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Review Article

Liposomes as Delivery Vectors for Proteins and Peptides: Advancements and Challenges

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ABSTRACT

A liposomal drug delivery system (LDDS) is a complex nano carrier system and an effective system for delivery of both large and small therapeutic components, ranging from protein and peptides to even very small nucleic acid molecules. It represents a promising approach for delivery of proteins and peptides, as these systems protect the sensitive biomolecules from degradation, provide controlled release, and improve their bioavailability. Instability, poor bioavailability and short half-life of the protein and peptides create a need of the liposomal delivery system which provides a sufficient protection against such challenges. Besides this, Liposomal delivery system offers various advantages including, overcoming of Blood Brain Barrier which is very helpful for achieving the neuronal target in protein and peptide delivery. Liposomes consist of a hydrophilic core which is always surrounded by a lipid bilayer and it can be prepared by various methods such as thin film hydration method, extrusion, reverse phase evaporation, sonication, microfluidic technology, dehydration and rehydration method, co-extrusion with lipid and protein solution, electrostatic method etc. Various novel excipients play a crucial role for the formulation of liposomes containing protein and peptides. Characterization of the formulated liposomal formulation is important aspect and various parameters such as stability, zeta potential, particle size, encapsulation efficiency, morphology assessment etc. are assessed for assuring the quality. There are various therapeutic purposes of liposomes such as cancer therapy, enzyme replacement therapy, vaccine and immunotherapy, hormone and peptide therapy and gene therapy. Modern pharmaceutics have experienced various advances in the delivery of protein and peptides through liposomal formulation creating potential opportunities for protein and peptide scientist in future.

INTRODUCTION

A liposomal drug delivery system (LDDS), a complex nano carrier system, is an effective system for delivery of both large and small therapeutic components; ranging from protein and peptides to even very small nucleic acid molecules. It employs liposomes, which are ball-like vesicles made of lipid bilayers that carry both hydrophobic and hydrophilic materials (Lasic, 1992).

Liposomes consist of a hydrophilic core which is always surrounded by a lipid bilayer as seen in figure 1 (Filipczak *et al.*, 2020). These vector systems can range from tens of nanometres to micrometres in size, and the bilayer's formulation can be tailored for particular therapeutic applications. Liposomes are generally composed of phospholipids with cholesterol, and occasionally, additional lipids or polymers to improve their stability and function (Adhikari & Pokhrel, 2024). Liposomes are of various types such as unilamellar liposomes which consist of single lipid bilayer, multilamellar liposomes which consists of multiple concentric lipid bilayers, larger

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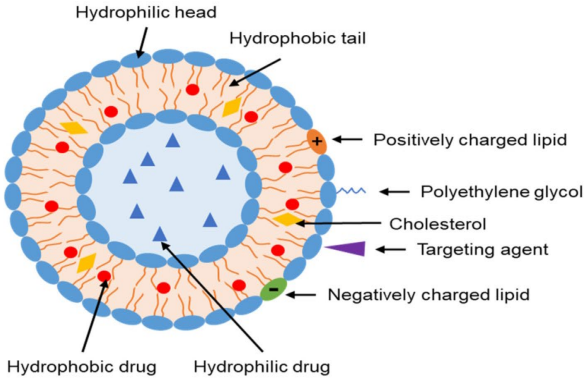

Figure 1: Basic liposomal structure (Duong *et al.*, 2023)

sized large unilamellar vesicles with single bilayer and small sized small unilamellar vesicles with single bilayer. Liposomal drug delivery systems (LDDS) represent a promising approach for the delivery of proteins and peptides, as they provide the protection from degradation, provide controlled release, and improve their bioavailability (Song *et al.*, 2022).

Importance of liposomal delivery system of protein and peptide drugs

Typically proteins and peptides delivery is hindered by different factors such as

Instability

Proteins and peptides are subjected to denaturation, aggregation, and degradation due to the various environmental factors such as pH, temperature and due to various enzyme activity (Cheison & Kulozik, 2017).

Poor bioavailability and short half-life

Proteins and peptides are often degraded in the gastrointestinal tract when they are given orally. Having the short half-life, many proteins and peptides are rapidly cleared from the body, requiring frequent dosing (Tang & Meibohm, 2006).

Liposomal delivery systems are essential for protein and peptide drugs as these biologics face different problems such as instability and low bioavailability and rapid clearance. Liposomes provides the flexible platform, which protects these molecules from degradation, increases half-life and bioavailability, and enables targeting. Liposomes provide controlled-release, reduced toxicity and enhancement of therapeutic potency, thus they are essential for the successful treatment of diseases such as cancer, diabetes, genetic and neurodegenerative disorders (Singh *et al.*, 2019).

Advantages of protein and peptide drug liposomal delivery

Protection from degradation and denaturation:

Proteins and peptides are highly affected by the different environmental conditions like temperature, pH and enzymatic degradation, resulting in loss of activity and/or complete degradation.

Liposomes basically provide a protective shield which prevent proteins and peptides from degradation by external factors, such as proteases (enzymes which digest proteins) and extreme conditions of the gastrointestinal tract (if taken orally) (Jash *et al.*, 2021).This protection is particularly important for proteins such as insulin, growth factors, or monoclonal antibodies that are typically not stable in aqueous conditions (Mitragotri *et al.*, 2014).

Enhanced bioavailability and absorption

When administered orally, proteins and peptides undergoes intestinal degradation, poor absorption and first-pass metabolism that result in poor oral bioavailability.

Enteric-coated liposomes protect the drugs from various digestive enzymes and facilitate its absorption through intestinal walls, increasing oral bioavailability (Maderuelo *et al.*, 2019).

Liposomal systems also increases parenteral bioavailability by causing increment in the circulation time and reducing clearance, thus allowing proteins and peptides for getting remain in the blood circulation for long-time which is enough to exert therapeutic effect (Drummond *et al.*, 2008).

Sustained release and long-term circulation

Prolonged half-lives are often required for efficacy of protein and peptide drugs, especially in case of chronic conditions or diseases such as hormone replacement therapy and cancer immunotherapy) (AlQahtani *et al.*, 2019).

Liposomes can be designed to allow for controlled or sustained release, reducing the need for frequent injections or doses. Proteins and peptide congener having relatively short half-lives and are rapidly cleared out of the bloodstream gets benefitted.

With modification of the liposome's surface with PEG (polyethylene glycol), the time of circulation of the liposomes can be increased even more, ensuring that therapy can be extended along the course of treatment and dosing frequency may be reduced (Woodle, 1993).

Targeted and site-specific delivery

Liposomes can be modified by conjugating targeting ligands (e.g. antibodies, peptides or aptamers) on their surface to achieve delivery to a specific tissue/cell as seen in figure 2 which is important for minimizing off-target effects and improving the therapeutic index for protein and peptide therapeutics (Anwar *et al.*, 2023).

Liposomal drug delivery systems precisely target tumour cells in cancer therapies protecting the healthy tissues from the toxicities of chemotherapeutic agents (Hossen *et al.*, 2019).

Liposomes can also be engineered for protein and peptide delivery to specific intracellular sites (e.g., endosomal

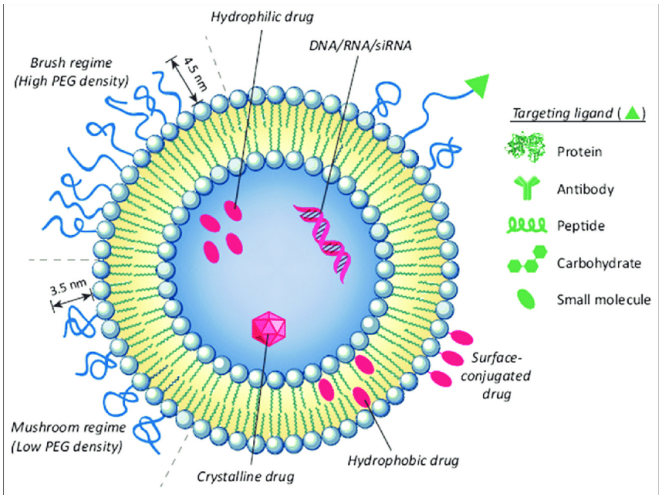

Figure 2: Ligand targeting in liposomes (Rafe, 2017)

or lysosomal targeting), allowing for better control over drug release with decreased systemic effects (Pearce *et al.*, 2012).

Overcoming the Blood Brain Barrier (BBB)

The blood-brain barrier (BBB) is considered to be a major obstacle in the administration of proteins and peptides for the therapy of different disorders in neurology or brain (e.g., Alzheimer's, Parkinson's disease or brain tumor) (Patel *et al.*, 2021).

Liposomal nanocarriers can also be modified with different ligands (i.e., transferrin or LRP-1 targeting peptides) that allow for brain-targeting capabilities and increased permeability of the liposomal system across the BBB (Gao *et al.*, 2022).

This ability is crucial for transporting protein-based therapeutics and peptide drugs to the brain where they are often required at low concentrations.

Decreased immunogenicity and toxicity

Many protein and peptide drugs can induce immune responses, particularly when they are perceived by the immune system as foreign and causes an allergic reaction in the body, and a consequently immunogenic effect is observed compromising the safety and therapeutic effect of the drug (Ju & Uetrecht, 2002).

Attachment of PEG molecules to liposomes i.e. PEGylation is commonly employed to decrease immunogenicity and to reduce the clearance through the reticuloendothelial system (RES) (d'Avanzo *et al.*, 2020).

Reduction of aggregation and degradation of proteins

Proteins and peptides delivered in solution tend to aggregate and this compromises their therapeutic efficacy and may lead to adverse side-effects (Acar *et al.*, 2017).

Liposomal delivery not only serves as a stable carrier of the protein/peptide molecule but also stabilizes it by keeping the protein or peptide within a lipid layer, preventing it from forming aggregates or changing the structure. Thus it helps in maintaining therapeutic efficacy (Sood & Panchagnula, 2001).

Co-administration/delivery of multiple therapeutic agents

It enables co-delivery of several drugs which enhances synergistically drug activities and improves therapeutic effects.

Therapeutic delivery of the proteins, peptides, genes and chemotherapeutic agents can be achieved simultaneously for the achievement of the combination therapy. It is especially effective in the cancer treatment in which targeted proteins and peptides drugs are combined with the chemotherapeutic agents and it is helpful for the enhancement of the therapeutic efficacy and overcoming of resistance (Qin *et al.*, 2018).

Preparation of liposomes

Thin film hydration method

In this method, chloroform or methanol are taken as organic solvent and lipid molecules (phospholipids, like phosphatidylcholine) are dissolved in it which is then evaporated under reduced pressure to create a thin film of lipid over the walls of a round-bottom flask. Thus formed film is then subjected to hydration with an aqueous solution that contains relevant protein or peptide. Multilamellar vesicles (MLVs) are formed which can subsequently be extruded or sonicated for a more homogeneous size distribution (Thabet *et al.*, 2022; Torres-Flores *et al.*, 2020).

This is the simplest and most reproducible technique and compatible for entrapment of hydrophilic as well as hydrophobic proteins/peptides. Large scale production of liposomal proteins or peptides can be done by this method. However, with this method, variation in the encapsulation efficiency can be observed and proteins and peptides are susceptible to the hydration conditions (that is, temperature, ionic strength) and denaturation may occur during the preparation (Thabet *et al.*, 2022).

Reverse-phase evaporation

It is a more sophisticated technique for generating liposomes with improved encapsulation efficiency for hydrophobic drugs, proteins, or peptides. First, the lipid phase is made solubilized in chloroform, an organic solvent and then combined with protein and peptide containing an aqueous phase. A water-in-oil emulsion is formed where aqueous phase is surrounded by the lipid phase. The organic solvent is then subjected to evaporation under reduced pressure that leads to liposome formation. The liposomes obtained are usually unilamellar (ULVs) with a much higher encapsulation efficiency.

This method helps for enhancement of encapsulation efficiency, particularly for hydrophobic macromolecules

(proteins or peptides) and highly stable liposomes are formed. However this technique is susceptible to the conditions during emulsion formation (e.g., emulsion stability, particle size). Residues of organic solvents can interfere with the integrity of proteins or peptides (Cortesi, 1999).

Extrusion

The extrusion method forces a liposome suspension through a membrane with a defined pore size (typically 100 nm to 200 nm). The liposomes are prepared first by any of the above methods (e.g., thin-film hydration).The suspension of multilamellar vesicles (MLVs) are formed which are repeatedly extruded through polycarbonate membranes resulting the small unilamellar vesicles (SUVs) (Olson *et al.*, 1979; Ong *et al.*, 2016).
The size of liposomes can be controlled through adjusted in membrane pores size and by adjusting the extrusion cycle number. This method produces uniform small liposomes with narrow size range and it is non-toxic and reproducible. However loss of the encapsulated protein/ peptide can occur during the extrusion step if the liposomal is of the fragile type (Ong *et al.*, 2016).

Sonication (probe or bath sonication)

In this method, cavitation bubbles within a liquid medium are generated through the use of high frequency sound waves, which create shear forces that, in turn, disperse lipid aggregates into liposomes. Lipid films or lipid solutions are combined with an aqueous solution comprising the protein or peptide and exposed to ultrasonic energy. Liposome preparation is subjected to sonication until the required size characteristics and homogeneity is obtained. This approach generally produces small unilamellar vesicles (SUVs). This technique is considered simple and fast method of preparation. However, high temperature and shear forces produced through sonication can denature proteins and there is lack of control over liposome size and stability (Hadian *et al.*, 2014).

Microfluidic technology

Microfluidics is a technique in which lipid and aqueous phases are filtered through microchannels under controlled conditions to create liposomes. At a controlled flow rate, lipid solutions and aqueous protein or peptide solutions are combined and driven through microchannels. This mixing leads to the generation of liposomes with controlled amount and homogeneity. Liposomes thus formed are often nanometer in size and highly encapsulated. Size, encapsulation efficiency, and formulation can be precisely controlled through this technique. However, specialized equipment are needed for the manufacturing and it can be difficult to scale up to larger production volumes.(van Swaay & DeMello, 2013; Yu *et al.*, 2009)

Dehydration-rehydration method

These techniques include the drying of liposome preparations and their subsequent rehydration. First liposomes are made using any of the method and then liposomes are dried by freeze-drying or by evaporation of aqueous phase under a low pressure. This creates dried liposomes which are then rehydrated using an aqueous solution that contains the protein or peptide of interest, leading to the reconstitution of the liposomes and the encapsulation of the protein or peptide(Visht *et al.*, 2014) Liposomal stabilization during storage can be achieved and delicate proteins and peptides can be encapsulated through this method, because the rehydration step can be finely controlled. However, this dehydration process can provoke partial aggregation or drug leakage of the drug-loaded vesicles. The dehydration process can induce conformational change of proteins/peptides, which decrease bioactivity.(Mugabe *et al.*, 2006)

Co-extrusion with lipid and protein solution

In this approach, the lipid solution and protein solutions are co-extruded through the membranes, which resulted in the formation of liposomes and encapsulated the protein or peptide. Lipids and proteins in a solvent are mixed and used for extrusion through the membrane to create liposomes. This method is suitable for proteins or peptides that are poorly encapsulated by other processes. However Optimization of lipid: protein ratio for maximum encapsulation is required. Shear-induced degradation of proteins may occur with extrusion.(Zupančič *et al.*, 2022)

Electrostatic assembly (Electrostatic liposome formation)

Here, negatively and positively charged lipids are added together with the oppositely charged proteins/peptides to form liposomes through electrostatic interactions. By using a combination of anionic and cationic lipids, positively charged proteins or peptides can easily be encapsulated. The electrostatic force between the lipids and biomolecules generates liposomes. This method is especially useful for proteins or peptides with a unique charge. However these electrostatic forces, when not properly controlled, may cause instability and/or release of the encapsulated material. This technique may not work well for proteins/peptides with relatively suboptimal charge characteristics.(Dua *et al.*, 2012)

Novel excipients in the formulation of protein and peptide liposomes

Novel excipients increases stability, improves bioavailability, and optimizes the active drug release profile in liposomal drug delivery development for protein and peptide.

Polymer-based excipients

- *Poloxamers (e.g., Poloxamer 188)*

They stabilize liposomes, reduce aggregation and enhance the encapsulation efficiency particularly of proteins and peptides.(Tian *et al.*, 2011)

- *PEGylated lipids (e.g., PEG-DSPE)*

Polyethylene glycol (PEG)-modified lipids can give stealth properties to liposomes, which can reduce the recognition of liposomes by the immune system and increase circulation time, which is important for protein/peptide delivery.(Milla *et al.*, 2012)

- *Chitosan*

It is a biopolymer which is charged positively that can enhance liposome stability, promote biological membrane permeability, and improve cellular uptake of protein and peptide-based agents.(Li *et al.*, 2015)

Lipids with special properties

- *Derivatives of phosphatidylcholine (PC)*

modified phospholipids (with longer or unsaturated tails) can provide greater stability and integrity to liposomes.(Farzaneh *et al.*, 2018)

- *Ceramide*

This spingolipid has been investigated for its ability to stabilize liposomes and increase the bioactivity of encapsulated peptides and proteins. (Watters *et al.*, 2012)

Charged lipids and surfactant

- *Dodecyl-β-D-maltoside*

This non-ionic surfactant has been used to stabilize liposomes and promote protein encapsulation.(Brea *et al.*, 2017)

- *Diacylglycerol (DAG)*

This is a lipid that is capable of improving liposomal formulation stability, in addition to promoting a better release of the peptides and proteins.(Papadopoulou *et al.*, 2024)

Surfactants and stabilizers

- *Tween 80*

A common emulsifier used in liposomal formulations, Tween 80 stabilizes lipid bilayers and prevents aggregation. (Cui *et al.*, 2022)

- *Albumin (e.g., Human serum albumin, HSA)*

It is used in some liposomal formulations as a stabilizer or to aid in the delivery of hydrophobic proteins/peptides and because it is naturally found in the blood, it can shut down circulation time.(Teixeira *et al.*, 2022)

Cryoprotectants and antioxidants

Tocopherol (Vitamin E), an antioxidant, can inhibit oxidative degradation of liposomal formulations of protein and peptides.(Srivastava *et al.*, 1989) Trehalose, a cryoprotectant, can help stabilise liposomal formations during lyophilisation processes, extending the shelf-life of protein- or peptide-based liposomes.(Ohtake & Wang, 2011)

Targeting ligands and functional excipients

- *Folate, transferrin, or antibody conjugates*

Liposomes can be modified with functional excipients to enhance target specificity towards cell types or tissues for delivering site-specific peptides and proteins.(Soni *et al.*, 2005)

- *Aptamers*

These are nucleic acid based ligands for targeting specific tissues or cells for effective delivery.(Soni *et al.*, 2005) However, the use of these new types of excipients can also address problems with liposomal delivery of proteins and peptides: instability, premature drug release, and poor bioavailability. The selection of excipient is based on the desired formulation objectives (e.g., improving drug encapsulation, stabilizing the active pharmaceutical ingredient, directing the delivery to the appropriate tissue or cell type).

Characterization of liposomes of protein and peptides

Characterization of liposomal formulations for proteins and peptides includes an intense assessment of key parameters to ensure their stability, encapsulation efficiency, and therapeutic efficacy. As for key characterization techniques, zeta potential and size distribution can be measured by dynamic light scattering (DLS), evaluating the uniformity of nanoparticles and their surface charge, which affects cellular uptake and stability. Ultrafiltration or centrifugation is used to evaluate encapsulation efficiency and drug loading, determining how much protein/peptide is encapsulated in the liposomes. Morphological analysis by electron microscopy (TEM or SEM) provides information about liposome structure and integrity, and stability studies at different temperatures or Lyophilization assesses aggregation or degradation resistance of formulations. Drug release kinetics is also evaluated in vitro; release kinetics will determine the release profile of the encapsulated proteins/peptides, which is very crucial for controlled delivery and sustained therapeutic activity. Finally, sterility and endotoxin testing is conducted to confirm the safety of the formulation for clinical use.(Dai *et al.*, 2006)

Therapeutic applications

- *Cancer therapy*

Liposomal formulations of therapeutic proteins (e.g., tumor necrosis factor-alpha, interleukins) or peptide-based drugs can be used for targeted cancer therapy, reducing systemic side effects.(Pandey *et al.*, 2016)

- *Enzyme replacement therapy*

In lysosomal storage diseases, liposomes can be used to deliver enzymes that are either deficient or dysfunctional in patients.(Santi *et al.*, 2020)

- *Vaccines and immunotherapies*

Liposomal systems have been employed to deliver a wide variety of proteins or peptides that serve as antigens to stimulate the immune system to combat disease, including cancer or infectious disease.(Schwendener, 2014)

- *Hormone and peptide therapies*

Liposomes are perfect carriers of peptides such as insulin or growth factors, protecting them from enzymatic degradation and allowing them to reach the bloodstream or target tissue effectively.(Jash *et al.*, 2021)

- *Gene therapy*

Liposomes are also used to deliver nucleic acids (such as mRNA or siRNA) together with proteins or peptides (Zylberberg *et al.*, 2017)

Besides these, there are various proteins and peptides which are developed in the form of liposomes using the different types of phospholipids as in Table 1.

Advances in liposomal delivery of protein and peptides

Liposomal drug delivery systems (LDDS) have led to the significant improvement in therapeutic efficacy, biological stability and targeted delivery. It emphasizes the specific difficulties associated with proteins and peptides, including instability, weak bioavailability, and rapid degradation.

Nanostructured lipid carriers (NLC) and lipid-core micelles

NLCs are a newer generation of liposomes which consist of a mixture of solid lipids and liquid lipids for the formation of matrix. They provide greater stability, improved encapsulation efficiency, and protection of sensitive proteins and peptides. These systems can be developed to formulate hydrophobic proteins peptides or hydrophilic ones providing an alternative to conventional liposomes. Lipid-core micelles encapsulate peptides and proteins in micellar structures. They are especially useful for solubilizing hydrophobic peptides and proteins by enhancing their solubility.(Izza *et al.*, 2022)

Surface modifications with targeting Ligands

Numerous studies have shown that the specific ligands attached to the surface of the liposomes drastically improve targeted delivery of proteins and peptides. Ligands may comprise antibodies, aptamers, peptides, or small molecules that are bound to specific receptors that are overexpressed on target cells or tissues. Monoclonal antibodies or tumor targeting are basically used to enhance the liposome targeting to cancer cells thereby reducing the systemic toxicity and improving the therapeutic efficacy. (Khan *et al.*, 2020)

PEGylation for prolongation of circulation time

One of the most important and widely used strategies to prolong the circulation time of liposomes is PEGylation. PEGylation decreases RES recognition and prevents early blood clearance. Stealth liposomes (liposomes that are conjugated with PEG) have been optimized in the last few years to minimize both immunogenicity and opsonization, which allows they can remain longer in the circulation and promotes the sustained release of the encapsulated protein or peptide.(Milla *et al.*, 2012)

Stimuli-responsive and pH-responsive liposomes

pH-responsive liposomes which have been studied to release drug payloads in acidic environments like the tumor (due to acidic tumor microenvironment) and inside lysosomes and endosomes. Advances in the design of stimuli-responsive liposomes have enabled the release of drugs in response to an external stimulus (e.g., temperature, light, magnetic field or enzymatic activity). (Lee & Thompson, 2017)

Liposome-based vaccine delivery for proteins and peptides

Liposomal vaccines are becoming one of the most developing drug delivery systems in recent years, especially for the delivery of protein-based vaccines or peptide antigens to initiate the immune response. These liposomal formulations have been developed to encapsulate antigens (peptides or recombinant proteins), allowing for improved presentation to the immune system, which may trigger humoral and cellular immunity. (Schwendener, 2014)

Challenges during formulation

Developing liposomal delivery systems for proteins and peptides has numerous challenges ranging

Table 1: Some Applications of Protein and Peptide Liposomes along with Phospholipids for development (Cui *et al.*, 2022)

S.N.	*Proteins/Peptides for Liposomal Formulations*	*Applications*	*Phospholipids used and Achievement*
	Insulin	Management of Diabetes	1,2-Dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG), Degradation of Insulin was reduced.
	Salmon Calcitonin	Treatment of Paget's Disease, Hypercalcemia, Postmenopausal Osteoporosis	Phosphatidylcholine (PC), Achievement of Higher Bioavailability
	Cy5-amine	Tissue imaging and detection	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), Longer residence time in the intestine.

from instabilities and sensitivities of these biologics. During the liposome preparation process, proteins and peptides are easily degraded, aggregated, and denatured, which usually leads to lower activity or poor encapsulation efficiency. Proteins and peptides are often hydrophilic, rendering them charge and thus difficult to co-encapsulate in the lipid bilayer; as a result, low drug loading with inefficient profiles of release are obtained. Liposomes, especially those incorporating sensitive biologics, are also prone to oxidation or hydrolysis, which makes it challenging to maintain their structural integrity over time. Moreover, it is necessary to design a formulation that will provide protection of the active drug from enzymatic degradation in vivo, remain stable before reaching the target site and be able to store multiple active ingredients. In addition, it is quite complex to achieve controlled, sustained release of the drug without causing adverse immune responses or having any effect on the pharmacokinetic profile of the drug during development process. Finally, the feasibility of scaling production of liposomal formulations, while preserving consistency and quality, represents technical and economical obstacles.(Sawant & Torchilin, 2012; Sercombe *et al.*, 2015)

Conclusion

Liposomal drug delivery system is one of the novel approaches in the protein and peptide drug delivery which offers various advantages despite of some challenges during the formulation. Various methods can be used for the preparation of the protein and peptide loaded liposomal formulation with the help of various novel excipients. Characterization of the formulation is crucial after development. Modern pharmaceutics have experienced various advances in the delivery of protein and peptides through liposomal formulation creating potential opportunities for protein and peptide scientist in future.

Authors Contribution

Both Authors have contributed for gathering the information, preparation of review articles and finalization for the publication.

Conflict of Interest

None

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