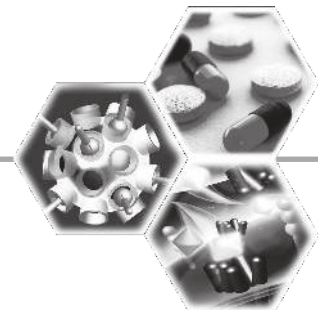

Recent developments in protein and peptide parenteral delivery approaches

Discovery of insulin in the early 1900s initiated the research and development to improve the means of therapeutic protein delivery in patients. In the past decade, great emphasis has been placed on bringing protein and peptide therapeutics to market. Despite tremendous efforts, parenteral delivery still remains the major mode of administration for protein and peptide therapeutics. Other routes such as oral, nasal, pulmonary and buccal are considered more opportunistic rather than routine application. Improving biological half-life, stability and therapeutic efficacy is central to protein and peptide delivery. Several approaches have been tried in the past to improve protein and peptide *in vitro*/*in vivo* stability and performance. Approaches may be broadly categorized as chemical modification and colloidal delivery systems. In this review we have discussed various chemical approaches such as PEGylation, hyperglycosylation, mannosylation, and colloidal carriers including microparticles, nanoparticles, liposomes, carbon nanotubes and micelles for improving protein and peptide delivery. Recent developments on *in situ* thermosensitive gel-based protein and peptide delivery have also been described. This review summarizes recent developments on some currently existing approaches to improve stability, bioavailability and bioactivity of peptide and protein therapeutics following parenteral administration.

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Advances in biotechnology have resulted in emergence of numerous protein and peptide therapeutics. The numbers of therapeutic proteins approved and under clinical trials are increasing exponentially. In March 2013, Pharmaceutical Research and Manufacturers of America's report on "Biologics medicine in development" listed over 900 new biologics targeting more than 100 diseases either in human clinical trials or under review by the US FDA. Among these, approximately 90 are recombinant proteins and over 300 are monoclonal antibodies [1]. Most of these drugs are under development for the treatment of cancer, diabetes, multiple sclerosis, infectious diseases and growth deficiencies.

Therapeutic proteins and peptides are highly potent molecules demonstrating specific mechanism of action. Unfortunately, clinical applications of these agents are hampered by numerous obstacles to their successful delivery. Although oral delivery is standard for administration of small molecules, it is not straightforward for administration of proteins and peptides. Intrinsic physicochemical and biological properties, including large molecular size, poor permeation through gastrointestinal membrane, poor stability attributed to low pH of gastric fluid and presence of proteolytic enzymes render oral delivery of proteins and peptides highly challenging [2]. Other noninvasive routes have also been investigated for protein and peptide delivery such as pulmonary, nasal, ocular, transdermal, buccal and vaginal. Among noninvasive routes, pulmonary and nasal are widely investigated. Pulmonary delivery offers several advantages such as high-surface area of the lungs, high vascularization, low thickness of the alveolar epithelium, rapid systemic absorption and evasion of first-pass metabolism. However, limited bioavailability of inhaled proteins due to degradation by proteases in the lungs and various clearance mechanisms in the respiratory tract with complication in design of inhalation devices and dry powder formulation of proteins outweighs the advantages of pulmonary delivery [3]. Exubera, the first inhaled insulin, was approved in early 2006 by the FDA. However in October 2007, Pfizer (NY, USA) took Exubera off the market due to poor market acceptance [4]. Also, intranasal administration is widely investigated due to avoidance of first-pass metabolism and direct brain delivery. However, bioavailability of nasally administered proteins is limited due to presence of metabolic enzymes in the nasal mucosal cavity, smaller surface area for absorption, poor permeation and fast mucociliary clearance [5].

Currently, marketed protein and peptide drugs are mostly administered by parenteral routes such as subcutaneous (s.c.), intramuscular or intravenous (i.v.) injections. In contrast to

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Key Terms

Nanoparticles: Nanoparticles are colloidal carriers with size ranging from 10 to 1000 nm.

Liposomes: Liposomes are bilayered vesicles/carrier systems composed of phospholipids of either synthetic or natural origin.

***In situ* thermosensitive gel:** Thermosensitive hydrogels refer to polymeric solutions which undergo sol–gel phase transition to form viscoelastic gel in response to changes in temperature.

PEGylation: Covalent conjugation of activated PEG to the therapeutic proteins or peptides of interest.

oral and pulmonary delivery, parenteral delivery can avoid biological barriers. However, there are several limitations to parenteral protein and peptide delivery [6]. The foremost challenge is the short *in vivo* half-life of these drugs usually in the range of a few minutes to a few hours following systemic administration. For example, tissue plasminogen activator (tPA) has an initial half-life of less than 5 min following systemic administration [7]. The short half-life of protein therapeutics is attributed to the enzymatic degradation and/or rapid renal clearance. To enhance therapeutic effectiveness, frequent administration is required, which leads to poor patient compliance [2].

The success of protein and peptide parenteral delivery relies on development of novel delivery approaches. Several strategies have been evaluated in an effort to overcome challenges associated with therapeutic protein and peptide delivery. The widely studied approaches can be generally categorized as chemical modifications and colloidal delivery systems [8,9].

Chemical modifications are employed to improve *in vivo*/*in vitro* stability and decrease clearance. Among chemical modifications, covalent conjugation of polymers such as PEG or polysialic acid to therapeutic proteins represent a relatively feasible and novel approach than the structural changes of proteins. Commonly employed formulation approaches include development of colloidal carriers as protein delivery systems such as microparticles, **nanoparticles**, **liposomes** and so forth. ***In situ* thermosensitive gels** have also emerged as novel approach for therapeutic proteins and peptides delivery. In addition, researchers have attempted to develop a combined approach utilizing two or more strategies. Together, all these approaches seek to achieve the following benefits: protecting protein/peptide from degradation; extending *in vivo* half-life; providing prolonged drug release; augmenting drug efficacy, while reducing side effects; reducing administration frequency and lowering drug dosage. Other advantages include alleviation of pain associated with frequent injections and significant reduction in cost of treatment [8,9].

This review describes recent developments in some colloidal carriers (microparticles, nanoparticles, liposomes, carbon nanotubes, micelles), *in situ* thermosensitive gels as well as chemical modification approach with special emphasis on **PEGylation**, hyperglycosylation and mannosylation for parenteral delivery of pharmaceutical proteins and peptides.

Chemical modifications

■ PEGylation

PEGylation is the most extensively studied approach for delivery of various proteins and peptides via parenteral routes. It exploits covalent conjugation of activated PEG to the therapeutic proteins or peptides of interest. PEGylation improves stability, pharmacokinetics, and therapeutic activity of protein and peptide drugs by altering various physicochemical properties such as molecular weight, size, solubility and steric hindrance [10]. Nonimmunogenicity, low protein/cellular adsorption among all known polymers used for drug delivery, nontoxicity, solubility in water, and FDA approval for injection with biotechnological drugs are some unique features of PEG that make them the most attractive polymers for parenteral delivery of proteins [11].

Mono-methoxy PEG (mPEG) is the most commonly used PEG molecule, which is a linear polyether diol with the chemical formula CH_3-$(OCH_2$-$CH_2)_n$-OH. In order to achieve ease and controlled conjugation, PEG has also been functionalized with amine binding terminal functional groups such as *N*-hydroxysuccinimide esters, *N*-hydroxysuccinimide carbonates, and aliphatic aldehyde, or thiol binding groups such as maleimide, pyridyl disulphides, and vinyl sulfonates [11]. In therapeutic proteins/peptides, amino groups (α-amino and ε-lysine amino) are the most suitable site of conjugation due to their prevalence (~10% of total amino acids present in protein) and negligible involvement in the active site of protein. Cysteine residues of proteins may also be used for this purpose. However, these are either very rare or present in active site of proteins. Therefore, it requires inserting these residues in proteins via protein engineering before conjugation. Sugar moieties of glycoproteins are another option that has also been used for PEG linkage [12–14]. PEG used for conjugation can be broadly divided into three groups depending on their architecture: linear, Y-shaped and branched (comb-shaped) PEG [11].

The polymeric chains of PEG molecules are very flexible, which gives larger hydrodynamic radius in aqueous media. In addition, their propensity of binding with two to three water molecules per ethylene oxide give a five to ten-times larger size as compared with globular proteins of equivalent molecular weights, which enhances systemic circulation of these therapeutics and decreases their renal filtration [12]. Moreover, PEGylation inhibits serum opsonin interaction with therapeutic proteins

and thus decreases cellular clearance by the reticuloendothelial system. PEGylation also inhibits protein degradation caused by interaction with cell-associated receptors and enzymes or specific cell-protein interactions. As a result of the aforementioned advantages, elimination of therapeutic proteins and peptides decreases significantly, which further decreases administration frequency and enhances their therapeutic efficacy [12]. For instance, efficacy of the tissue inhibitor of metalloproteinases-1 (TIMP) is limited because of short plasma half-life. To improve *in vivo* performance, a modified recombinant human TIMP-1 (rhTIMP-1) was PEGylated on lysine residues [15]. A mixture of mono- and di-PEGylated rhTIMP-1 species were generated by conjugating with 20 kDa mPEG chains. The conjugates retained complete inhibitory activity toward the MMP-3 catalytic domain and also partial inhibitory activity toward full length MMP-9. *In vivo* pharmacokinetic studies in mice demonstrated that PEGylation extended the plasma half-life of rhTIMP-1 from 1.1 to approximately 28 h. *In vitro* biological assays suggested that 20 kDa mPEG chains inhibited both MMP-dependent cancer cell invasion and tumor cell associated gelatinase activity. Study results indicate that PEGylated TIMP-1 has the potential for further development as an anticancer recombinant protein therapeutic. In addition, this strategy may find clinical applications in the treatment of other diseases [15].

In another study, PEGylation has been used to improve *in vivo* performance of recombinant human growth hormone (hGH) by enhancing its systemic circulation time. In this approach, PEG was conjugated to hGH by two methods: microbial transglutaminase mediated PEGylation to yield PEG-Gln141-hGH, and covalent PEG conjugation to α-amine residue of Phe1 to yield PEG–Nter–hGH. Both the strategies yielded monoconjugated PEGylated hGH with preserved native secondary structures. *In vivo* studies in rats demonstrated that both PEGylated products significantly improved systemic circulation and exerted a 4.5-fold increase in half-life relative to unmodified hGH. These results indicate that site-specific PEGylation of hGH helps to generate conjugates with prolonged half-life relative to native protein (hGH) [16].

Similarly, improvement in the systemic circulation half-life of recombinant human thyroid stimulating hormone (rhTSH or Thyrogen®) was studied following PEGylation. Thyrogen is indicated for diagnosis and treatment of thyroid cancer with a multidose regimen owing to short-circulating half-life. To reduce dosing frequency and extend duration of action, PEGylation strategy was attempted [17]. This study demonstrated successful PEGylation of a cysteine-knot with higher-reaction yield (approximately 85%) for monoPEGylated rhTSH. *In vivo* studies in rats demonstrated that 40 kDa PEG αG22C rhTSH had prolonged duration of action relative to unmodified rhTSH. Applicability of PEGylation in enhancing systemic half-life of proteins and peptides has also been established in several other studies. As a result, several therapeutic proteins are now being marketed just because of an increase in circulation half-life by PEGylation leading to enhanced therapeutic effect and decreased administration frequency.

Shielding of antigenic determinants of protein drugs by PEGylation inhibits their recognition from antibodies and thus may reduce immunogenicity. To demonstrate this phenomenon, Freitas *et al.* have PEGylated recombinant uricase (UC-r) with either methoxyPEG-4, 6-dichloro-*s*-triazine or methoxypolyethilenoglycol-*p*-nitrophenyl-carbonate. In result, PEGylation of UC-r with methoxypolyethilenoglycol-*p*-nitrophenyl-carbonate and methoxy PEG-4, 6-dichloro-*s*-triazine retained the enzyme activity (87 and 75%, respectively) and significantly reduced the immunogenicity of native UC-r in Balb/c mice. Also, PEGylation did not induce any detectable antibody response [18].

Types of linkers between PEG and protein may govern pharmacokinetic and pharmacodynamic behavior of PEGylated protein. Investigations were made to study the effect of linkers using a thrombolytic agent SAK, used for the treatment of myocardial infarction. Linkers such as phenyl, propyl and amyl moieties were employed to conjugate the C-terminus of protein and PEG. Both propyl and amyl linkers led to loose conformation while phenyl linkers induced dense PEG conformations. Moreover, phenyl conjugated PEGylated proteins demonstrated extensive shielding for domains adjacent to the C-terminus but barely shielded its bioactive domain. *In vivo* studies demonstrated dense PEG conformations to be more efficient in maintaining bioactivity, prolonging plasma half-life, lowering proteolytic sensitivity, and immunogenicity relative to loose conformations [19].

PEGylation is also reported to have a beneficial effect on improving physicochemical stability of proteins. It shows protecting effects on proteins postexposure to organic solvents.

Key Term

Glycosylation: It may be defined as a co- or posttranslational enzymatic process that conjugates proteins, lipids or other organic molecules to polysaccharides to form a glycoconjugate.

Bacteriorhodopsin, a membrane protein, when modified with mPEG, demonstrated enhanced stability against high concentrations of ethanol (30%) and electromagnetic radiation (photochromism). On the other hand, unmodified proteins instantaneously denatured in 30% ethanol [20]. PEGylation also enhances protein pH and thermal stability [21,22].

In vivo fate of PEGylated proteins has also been examined by several investigators. Elliott *et al.* conducted studies to delineate the relationships among pharmacology, toxicology and immunogenicity of PEGylated proteins utilizing multiple analytical methodologies [23]. Investigators utilized two independent analytical approaches, gel electrophoresis and NMR spectroscopy, to find out the biological fate of a 40 kDa PEG-insulin after i.v. administration to rats. The PEG moiety was evident for more than several weeks in both serum and urine samples. On the contrary, insulin moiety displayed shorter half-life than PEG indicating *in vivo* bond cleavage. It is imperative to investigate bond cleavage and identification of end products present in biological fluids since the fragments may have toxicological effects due to unconjugated PEG accumulation or immunogenic recognition of PEGylated protein [23]. The authors showed that the PEG-protein conjugate was not intact throughout the course of analysis. A free PEG moiety was found to persist in systemic circulation and in tissues. The authors suggested that further studies are required to understand the immune recognition of the cleaved PEGylated protein and its degradants/products. The combination of assays utilized in this study with an animal model provided the basis for translation to clinical studies for the patients exposed to PEGylated protein products. This study demonstrated the dual analytical platform (NMR and gel-based technology) allowing pharmacokinetic profiling of PEGylated proteins, and *in vivo* integrity estimation for the PEGylated protein conjugates.

Notwithstanding potential benefits, the shielding effect of PEGylation can sometimes compromise the therapeutic activity of proteins and peptides. Steric hindrance caused by higher PEGylation can reduce access of the active site to the receptors and can decrease the therapeutic response. However, at the same time, shielding protects the drug from enzymatic degradation and enhances systemic circulation, which is responsible for increased therapeutic outcome. This balanced benefit can be observed with PEGylated IFN α-2a (PEGASYS®, Roche USA, NJ, USA). It only retains 7% of antiviral activity compared with its native form. However, because of PEGylation, it shows improved pharmacokinetics in hepatitis C patients [24].

Several PEGylated proteins are commercially available such as Adagen®, Oncaspar®, PEG-Intron®, PEGASYS, Neulasta®, Somavert®, Mircera® and Cimzia® [25]. **Table 1** shows recent developments in PEGylation of protein and peptide therapeutics.

■ Hyperglycosylation

Glycosylation has been one of the highly studied subjects for protein and peptide delivery. It is a co- or post-translational enzymatic process that conjugates proteins, lipids or other organic molecules with polysaccharides to form a glycoconjugate [2]. The nature of the carbohydrate conjugated to the protein determines and regulates its structure, function, activity, immunogenicity and pharmacokinetic profile. Attempts have been made to hyperglycosylate therapeutic proteins to improve pharmacokinetic behavior. It closely resembles to PEGylation. The advantage of hyperglycosylation over PEGylation includes the biodegradable nature of carbohydrates, whereas intact PEG is eliminated from the body with no biodegradation. Similar to PEGylation, it serves to reduce interactions with the clearance process and antigen presenting cells, leading to prolonged systemic circulation and reduced immunogenicity. Hyperglycosylated proteins are expected to possess reduced or equivalent activity to their native counterparts.

Hyperglycosylation can be performed by either *in situ* chemical reactions or site-directed mutagenesis. The process may result in either N-linked or O-linked protein glycosylation. In N-linked glycosylation, the saccharide chain is attached to asparagine of tripeptide sequence Asn-X-Ser/Thr, where X represents an amino acid other than proline. On the other hand, O-linked oligosaccharides are not site-specific sequences but are generally found conjugated to either serine or threonine [8]. For hyperglycosylation, polysialic acid (PSA), an endogenous oligosaccharide, is often selected. In certain bacterial cell walls, PSA is present, which is identical to the human counterparts. PSA present in the bacterial cell wall prevents recognition by the human immune system, which makes it an ideal candidate for protein conjugation [37]. PSA is highly hydrophilic and covalently conjugates ε-amino acids of lysine following reductive amination reactions [37,38]. It is available in different

Table 1. Recent developments in PEGylation of protein and peptide therapeutics.

Protein/peptide	Molecular weight	Remarks	Ref.
IFN-α2	19.3 kDa	Improved lymphatic uptake and significantly increased antitumor efficacy of interferon against axillary metastases of highly lymph metastatic variant of human breast MDA-MB-231 carcinoma	[26]
Filgrastim (methionyl human rh-Met-G-CSF), BK0026	NA	PEGylation improved stability for more than 24 months at 4–8°C	[27]
TRAF 6	~3491 Da	Improved bioavailability and enhanced uptake in bone marrow	[28]
Uricase	127.6 kDa	PEGylation extended elimination half-life (191.5 h) with reduced clearance rate	[29]
IFNβ-1A	22.5 kDa	PEGylation developed convenient dosage regimen for multiple sclerosis with similar efficacy, safety and tolerability to that of the parent protein (unconjugated)	[30]
IGF-I	7649 Da	Single subcutaneous injection of PEGylated IGF-I resulted in steady state levels in brain tissues and cerebrospinal fluid relative to IGF-1 and reverted deficits in IGF-1 signaling, synaptic protein and cognitive performance	[31]
Erythropoietin	35 kDa	PEGylation retained similar biological activity to that of erythropoietin and demonstrated fivefold longer terminal half-life in rats	[32]
IFNγ	~15.6 kDa	PEGylated IFNγ conjugates revealed an increased plasma half-life, decreased renal clearance and higher liver accumulation than the unmodified IFNγ after i.v. administration in mouse	[33]
Recombinant human OPG	90 kDa	The half-life and AUC of poly(PEG)-OPG (comb shaped) were comparable with those of linear PEG-OPG derivatives. The poly(PEG)-OPG showed the strongest inhibitory effect on bone resorption activity in ovariectomized rats	[34]
C-peptide	NA	PK study was conducted in beagle dogs using two representative PEGylated C-peptides of different molecular weights (a 20 kDa linear PEG (Example 3) and a 40 kDa branched PEG. Improved PK profile was observed relative to C-peptide alone which was undetectable after half day	[35]
Protein of canine urate oxidase	NA	PEGylated dog source urate oxidase analog protein demonstrated prevention of primary uric acid induced nephropathy in rats with i.v. administration. Following subcutaneous administration of urate oxidase analog, 76.3% bioavailability was achieved	[36]

i.v.: Intravenous administration; NA: Not available; OPG: Osteoprotegerin; PK: Pharmacokinetic.

sizes and its clearance depends on type and molecular size of the polymer. Hence, it is possible to tailor the rates of clearance of peptides or proteins optimally. The large-molecular-weight PSA are suitable for the delivery of low-molecular-weight drugs and peptides, while those with lower molecular weight could be used for large proteins as well as particulate drug-delivery systems **(FIGURE 1)** [37,38].

Colominic acid is an example of a short/low molecular mass PSA derivative. It was employed to improve pharmacokinetics of enzymes such as catalase and asparaginase [39,40]. Asparaginase (*Erwinia carotovora* L-asparaginase) was covalently coupled to colominic acid by reductive amination. The glycosylated asparaginase demonstrated improved *in vivo* half-life by three- to four-fold in mice with reduction in immunogenic response. Initial asparaginase activity for the glycosylated construct was retained within 82–86% that of the native form [41]. These results foster polysialylation as an alternative means for improving therapeutic response and pharmacokinetic profile of asparaginase or other therapeutic proteins. Similarly, insulin was also polysialylated with colominic acid [42]. *In vivo* studies in mice demonstrated a three- to four-fold prolonged glucose suppression relative to its native form. Considering the result, polysialylation may offer a promising strategy to enhance therapeutic levels of insulin.

Bolt *et al.* showed that hyperglycosylation of recombinant Factor IX, a blood coagulation factor used to treat Hemophilia B, improved and prolonged its systemic circulation time relative to native protein [43]. Pharmacokinetic profile of recombinant human IFN-α2 (rhIFN-α2) was also remarkably improved by *N*-glycosylation. Following s.c. administration to rats, there was a 25-fold increase in the elimination half-life and a 20-fold decrease in the systemic clearance rate in

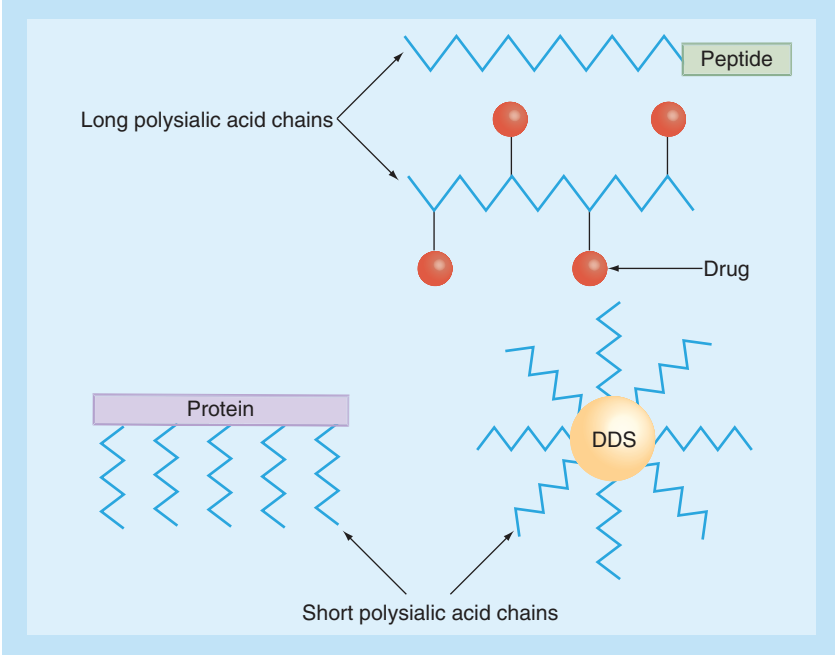

Figure 1. Polysialylated constructs.
DDS: Drug delivery systems.
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case of *N*-glycosylated variants, compared with the nonglycosylated rhIFN-α2 [44].

Hyperglycosylation is also reported to improve physical and chemical stability of proteins under different environmental conditions. The rhIFN-α2b with four *N*-glycosylation sites showed improved stability against acidic pH, thermal stress, and repetitive freeze–thaw cycles in comparison with nonglycosylated rhIFN-α2b [45]. Glycoengineering might be a promising strategy for protecting proteins from inactivation under stress conditions and for overcoming aggregation problems throughout the production and storage conditions. Attached carbohydrates may stabilize protein by several mechanisms. Proposed mechanisms include the formation of hydrogen bonds with a polypeptide backbone or surface hydrophilic amino acids and steric interaction with the adjacent peptide residues. Also, enhanced protein hydrophilicity by sialic acids may make intermolecular interaction unfavorable, abolishing the formation of stress-induced aggregates [45].

Other studies indicated that any alterations in the endogenous glycosylation pattern may have a drastic impact on biophysical properties of proteins. Kosloski *et al.* demonstrated that removal of N-linked polysaccharide from coagulation Factor VIII (FVIII) led to loss of protein activity (>30%) [46]. There was no increase in immunogenicity observed with deletion of glycosylation.

Few products are currently on the market based on hyperglycosylation. An example of such product is darbepoetin α, (Amgen, CA, USA), a hyperglycosylated form of recombinant human erythropoietin (rhu-EPO). It differs from rHu-EPO in that it contains five N-linked oligosaccharide chains and has a carbohydrate composition of 51%. After a single i.v. administration in humans, darbepoetin α exhibits an approximately three-times longer half-life and significantly increased AUC than that of rHu-EPO [47].

Other products containing polysialylated forms, based on PolyXen® technology, include erythropoietin, GCSF, IFN-α-2b and insulin (SuliXen®, a long-acting insulin). These are in various stages of preclinical and clinical evaluation [48].

■ Mannosylation

Mannose receptor-targeted delivery of proteins and peptides can be achieved by conjugation with mannose (mannosylation). Mannose receptors are reported to be expressed on Kupffer cells, macrophages, alveolar, monocyte-derived dendritic cells and subsets of vascular and lymphatic endothelial cells [49]. Mannosylated proteins can be recognized by mannose-specific lectins, namely, mannose receptors and MBPs. In a recent study, Opanasopit *et al.* investigated *in vivo* (in male ddY mice) recognition of mannosylated proteins by hepatic mannose receptors and MBP [49]. Different mannosylated proteins (Man_{17}-superoxide dismutase [SOD], Man_{21}-SOD, Man_{12}-bovine serum albumin [BSA], Man_{16}-BSA, Man_{25}-BSA, Man_{35}-BSA, Man_{46}-BSA and Man_{32}-IgG and Man_{42}-IgG) with increasing mannose residues (mol/mol) or surface density were used for the study. Furthermore, these proteins were radiolabeled with ^{111}In, solubilized in saline and injected into mice with increasing doses (0.05, 0.1, 1, 10 or 20 mg/kg) via lateral tail vein. Results demonstrated that all three different mannosylated proteins were taken up mainly by liver and uptake saturated with increasing doses. Of the different mannosylated protein derivatives, ^{111}In-Man-SOD, ^{111}In-Man_{12}-BSA and ^{111}In-Man_{16}-BSA demonstrated dose dependent pharmacokinetic profile, whereas other derivatives showed slow hepatic uptake at <1 mg/kg. Also, this study demonstrated that *in vivo* recognition of serum mannose binding protein has a stronger cluster

or surface density effect than that of mannose receptors. The authors speculated that differences observed in recognition are due to the unique arrangement of carbohydrate recognition domains on each mannose-specific lectin that is available for mannosylated ligand recognition [49].

Mannosylated proteins are shown to demonstrate better pharmacological and pharmacokinetic properties. As an example, carbohydrate conjugated derivatives of SOD (carboxymethyl, diethylaminoethyl dextrans, galactosylated and mannosylated) have been synthesized and evaluated in rats. Mannosylated SOD targeted to parenchymal and nonparenchymal cells, respectively, resulted in higher inhibitory effects. Results demonstrated that mannosylated SOD were useful in prevention of hepatic ischemia/reperfusion injury [50,51]. In another study, Kel and co-workers synthesized and demonstrated the inhibitory effect of soluble mannosylated epitope of proteolipid protein (M-PLP139–151, a protein derivative) in an experimental autoimmune encephalomyelitis (EAE) mice model [52]. EAE is a disease that is mediated by autoreactive myelin-specific T-cell. It was demonstrated that the protein derivative had a significant reduction of EAE in mice when administered before onset of clinical symptoms. On the other hand, protein derivative administration in an established EAE disease did not have pronounced effect, suggesting that the protein derivative is effective during/before the onset of disease symptoms that involved (re)activation of autoreactive T-cells [52].

Colloidal delivery systems

Colloidal drug delivery systems have gained increasing popularity due to their enormous applications in protein and peptide delivery. Colloidal carriers such as microparticles, liposomes and nanoparticles are widely investigated for the parenteral delivery of protein and peptide therapeutics [9,53,54]. Advantages of these carrier systems include protection of sensitive proteins, prolonged release, reduction of administration frequency, patient compliance and controlled plasma levels. **Table 2** lists recent developments and patents for protein and peptide therapeutics encapsulated in microparticles, liposomes and nanoparticles. Recently, carbon nanotubes and micelles have also gained interest as a carrier for protein and peptides. **Figure 2** depicts colloidal carriers for protein and peptide parenteral delivery.

■ Microparticles

Over the decades, microparticles composed of biodegradable polymers have been studied extensively for the delivery of numerous protein and peptide therapeutics. An ideal microparticle formulation should have reasonably high protein-loading capacity, encapsulation efficiency and provide sustained release of biologically active proteins [55]. Numerous biodegradable and biocompatible polymers, either of natural or synthetic origin, were studied over the years for achieving ideal microparticles formulation. Starch, alginate, collagen, poly (lactide-*co*-glycolide) (PLGA), polycaprolactones (PCL) are some of the more commonly employed biodegradable polymers for controlled systemic delivery of proteins and peptides [56]. Among these polymers, PLGA (copolymers of D,L-lactic and glycolic acid) has been immensely studied for proteins and peptides delivery because of long safety history [57,58]. The success of PLGA based microparticles in protein and peptide delivery can be evidenced by marketed formulations such as Lupron Depot®, Decapeptyl®, Sandostatin LAR® Depot, and Somatuline® LA [56].

For systemic delivery, biodegradable microparticles offer a distinct advantage as depot formulation providing controlled release over several days to months [58]. Rate and extent of drug release from microparticles may depend on composition of polymer and fabrication methods. Therefore, to provide prolonged and complete release, researchers have focused toward development of different types of polymers and microparticles fabrication methods. Schwach *et al.* screened various type of microparticles loaded with a highly potent peptidic gonadotropin releasing hormone antagonist (degarelix) for the prostate cancer indication in a castrated male rat model [59,60]. For screening, investigators have prepared degarelix-loaded microparticles utilizing three different formulation technologies: spray drying, microextrusion and double emulsion methods. Following s.c. injection, microparticles prepared by spray drying and microextrusion exhibited comparable potencies while double emulsion microspheres were significantly less potent in terms of both onset and duration of inhibition of luteinizing hormone (LH) secretion. At higher doses, prolonged LH inhibition was observed for spray-dried microparticles (36-day LH inhibition) and microextruded microparticles (21-day LH inhibition) compared with those prepared by double emulsion (14-day LH inhibition).

Table 2. Recent developments and patents in microparticles, liposomes and nanoparticles for parenteral delivery of protein and peptide therapeutics.

Protein/peptide	Molecular weight	Polymer	Remarks	Ref.
Rat GDNF	26 kDa	PLGA	*In vitro*-release studies revealed controlled release of glycosylated GDNF from microparticles for at least 40 days. In addition, the released GDNF was biologically active in PC-12 bioassay.	[69]
MEP421(Asn–Leu–Pro–Arg acetate; braintide)	NA	PLGA	MEP421 loaded PLGA microspheres showed sustained drug release over 30 days under *in vitro* conditions. The good correlation was obtained between *in vitro* and *in vivo* release following s.c. injection in rats	[70]
Mono-PEGylated GHRP-6	Mono-PEG-GHRP-6 conjugates PEG-1k, -2k and -5k showed average molecular weight of 1757, 3100 and 6316 Da	PLGA	The mono-PEG-GHRP-6 loaded microspheres showed a lower initial burst release compared with native GHRP-6 microspheres. Size of attached PEG showed effect on release period of the GHRP-6. The time points for reaching more than 90% release of the mono-PEG-1K-GHRP-6, mono-PEG-2K-GHRP-6 and mono-PEG-5K-GHRP-6 microspheres were 15, 20 and 30 days, respectively.	[71]
TGF- β1	24 kDa†	Chitosan–PCL copolymer	The initial fast release of TGF-β1 from different microspheres could be significantly reduced by increasing PCL content in chitosan–PCL copolymer	[72]
GLP-1	3297.6 Da‡	PLGA	After s.c. administration, GLP-1-loaded microspheres achieved controlled release for 28 days *in vivo* and exhibited sustained long-term pharmacological efficacy to decrease blood glucose level in STZ-induced diabetic mice model	[73]
Insulin	NA	PLGA	In STZ-induced diabetic rats, DIC-loaded PLGA microspheres controlled blood-glucose levels and maintained lower glucose levels without a loss of body weight compared with insulin loaded PLGA microspheres	[74]
Basal insulin	5 kDa	p(CPP:SA)	A s.c. administration of CPP:SA 50:50 microspheres in diabetic rats released insulin over a month. Moreover, the released insulin was bioactive as evidenced by the lowering of blood glucose levels	[75]
Exenatide (a synthetic exendin-4)	4186.6 Da	PLGA	The results of pharmacodynamics studies in STZ-induced diabetic mice suggested that, one-time injection of exenatide microspheres demonstrated a similar hypoglycemic effect to exenatide solution injected twice daily within 30 days	[76]
FGF-1 and Neuregulin-1	NA	PLGA	A significant improvement in cardiac function was detected in rats treated with growth factor-loaded microparticles	[77]
Vasoactive intestinal peptide	3325.84 Da	Egg yolk phosphatidylcholine, cholesterol, egg yolk phosphatidylglycerol, and PEG grafted to distearoyl-phosphatidylethanolamine	A s.c. administration of vasoactive intestinal peptide self-associated with sterically stabilized liposomes normalized mean arterial pressure in spontaneously hypertensive hamsters. On the other hand, vasoactive intestinal peptide alone (0.1 nmol) and empty sterically stabilized liposomes had no significant effects on mean-arterial pressure	[78]
Insulin	5733.49 Da‡	DOPE, DOTAP, cholesterol	*In vitro* release of insulin was prolonged by cationic liposomes	[79]

DIC formed between insulin and a copolymer composed of branched oligoethyleneimine, mPEG and poly(L-histidine).
†From R&D catalog.
‡From Sigma-Aldrich catalog.
CPP:SA; poly 1,3-bis-(p-carboxyphenoxy) propane-co-sebacic acid; DC–Chol: 3β-[N-(N′,N′-dimethylaminoethane)-carbamoyl]–cholesterol; DIC: Dual-interaction complex; DOPE: Dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP: Dioleoyl-3-trimethylammonium-propane; DPPG: 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol; ECP: Extracellular proteins; EPC: Egg lecithin; NA: Not available; MVL: Multivesicular liposome; PCL: Polycaprolactones; PGA: Poly(glycolide); PLA: Polylactic acid; PLGA: Poly(lactide-co-glycolide); rHV2: Recombinant hirudin variant-2; s.c.: Subcutaneous; SLN: Solid lipid nanoparticles; STZ: Streptozotocin.

Table 2. Recent developments and patents in microparticles, liposomes and nanoparticles for parenteral delivery of protein and peptide therapeutics (cont.).

Protein/peptide	Molecular weight	Polymer	Remarks	Ref.
ECP antigen of *Aeromonas hydrophila*	NA	ECP, chitosan	Antigen encapsulated in chitosan-coated liposome was more stable and induced a better immune response. It demonstrated more enhanced adaptive and innate immune responses than Freund's incomplete adjuvant–ECP at 21, 42 and 63 days postimmunization in rabbit	[80]
IFN-α-2b	NA	Soybean, lecithin, cholesterol	After intramuscular injection in Wistar rats, for liposomal IFNα-2b, apparent terminal half-life was 2.3-times longer than that of IFNα-2b in solution	[81]
LXT-101 (Ac-D-Nal-DPhe(4-Cl)-D-Pal-Ser-Mop-D-Pal-Leu- Arg-Pro-D-Ala·NH2)	NA	Phosphatidylcholine-rich fraction of EPC, DPPG, triolein, cholesterol	Pharmacokinetic studies in Wistar rats demonstrated that LXT-101 from MVLs exhibited about 2.2-fold increase in bioavailability and 19.3-fold increase in mean residence time compared with the normal solution following s.c. injection	[82]
rHV2	NA	Stearylamine, cholesterol, egg phosphatidylcholine, DC–Chol	Cationic liposomes induced sustained release of rHV2 in plasma, significantly prolonged the antithrombotic efficacy and plasma level of rHV2 after intravenous injection in rats in comparison with neutral lipid liposomes	[83]
Insulin	NA	PEG, PLA	A s.c. administration to fed diabetic rabbits with nanoparticles containing 50 IU of insulin load per kg body weight controlled the blood glucose level within the physiologically normal range of 90–140 mg/dl, and provided prolonged effect for more than 7 days	[84]
L-asparaginase	~128 kDa	PLGA	Nanoparticles demonstrated sustained release of native as well as PEG–conjugated asparaginase with 66.66 and 44.45% release in 28 days, respectively. The PEG conjugation protected the enzyme and prevented it from denaturation during encapsulation	[85]
Catalase	NA	Lecithin, triglyceride	Catalase loading into SLN demonstrated significant protection against proteolytic degradation. Moreover, SLN sustained the release of catalase	[86]
Catalase	NA	Soybean phosphatidylcholine, tripalmitin	Catalase entrapment was 77.9 ± 1.56% into SLN. Catalase release from SLN was sustained and catalase was released up to 20% within 20 h. Catalase-loaded SLN stably retained 30% of H_2O_2-degrading activity for at least 24 h in a proteolytic environment. On the other hand, free catalase lost its activity within 1 h	[87]
Insulin	NA	γ-PGA	A s.c. administration of insulin nanoparticles (γ-PGA) to mice appeared in rat blood in the very early time interval and started to taper off after 3 h	[88]
Peptide GAP-107B8.107	NA	EPC, egg phosphatidylglycerol	Studies in rats with bolus intravenous injection demonstrate that PEGlylated liposomal formulation significantly extended the half-life (8–26-times) of the peptide GAP-107B8.107, increase the *in vivo* stability, systemic exposure (>90-times that found for free peptide) and reduce clearance rates (>ten-times).	[89]

DIC formed between insulin and a copolymer composed of branched oligoethyleneimine, mPEG and poly(L-histidine).
†From R&D catalog.
‡From Sigma-Aldrich catalog.
CPP:SA; poly 1,3-bis-(p-carboxyphenoxy) propane-co-sebacic acid; DC–Chol: 3β-[N-(N′,N′-dimethylaminoethane)-carbamoyl]–cholesterol; DIC: Dual-interaction complex; DOPE: Dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP: Dioleoyl-3-trimethylammonium-propane; DPPG: 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol; ECP: Extracellular proteins; EPC: Egg lecithin; NA: Not available; MVL: Multivesicular liposome; PCL: Polycaprolactones; PGA: Poly(glycolide); PLA: Polylactic acid; PLGA: Poly(lactide-co-glycolide); rHV2: Recombinant hirudin variant-2; s.c.: Subcutaneous; SLN: Solid lipid nanoparticles; STZ: Streptozotocin.

Table 2. Recent developments and patents in microparticles, liposomes and nanoparticles for parenteral delivery of protein and peptide therapeutics (cont.).

Protein/peptide	Molecular weight	Polymer	Remarks	Ref.
Exenatide	NA	PLGA	The efficacy of exenatide microspheres was assessed in male C57 mouse via s.c. injections of exenatide solution and microspheres. Blood glucose concentrations of groups of microspheres were significantly lower than the control group over 10 days	[90]
Goserelin	NA	PLGA	Goserelin-loaded microparticles were prepared with PLGA following the emulsion evaporation method. Microparticles exhibited decreased initial drug release amounts and superior long-term continuous release properties	[91]

DIC formed between insulin and a copolymer composed of branched oligoethyleneimine, mPEG and poly(L-histidine).
†*From R&D catalog.*
‡*From Sigma-Aldrich catalog.*
*CPP:SA; poly 1,3-*bis*-(p-carboxyphenoxy) propane-co-sebacic acid; DC–Chol: 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl]–cholesterol; DIC: Dual-interaction complex; DOPE: Dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP: Dioleoyl-3-trimethylammonium-propane; DPPG: 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol; ECP: Extracellular proteins; EPC: Egg lecithin; NA: Not available; MVL: Multivesicular liposome; PCL: Polycaprolactones; PGA: Poly(glycolide); PLA: Polylactic acid; PLGA: Poly(lactide-co-glycolide); rHV2: Recombinant hirudin variant-2; s.c.: Subcutaneous; SLN: Solid lipid nanoparticles; STZ: Streptozotocin.*

Also, with s.c. injection, more prolonged LH inhibition was observed for degarelix loaded microparticles compared with degarelix solution [59,60]. In another study, biodegradable microspheres have been developed for attaining sustained release of recombinant IFNα-2b. IFNα-2b is used for the treatment of hepatitis C virus infection and some cancers such as hairy cell leukemia and lymphoma. It requires long term frequent injections because of short *in vivo* half-life, which produces fluctuation in serum concentrations and adverse effects. In order to improve therapeutic index, Li *et al.* have prepared IFNα-2b loaded microspheres [61]. Investigators have utilized a mixture of PLGA and poly (ethylene glycal/butylenes terephthalate) (9:1, w/w) as a microsphere matrix to achieve an optimal release profile. For preparing microspheres, first gelatin microparticles containing IFNα-2b were prepared, which were further encapsulated into poly (ethylene glycal/butylenes terephthalate) microspheres. The microspheres have demonstrated an ideal *in vitro* zero-order release profile with the cumulative release of 83.06% at day 23. Following a single s.c. injection of IFNα-2b loaded microspheres, sustained steady state plasma levels of active IFNα-2b were attained for over 13 days. On the other hand, s.c. injection of IFNα-2b solution demonstrated maximum plasma concentration (C_{max}) of 182.59 IU/ml at 1 h, which rapidly declined to 3 IU/ml at 12 h **(Figure 3A & B)**.

Microparticles are promising depot formulations for macromolecules. However, retention of protein stability during microparticles fabrication is a major concern. Proteins incorporated into microparticles are prone to denaturation at aqueous-organic interface during formulation development resulting in loss of protein bioactivity [57]. If such degraded protein is released in plasma, it can exert numerous immunological reactions [57]. Therefore, the central goal of most of the research is currently focused on stabilization of proteins during microparticle preparation, storage and release. Different stabilizing excipients have been explored and can be used to prevent aggregation and unfolding of therapeutic proteins. PEG and sugars have demonstrated excellent protein stabilizing effect. For instance, addition of PEG (MW 5000) and maltose with α-chymotrypsin in a primary emulsion has significantly reduced its aggregation and inactivation [62].

Protein stability can also be achieved through judicious choice of microparticles fabrication methods. To prevent degradation

at aqueous-organic interface, nonaqueous methodology called ProLease® technology was introduced [63]. In this method, first protein is dissolved in an aqueous solvent and stabilized by complexation with cation Zn^{2+}. The complex is micronized and lyophilized to obtain protein particles of 1–6 µm. Later, particles are added to polymer solution (polymer dissolved in an organic solvent) containing Zn^{2+}. Microspheres are formed by creating droplets through an ultrasonic nozzle and immediately freezing it. Organic solvent is then extracted by mixing with either ethanol or a mixture of ethanol and hexane or pentane. Finally, microspheres are lyophilized to get free flowing powder [63]. Nutropin Depot®, a depot formulation of recombinant human growth hormone, was developed using ProLease technology [64].

Protein in solid state can also be encapsulated using solid-in-oil-in-water (s/o/w) method. Either spray- or spray-freeze-dried protein or protein-loaded solid nanoparticles can be encapsulated in microspheres using s/o/w method. However, later approaches showed more advantages in terms of increasing the encapsulation efficiency, reducing burst release, improving release profiles, and enhancing protein activity during release. Encapsulation of lysozyme-loaded solid nanoparticles in PLGA microspheres by s/o/w protocol lead to good stability after encapsulation and release [65]. Similarly, Brenda *et al.* have developed polymeric microspheres encapsulating protein nanospheres for HRP. For preparing protein nanospheres, HRP was co-lyophilized with methyl-β-cyclodextrin (MβCD). The HRP/MβCD powder was suspended in ethyl acetate in order to dissolve MβCD followed by sonication for 30 s. The solid protein nanoparticles were collected by centrifugation following removal of ethyl acetate containing MβCD. The formed nanoparticles were encapsulated into PLGA microspheres by s/o/w method [66]. Investigators have also prepared lyophilized HRP loaded PLGA microspheres. After encapsulation, the specific activity of HRP could be completely recovered (100 ± 4%) for HRP nanoparticles but dropped to 87 ± 2% for lyophilized HRP. Similarly, the residual HRP activity subsequent to 24 h of *in vitro* release was significantly higher for the encapsulated nanoparticles (100 ± 1%) than that of the lyophilized HRP loaded formulation (65 ± 9%). The attributed reason is decreased aggregation formation in the case of HRP protein nanospheres (3 ± 2%) loaded microparticle compared with lyophilized HRP (12 ± 1%).

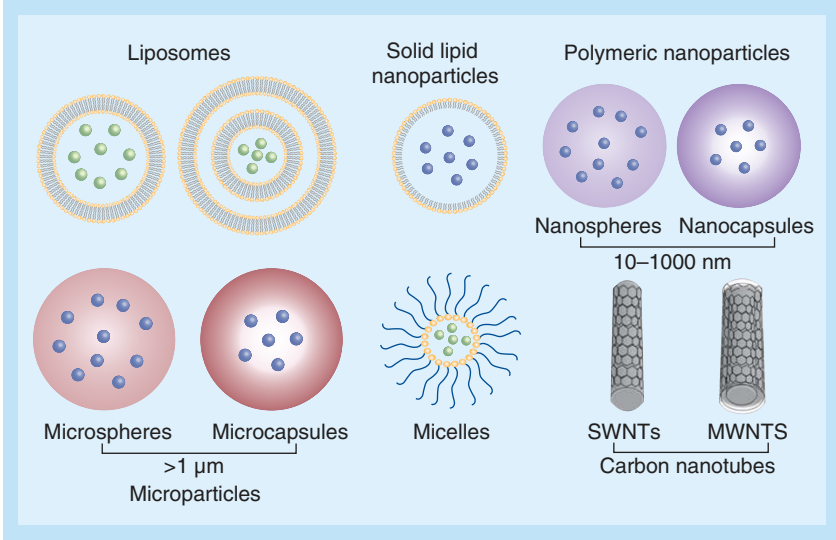

Figure 2. Colloidal carriers for protein and peptide parenteral delivery.
MWNT: Multiwalled carbon nanotubes; SWNT: Single-walled carbon nanotubes.

Microspheres loaded with protein nanoparticles showed sustained release for 25 days. The use of protein nanoparticles revealed substantial advantages over a lyophilized protein formulation.

PEGylated proteins are also formulated into microparticles. Encapsulation of PEGylated proteins resulted in high encapsulation efficiencies and lower initial burst release than native protein-loaded microspheres. PEGylated TNF-related apoptosis-inducing ligand (PEG-TRAIL) exhibited approximately twofold higher encapsulation efficiency in PLGA microspheres than TRAIL. The calculated burst release from PEG-TRAIL microspheres and TRAIL microspheres was 15.8 and 42.7%, respectively. Further, *in vivo* studies demonstrated better pharmacokinetic and antitumor efficacy with PEG-TRAIL microspheres than TRAIL microspheres **(FIGURE 4)** [67].

In another study, Hinds *et al.* have encapsulated PEGylated-insulin into PLGA microparticles [68]. Low burst release was observed under *in vitro* conditions and a nearly complete release of PEG–insulin was achieved from microparticles. Also, insulin biological activity was preserved subsequent to PEGylation and PLGA encapsulation. A single s.c. injection of PEG–insulin microspheres in to diabetic rats lowered the serum glucose levels to values of <200 mg/dl for approximately 9 days. Aforementioned studies suggest that the combination of two complementary technologies (i.e., PEGylation and microencapsulation) may have great potential for sustained delivery of therapeutic proteins and peptides.

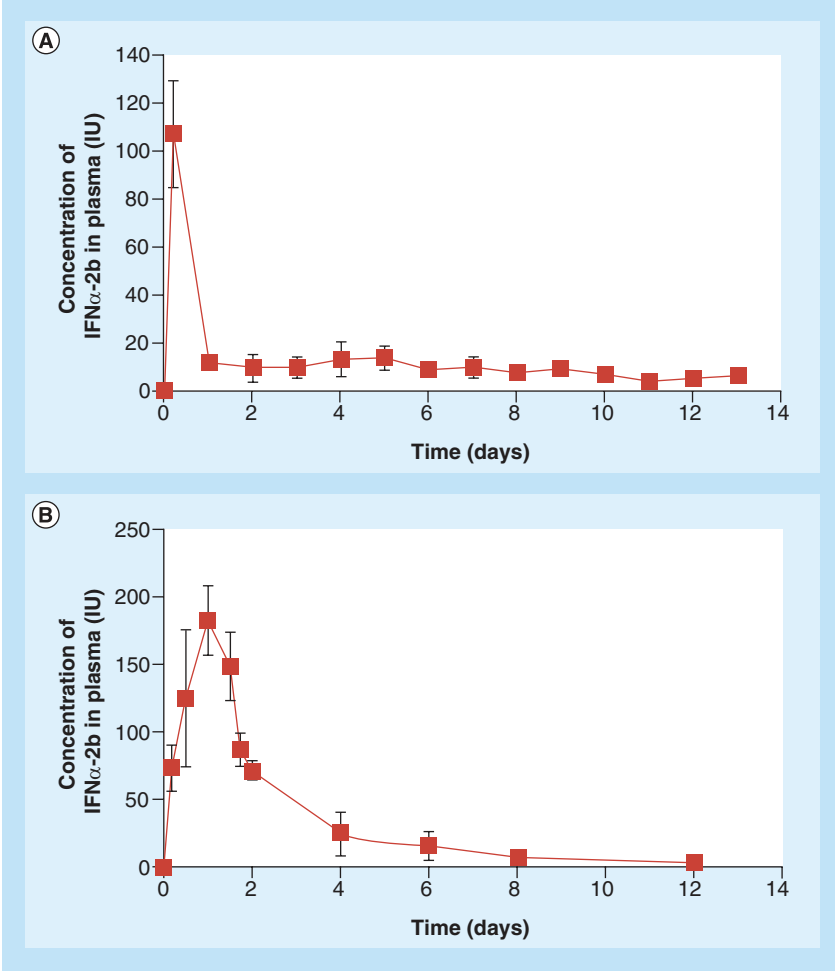

Figure 3. Release profile of IFNα-2b. (A) Release profile of the optimized IFNα-2b microspheres in rats. **(B)** Profile of IFNα-2b concentration following administration of IFNα-2b solution in rats. Six rats were administrated with a single subcutaneous dose of 200,000 IU.
Reproduced with permission from [61].

■ Nanoparticles

Nanoparticles are colloidal carriers with size ranging from 10 to 1000 nm [92], and can be fabricated from lipids, polymers or metal. For protein and peptide delivery, polymeric and solid lipid nanoparticles (SLNs) have been widely investigated. Polymeric nanoparticles are fabricated from natural or synthetic polymers, such as chitosan, alginate, PCL, polylactic acid (PLA), poly (glycolide), PLGA [93]. Polymeric nanoparticles can be generated as nanospheres or nanocapsules using different fabrication method. In nanospheres, drug molecules are uniformly distributed into polymeric matrix while nanocapsules carry drug molecules confined within a polymeric membrane [92,94]. A detailed methodology for proteins and peptides loaded nanoparticle preparation is discussed elsewhere by Yadav *et al.* [93]. Similar to microparticles, nanoparticles can protect biologics from degradation, prolong *in vivo* half-life and provide long term drug release. Nonetheless, few research attempts have been made in development of polymeric nanoparticles for parenteral delivery of proteins and peptides. In a recent study, PLGA nanoparticles were developed and characterized as a sustained release system for salmon calcitonin (sCT) [95]. Nanoparticles were prepared by a double emulsion solvent evaporation method employing Eudragit RS and PLGA. It was demonstrated that sCT was incorporated into nanoparticles with encapsulation efficiencies in the range of 69–83%. *In vitro* release studies demonstrated that NPs composed of Eudragit RS and PLGA/Eudragit RS provided faster drug release while the sCT release from pure PLGA nanoparticles was very slow and reached only 20% after 4 weeks. After s.c. administration of PLGA nanoparticles in Wistar rats, an elevated serum sCT levels were sustained for 3 days with higher bioavailability compared with sCT solution.

Proteins released from nanoparticles formulation are expected to retain its native structure and biological activity and hence stabilizers can be added during preparation. For the delivery of insulin, Kumar *et al.* developed insulin-loaded PLGA nanoparticles with different stabilizers such as pluronic F68, trehalose and sodium bicarbonate utilizing double emulsion solvent evaporation method [96]. The co-encapsulation of stabilizers resulted in diminished encapsulation efficiency. However, pharmacodynamic studies suggested that these nanoparticles provide sustained insulin release and prolonged reduction of blood glucose in streptozotocin (STZ) induced diabetic rats.

In recent studies, hydrophobic ion-pairing (HIP) complexation approach has been applied to enhance protein stability and increase encapsulation efficiency into nanoparticles. In this approach, ionizable functional groups of a drug molecule are complexed with ion-pairing agents (e.g., surfactant or polymer) containing oppositely charged functional groups leading to formation of HIP complex [97]. In this approach, hydrophilic protein molecules exist in a hydrophobic complex form. As a result, its partition into the polymeric matrix can be significantly enhanced during encapsulation. Moreover, HIP complexation involves ionic interactions and obviate the use of any covalent modification

in proteins to impart more hydrophobicity [97]. This approach has been studied for formulating nanoparticles loaded with various protein- and peptide- based therapeutics such as insulin, melittin and leuprolide [98–100].

Recently, Gaudana *et al.* prepared a HIP complex of lysozyme using dextran sulphate as a complexing polymer [101]. The HIP complexation did not cause any loss in enzymatic activity of lysozyme. Furthermore, nanoparticles containing lysozyme in the HIP complex form were developed utilizing spontaneous emulsion solvent diffusion method. Prepared nanoparticles sustained release of lysozyme for 30 days and released lysozyme maintained its enzymatic activity. In another study, Shi *et al.* hydrophobically ion-paired insulin with sodium dodecyl sulfate (SDS) [98]. The formed insulin–SDS complex was further loaded into PLGA nanoparticles by a modified spontaneous emulsion solvent diffusion method. In comparison with a conventional method, direct dissolution of SDS-paired insulin in nonaqueous organic phase led to an increase in drug recovery from 42.5 to 89.6%. Hypoglycemic activity of insulin was unaltered after ion-pairing with SDS. Moreover, after s.c. administration, the hypoglycemic activity of SDS-paired insulin was slightly higher than that of free insulin in mouse model.

In a recent study, Peng *et al.* have developed an insulin–phospholipid complex by anhydrous co-solvent lyophilization process [102]. This complex was loaded in biodegradable poly(hydroxybutyrate-*co*-hydroxyhexanoate) nanoparticles (INS–PLC–NPs). The insulin–phospholipid complex significantly enhanced lipophilicity of insulin and facilitated encapsulation into nanoparticles with encapsulation efficiency of 89.73%. INS–PLC–NPs provided sustained insulin release over 31 days. Moreover, hypoglycemic effect in STZ induced diabetic rats lasted for more than 3 days following s.c. injection of INS–PLC–NPs, which was significantly prolonged compared with administration of insulin solution **(FIGURE 5)**. Pharmacological bioavailability of INS–PLC–NPs relative to insulin solution was over 350% [102]. Therefore, the INS–PLC–NPs system may be highly promising as a long-term insulin release formulation, which may significantly improve patient compliance. Even though numerous research studies exploited polymeric nanoparticles application for parenteral controlled delivery of proteins and peptides, most of the research has been directed toward noninvasive delivery of protein and peptide therapeutics.

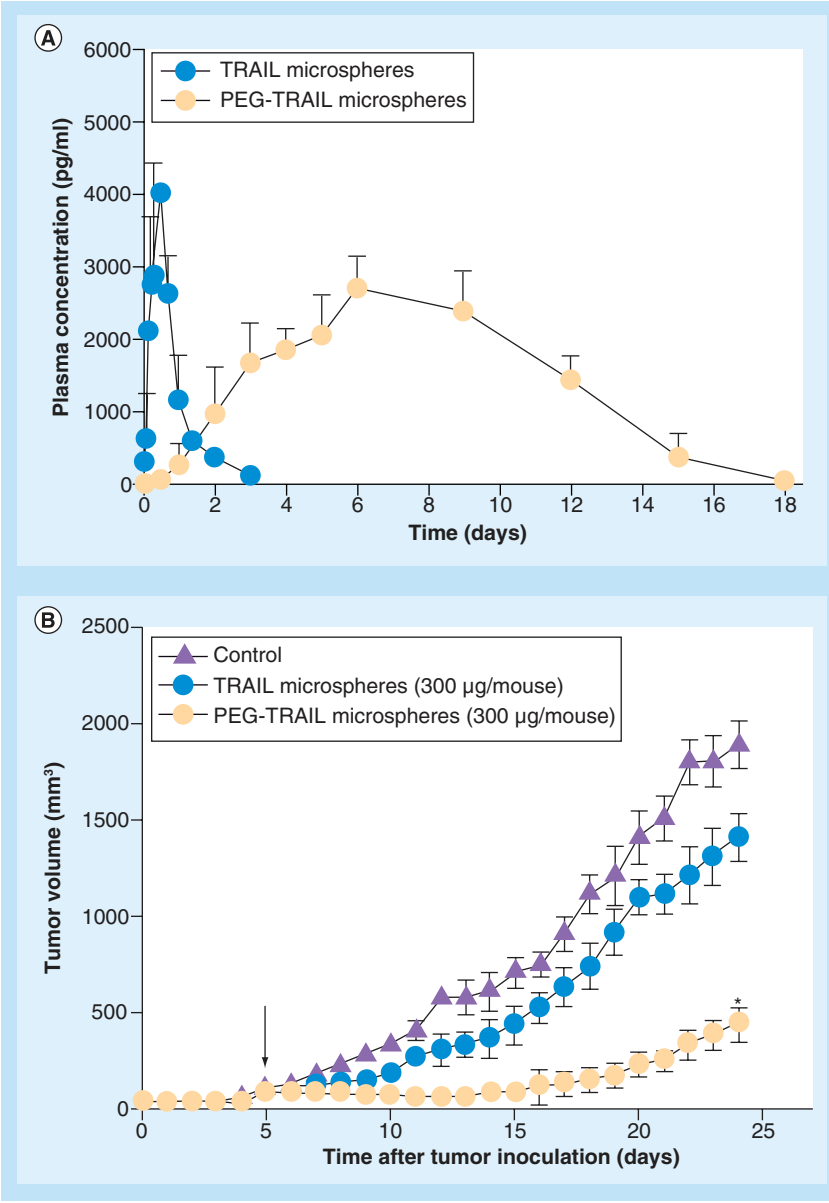

Figure 4. Pharmacokinetic and pharmacodynamic of TNF-related apoptosis-inducing ligand or PEG-TNF-related apoptosis-inducing ligand microspheres. (A) *In vivo* pharmacokinetic profiles of TRAIL or PEG-TRAIL microspheres after subcutaneous administration (100 µg/rat; n = 5); **(B)** Tumor growth suppressions by TRAIL or PEG-TRAIL microspheres (300 µg/mouse, subcutaneous).
TRAIL: TNF-related apoptosis-inducing ligand.
Reproduced with permission from [67].

SLNs are colloidal systems being explored as an alternative to polymeric nanoparticles, liposomes, emulsions for protein and peptide delivery [103,104]. SLNs provide dual advantage of both polymeric nanoparticles and liposomal properties. Also, these carriers provide unique advantages such as high entrapment of

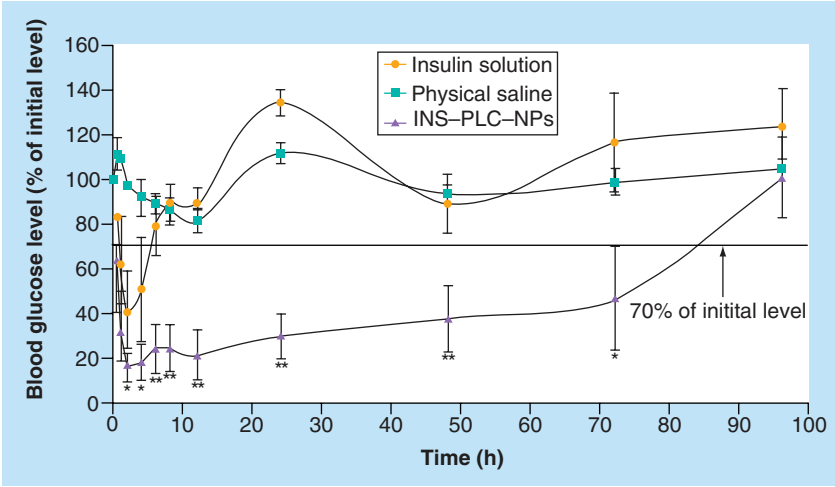

Figure 5. Blood glucose level–time curve after subcutaneous injection of insulin solution at a dose of 1 IU/kg, in saline and INS–PLC–NPs at a dose of 4 IU/kg to diabetic male Sprague–Dawley rats (220–280 g). Data presented as mean ± SD (n = 5).
*p <0.05; **p <0.001.
INS–PLC–NPS: Poly(hydroxybutyrate-*co*-hydroxyhexanoate) nanoparticles.
Reproduced with permission from [102].

hydrophobic drugs, easy production and scale up, improved drug stability, controlled drug release, and improved bioavailability. In addition, SLNs have excellent biocompatability and biodegradability over other carrier systems [105,106]. In particular, hydrogenated castor oil, PEGylated chitosan, tristearin, Tween®-80, phosphatidylcholine, soybean lecithin, middle chain triacylglycerols, poloxamer, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, cholesteryloleate, and sodium stearate are commonly employed in the SLN preparation. Salmaso *et al.* prepared insulin loaded solid lipid particles with tristearin, Tween®-80, phosphatidylcholine and 5 kDa PEG (if different weight ratio) following supercritical gas-assisted melting atomization process [107]. Optimized insulin particles were evaluated for hypoglycemic effect in a diabetic mouse model. For *in vivo* studies, solid lipid particles were extracted for insulin and this insulin was injected subcutaneously into diabetic mice. Insulin extracted from nanoparticles demonstrated similar hypoglycemic effect to native insulin in solution. Results indicated that the process of manufacturing did not impair protein biological activity (hypoglycemic activity). Also, solid lipid particles provided sustained release of insulin for more than 50 h *in vitro*. In another study, Huang *et al.* encapsulated a hemolytic peptide drug, melittin, in the lipid nanoparticles and attempted to deliver drug to melanoma cancer tissues [108]. Lipid particles were prepared with 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and cholesteryloleate. In this study, the authors designed and prepared a hybrid cytolytic peptide, α-melittin, in which the N-terminal of melittin was conjugated to C-terminus of amphipathic α-helical peptide (FAEKFKEAVKDYFAKFWD-NH2) via a Gly-Ser-Gly linker. This α-melittin demonstrated a strong α-helical configuration that allowed interactions with phospholipids and self-assembling into lipid nanoparticles. Results demonstrated that α-melittin lipid nanoparticles efficiently shielded the new hybrid peptide charge that resulted in neutral nanoparticles and reduced the cytotoxicity. *In vivo* studies were conducted in C57/BL6 mice by injecting α-melittin lipid nanoparticles via tail vein injection. Tumor volumes differed significantly for α-melittin lipid nanoparticles (48.89 ± 29.50 mm^3), α-peptide (322.20 ± 49.84 mm^3) and phosphate-buffered solution (PBS) group (389.23 ± 49.84 mm^3). *In vivo* results demonstrated 82.8% tumor inhibition rate relative to PBS treated group. Also, the authors demonstrated that the hybrid peptide encapsulated nanoparticles reduced liver injury. The new hybrid peptide was found to be safe relative to melittin.

Hydrophobic peptide therapeutics are also being encapsulated in SLNs. Cyclosporine-A (Cys-A) is a cyclic peptide comprising of 11 amino acids, mostly used as an immunosuppressant. To prepare particles, soybean lecithin, poloxamer and PEGylated chitosan were used. Cys-A loaded PEGylated chitosan modified SLNs and chitosan modified SLNs were prepared using an emulsification/solvent evaporation method [109]. *In vivo* studies were conducted with single i.v. injection of nanoparticles (equivalent to 5 mg Cys-A) to rabbits, where Cys-A dissolved in a mixture of ethanol and cremophor served as control. Pharmacokinetic parameters demonstrated that PEGylated chitosan coated SLNs were superior to chitosan modified SLNs and Cys-A solution. The AUC for PEGylated chitosan modified SLNs, chitosan modified SLNs and Cys-A solution was found to be 931.58 ± 2293.16 (μg/ml)*h, 50.26 ± 7.47 (μg/ml)*h and 36.24 ± 6.19 (μg/ml)*h, respectively. The elimination half-life for nanoparticles was approximately 21-times longer than the Cys-A solution. The study results indicate that SLNs can encapsulate Cys-A, sustain its release, prolong systemic circulation, and improve bioavailability.

Several novel strategies have been applied to entrap hydrophilic peptides in the lipid core of SLNs that includes HIP complexation of peptides with counter ions (SDS, dioctyl sodium sulfosuccinate) and development of gel-core SLNs. Gallarate *et al.* loaded insulin and leuprolide (model peptides) via HIP complexation into a SLN following coacervation technique [110]. To prepare SLN, initially peptide drug ion pairs were prepared. Peptides were mixed with counterion solutions at various ratios (peptide:counterion) and precipitants were extracted following centrifugation and lyophilization. For insulin and leuprolide, SDS and dioctyl sodium sulfosuccinate (AOT) were used as counterion, respectively. The peptide precipitate was dissolved in ethanol and further used in SLN preparation. Results demonstrated high entrapment efficiency (>85%) for both insulin and leuprolide. The thermal degradant of insulin (A21-deamido insulin) was quantified to be <5% indicating the chemical integrity of insulin maintained after coacervation process. Furthermore, *in vitro* release studies were conducted and reported only for leuprolide formulation following two methods: the test tube method and diffusion through a hydrophilic membrane. In the test tube method, *in vitro* release studies were conducted in decanol as organic solvent with 0.05% leuprolide AOT ion pair loaded in 1% SLN stabilized with 0.5% polyvinyl alcohol (PVA; PVA9000 and PVA85000) and 0.05% leuprolide AOT 1:2 ion pair aqueous solution. Results demonstrated that more than 80% of leuprolide was released in 7 h from the HIP complex into organic solvent. The faster release may be attributed to hydrophobic nature of the HIP complex. Nevertheless, ion-pair loaded in SLN and surface stabilized with PVA9000 and PVA85000 sustained the peptide release. Different grades of PVA that covers SLN surface influenced the release but to a low extent, probably owing to surface adsorbed drug interaction with PVA. The second set of experiments (diffusion through hydrophilic membrane) was conducted in aqueous media (drug receiving chamber). *In vitro* release studies were conducted for, 0.05% leuprolide AOT-loaded SLN stabilized with 0.5% PVA85000, 0.05% leuprolide AOT 1:2 ion pair aqueous solution as blank solution and 0.05% leuprolide aqueous solution. Results indicate that approximately 80% of leuprolide was released from aqueous leuprolide solution in 7 h, whereas the ion pair and ion pair loaded in SLN (sterically stabilized with PVA) demonstrated sustained release with 15 and 5% leuprolide release at 24 h, respectively. The study demonstrates that SLN can entrap peptides, improve peptide stability and sustain the release of peptides. Also, the study demonstrates a new coacervation method to entrap hydrophilic peptide drugs into SLN. In another study Yang *et al.* reported the loading of thymopentin and insulin peptides into gel-core-SLN [111]. Nanoparticles were prepared following double emulsion and thermal sensitive gel technology. Pluronic F127 and glyceryl palmitostearate, glyceryl tripalmitate, cetyl palmitate were used to form gel-core in SLN. It is hypothesized that hydrogel forms SLN core with lipid surface. Thymopentin and insulin were loaded into gel-core–SLN. The novel gel-core–SLN carrier system demonstrated higher entrapment efficiency than conventional SLNs. Higher entrapment (~62%) was achieved with glyceryl tripalmitostearate for insulin and thymopentin in gel-core–SLN relative to other glyceryl derivatives. *In vitro* release studies for thymopentin and insulin were conducted where conventional SLN served as control. Results demonstrate that gel-core–SLN sustained the release for both peptides. Thymopentin and insulin release was approximately 25 and 15%, respectively in 24 h, whereas the conventional insulin and thymopentin SLN released approximately 50% of both the drugs in 24 h. Study results indicate that gel-core–SLN demonstrate high-peptide entrapment, reduce burst release and further sustain the release of peptide therapeutics from the gel-core.

■ Liposomes

Liposomes are bilayered vesicles with an aqueous core enclosed by phospholipid membrane. These lipid vesicles are composed of phospholipids of either synthetic or natural origin and vary in size from 20 nm to several hundred micrometers. Depending on the preparation method, liposomes can be small unilamellar vesicles (25–50 nm), large unilamellar vesicles (100–200 nm), giant unilamellar vesicles (1–2 µm) and multilamellar vesicles (MLV; 1 µm–2 µm) [112,113]. These vesicles are highly biocompatible and less toxic in nature as phospholipids used in fabrication are the components of biomembranes. Like polymeric carriers, liposomes also provide advantages of protecting therapeutic proteins from *in vivo* degradation, prolonging half-life, longer systemic circulation time and higher bioavailability. Furthermore, it is customary to develop liposomes with desired

properties such as cationic, PEGylated liposomes by varying lipid compositions [114]. For successful delivery, proteins and peptides can be either encapsulated into liposomes or adsorbed on the surface. Protein cargos loaded in liposomes have demonstrated excellent therapeutic efficacy on parenteral administration. For instance, persistence hypoglycemic effect was observed with insulin entrapped in liposome after s.c. injection to alloxan and STZ induced diabetic dog models [115]. In another study, to improve therapeutic activity of IL-2, Kanaoka *et al.* have developed liposomal IL-2 by mixing DSPC–DSPG liposome suspension and lyophilized IL-2. Under optimal condition, IL-2 was strongly and almost completely adsorbed onto liposomes. This liposomal IL-2 displayed improved therapeutic effect against experimental M5076 metastases, after s.c. administration in mice. There was approximately eightfold enhancement in mean residence time of IL-2 in the systemic circulation when formulated as liposomes [116].

The size and surface properties are key parameters that control formulation efficacy and hence need to be considered and optimized while designing liposomal formulation. For instance, most prominent hypocalcemic effect was observed with large MLV containing calcitonin than small unilammelar vesicles. These results suggest the importance of size in designing liposomal formulation [117]. Both unilamellar and multilamellar liposomes provide sustained release from several hours to days after intravascular administration. The prolonged drug release can be achieved by multivesicular liposomes, also known as DepoFoam® technology. Unlike ULV and MLV, these DepoFoam particles (or multivesicular liposome) are composed of nonconcentric multiple aqueous chambers surrounded by a network of lipid layers. This arrangement confers an increased level of stability and longer duration of drug release [118]. DepoFoam particles offer advantages of high drug loading, sustained drug release and prolonged therapeutic activity. Encapsulation of PEGylated proteins into multivesicular liposomes is reported to provide more sustained release compared with native proteins [119]. The DepoFoam formulation loaded with human apoE-18A (hE-18A) chimera (DepohE-18A) yielded sustained release of hE-18A in human plasma as well as PBS for 7 and 8 days, respectively under *in vitro* conditions. Moreover, post single s.c. administration of DepohE-18A in apoE-null mice produced cumulative 18% decrease in serum cholesterol levels after 6 days and also cholesterol levels remained low even at 8 days. On the contrary, an equivalent dose of free peptide showed maximal cholesterol lowering in 4 h, followed by a rapid decline in efficacy within 24–48 h [120]. DepoFoam formulations for insulin, IGF-I, leuprolide, progenipoietin, leridistim, IFNα-2b and octreotide have also been developed and characterized [121–126].

For parenteral delivery, PEGylated liposomes have shown more improvement in systemic circulation time relative to conventional liposomes. Surface modification of liposomes with PEG interferes with recognition and uptake by reticuloendothelial system and thereby provides extended circulation time. Recently, Kim *et al.* developed liposomal formulation to prolong *in vivo* half-life of tPA, a widely used thrombolytic agent [127]. Liposomes were prepared by lipid film method with egg phosphatidylcholine (EPC), cholesterol and sodium cholesterol-3-sulfate. To prepare PEGylated liposomes (PEGLip), distearolyphosphatidyl ethanolamine-*N*-PEG 2000 (DSPE-PEG [2000]) was included during preparation. *In vivo* pharmacokinetic profile of tPA following parenteral administration in rats was improved by both conventional (egg phosphatidylcholine liposomes) and PEGylated (EPC–PEG) liposomes **(Table 3)**. Encapsulation of tPA into EPCL and EPC–PEG liposome extended half-life of tPA by 16- and 21-times relative to free tPA [127].

Another liposomal formulation technology known as (PEGLip) technology has also demonstrated promising results for proteins and peptides delivery. PEGLip technology involves noncovalent, high-affinity binding of proteins and peptides containing specific amino acid sequence to the outer surface of PEGlip [128]. This association is reported to enhance pharmacodynamic properties of the formulated proteins. For instance, Yatuv *et al.* developed PEGLip for the delivery of FVIII for the treatment of Hemophilia. Efficacy of developed formulation was assessed in both preclinical and clinical studies. PEGLip formulation of both recombinant and plasma derived forms of FVIII extended hemostatic efficacy *in vivo,* which was further demonstrated by better survival of hemophilic mice following tail-vein transection. Mice that received PEGLip-FVIII bled less and survived significantly longer than standard FVIII or saline treatment groups. PEGLip-FVIII also provided significantly extended protection from bleeding in human subjects than the standard FVIII. A single prophylactic injection of PEGLip-FVIII at

Table 3. Pharmacokinetic parameters after intravenous administration of three tissue plasminogen activator formulations to rats (n = 5).

Pharmacokinetic parameters	Formulations		
	Control	EPCL	EPC–PEGL
$t^{1/2}$ (min)	5.87 ± 4.95	50.03 ± 14.87	132.61 ± 20.26
AUC (IU h/ml)	17.07 ± 6.69	274.61 ± 50.96	355.41 ± 109.13
AUMC (IU h^2/ml)	1.49 ± 1.09	330.27 ± 168.43	1038.39 ± 418.22
MRT (min)	5.08 ± 3.09	69.79 ± 21.35	171.92 ± 36.99
CL (ml/min/kg)	9.18 ± 4.73	0.48 ± 0.07	0.38 ± 0.17
Vd_{ss} (ml/kg)	42.04 ± 16.56	32.49 ± 6.81	63.70 ± 19.24

AUMC: Area under the first moment plasma concentration–time curve; CL: Total body clearance; EPC–PEGL: Liposomes composed of egg phosphatidylcholine:cholesterol:sodium cholesterol-3-sulfate:distearolyphosphatidyl ethanolamine–PEG; EPCL: Liposomes composed of egg phosphatidylcholine:cholesterol:sodium cholesterol-3-sulfate; MRT: Mean residence time; $t^{1/2}$: Terminal half-life Vd_{ss}: Volume of distribution at steady state.
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25 IU/kg resulted in a mean bleed-free interval of 10.9 days, while it was 5.9 days with standard FVIII. Likewise, administration at 35 IU/kg PEGLip-FVIII also resulted in a prolonged mean bleed-free interval of 13.3 days, which was significantly higher compared with standard FVIII (7.2 days) [129].

Several studies have demonstrated usefulness of liposomal formulation in enhancing efficacy of protein and peptide therapeutics. However, several critical issues need to be resolved such as cost, stability of formulation and protein payloads, and scale-up to advance liposomal formulation to clinic.

■ Carbon nanotubes

Carbon nanotubes (CNTs) are hollow cylindrical nanostructures consisting of hexagonal arrangement of sp2-hybridized carbon atoms. Based on rolling single or multiple layers of graphene sheets, CNTs are generally categorized as single-walled CNTs (SWNTs) and multi-walled CNTs (MWNTs). CNTs as carriers offer numerous advantages, such as large surface area, ligand functionalization for targeting and propensity to internalize into a wide variety of cell types [130,131].

Proteins as a result of larger molecular weight could not penetrate through biological barriers. CNTs have been applied as a molecular transporter for protein delivery *in vitro* [132]. For this purpose, proteins are adsorbed on the sidewalls of acid-oxidized SWNTs by means of hydrophobic interactions. In a study by Kam *et al.* SWNT-biotin-strepavidin conjugate delivered strepavidin into cells and exhibited toxicity to HL60 cells [133]. In a similar study, proteins such as cytochrome C (12 kDa), Alexa-fluor® BSA, (66 kDa), fluorescein isothiocyanate-labeled human IgG (150 kDa), and protein A (SpA 42 kDa) are reported to be readily transported into various mammalian cells by CNTs [134]. In another study, MWNTs were used as a carrier for the recombined RTA. The toxin protein RTA–MWNT conjugate was found to translocate to the cytoplasm of various cell lines and induced cell death. The conjugate showed approximately three-times higher cell death rates for HL7702, HeLa, MCF-7, L-929, and COS-7 cells relative to RTA alone [135].

Several studies have demonstrated that peptide–carbon nanotube complexes can enhance the immune (antibody) response against peptides [136–139]. In a recent study, application of CNTs as a carrier to deliver peptide antigen into dendritic cells and to achieve enhanced IgG response to tumor-associated antigens has been studied by Villa *et al.* [140]. For this purpose, a 19 amino acid peptide named WT1Pep427 derived from WT1 (Wilm's tumor antigen) protein was covalently conjugated to SWNT scaffolds. The immunogenic features of peptide–CNT conjugates were subsequently assessed *in vivo*. Immunization of BALB/c mice with peptide-SWNT constructs elicited high antibody responses in comparison with the free peptide [140].

■ Micelles

Micelles are the nano-scaled constructs formed by self-assembly of amphiphilic molecules. In general, regular micellar nanoconstruct comprise of inner hydrophobic core and outer hydrophilic corona [141]. With the ongoing research efforts in drug delivery, these nanoconstructs are being widely explored as carrier systems for macromolecules (protein and/or peptide) because of various advantages such as: ability to encapsulate macromolecules in their core; ensure

chemical and physical stability for encapsulated macromolecules; development of formulation with a small polydispersity index; optimal lipid concentration conserves peptide structure during lyophilization; sustained release of encapsulated drugs and improved drug pharmacokinetic profile [142,143]. To encapsulate protein/peptide drugs, phospholipids such as DSPE-PEG [2000], co-polymeric systems PEG-PE, PLA-PEG and hyperbranched poly([amine-ester]-*co*-[D,L-lactide]) and polyion complex micelles have been investigated [142,144–146].

Studies have been conducted to improve the stability and bioactivity of peptides with micelles. Examples of such studies include pancreatic polypeptide (PP), and neuropeptide Y (NPY) loaded micelles [147,148]. Banerjee *et al.* encapsulated an endogenous peptide hormone, PP, in a phospholipid micellar formulation [147]. PP is secreted by F cells of the islets of langerhans of pancreas. It holds glucoregulatory function and therefore has significant use in the treatment of diabetes and obesity. DSPE-PEG [2000] was used to encapsulate PP and develop sterically stabilized phospholipid micellar (SSM) system. SSM size and distribution studies demonstrated that in absence of SSM a bimodal large heterogeneous PP aggregates (98.3 ± 17.9 nm and 773.3 ± 163.45 nm) were observed. However, in the presence of SSM, a monomodal distribution was observed with a mean diameter of 13.5 ± 0.7 nm. Secondary structure of PP was characterized with circular dichroism, which demonstrated that PP secondary structure was retained in presence and absence of SSM. Proteolytic degradation of PP in the presence of trypsin indicated the approximately 2.5-fold improvement in stability when PP is associated with SSM. Furthermore, *in vitro* bioactivity studies for PP–SSM were conducted in SK-N-MC cells to determine the inhibition of cAMP production in cells. Results demonstrated that PP retained its α-helical conformation, activity (when in micelles) and SSM did not hamper peptide interaction with its receptor. Similar studies were conducted by Kuzmis *et al.* to improve the stability and bioactivity of NPY with SSM prepared with DSPE-PEG [2000] [148]. NPY in saline formed large aggregates (557 ± 100 nm) but the mean hydrodynamic diameter of NPY in SSM was monomodal (14 ± 3 nm). Circular dichroism spectroscopy revealed that NPY peptide retained α helical structure indicating that peptide is stabilized in SSM. *In vitro* bioactivity in human SK-N-MC brain neuroepithelioma cells with NPY–SSM demonstrated similar results to the earlier studies. Cyclic AMP content was decreased significantly indicating that SSM construct retained stability and biological activity of NPY.

Protein and peptide therapeutics suffer from rapid *in vivo* degradation, poor pharmacokinetics and stability. To improve *in vivo* pharmacokinetics and stability, Yanan *et al.* encapsulated recombinant human erythropoietin (rhEPO) in PLA-PEG micelles [142]. PLA-PEG co-polymers with varying average molecular weights (PLA_{10000}–PEG_{3600}; PLA_{5000}–PEG_{3900}; and PLA_{2000}–PEG_{3800}) were employed for *in vivo* studies [142]. *In vivo* studies were conducted in Sprague-Dawley rats with i.v. administration of rhEPO loaded PLA-PEG micelles, where native rhEPO served as control. Pharmacokinetic parameters revealed that AUC for rhEPO micelles was twice that of native rhEPO. T_{max} and C_{max} were 4.36 h and approximately 40 ng/ml, respectively for rhEPO micelles in comparison to native rhEPO (~2 h and ~12 ng/ml). Of the three co-polymers, PLA_{3900}-PEG_{5000} demonstrated promising results as a vehicle for rhEPO delivery.

Similarly, human GLP-1 suffers from poor *in vivo* stability and performance. Lim *et al.* studied GLP-1 to improve its *in vivo* half-life and down regulate anti-inflammatory response with self-associated PEGylated phospholipids (DSPE-PEG [2000]) that form GLP1-SSM constructs [149]. Prepared GLP-1-SSM constructs had a hydrodynamic size of approximately 15 nm. *In vivo* studies were conducted with murine mice model of acute lung injury (lipopolysaccharide induced lung injury) for 12 h. Increasing peptide doses (5, 15 or 30 nmol of GLP-1/mouse) were administered subcutaneously where methylprednisolone (3 mg/kg) was used for comparison. To access the *in vivo* magnitude of GLP-1-SSM in down regulating lung inflammation parameter such as neutrophil cell count, proinflammatory cytokines (IL-6) levels in bronchoalveolar lavage fluid and MPO were selected. Results demonstrated a dose dependent suppression of neutrophil count with GLP-1-SSM whereas the GLP-1 in saline had no effect. Similarly, increasing dose significantly lowered MPO activity in murine lung. There was no statistically significant difference observed with increasing GLP1–SSM dose among bronchoalveolar lavage fluid IL-6 concentrations. Results show that SSM significantly improved *in vivo*

GLP-1 half-life and suppressed inflammation in a dose dependence manner [149].

In recent studies, polyionic complex (PIC) micelles have been developed to improve peptide stability and biological activity. PIC micelles are formulated with self assembly of PEG block copolymer that contain charged inner segment with an oppositely charged protein. The outer corona of PIC micelles is occupied by PEG chain. Heffernan *et al.* prepared CAT-loaded disulfide-crosslinked polyion micelles (DCPM) [144]. A disulfide linkage was selected for cross-linking because disulfide are relatively stable in serum and extracellular fluid but are cleaved under intracellular reducing conditions. PEG-poly-L-lysine (PEG-PLL) amines were modified with linkable dithiopyridine groups, which preserved the positive charges on PLL chain and generated PEG-PLL-dithiopyridine). CAT loaded DCPM with a size range of 40–400 nm were prepared. SDS–PAGE gel demonstrated a 93% CAT encapsulation in DCPMs with maintenance of CAT enzymatic activity. Results demonstrate that DCPMs may be employed as an attractive delivery platform for enzymes and other proteins that are prone to loss of function upon exposure to organic solvents. Moreover, these DCPMs can be prepared under sterile conditions by simple mixing and can encapsulate proteins with neutral isoelectric points due to covalent tethering of the protein in the micellar core. Similarly, Wang *et al.* prepared recombinant hirudin (rHV2) loaded PIC micelles (mPIC) targeting platelets [150]. mPIC were prepared with methylPEG-grafted-chitosan or Arg-Gly-Asp (RGD)–PEG-grafted-chitosan. Size of mPIC and RGD–PIC micelles was found to be 30.9 ± 0.5 nm and 41.9 ± 1.8 nm indicating RGD–PIC micelles were significantly larger. The entrapment efficiency for mPIC and RGD–PIC was 80% and 84%, respectively. *In vivo* pharmacokinetic studies were conducted in Sprague-Dawley rats with i.v. administration of micelles and rHV2 solution. The AUC of rHV2 solution, mPIC micelles and RGD–PIC micelles were approximately 902 min µg/ml, approximately 1735 min µg/ml and approximately 1376 min µg/ml, respectively. Results demonstrated that mPIC micelles significantly retarded clearance of rHV2 (~1.1 ml/min/kg) relative to solution (~2.2 ml/min/kg). Study results demonstrated that mPIC micelles hold a significant advantage favoring pharmacokinetics of rHV2 and may aid in improving *in vivo* stability and pharmacokinetic profile of other protein drugs.

■ *In situ* thermosensitive gels

Thermosensitive hydrogels refer to polymeric solutions which undergo sol–gel phase transition to form viscoelastic gel in response to changes in temperature. It represents an attractive alternative to particulate delivery approaches as parenteral depot systems. These thermosensitive polymers form temperature dependent micellar aggregates which undergo gelation after further temperature increment due to aggregation or packing [151]. For delivery, the active agent is mixed with polymer in the solution state. The solution, following administration, forms *in situ* gel depot at physiological temperature in which active agent remains entrapped. The entrapped cargo is released from the depot over a period of time providing extended release. The main advantage of thermoreversible polymeric system is release kinetics can be modulated by adjusting copolymer composition. Delivery can be extended from weeks to months after a single injection. Several thermoreversible block copolymers composed of PEG, PCL, PLA, poly(glycolide), PLGA, poly (*N*-isopropylacrylamide), polyethylene oxide, and chitosan were studied as controlled release carrier for delivery of protein and peptide therapeutics [152]. Recent developments in thermosensitive gel formulations for the parenteral delivery of proteins and peptides are summarized in **TABLE 4**.

Thermosensitive copolymers composed of PLGA and PEG have been widely studied for delivery of proteins and peptides due to their biocompatibility and biodegradability. These thermosensitive copolymers demonstrated promising results for the sustained delivery of protein and peptide drugs. For instance, Chen *et al.* investigated the suitability of PLGA–PEG–PLGA triblock copolymer as a matrix material for a controlled release system for porcine growth hormone (pGH). The *in vivo* absorption profile of pGH from thermosensitive polymeric gel formulation was evaluated in New Zealand White rabbits after s.c. injection. In the control group, pGH aqueous solution was administered intravenously and subcutaneously. After i.v. administration, high pGH blood levels were achieved initially, which diminished over 12 h period. Similarly, s.c. administration of pGH aqueous solution resulted in an initial high pGH blood levels for 2 h which gradually declined over 24 h periods. On the contrary, single s.c. injection of polymer formulations provided near steady state serum levels of exogenous pGH (3–7 ng/ml for high dose and 2–4 ng/ml for low dose)

Table 4. Recent developments on proteins and peptides parenteral delivery from *in situ* thermosensitive gels.

Peptide/protein	Molecular weight	Gelling polymer	Remarks	Ref.
Insulin	5807.6 Da	PLA–PEG–PLA	*In vitro* release studies demonstrated insulin release from the delivery systems over a period of 3 months. Moreover, after s.c. administration in diabetic rats, more elevated insulin level and corresponding decreased blood glucose level was achieved compared with control group	[162]
Insulin	NA	Chitosan/glycerol-phosphate	The increase in Gp salt and initial insulin concentration decreased the insulin release rate form chitosan/Gp gel. Also, there was no deleterious effect of chitosan/Gp gel on insulin stability	[163]
Insulin	5807.8 Da	D,L polylactide in triacetin	Following s.c. administration in rats, controlled release of insulin with reduction in blood glucose for approximately 1 month was observed	[164]
bFGF	NA	PEG–PCL–PEG	Incorporation of bFGF into the NPs/hydrogel composite significantly improved its immunogenicity and strong humoral immunity was maintained for longer than 12 weeks	[165]
bFGF	NA	PEG–PCL–PEG	The immunogenicity of bFGF was improved significantly after encapsulation into the hydrogel. Strong humoral immunity was maintained for more than 14 weeks in case of bFGF-hydrogel	[166]
Salmon calcitonin	3431 Da	mPEG–PLGA–mPEG	Polymeric formulations controlled the release of salmon calcitonin over approximately 20–40 days and prevented methylprednisolone acetate induced osteoporosis *in vivo*	[167]
hGH	20 kDa	Cationic poly(organophosphazenes)	A single s.c. administration of hGH/cationic polymer conjugate induced the elevated plasma level of hGH until 5 days and also elevated plasma level of IGF-1 as a function of free hGH until 13 days, in male cynomolgus monkeys	[168]
hGH	NA	Poly(organophosphazene)–polyethylenimine	In pharmacokinetic studies in Sprague-Dawley rats, PEI-conjugate hydrogel/hGH complexes extended release of hGH and increased half-life and AUC compared with hGH administration alone	[169]
Insulin	6 kDa	PLA–PEG–PLA	The combination approach of chitosan–zinc–insulin complexes with thermosensitive gel significantly reduced initial burst release as compared with zinc–insulin and insulin alone	[170]
hGH	20 kDa	Poly(organophosphazene)	In a pharmacokinetic study in rats, the hGH protamine sulfate PECs-loaded hydrogel extended half-life by 13-fold compared with hGH solution	[171]
Bee venom peptide	NA	PLGA–PEG–PLGA	Bee venom peptide was released from the copolymer-based hydrogels over 40 days *in vitro*	[172]

GP: Glycerol-phosphate; hGH: Human growth hormone; mPEG: methoxy PEG; NA: Not available; NP: Nanoparticle; PCL: Polycaprolactones; PEI: Polyethyleneimine; PLA: Polylactic acid; PLGA: Poly (lactide-co-glycolide; s.c.: Subcutaneous.

for nearly 4 weeks. There was approximately a 5 to 15-fold increment in absolute bioavailability of pGH from the thermosensitive polymer based systems relative to s.c. aqueous solution [153].

Pluronic F-127 (PF127) is another thermosensitive triblock copolymer consisting of a central hydrophobic block of polypropylene glycol flanked by two hydrophilic blocks of PEG. PF-127 thermosensitive gel has shown to prolong the release of various proteins and also extended stability [154,155]. In a recent work, Akash *et al.* have evaluated sustained delivery of IL-1 receptor antagonist (IL-1Ra) from PF127 based thermosensitive gel both *in vitro* and *in vivo* [156–158]. IL-1Ra is a naturally occurring anti-inflammatory antagonist of IL-1β approved recently for treatment of Type 2 diabetes mellitus. However, due to short half-life of IL-1Ra, administration at higher doses with frequent dosing intervals is required. The PF127 thermosensitive gel improved stability of IL-1Ra. Stability of drug was confirmed using differential scanning calorimeter analysis, fourier transform infrared spectroscopy and SDS-PAGE. The gel

formulation increased the thermostability of IL-1Ra significantly and there was no deleterious effect of the polymer on the structure of IL-1Ra. Furthermore, results of the SDS-PAGE analysis confirmed the stability of IL-1Ra in gel formulation. Subsequent to s.c. injections, 25% PF127 gel significantly sustained the plasma concentration of IL-1Ra for a longer period of time as compared with 20% PF127 gel and/or pure IL-1Ra solution in Wistar rats. The maximum plasma concentration (C_{max}) of IL-1Ra loaded in 25% PF127 gel was achieved at 4 h compared with 15 min for pure IL-1Ra solution. The plasma half-life of IL-1Ra from PF127 gel formulation was significantly higher (12.53 h) compared with that of pure IL-1Ra solution.

Recently, researchers have introduced concept of polyelectrolyte complexes for sustaining release of proteins from gel. These complexes are larger than the original protein and restrict the diffusion of protein from delivery systems and thereby reduce the initial burst release. Park *et al.* have developed a combined system of polyelectrolyte complex (PEC) with biodegradable, and thermosensitive poly(organophosphazene) hydrogel for a controlled and sustained delivery of hGH **(FIGURE 6)** [159]. The hGH loaded PECs were induced by mixing solutions of polycations (PLA, poly-L-lysine) and hGH. PLA showed a complex formation with hGH even at a small weight ratio in contrast to PLL. The size of PLA/hGH complexes were increased with increasing amounts of PLA, and at a weight ratio of ten, the size was largest (3346 nm). All PECs loaded hydrogels showed a slower release rate than hGH alone loaded hydrogel under *in vitro* conditions. In the pharmacokinetic study in Sprague-Dawley rats, the PEC-loaded hydrogel showed a more prolonged hGH release than the PEC and hGH solution alone. Single

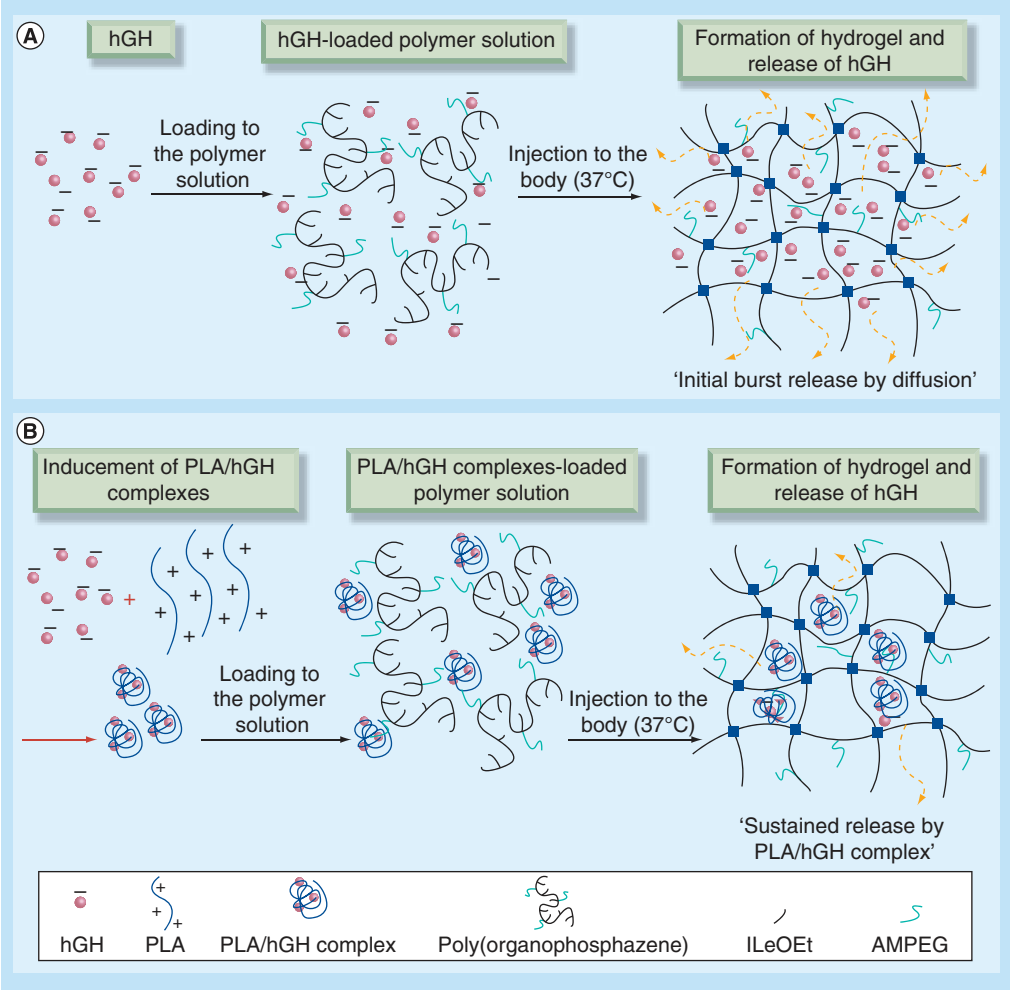

Figure 6. PLA/hGH polyelectrolyte complex-loaded hydrogel.
AMPEG: α-Amino-ω-methoxy-PEG; ILeOEt: L-isoleucine ethyl ester; PLA: Polylactic acid.
Reproduced with permission from [159].

administration of the PEC-loaded hydrogel resulted in the sustained release of hGH for 5 days, which clearly indicates the potential of PEC-loaded poly(organophosphazene) hydrogel for an effective sustained delivery of hGH [159].

The combination of *in situ* gel forming system with other delivery approaches demonstrated more success for proteins and peptides delivery. For instance, in a recent study by Jabarian *et al.* attempted to develop sustained delivery system of octreotide by the combination of PEGylation technology with *in situ* gel forming drug delivery system [160]. The authors have synthesized PEGylated octreotide and compared its efficacy with original octreotide from a poloxamer based *in situ* gel. The developed PEGylated octreotide maintained full biological activity and demonstrated higher serum half-life relative to the original octreotide. The pharmacokinetics of a octreotide and PEGylated octreotide from *in situ* gel forming system and octreotide solution were evaluated after s.c. injection to male rats. Serum concentration–time profiles of octreotide and PEGylated octreotide are shown in **Figure 7**. $AUC_{(0-\infty)}$ for the octreotide loaded gel formulation (187.41 ng day/ml) was six-times relative to octreotide solution (706.99 ng h/ml [29.45 ng day/ml]). Improved bioavailability was attributed to the ability of thermoresponsive gel formulation to retard peptide release. Moreover, higher $AUC_{(0-\infty)}$ was achieved with PEGylated octreotide gel formulation (348.84 ng day/ml) relative to octreotide gel formulation (187.41 ng day/ml). The results can be explained by the combined effect of PEGylation, which improved pharmacokinetic properties of octreotide, and gel formulation, which provided sustained release [160].

In another study, Hiwale and co-workers studied combined formulation of gelatin microspheres and thermoreversible gel as a drug-depot system for lysozyme [161]. Gelatin microspheres (GMs) loaded with lysozyme were prepared using two different cross linking agents: D-glucose and glutaraldehyde. Release profile of lysozyme from both microspheres was very similar and slower than the lysozyme solution. Suspension of the microspheres in the PF127 gel strongly affected release of lysozymes. Remarkably, the PF127 matrix controls both the rate of GM swelling and rate of lysozyme release, which leads to an almost linear release profile from both aqueous solution and GMs. *In vitro* release of lysozyme, from both types of cross-linked GMs, was successfully controlled when suspended in PF127 gel. Results demonstrate the potential application of this new combined formulation as a proteins and peptides depot system [161].

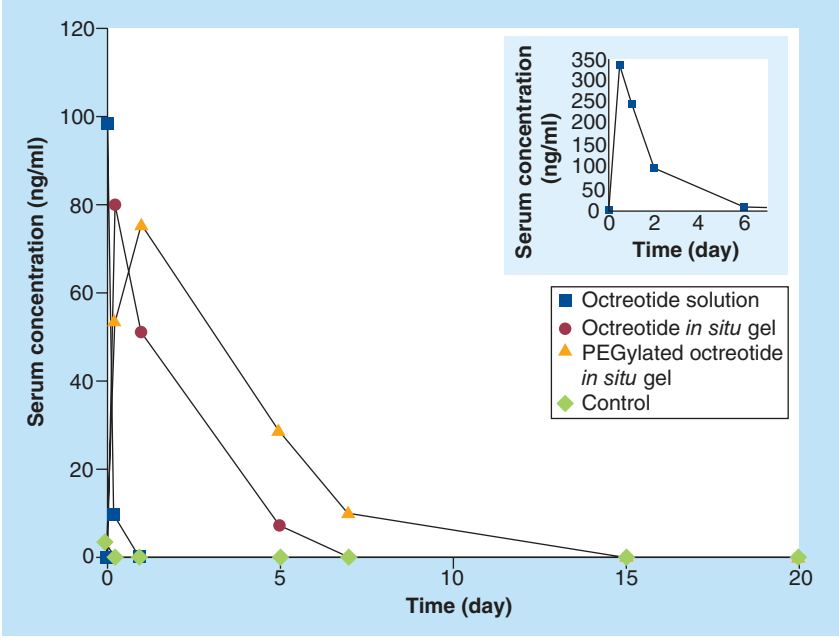

Figure 7. Comparative *in vivo* release profiles of PEGylated octreotide and octreotide loaded into *in situ* gels, octreotide solution (insert) and placebo *in situ* forming gel as control (n = 4).
Reproduced with permission from [160].

Regulatory guidelines

The FDA regulates the premarket testing and marketing approvals for all the peptide and protein drugs with empowers granted to it by the Federal Food, Drug and Cosmetic Act (FD&C Act, 1938) and its amendments [173]. Any drug or drug product to be approved for human use requires FDA clearance. In order to obtain approval for peptide and protein drugs, FDA requires evaluation for drug and drug-product safety and efficacy.

Immunogenicity of protein therapeutics may pose a problem for both product efficacy and patient safety. Immunologically based adverse events such as anaphylaxis and cytokine release hormone have limited sponsors from further development and put them to terminate the development of therapeutic protein products. On the other hand, unwanted immune responses to therapeutic proteins may also neutralize the biological activity of these protein drugs and consequently, provoke adverse reactions. In addition, cross reaction of immune system to an endogenous protein counterpart

may raise severe adverse effects [174]. In the current 2013 draft, descriptions about the product- and patient-specific-factors that may affect immunogenicity of therapeutic protein products have been described. Patient-related factors include dose, frequency and duration of treatment, route of administration, immunological status and competence of patient, genetic status and status of immune tolerance. It has been postulated that intradermal, and s.c. injections are associated with increased immunogenic response due to increased sensitization effect. On the other hand, the i.v. route is considered to be the safest and elicit negligible immune response. Product related factors include product origin, molecular structure, aggregation, modifications such as glycosylation or PEGylation, impurities with adjuvant activity, immunomodulatory properties of the product, formulation excipients, product container closure and product custody.

As a result of several clinical consequences ranging from transient antibody responses, with no apparent clinical manifestations to life-threatening and catastrophic reactions, the FDA encourage manufacturers to elucidate underlying immunologic mechanism for related adverse events during the protein product development. Also, the FDA draft includes recommendations to the sponsors and researchers that may help them to reduce the likeliness of developing an immune response with the protein drug or drug product. To develop a better protein drug candidate or drug product and to eliminate any toxic formulation for human use, the FDA has recommended conducting preclinical and toxicological studies [175].

Conclusion

Therapeutic proteins and peptides are gaining tremendous importance in today's healthcare system. In recent years, numerous therapeutically potent protein and peptide drugs have been developed. Although highly potent, one of the major challenges to the successful clinical use of these therapeutics is lack of an effective delivery method. Abundant efforts have been put toward delivering protein and peptide therapeutics via noninvasive routes, still parenteral delivery remains upfront. In the past few years, significant progress has been made to improve parenteral delivery of proteins and peptides using various delivery technologies such as microparticles, liposomes, nanoparticles, *in situ* thermosensitive gels as well as by chemical modification via PEGylation and hyperglycosylation. Extensive research efforts made several parenteral products clinically available utilizing these strategies. These technologies have improved therapy, reduced cost of therapy and improved patience compliance. However, the numbers of clinically available products are fewer compared with the extensive efforts put towards the development of proteins and peptides delivery systems. The complexity of scale-up, difficulty in preserving bioactivity of proteins and peptides and high cost of manufacturing are hampering successful development of such formulations. Hence, there is an unmet need in the development of novel approaches or further improvements in existing approaches to achieve acceptable and cost effective parenteral delivery of proteins and peptides.

Future perspective

Tremendous efforts have been made in the last decade with chemical modifications, colloidal and thermosensitive hydrogel based delivery systems to improve *in vivo* half-life, stability and efficacy of protein and peptide therapeutics. Still, there requires a great deal of research and development to make them feasible for commercialization. Chemical modification may help to improve stability, *in vivo* circulation time, reduce toxicity of peptide drugs (α-melittin) to normal cells/tissues and enhance drug efficacy in diseased cells. However, protein/peptide chemical modification lacks sustained drug release and specificity. In such scenario, conjugation of a targeting moiety to the PEGylated protein/peptide may prove to be safer, specific in drug delivery, more efficacious and improve patient compliance. Carrier systems like micro/nanoparticles and thermosensitive hydrogels sustain drug release and provides stability and protection from enzymatic degradation. Some of these products have successfully made it to market. However, delivery of drugs in therapeutic concentrations to its site of action, especially when the target is out of systemic circulation, still remains a challenge to pharmacologists. Furthermore, to improve specificity of carrier systems and deliver protein/peptide payloads to diseased tissue in therapeutic concentrations, targeting moieties may be conjugated to the carrier surface. Dual strategy such as protein/peptide chemical modification followed by encapsulation into a carrier system may further improve

their release kinetics, *in vivo* half-life, circulation time and stability. Moreover, protein/peptide-loaded targeted-carrier systems may be dispersed in the hydrogel system, which forms depot and further sustain protein/peptide drug release. Advanced techniques and methods are required to access the protein/peptide stability in the carrier system. Another major area requiring development is *in vitro/in vivo* correlation. An interaction between the drug delivery scientists and immunologists necessitates the understanding of the carrier system drug release and its presentation to immune system. The guideline from the International Conference on Harmonisation and 2013 draft released by the FDA may help researchers to have a concrete research strategy to develop a better protein/peptide drug product and evaluate formulation and drug stability under various conditions. These guidelines may help researchers and investors to accelerate the product development and approval process allowing more protein and peptide products to reach market.

Financial & competing interests disclosure

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Executive summary

Chemical modifications

- The protein modifications widely studied are PEGylation, hyperglycosylation and mannosylation.
- PEGylation may improve stability, pharmacokinetics, and therapeutic activity of protein and peptide drugs.
- The biodegradable nature of carbohydrates (hyperglycosylation) is advantageous over PEG (PEGylation). Polysialic acid is widely used for hyperglycosylation.

Colloidal delivery systems

- Colloidal carriers such as microparticles, liposomes, nanoparticles, carbon nanotubes, and micelles are reported for protein and peptide delivery.
- Microparticles, nanoparticles and liposomes can provide sustained release of protein and peptide therapeutics.
- Hydrophobic ion-pairing complexation approach has been applied to enhance protein stability and increase encapsulation efficiency into polymeric and solid lipid nanoparticles.
- PEGylated liposome technology has demonstrated promising results for proteins and peptides delivery.
- Carbon nanotubes have been applied as a molecular transporter for protein delivery.
- Polyionic complex micelles demonstrated improvement in *in vivo* stability and pharmacokinetic profile of protein drugs.

In situ *thermosensitive gels*

- Polyelectrolyte complexes provided a more prolonged release from thermosensitive gel over native proteins.
- Combination of *in situ* thermosensitive gels with other delivery approaches demonstrated more success over single approach.

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