0.25	0.5 – 1.5	Asparagine	Lysine	Proline	-
Aspart	0.25	0.5 – 1.5	Asparagine	Aspartic acid	Lysine	-
Glargine	2 – 4	24	Glycine	Proline	Lysine	Arginine–arginine
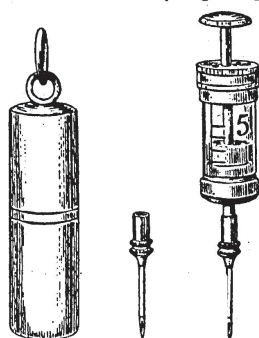
Figure 5. Joseph Wood in 1853 invented the first hypodermic syringe. Used with permission of the New York Academy of Sciences [62,63].

7.2 Glycosylation

Darbepoetin-α is an erythropoiesis-stimulating protein similar to recombinant human erythropoietin [56]. It is produced in Chinese hamster ovary cells by recombinant DNA technology. It differs from human erythropoietin by the addition of two N-linked oligosaccharide chains and an increase in the number of sialic acid residues from 14 in human erythropoietin compared with 22 sialic acid residues in darbepoetin-α. These two additional sites result from amino acid substitutions in the peptide backbone, which do not interfere with receptor binding. The carbohydrate chains increase the molecular weight of the glycoprotein from ~ 30,000 Da for human erythropoietin to 37,000 Da for darbepoetin-α. Amgen markets darbepoetin-α as Aranesp®. It has a threefold longer terminal half-life in humans than erythropoietin, leading to a decrease in frequency of administration and potentially greater patient compliance.

7.3 Amino acid substitutions

Recently, several novel insulin formulations have been developed that dramatically affect insulin's pharmacokinetic properties. This has been accomplished by substituting some amino acids in the primary structure of the protein in a manner that does not change the biological activity of the molecule. **Table 5** summarises these amino acid substitutions and their effect on the pharmacokinetics relative to human insulin.

7.3.1 Rapid-onset insulins

Insulin is composed of two polypeptide chains: A and B. Chain A consists of 21 amino acids and chain B consists of 30 amino acids. In human insulin, amino acids 28 and 29 on the B-chain are $proline_{28}$ and $lysine_{29}$, respectively. Eli Lilly has developed and marketed a fast onset-of-action insulin called LysPro insulin. In this insulin formulation, two amino acids have been reversed from native human insulin so that lysine appears in position 28 and Proline is found at position 29 [58]. This form of insulin favours the more soluble monomeric structure and hence it is available more rapidly to control postprandial glucose compared with regular human insulin. NovoNordisk developed insulin-aspart. This analogue accomplishes the same rapid onset-of-action effect as the $proline_{28}$ $lysine_{29}$-insulin by simply substituting the $proline_{28}$ with aspartic acid in the same position of the B-chain [59].

7.3.2 Slow-release insulin

Another amino acid substitution can actually achieve the opposite effect. Insulin glargine is a 24-h long-acting recombinant insulin analogue produced by Aventis [60]. Insulin glargine differs from human insulin, in that on the A-chain the asparagine amino acid is replaced with a glycine at position A21. In addition, two arginine amino acids are added to the C-terminus of the B-chain. Insulin glargine is designed to have low aqueous solubility at physiological pH. This insulin analogue is injected in aqueous solution at pH 4. On subcutaneous injection, it precipitates at physiological pH and forms a slow-dissolving depot of hexameric insulin. Studies have shown a relatively constant 24-h concentration profile, with no pronounced insulin peak [61]. This profile helps mimic physiological basal insulin release.

Thus, chemical alterations of existing proteins have resulted in dosage forms with significantly altered pharmacokinetics compared with their native molecules. These methods are not panaceas, for pegylation and amino acid substitutions can alter the biological activity of the molecules, their toxicities or their bioavailabilities.

Table 6. Alternative protein delivery technologies.

Non-invasive routes	Molecule applicability	Comments
Nasal	Small peptides	Low bioavailability, need for enhancers
Oral	Small peptides	Low bioavailability, limited to low-dose drugs, hepatic route may be advantageous for insulin
Pulmonary	Peptides, proteins	10% bioavailability, effective protein delivery shown, questions about chronic delivery for some drugs
Buccal	Peptides, proteins	Uncertain reproducibility, bioavailability
FcRn	Large molecular weight proteins, peptides	Antibody-Fc region drug link, enabling the active receptor-dependent uptake. Significant bioavailability, early-stage technology
Sustained-release technologies		
Amino acid substitutions	Peptides, proteins	Several successful examples, bioavailability can be preserved, limited to small, but significant, changes in half-life. Selective drug applicability, potential immunogenicity, carcinogenicity
Pegylation/ glycosylation	Peptides, proteins	Potential for altering bioavailability, Increased biological half-life for several days. Chemistry not compatible with all proteins
Biodegradable polymer delivery	Proteins, peptides	Drug must have wide therapeutic index. Loadings are generally low. Protein stability issues limits potential drug candidates. Difficult manufacturing

8. The needle and syringe

The invention of the first hypodermic syringe very similar to what we use today is attributed to Alexander Wood (**Figure 5**). In 1853, Wood experimented with the use of a hollow needle for the more effective subcutaneous administration of morphine in the treatment of neuralgia [62]. Injections of drugs such as morphine were initially targeted at treating local pain among individuals. However, it soon became apparent that there was systemic absorption of these drugs as ailments far removed from the site of injection also benefited. Ultimately, this observation would become the basis of all injection therapies.

Today virtually all protein drugs are administered in aqueous solutions using needles and syringes. The quality of today's disposable needles is such that the pain perception is almost negligible. Insulin needles today are available in tiny 31-gauge sizes. One study was conducted to assess the pain associated with insulin injection. Injection pain was assessed in 39 Type 1 diabetic patients. The results of the study were that injections were relatively painless, with an average (median) pain score of < 10% of maximum pain. The investigator concluded that insulin injection and blood glucose self-monitoring in general is not very painful [64,65]. There is no doubt that many people have a psychological aversion to injections with a hypodermic syringe and needle. However, studies with insulin and growth hormone injections to children suggest that most proteins injected in aqueous solution are in fact painless.

9. Conclusion

Therapeutic proteins are becoming more important in an ever-increasing part of the healthcare system. The biotechnology boom has created new classes of molecules that are being used to treat diseases that were incurable 10 years ago. The structural and therapeutic properties of these molecules make them more dependent on drug delivery technologies so that they can achieve their maximum effectiveness. However, formulating proteins such that they maintain their stability and that they are delivered within their efficacious and safe target doses remains a challenge. The protein drug delivery systems reviewed in this article have been divided into two broad groups. One set of technologies has focused on successful non-invasive means of transporting therapeutic proteins to the body. The second set of technologies is generally aimed at extending the activity of the molecules once administered. **Table 6** summarises the various technologies investigated for protein delivery.

The commercial success of protein delivery via the oral or nasal routes has been limited to four molecules. These compounds are generally small peptides with relatively low daily doses. There has not been much success with larger proteins due to the relatively low bioavailability observed for the oral and nasal routes. Therefore, with today's technologies, one

must remain sceptical with regard to oral delivery of proteins with a molecular size of insulin and greater.

Insulin delivery will remain the focus of many researchers and companies looking to solve the protein delivery problem. This is only natural, as the number of patients afflicted with diabetes in the world continues to increase. The pulmonary route for non-invasive protein delivery has received much-deserved attention during recent years. Pulmonary delivery of insulin has been shown to be effective and well tolerated by patients participating in clinical trials. Reservations about untoward effects of pulmonary insulin or other proteins will need to be weighed against the benefits. A pulmonary dosage form is likely to result in significantly greater compliance among Type II diabetics who are hesitant to self-administer multiple daily injections. The latest statistical estimates indicate there are ~ 171 million people diagnosed with diabetes worldwide, and that number is expected to rise by 237 million by the year 2030. The economic costs to the healthcare system of delivering insulin at 10% bioavailability to such a large population will also need to be assessed. Furthermore, the ability of the pharmaceutical industry to meet the demand for a potentially huge product demand also will need to be determined. The set up costs for recombinant protein production are huge. A large scale manufacturing facility requires a minimum bioreactor volume of 10,000 litres. This takes 3 – 5 years to construct and costs $250 – $500 million dollars [66]. This represents considerable risk for the pharmaceutical company when the outcome of clinical trials is uncertain.

10. Expert opinion

The limitation of polymeric drug delivery to only small peptides with wide therapeutic indices has been disappointing, but it highlights the complexity of pharmaceutical product development compared with academic proof of principle. One important lesson from the Nutropin Depot experience is that the presumed advantages of sustained-release dosing need to be critically compared with chronic, but virtually painless, daily injections with 30-gauge needles. Value-added technologies need to assess the 'big picture' early in product development in order to avoid the pitfalls common to new drug development.

The mixed successes seen thus far in altering proteins' half-lifes by polymer matrix formation or by chemical changes to native molecules are likely to be dictated by the nature of the newer protein-based molecules being submitted for regulatory approval. Most of the newer protein-based compounds in the biotech pipelines are monoclonal antibodies. These molecules have molecular weights in the 150,000 Da range and, therefore, are probably not likely candidates for non-invasive delivery routes. These molecules are also particularly sensitive to changes in tertiary structure, which might affect their binding to target receptors. Furthermore, the doses of these monoclonal antibodies are very high. For example, infliximab is dosed at 350 mg by injection to treat rheumatoid arthritis and Crohn's disease [67]. Rituximab is infused intravenously at a dose of ~ 750 mg in order to treat B-cell non-Hodgkin's lymphoma [68]. Bevacizumab is a recombinant humanised monoclonal IgG1 antibody that binds to and inhibits the biological activity of human vascular endothelial growth factor [69]. It is used to treat patients with metastatic carcinoma of the colon and rectum. It is also dosed at about 350 mg i.v. per infusion. These high doses are likely to preclude any formulation changes that significantly increase the mass of injected material that would make the drug's administration very uncomfortable for the patient. Thus, polymeric delivery systems for such macromolecules are unlikely. There will be a need for novel monoclonal antibody formulation development to allow the injection of such large doses. Pegylated forms of some of these monoclonal antibodies are likely to join the approval of traditional pegylated proteins. Similarly, peptide substitutions in other recombinant proteins will probably result in some new and interesting pharmacokinetic properties for analogues of old drugs. The small efficiency increases in non-invasive approaches to deliver proteins will probably never be applicable to the vast majority of protein drugs. This will preserve the role of the needle and syringe for the foreseeable future.

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Patents

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