

Chapter 6

Liposome Nanocarriers for Peptide Drug Delivery



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Abstract Peptide drugs have limitations, including low membrane permeability, inability to cross the blood-brain barrier, low stability towards enzymatic degradation, lower plasma half-life, and low oral bioavailability. Nanoparticles can effectively encapsulate various hydrophobic and hydrophilic drugs, protein-based drugs, peptides, and nucleic acids. Entrapment of these drugs can improve their solubility and stability. Nanoparticles can be developed to release the drug at the target site using stimulus trigger release. The different nanoparticles-based system serves this purpose, and they can be broadly classified as dendrimers, micelles, liposomes, carbon nanotubes, and quantum dots. Therapeutic peptides can be incorporated inside the liposomes to enhance stability and better tumor accumulation. Peptide-based liposomes can successfully target the tumor cells and lower the off-target effects of chemotherapeutics. Toxicity to normal cells caused by anticancer therapeutics has been dramatically reduced by the use of peptide-based liposomes. This chapter covers the fundamentals of incorporating peptides in liposomal particles and characterizing them using different methods. Examples of peptide-based liposomal delivery is also discussed.

Keywords Nanoparticles · Liposomes · Peptides · Drug delivery · Targeted liposome

6.1 Introduction

Nanotechnology can be defined as a branch of science based on the development of technology in the synthesis, manipulation, and study of materials and devices in the nanometer size range. Nanotechnology is applicable to a wide range of disciplines from basic materials science to personal care and therapeutic and diagnostic applications (Park 2007). Significant research and development for the medical application of nanotechnology (nanomedicine) in the last decades has provided a wide range of biomedical applications (Kawasaki and Player 2005), including diagnosis and as a tool for the treatment of various diseases (De Jong and Borm 2008). The development of novel approaches to effective drug delivery is one of the most promising applications of nanomedicine. The prospect of nanoparticles from adenoviral

vector to lipid capsules as nanocarrier in vaccines against Covid-19 (Tenchov et al. 2021; Shin et al. 2020) has shown the tremendous impact and success of nanomedicine.

Approval of medicinal use of insulin in 1922 as the first peptide-based treatment for diabetes had opened the immense potential of peptides as therapeutics in different diseases. Several peptide drugs are on the market, and many more are in clinical development (Muttenthaler et al. 2021; Henninot et al. 2018). Peptide drugs offer several advantages such as ease of synthesis, low costs, low immunogenicity, natural biological messengers of various pathways, and targeting of protein-protein interactions.

6.1.1 Limitations of Peptides as Therapeutics

Along with so many advantages of peptides, their limitations such as low membrane permeability, inability to cross the blood-brain barrier, low stability towards enzymatic degradation, lower plasma half-life, and low oral bioavailability are the hurdles for peptide-based drug development (Cao et al. 2019). *In vivo* stability is one of the major barriers to peptide drug delivery. Peptides' bioavailability is reduced through degradation by various protease enzymes along with other digestive enzymes. Various processes are being explored to increase the plasma stability of peptides to render enough therapeutic efficacy (Otvos Jr and Wade 2014). Peptides have only 1% oral bioavailability, with few exceptions (e.g., cyclosporine A) (Zhou and Po 1991). Along with cellular proteases, proteases such as trypsin, pancreatic esterase, and α -chymotrypsin, secreted from the pancreas, are present in large quantities in the small intestinal lumen. These proteases are responsible for the degradation of the majority of peptides (Vlieghe et al. 2010). Due to the size of the peptide molecules and the polar nature of peptide bonds, permeability across the cell membrane becomes challenging. Absorption of a peptide across the intestinal barrier can occur by (i) passive diffusion through the lipid layer, (ii) the paracellular pathway, and (iii) transporters [e.g., peptide transporter 1 (PEPT1), vitamin B12 transport system] (Edmonds and Price 2013). Various methods are being investigated to increase peptides' plasma stability to have enough biological effect. PEGylation, lipidation, and glycosylation processes have shown a sufficient effect on peptide stability (Morimoto 2017). Several formulation strategies have been considered to tackle the poor oral bioavailability of peptides and proteins (Vlieghe et al. 2010). One of these strategies is the use of substances that can assist the absorption of drugs, enhancing oral bioavailability. Various absorption enhancers have been examined for the improvement of peptide and protein absorption; these can be categorized into cationic and anionic agents, surfactants, bile salts, fatty acids, chelating agents, acylcarnitines, and their derivatives (Renkuntla et al. 2013). Synergistic effects can be observed from combination of these enhancers rather than a single enhancer. Co-administration of protease inhibitors can prevent the degradation of protein and peptides in the gastrointestinal tract and reduce enzymatic blockade.

Inhibitors for major digestive enzymes such as aprotinin and inhibitors for aminopeptidase, namely bestatin, puromycin, and boroleucine, have been used widely to prevent the degradation of peptides *in vivo* as well as for oral delivery of peptides. Physiologically responsive hydrogels can protect peptide degradation through their three-dimensional mesh-like structure and are also capable of reacting to surrounding stimuli such as ionic strength difference, temperature, and pH alterations (Lowman et al. 1999). The oral bioavailability of protein and peptide therapeutics can be supported through mucoadhesive polymer systems, which contain natural or synthetic polymers that enable them to adhere to the mucin layer on the mucosal epithelium. Incorporation of cyclodextrins with a hydrophobic interior and a hydrophilic outer side (Challa et al. 2005; Kanwar et al. 2011) has the potential to interact with guest molecules and serve as a drug delivery vehicle for large molecules like peptide or protein (Irie and Uekama 1999; Renukuntla et al. 2013).

6.2 Nanoparticle in Drug Delivery

Nanoparticles can effectively encapsulate various hydrophobic and hydrophilic drugs, protein-based drugs, peptides, and nucleic acids. Entrapment of these drugs can improve their solubility and stability. Nanoparticles can be developed to release the drug in the target site by the use of stimulus trigger release. Nanoparticles can also be functionalized on their surface by peptides, antibodies, and aptamers for active targeting and for diagnostic purposes (Kim et al. 2006; Sonju et al. 2021). In addition to this, nanoparticles can be designed to circulate in the blood for a longer time, improving the biodistribution properties of the drugs. Due to their size, nanoparticles can easily pass through the endothelium and accumulated in inflammatory sites like tumors (Moghimi et al. 2001). This property of nanoparticles makes them effective nanocarriers and also reduces the toxicity of free drugs resulting from off-target effects (Singh and Lillard Jr. 2009). The different nanoparticles-based systems can be broadly classified as dendrimers, micelles, liposomes, carbon nanotubes, and quantum dots.

Advantages of Nanoparticles as Carriers of Peptides

- The incorporation of peptides in nanoparticles improves the stability as it protects the peptide from enzymatic degradation
- Nanoparticles as nanocarriers of a peptide can be used for control release and for targeting specific effects.
- Oral delivery of peptides using a nanoparticle drug delivery system is a promising platform for peptide therapeutics.
- Nanoparticles as nanocarrier of peptide drugs can change/enhance the biodistribution and pharmacokinetic properties.
- Nanoparticles can incorporate peptides as cargo or as a targeting agent.

6.2.1 *Dendrimers*

Dendrimers are radially symmetric well-defined artificial molecules with a symmetric core, an inner shell, and an outer shell. Dendrimers are hyper-branched structures characterized by a high number of functional groups and a compact molecular architecture. The molecular structure of dendrimer consists of the central core with a single atom or group of atoms from which the branches of atoms, also called dendrons, are produced by various chemical reactions to form a homogeneous and the monodisperse structure consisting of tree-like arms or branches (Tomalia and Fréchet 2002; Abbasi et al. 2014). End groups in dendrimers can easily be functionalized and facilitate modifications of physicochemical and biological properties. Dendrimers have emerged as a new class of nano-sized molecules with tremendous application in anticancer therapies and diagnostic imaging (Srinivasa-Gopalan and Yarema 2007; Stiriba et al. 2002). Peptide dendrimers are being widely researched for their applications in various fields, including as biomedical diagnostic agents and as delivery vehicles for vaccines, drugs, and genes (Sadler and Tam 2002).

6.2.2 *Micelles*

Conventional micelles are defined as a collection of amphiphilic surfactants that aggregate spontaneously in an aqueous solution to form a vesicle with a hydrophobic core. It can incorporate hydrophobic drugs in the inner core (Rangel-Yagui et al. 2005). Alternatively, polymeric micelles are formed by the spontaneous arrangement of amphiphilic co-polymers in an aqueous solution with a hydrophobic core and a hydrophilic shell. Polymeric micelles can incorporate a hydrophobic drug in the core and can also be coupled with the targeting ligands such as peptide antibodies on its shell for specific cell targeting and enhancing the cellular uptake of the incorporated drugs (Amin et al. 2017). These nanoparticles have been widely studied for their role as anticancer drug delivery system. Phospholipid micelles are widely used for peptide drug delivery. Glucagon-like peptide 1, glucose-dependent insulinotropic peptide, and neuropeptide Y are some of the peptides that are delivered using the micellar nanocarrier (Esparza et al. 2019). A chitosan-based micelle using N-octyl-N-arginine chitosan (OACS) was developed for insulin oral delivery (Zhang et al. 2013).

6.2.3 *Carbon Nanotubes*

Carbon nanotubes (CNTs) are cylindrical molecules composed of carbon atoms and can be described as graphene sheets rolled into a single or multiwall seamless cylinder. The diameters of CNTs vary from a few to hundreds of nanometers with a

length/diameter ratio of higher than 106 with exceptional thermal, mechanical, optical, and electrical properties (Roldo 2016). The single-walled CNTs have gained wide popularity as a drug delivery system due to their high cargo loading capacity, intrinsic stability, prolonged circulation time, and enhancement of bioavailability of the incorporated drug. Single-walled CNT-based nanomaterials have been reported to be drug delivery vehicles for nucleic acids, proteins, and drug molecules. These nanotubes have also been functionalized by antibodies for enhancing the uptake and site-specific anticancer drug delivery (Mahajan et al. 2018). Along with the carbon nanotubes, peptide-based nanotubes are also gaining popularity. The use of synthetic polypeptides, short Fmoc-dipeptides, cyclic peptides of alternating D- and L-amino acids, and preassembled bundles of α -helices forming peptide-based nanotubes has been reported (Burgess et al. 2015; Ghadiri et al. 1993; Rho et al. 2019; Hartgerink et al. 1996).

6.2.4 *Quantum Dots*

Quantum dots (QD) are inorganic fluorescent semiconductor nanoparticles with very unique optical and electronic properties. They exhibit high photostability, size-dependent optical properties, high brightness, and large Stokes shift, making them a better choice over organic dyes. Quantum dots consist of an ultra-small core with a size ranging from 1.5 to 10 nm of a semiconductor material (e.g., cadmium selenide (CdSe)) that is surrounded by another layer of semiconductor usually made of zinc sulfide (ZnS). The inner core and semiconductor layer are encapsulated by a cap from the outside that is made of different materials (Lombardo et al. 2019). QDs are used as a fluorescent agent for disease diagnosis and in various cellular and in vivo assays (Maxwell et al. 2020). Quantum dots have several features such as small size versatile surface chemistry with unique optoelectrical properties, which make them an excellent agent for real-time monitoring and tracking of nanoparticles in an in vivo model without significant alteration of nanocarrier (Probst et al. 2013). Recently, QDs conjugated with peptides have been developed for the enhancement of activity, site-specific action, and drug delivery. Peptide nanofibers with graphene quantum dots are evaluated for both targeting and imaging of tumor cells (Su et al. 2015). A peptide-carbon QDs conjugate derived from human retinoic acid receptor responder protein 2 has been used against both antibiotic-resistant gram-positive and gram-negative pathogenic bacteria (Mazumdar et al. 2020).

6.2.5 *Liposomes*

Liposomes are widely studied and one of the most well-characterized nanoparticle-based systems. A hydrophobic molecule can be incorporated into the lipid bilayer, whereas a hydrophilic drug can be entrapped into the core of the liposome.

Liposomes are used as nanocarriers of drugs for different diseases such as cancer, hepatitis A, and fungal and bacterial infections (Beltrán-Gracia et al. 2019). Surface-functionalized liposomes are used for site-specific delivery of drugs. Peptides, proteins, antibodies, and carbohydrates molecules can be coupled on the surface of liposomes for targeting designated cell types which overexpress specific types of receptors. Recently approved vaccines for COVID-19 from Pfizer-BioNTech, Moderna uses liposome technology for the delivery of RNA-based vaccines (Polack et al. 2020; Dagan et al. 2021). In this chapter, we will focus on peptide-based liposomes.

6.3 Liposome

Liposomes as a delivery vehicle for peptide and protein drugs and proteins are extensively studied. Liposome surface can be easily functionalized by peptides for targeting specific cell types, or they can be used as cargo for hydrophilic and hydrophobic peptides for specific site delivery. Peptide-based liposomes have the ability to lower the off-target effects, enhancing the stability of peptides and having better cells and tissue permeability.

Liposomes are spherical nanoparticles with an aqueous core and a lipid bilayer. These are formed naturally when lipids are stirred into an aqueous media, resulting in a population of vesicles with diameters from nanometers to micrometers. The water molecules reject the hydrocarbon tails, which point in the same direction; however, the lipid head groups are drawn to water molecules and organize themselves in such a way that they point into the aqueous compartment (Fig. 6.1) (Lopes 2013).

The inner layer's head groups point in the direction of the intravesicular fluid, while the tails point away. As a result, one layer's hydrocarbon tails point toward the outer layer's hydrocarbon tails, creating a natural bilipid membrane (Raffa et al. 2010). Liposomes can contain drugs with a wide variety of lipophilicities in the

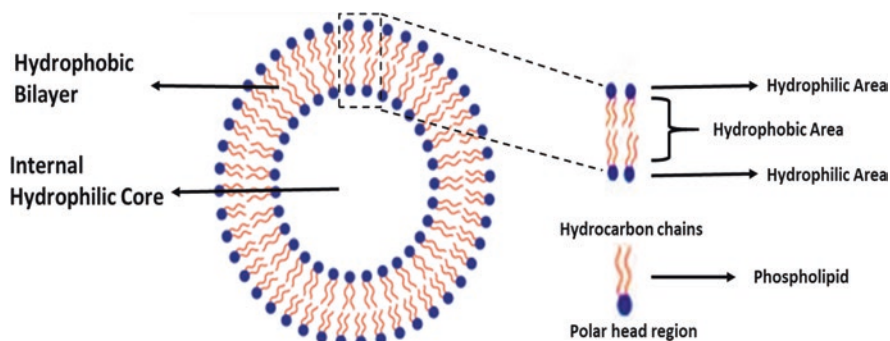


Fig. 6.1 Basic structure and composition of liposomes

lipid bilayer, the enclosed aqueous volume, or the bilayer interface (Huwlyer et al. 2008). Liposomes are usually prepared from natural or synthetic lipids, and the ingredients of liposomes are not limited to lipids and can also be formed from polymers (Meerovich and Dash 2019). Liposomes are biocompatible, biodegradable nanostructures made out of natural or synthetic lipids or polymers that can be used in biomedical research. One of the remarkable properties of liposomes is the ability to compartmentalize and dissolve both hydrophilic and hydrophobic molecules. Because of these properties, liposomes find applications in drug delivery (Çağdaş et al. 2014).

6.4 Formulation and Manufacturing Strategies of Liposome

Liposomes are made up mostly of phospholipids. These biomolecules are also key components in the construction of biological membranes. They have a polar head (water-soluble hydroxy groups) and an insoluble base, making them amphiphilic molecules. Liposomes may be zwitterionic, charged positively or negatively, or uncharged. The polar head charge is fully responsible for this. Liposomes are usually prepared for two kinds of lipids: naturally occurring or synthetic lipids (consisting of a phosphorus polar head and a glycerol backbone) and sterols (e.g., cholesterol) (Chowdhury 2008).

Liposomes also contain cholesterol, which is an important ingredient. It affects the characteristics of the lipid bilayer in liposomes in a modulatory way. It can modulate the stoutness of the liposome structure and enhance the packing between phospholipid molecules (Briuglia et al. 2015), resulting in a more ordered conformation and lower micropolarity in the aliphatic tail region (Liu et al. 2017); neighboring molecules (particularly water-soluble compounds) have less bilayer flexibility (Tarun and Goyal Amit 2014) and increased bilayer microviscosity (Olusanya et al. 2018). Cholesterol is also needed for liposomal membrane structural stability, which helps to keep the liposome stable in intestinal environmental stress (Liu et al. 2017).

Liposomes can be functionalized with varieties of biomolecules or small molecules (PEGs, aptamers, antibodies, proteins, peptides, ligands, sugars, or small molecules) for targeting effects (Fig. 6.2). To render special targeting properties, surface functionalization of liposomes can be used (Riaz et al. 2018). The preparation process has a significant impact on the characteristics of the processed liposomes. While liposome formation may occur spontaneously, mechanical agitation is often necessary. Various preparation procedures have been created in order to have control over the size and form of the liposomes generated, increase the efficacy of trapping the target molecules, and avoid the eventual leaking of encapsulated compounds from liposomes (Çağdaş et al. 2014).

Before one selects the type of liposome and components required, there are a few factors to consider: (1) liposomal components and the physicochemical properties of the material to be entrapped, (2) the ideal concentration of the encapsulated item

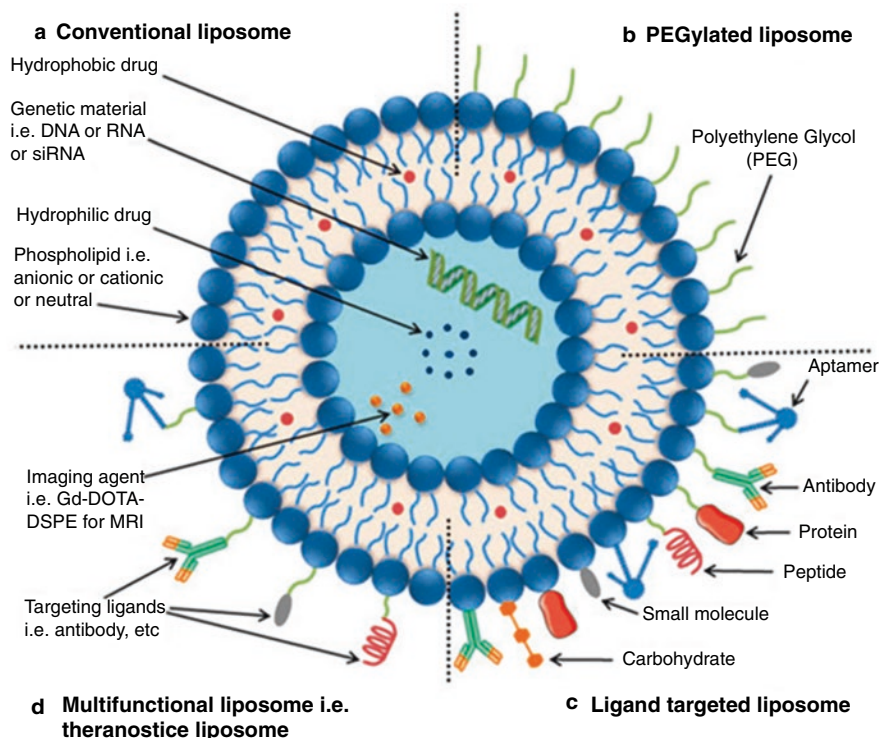


Fig. 6.2 Liposomes: Conventional and functionalized liposomes: (a) Phospholipid-based liposomes, (b) PEGylated/stealth liposomes with a layer of polyethylene glycol (PEG), (c) targeted liposomes with a specific ligand to target a disease site, and (d) multifunctional liposomes that can be used for diagnosis and treatment. (Reproduced from Creative Commons Attribution License) (Riaz et al. 2018)

and its possible toxicity, (3) the features of the media in which the liposomes are suspended (4) extra processes engaged during application (liposome transport), and (5) the optimal size, polydispersity, and shelf-life of the liposomes, (6) the possibility processing and the reproducibility of effective and efficient liposomal products across batches (Gomez-Hens and Fernandez-Romero 2006; Dua et al. 2012). Liposome size is an important factor in achieving effective drug accumulation at the target location and preserving liposome circulation half-life for in vivo drug delivery. The size and quantity of bilayers in the produced liposome are also related to the volume of encapsulated medication. Depending on the goal of the formulation, several liposome preparation methods may be utilized. Lipid hydration and the replacement of organic solvents with an aqueous medium are the most extensively utilized liposome production procedures (reverse-phase evaporation and organic solvent injection). According to Bangham's procedure, lipids are dissolved in a suitable organic solvent such as chloroform or methanol. The solvent is then evaporated

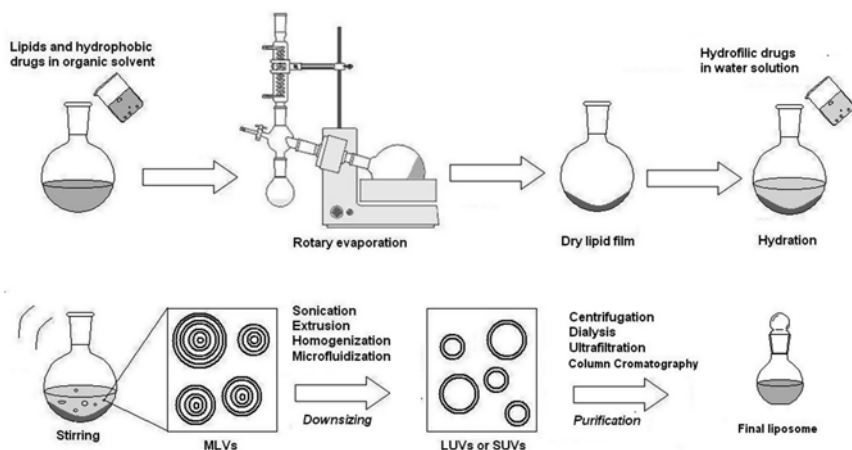


Fig. 6.3 Lipid hydration accompanied by vortex or manual stirring represents liposome production. (Reproduced from Creative Commons Attribution License) (Lopes 2013)

using rotary evaporation under reduced pressure before a thin layer is produced. The thin film is then hydrated in an aqueous solution at a temperature above phase transition to producing multi-lamellar vesicle (MLV) liposomes (Fig. 6.3). Lipophilic drugs are typically incorporated by co-dissolution with lipids (Wagner and Vorauer-Uhl 2011). Drugs that are hydrophilic dissolve in an aqueous medium or buffers. Amphiphilic drugs can be dissolved in both mediums. The creation of large vesicles (MLV) with a heterogeneous size distribution will occur during the liposome preparation process; and large liposomes can be made into small unilamellar vesicles by using a vesicle size reduction process.

6.5 Characterization of Liposomes

Characterization of liposomes involves various attributes such as encapsulation of drug, nanoparticle morphology, shape, size, surface charge (zeta potential), physical and chemical stability, and release of encapsulated drugs by *in vitro* studies (Fig. 6.4). Dialysis, ultra-centrifugation, ultrafiltration, and solid-phase extraction aid in the removal of the unencapsulated drug. Then, the encapsulated drug can be quantified using various methods such as fluorescent-based spectroscopy, RP-HPLC, capillary electrophoresis (CE), and field-flow fractionation (FFF).

The morphology of liposomes is determined by transmission electron microscopy (TEM), scanning electron microscopy (SEM), and cryo-TEM. TEM is the most frequently used microscopy technique to study the morphology of nanoparticles (Henry 2005; Kuntsche et al. 2011). These techniques are used to study the spherical shape of liposomes as well as detailed structural information of lipid layers (Tonggu and Wang 2020). Microscopy techniques are great tools to determine

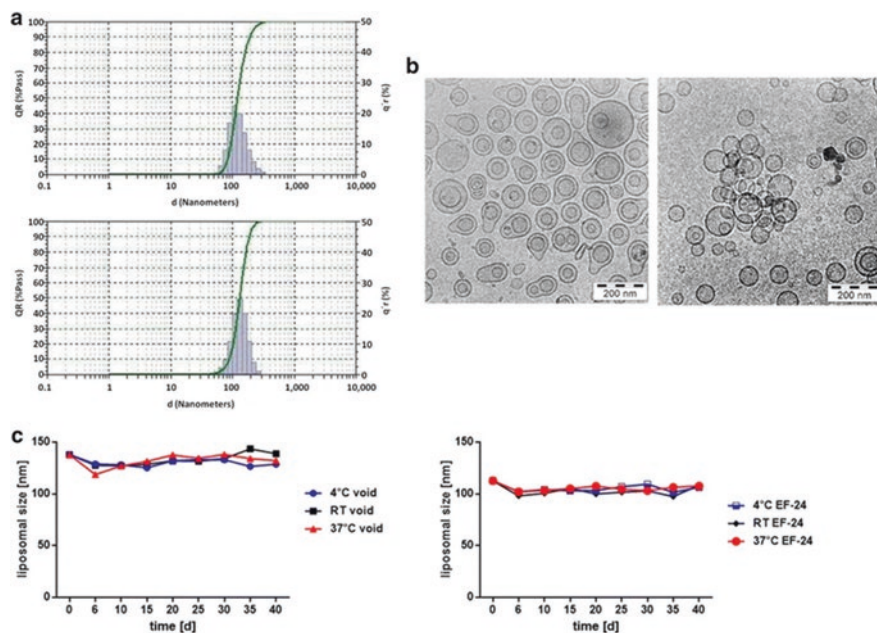


Fig. 6.4 Synthesis and characterization of void and EF24-containing PEGylated liposomes. Pegylated liposomes synthesized using a lipid hydration method were further characterized using DLS and TEM. (a) DLS of void and EF24-loaded liposomes revealed a narrow size distribution with an average diameter of less than 150 nm, (b) transmission electron microscopy of void (left panel) and EF24-containing liposomes (right panel) demonstrated spherical morphology and an average diameter of around 120 nm, in line with the data obtained by DLS, (c) the stabilities of void and EF24-loaded liposomes were determined at three different temperatures (4, 20, and 37 °C) using DLS over 40 days. (Reproduced from Creative Common Attribution License) (Bisht et al. 2016)

particle morphology but provide very little information about particle size and its distribution (Robson et al. 2018; Fan et al. 2021).

The particle size of liposomes is usually determined by using dynamic light scattering (DLS). DLS aid in the characterization of liposomes by providing information about mean particle size, particle size distribution, zeta potential, and polydispersity index. The particle size of the liposome is an important parameter as it has to be optimized depending on the targeted site and delivery method. Liposomes for antitumor drug delivery are usually in size range of ≤ 100 nm for better drug permeation into the tumor micro-environment and local tumor tissues (Nagayasu et al. 1999; Danaei et al. 2018). The optimum size of liposomes depends on the targeted tumor size, stage, and location. The large particle size of liposomes may not show the intended therapeutic effect because of poor permeability and phagocytosis by immune system cells. The particle size of liposomes can be controlled by using the techniques such as, sonication, homogenization, and extrusion (Mozafari

2005a; Mozafari 2005b). These techniques ensure the liposomes with small particle size (<200nm) and uniform size distribution.

Another important attribute of liposomes is the surface charge. Surface charge of the liposomes is determined by the phospholipid head groups that can incorporate positive or negative charges. The surface potential of nanoparticles provides information about the intraparticle interactions strength, adsorption of counterions, and particle stability (Fan et al. 2021). Surface potential, also termed as zeta potential, should be optimum to maintain the repulsion between the particles and uniform suspension of particles. Usually zeta potential of < -30 mV or > 30 mV is considered optimum for preventing the aggregation of particles in suspension (Samimi et al. 2019). Liposomes should be able to retain the drug during storage and in vivo circulation before delivering the drug to the desired site (Shen and Burgess 2013; Wang et al. 2014).

In vitro drug release from the liposomes can be determined by employing different methods such as sampling and separation (SS), dialysis membrane (DM), continuous flow (CF), or combined approaches (Fan et al. 2021). Among them, the dialysis membrane method is widely used for the determination of the drug release from the liposome. DM approach employs a dialysis sac in which the liposomes are kept, and this dialysis sac is immersed in the buffer. The dialysis membrane with an appropriate molecular weight cut-off (MWCO) should be selected based on the entrapped drug molecular weight. The amount of drug in the buffer outside of the dialysis sac is quantified at different time intervals to obtain the in vitro release of the drug from liposomes (Fan et al. 2021).

6.6 Liposome Stabilization Strategy with Lyophilization

Liposome stability is a major concern when developing them for pharmaceutical applications. Liposomes' increased bilayer permeability can lead to drug leakage, vesicle aggregation/fusion, and precipitation (Du Plessis et al. 1996). The most frequent strategy to improve liposome stability is to design an appropriate formulation, which entails selecting the right lipid composition and concentration as well as incorporating other substances to lengthen shelf life. Cholesterol and its derivatives, for example, can decrease lipid bilayer permeability. Antioxidants and metal chelators can be added to enhance the stability since unsaturated lipids are prone to peroxidation (Abdelwahed et al. 2006a, b; Chen et al. 2010). Furthermore, the presence of oxygen, both in the form of dissolved oxygen and in the container's headspace, must be avoided. When liposomes are hydrolyzed in aqueous dispersion, lysophospholipids and fatty acids are produced. This reaction is catalyzed by hydroxyl and hydrogen ions, and it can be slowed down by adjusting the pH or by adding a neutral buffer (Zhang and Pawelchak 2000). Liposome stabilization can be achieved using a variety of techniques, including lyophilization, and spray-drying, as well as formulation optimization. Lyophilization is the most popular procedure for increasing

the shelf life of liposomes, especially for thermosensitive drugs contained inside them (Chen et al. 2010).

Lyophilization, also known as freeze-drying, is a sophisticated drying method used to transform labile material solutions into solids that are stable enough for distribution and storage. Lyophilization is an industrial procedure that involves sublimating and desorbing the water from a frozen sample using a vacuum system. Nonetheless, when performed without the required stabilizers, this process causes a wide range of stress, including fusion and drug degradation, during the freezing and drying steps (Abdelwahed et al. 2006a, b; Abdelwahed et al. 2006a). Cryoprotectants, such as saccharides and their derivatives (e.g., sucrose, trehalose, hydroxypropyl—cyclodextrin (HP—CD), are used to keep the vesicles stable throughout freeze-drying (Bendas et al. 1996; Mohammed et al. 2006). Sugars stabilize membranes by replacing water, which is the most well-known and widely accepted mechanism. The protective effect is thought to be based on complicated and particular interactions between phospholipids and sugars. Experiments have shown that interactions take place by a hydrogen bond between the sugars' hydroxyl groups and the phosphate groups on the bilayer surface. In summary, sugars limit water-phospholipid interactions (Chen et al. 2010; Santivarangkna et al. 2008). Among different sugars, trehalose has been detected to enter the membrane and establish hydrogen bonds with the carbonyl groups of phospholipids (Diaz et al. 1999; Luzardo et al. 2000; Villarreal et al. 2004). As a result, trehalose seems to have a greater affinity for phospholipid bonding and is considered an excellent cryoprotectant (freeze-protectant) for liposomes. When liposomes were freeze-dried in the presence of adequate amounts of trehalose (a carbohydrate found in high concentrations in organisms), it was observed that they preserved up to 100% of their original contents. Proper controlled lyophilization of liposomes can be achieved using freeze-driers in various sizes, from portable laboratory versions to large commercial devices (Akbarzadeh et al. 2013; Awada et al. 2004).

6.7 Liposomal Nanocarrier System for Peptide Drug Delivery

Peptide drugs can be incorporated into the liposome in various ways, and it depends on the function of the peptide as well as the characteristics of the transported peptide drug. Hydrophobic peptides are entrapped into the liposomal bilayer and typically dissolved into the organic solvent before forming a thin lipid layer in the thin layer hydration method. Hydrophilic peptides can be entrapped into the hydrophilic compartment of the liposome. Targeting peptides are attached to the outer layer to facilitate the selective delivery of the liposomal carrier. One such example is the study reported on peptide S1 that could specifically bind to the vascular epithelial growth factor receptor 2 (VEGFR2) used to target liposomes to the cells that over-express VEGFR2. S1 peptide incorporated liposomal formulation was found to be an efficient nanoscale drug delivery device *in vitro* and *in vivo* (Han et al. 2016).

The peptide entrapped in the liposome can be measured through the process called the entrapment efficiency method. In this process, the liposome solution can be dissolved in a suitable organic solvent to extract the entrapped peptide and then analyzed with suitable detection methods and compared with the loading solution to get the percentage of the entrapped peptide content into the liposomal compartment. Entrapment efficiency can be measured using the following formula:

$$\text{Entrapment efficiency} = \frac{\text{Drug content in the liposome formulation}}{\text{Drug content in the loading solution}} \times 100\%$$

The release profile of the liposome is an important characteristic of the liposomal formulation that can be assessed through different release study methods, and the dialysis process can be a reliable way for this process. Specific molecular cutout dialysis bags can be used, and buffer media can be utilized for the assessment of the release property of the liposome solution. Rezaei et al. performed the dialysis method to investigate the *in vitro* release profile of the peptide from encapsulated. The cumulative percentage release of the liposomal samples is given as a summary of their release behavior. Under the test conditions, the peptide's release rate was gradual and desirable (Rezaei et al. 2020).

Liposomal drug release can be increased or decreased with the utilization of different phospholipid properties and concentrations through maintaining peptide to lipid ratio (Li et al. 2015). To test the pharmacological availability of liposomes with phosphatidylethanol, Kisel et al. developed three types of liposomal formulations with insulin: (i) dipalmitoyl phosphatidylcholine/dipalmitoyl phosphatidylethanol (1:1 w/w) liposomes, (ii) dipalmitoyl phosphatidylcholine/dipalmitoyl phosphatidylethanol/palmitoyl–stearoyl sucrose (1:1:0.2) liposomes, and (iii) liposomes composed of natural phosphatidylcholine and phosphatidylinositol (1:1). Hyperinsulinemia was seen after oral administration of all liposomal species in animal model studies. Hyperinsulinemia was accompanied by a drop in blood glucose content when liposomes containing dipalmitoyl phosphatidylethanol were used (Kisel et al. 2001). In male Wistar rats, Katayama et al. found that with intragastric delivery of positively charged double liposomes (DL) prepared with stearylamine (SA) and phosphatidylserine (PS) using the glass filter method as insulin carriers in combination with aprotinin, a protease inhibitor resulted in notable hypoglycemic effects (Katayama et al. 2003). Aprotinin was conjugated with chitosan to create a polymer–protease inhibitor with a positive charge. Liposomes were prepared with 1- α -distearoylphosphatidylcholine (DSPC), dicetylphosphate (DCP), and cholesterol (molar ratio: 8:2:1) to acquire negative charges. A formulation was prepared with polyelectrolyte complexes between negatively charged multilamellar vesicles (MLV) and positively charged chitosan–aprotinin conjugate to improve systemic uptake of therapeutic peptides after oral administration. *In vitro*, it was shown that chitosan–aprotinin inhibited trypsin substantially at doses of 0.05% and 0.1%, but no inhibition was shown in the presence of 0.1% chitosan (Werle and Takeuchi 2009). In another study, a new form of liposome containing tetraether lipids (TELs) produced from archaea bacteria may enhance oral peptide delivery. All liposomal

formulations were prepared by the film method with a mixture of host lipids, e.g., egg phosphatidylcholine (EPC) or dipalmitoyl phosphatidylcholine (DPPC), tetraetherlipids (TEL), and other lipids, e.g., cholesterol. The film technique was used to make liposomes, which were then extruded. Photon correlation spectroscopy revealed the presence of vesicles with sizes ranging from 130 to 207 nm (Parmentier et al. 2011). Niu et al. showed that insulin oral bioavailability was considerably improved when liposomes containing bile salts (BS-liposomes) were used for the formulation of recombinant human insulin (rhINS). By using the reversed-phase evaporation method, BS-liposomes containing sodium glycocholate (SGC), sodium taurocholate (STC), or sodium deoxycholate (SDC) were prepared. These bile salt containing liposomes exhibited increased residence duration and penetration through biomembranes and increased absorption in a model system. Based on their studies Niu et al. conclude that enhanced absorption of insulin-loaded liposomes may be due to mechanisms of trans-enterocytic internalization of liposomes. (Niu et al. 2014). In another study, liposomal vasoactive intestinal peptide (VIP) inhalation therapy was used as a potential therapeutic option for severe lung disorders. It was observed that in the lungs, VIP has a relatively brief time of action. To enhance the activity and duration of action of VIP in the lungs, Stark et al. created a liposomal drug delivery method for VIP and evaluated it for its ability to protect VIP against enzymatic cleavage. The liposomal formulation components were polyethyleneglycol conjugated distearyl-phosphatidylethanolamine (DSPE-PEG2000)-lyso-stearyl-phosphatidylglycerol (lyso-PG-palmitoyl-oleoyl-phosphatidylcholine (POPC) (1,7.5:11). When these formulations were evaluated in a model system, it was found that the free VIP was quickly digested, but liposomal-associated VIP showed relatively enhanced stability (Stark et al. 2008) (Table 6.1).

Liposomes as a nanocarrier are being used for peptide drug delivery in various ways. Oral administration of liposomes with peptide cargos was evaluated using various techniques. Muramatsu et al. found that soybean sterol containing insulin liposomes were able to lower blood glucose levels in rats for up to 21 h after injection (Muramatsu et al. 1996). Liposomes with peptide loads for buccal distribution have been the subject of research. In normotensive/spontaneously hypertensive hamsters, Suzuki et al. found significant vasodilation after administration of free vasoactive intestinal peptide (VIP) and significantly prolonged vasodilation with VIP liposomes, indicating that VIP-induced vasodilation is impaired in situ in essential hypertension and is restored by encapsulation into liposomes (Suzuki et al. 1996; Sejourne et al. 1997). Liposomes with peptide cargos for pulmonary administration also had been investigated. In the rat lung, Huang et al. found that pulmonary distribution of insulin-loaded liposomes resulted in prolonged effective hypoglycemia, which was not seen with a mix of free insulin and empty liposomes (Huang and Wang 2006; Bi et al. 2008). Intravenous administration of liposomal formulation containing peptides was also investigated. Using iodine-labeled VIP liposomes, Refai et al. found that VIP liposomes were better absorbed by the rat lung than free VIP (Refai et al. 1999). The inhalation of vasoactive intestinal peptide (VIP) has been proposed as a viable therapeutic option for a variety of lung disorders, including asthma and pulmonary hypertension. Due to fast enzymatic

Table 6.1 Peptide liposomes composition, preparation, and characteristics

Name	Composition	Preparation method	Particle size	Indication	Ref.
Liposome-entrapped insulin	Liposomes were prepared by mixing phosphatidylcholine and phosphatidylinositol ('fluid' 1:1 w:w) or dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidylethanol ('solid', 1:1 w:w) or the latter supplemented with palmitoyl–stearoyl sucrose (1:1:0.2 w:w:w) in chloroform	Thin-film hydration method	50–250 nm	Hypoglycemic effect	Kisel et al. (2001)
Double liposomes (DLs) containing insulin	26 mM H-soyaPC was dissolved alone or with 2.6 mM SA or PS as lipids with electrical charges in chloroform	Glass filter method	2–9 μ m	Hypoglycemic effect	Katayama et al. (2003)
Oral peptide delivery with chitosan–aprotinin-coated liposome	Anionic multilamellar liposomes (MLV) consisting of DSPC, DCP, and Chol (molar ratio: 8:2:1)	Thin-film hydration method	3 and 4.5 μ m	Novel polymer protease inhibitor-based delivery systems	Werle and Takeuchi (2009)
Octreotide peptide oral delivery with tetraether lipids (TELs)	A mixture of host-lipids, e.g., EPC or DPPC, TEL, and other lipids, e.g., cholesterol was dissolved in chloroform/methanol (8:1)	Thin-film hydration method	130–207 nm	Acromegaly, Gastrointestinal disorders, and psoriasis	Parmentier et al. (2011)
Recombinant human insulin (rhINS)-loaded BS-liposome	Soybean phosphatidyl choline (SPC) and bile salts (SGC/STC/ SDC) were dissolved in 5 mL absolute ether with a molar ratio of 4:1, into which 1 mL rhINS solution in citric–Na ₂ HPO ₄ buffer (4 mg/mL, pH = 3.0) was added	Reversed-phase evaporation method	150 nm	Hypoglycemic effect	Niu et al. (2014)
Inhalation administration of vasoactive intestinal peptide (VIP)	Stock solutions of DSPE PEG2000, lyso-PG, and POPC in pure chloroform or chloroform-methanol mixtures, respectively, were made	Thin-film rehydration method	95 nm	Cystic fibrosis, ulcerative colitis, and primary pulmonary hypertension	Stark et al. (2008)

breakdown in the airways, peptides have a short half-life, which limits their medicinal utility. As a result, Hajos et al. created unilamellar nano-sized VIP-loaded liposomes (VLL) and found that by creating a “dispersible peptide depot” in the bronchi, the liposomes have the potential to enhance VIP inhalation treatment. As a result, exposure to cells, i.e., direct ligand-receptor interactions, might stimulate the release of VIP from liposomes. A schematic diagram of the model proposed for the stability of VIP against enzymatic degradation is shown in Fig. 6.5 (Hajos et al. 2008).

Peptides encapsulated in liposomes are shielded from the inactivating effects of environmental factors, thus causing no adverse side effects. Liposomes provide a unique method of delivering peptides into cells or even individual cellular compartments. Liposome size, charge, and surface qualities can easily be changed by

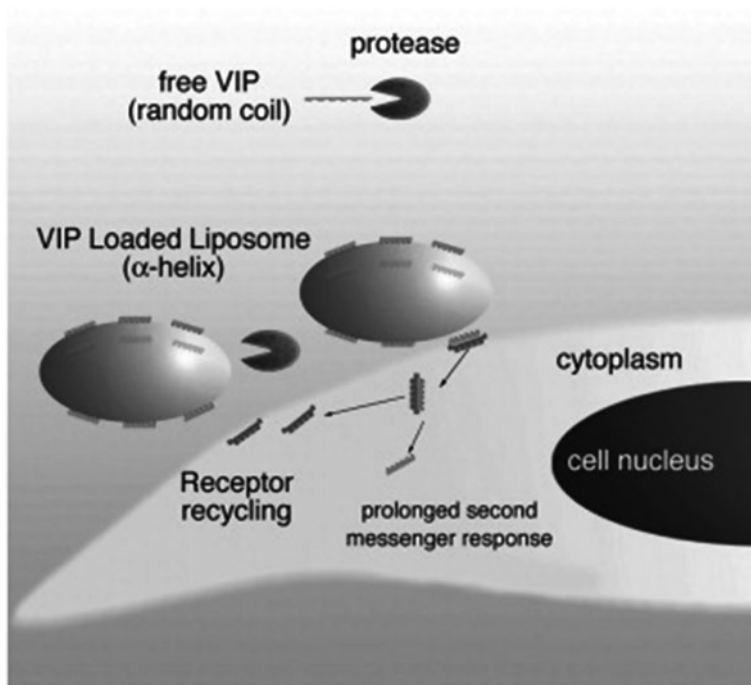


Fig. 6.5 Suggested fusion model of VLL Free VIP (random coiled) can easily be degraded by proteases on the way to the receptor. VIP from VLL is protected against proteases. VLL show no peptide leakage during storage, but electrostatically bound VIP may become released by direct contact and binding to the receptor. Following the ligand-receptor-complex internalization, and intracytoplasmic complex disintegration, the receptor is recycled to the cell surface to bind new upcoming VIP molecules. In addition to the protection by liposomes per se, the alpha helical conformation of VIP induced by negatively charged liposomes may convey further degradation protection; moreover, it is preferred VIP-conformation for receptor binding. Reprinted from Publication Inhalable liposomal formulation for vasoactive intestinal peptide Hajos et al. Vol357 p286-294 (2008) International Journal of Pharmaceutics, with permission from Elsevier (Hajos et al. 2008)

simply adding new chemicals to the lipid mixture before liposome formation and/or using different preparation procedures (Adibzadeh et al. 1992). The characteristics of the carrier are then exploited to modulate drug distribution rather than the physicochemical properties of the medicinal ingredient (Çağdaş et al. 2014). Liposomes have different advantages in peptide drug delivery and peptide-based targeted therapy leading to enhanced efficacy with reduced toxicity (Swaminathan and Ehrhardt 2012). They play a role in improving drug solubility (Mohammed et al. 2004), working as a sustained release system (Mukherjee et al. 2007), offering targeted drug delivery (Sonju et al. 2021), reducing drug toxicity (Naik et al. 2021), providing support against drug degradation (Cristiano et al. 2017), enhancing API circulation half-life (Allen et al. 2006), overcoming multidrug resistance (Matsuo et al. 2001), improving the therapeutic index of the entrapped drug (Wang et al. 2010), and shielding APIs against their neighboring environment (Park et al. 2011a).

Positive surface charge insulin-loaded liposomes display greater resistance to trypsin digestion than negative and neutral surface charged insulin-loaded liposomes, according to Kato et al. (Kato et al. 1993). Corona-Ortega et al. found that cationic/positively charged liposomes had better cell adhesion than neutral/negative IL-2-encapsulated liposomes (Corona-Ortega et al. 2009). Law et al. conducted a thorough investigation into formulation production parameters, with the liposomal charge being a key parameter under investigation in salmon calcitonin-loaded liposomes, where a negative surface charge allowed greater cargo encapsulation over neutral and positive surface charges (Law and Shih 2001). Some payloads exhibit interaction with lipid membrane because they are either lipophilic or amphiphilic. Peptides belong to the latter group and studies demonstrating their interaction with a lipid membrane (Stark et al. 2007; Neville et al. 2000; Joffret et al. 1990; Arien et al. 1995). Following cholate-induced disruption of sCT liposomes, Arien et al. tried to explain why the oral administration of calcitonin (CT)-loaded liposomes resulted in a hypocalcemia in animal model. Based on their studies the authors conclude that CT-lipid complex formed protects the peptide from trypsin digestion (Arien et al. 1995). VIP interaction with lipid bilayer was reported by Stark et al. (Stark et al. 2007), and IL-2 interaction with lipid bilayer was reported by Neville et al. (Neville et al. 2000), all of which were verified by freeze-fracture microscopy. Joffret et al. hypothesized that IL-2 contact with liposomal bilayers resulted in increased proliferation of cytotoxic T cells after administration of IL-2 liposomes (Joffret et al. 1990). The drug loading process determines the payload's encapsulation efficiency and is thus a relevant parameter. The association of insulin with the liposomal membrane is promoted in the presence of a transmembrane gradient, according to Hwang et al., and this is not optimal for insulin loading (Hwang et al. 1999). In contrast to the transmembrane gradient process, the reversed step evaporation method resulted in a twofold rise in insulin encapsulation into liposomes (Hwang et al. 2000). In vitro studies have shown that including permeation enhancers into liposomal formulations has a positive influence. Degim et al. and Maitani et al. demonstrated increased insulin permeability through Caco-2 cell monolayers and rabbit nasal mucosa linked to diffusion cells prepared with Caco-2 cell monolayers (Degim et al. 2004; Maitani et al. 1992). In the presence of sodium

taurodeoxycholate, Song et al. demonstrated improved sCT permeation through Caco-2 cell monolayers (Song et al. 2005). PEGylation has many benefits, including the avoidance of opsonization (in vivo) and, in several situations (formulation related), increased payload encapsulation (Immordino et al. 2006). Park et al. found that PEGylated liposomes encapsulated insulin more effectively than non-PEG counterparts (Park et al. 2011a; b). According to Kedar et al., IL-2-encapsulated PEG liposomes interacted better with cells in vitro than their non-PEG equivalent (Kedar et al. 2000).

6.8 Types of Liposome

6.8.1 Active Targeting Liposomes

The main limitation of conventional liposomes is the off-target effects. This problem can be addressed by the development of actively targeted liposomes. Liposomes serve as an attractive, active targeting drug delivery system as their surface can be modified by various ligands such as small molecules, aptamers, antibodies, and peptides (Byrne et al. 2008). These ligands can specifically target the cancer cell, which overexpresses the corresponding receptors. Tumor cells are found to overexpress receptors like EGFR, HER-2, transferrin receptor, folate receptors, integrins, and so on, depending on tumor types. Targeting these overexpressed receptors by conjugating ligands on the liposomal surface can result in tumor-specific targeting and drug delivery (Torchilin 2007). Actively targeting liposomes can be developed to target tumor cell surface receptors like EGFR, HER-2, or tumor microenvironment/vasculature like VEGF, matrix metalloproteinases, and $\alpha\beta$ -integrins (Deshpande et al. 2013). Peptides serve as an excellent targeting agent because of their specificity, easy synthesis, low costs, lower immunogenicity, and ease of conjugation on the liposomal surface. Additionally, antitumor peptides as ligands on liposomal surfaces incorporating cytotoxicity can have better tumor accumulation and better cytotoxicity towards tumor cells. Use of cell-penetrating peptides in liposomes aid in better penetration of the drug into tumor cells (Ye et al. 2016).

6.8.2 Stimuli-Responsive Liposomes

Conventional liposomes have some limitations in terms of drug release, as the drug may get released before it reaches to target site and may not accumulate at the target site. This challenge is addressed by the development of stimuli-responsive liposomes (An and Gui 2017). The stimuli may be presented to liposomes by the target site microenvironment (e.g., pH, redox potential, enzyme) or applied externally from the outside (e.g., hyperthermia, ultrasound, magnetism). Various

stimuli-responsive liposomes such as pH-sensitive, thermosensitive, magnetic field-sensitive, and ultrasound-sensitive liposomes are developed (Torchilin 2009; An et al. 2010; Amstad et al. 2011). Stimuli-responsive liposomes, after reaching the target site, undergo changes in the composition or structure of the bilayer in the presence of the stimuli leading to the release of drug at the target site. Thus reducing the premature release of the drug and increasing the site-specific targeting with reduced off-target effects of liposomes (Andresen et al. 2005).

6.8.3 Temperature-Sensitive Liposomes (Thermosensitive Liposomes)

Thermosensitive lipids and polymers with a low critical solution temperature (LCST) are used to prepare the thermosensitive liposomes. These liposomes can be used in the site-specific delivery to the tumor sites where hyperthermia is observed compared to normal tissues (Danhier et al. 2010). An increase in temperature changes disrupts the lipid bilayer structure of liposomes, releasing the drug in the tumor sites (Chountoulesi et al. 2017). Heat can also be applied externally on tumor sites which trigger the release of drugs from thermosensitive liposomes.

6.8.4 pH-Sensitive Liposomes

The tumor microenvironment has lower pH compared to the normal cell environment due to the high rate of glycolysis in cancer cells generating excessive metabolites like lactic acid and CO₂ (Cardone et al. 2005). This lower pH of the tumor microenvironment can be exploited for effective delivery and targeting of the targets by the development of pH-sensitive liposomes. pH-sensitive liposomes are composed of pH-sensitive lipids that show structural integrity in normal pH but destabilize the lipid bilayer as soon as it is exposed to lower pH around 6–6.5 (Felber et al. 2012; Cardone et al. 2005). This facilitates the drug release more in tumor cells from pH-sensitive liposomes. A pH-sensitive liposome is composed of pH-sensitive lipids like DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) and a weakly acidic amphiphile, such as cholesteryl hemisuccinate (CHEMS) with polymorphic phase behavior, and adopts the hexagonal state at lower pH compromising the lipid bilayer integrity and facilitates the drug delivery (Soares et al. 2011). Dual targeting liposomes by the use of a pH-sensitive approach are studied for their target-specific effect and delivery. The use of pH-dependent peptides, cell-penetrating peptides, and antimicrobial peptides coupled to the pH-sensitive lipids/polymers to trigger the drug release from liposomes are also being developed for targeting tumor cells (Zhao et al. 2016; Ding et al. 2017; Zhang et al. 2016).

6.8.5 Magnetic Field-Responsive Liposomes

Magento-liposomes (MLs) incorporate magnetic and metallic nanoparticles such as Fe_3O_4 and can serve as an excellent agent for diagnostic and therapeutic purposes. MLs are responsive to the externally applied magnetic field and help to maintain the liposomes at the target site for complete release of the drug and enhance the site-specific delivery of the drug. MLs which incorporate metallic nanoparticles can play a dual role as a hyperthermia agent or as a nanocarrier for drug delivery or a combination therapy for cancer treatment (Anilkumar et al. 2019). Magnetic nanoparticle-loaded thermosensitive liposomes with near-infrared (NIR) laser-triggered release of doxorubicin in tumor cells showed that MLs can be used as combined photothermal–chemotherapy of tumors (Shen et al. 2019).

6.8.6 Ultrasound-Responsive Liposomes

Ultrasound-responsive liposomes encapsulate the drug along with a small amount of air which makes them acoustically active on ultrasound stimulation. These liposomes are suitable for ultrasound imaging and for controlled localized drug delivery on ultrasound stimulation in different conditions like cancer, thrombus, arterial stenosis, myocardial infarction, and angiogenesis (Huang 2010). These echogenic liposomes are prepared from different methods, including lyophilization and pressurization. The application of high-intensity-focused ultrasound (HIFU) induces local hyperthermia resulting in the release of drug contents from liposomes. Various studies of ultrasound-responsive liposomes, including cell-penetrating peptides and conjugated doxorubicin, showed enhanced cytotoxicity towards cancer cells upon ultrasound stimulation (Xie et al. 2016; Lin et al. 2016).

6.8.7 Light-Sensitive Liposomes

Light of a certain wavelength can trigger the release of drug contents from light-sensitive liposomes with a high level of control. These liposomes consist of photosensitizer (photoactive molecules) that generates singlet oxygen and other reactive oxygen species upon exposure to a particular wavelength of light resulting in disruption of lipid bilayer membrane and release of entrapped drug (Miranda and Lovell 2016; Prasmickaite et al. 2002). Photosensitizers like porphyrin derivatives, chlorins, phthalocyanines, and porphycenes are incorporated in the lipid bilayer or conjugated to the lipids for the light-sensitive effect (Enzian et al. 2020; Yavlovich et al. 2010). Various adjustable factors like wavelength, duration, light intensity, and photosensitizer concentrations make these photosensitive liposomes an attractive drug delivery system with a high level of temporal and spatial control of drug release

and easy optimization with a broad range of applications (Yavlovich et al. 2010). Various photosensitive liposomes incorporating drugs are studied for precise and control delivery to the target site by adjustable near-infrared (NIR) light irradiation (Sun et al. 2016). Photosensitive liposomes consisting of gold nanoparticles are gaining popularity as they can use NIR as a light source for deep tissue penetration and lower phototoxicity (Mathiyazhakan et al. 2018).

6.9 Limitation of Liposomes

6.9.1 *Reticuloendothelial System (RES) and Liposome Clearance*

The reticuloendothelial system is present in primary organs such as the liver, spleen, kidney, lungs, lymph nodes, and bone marrow. Maximum liposomal uptake is observed in the liver, followed by the spleen, which aids in the removal of liposomes from circulation. Macrophages present in the RES eliminate the liposomes. RES elimination of liposomes is addressed by the conjugation of PEG polymers to the lipid membrane. PEG helps prolong the circulation time of liposomes and prevents elimination by RES by steric stabilization of liposomes (Ishida et al. 2001).

6.9.2 *Accelerated Blood Clearance Phenomenon*

Repeated injection of PEGylated liposomes may result in loss of their long circulation time properties, which in turn leads to rapid blood clearance. This phenomenon is known as accelerated blood clearance (ABC) (Dams et al. 2000; Ishida et al. 2003). The exact mechanism of ABC of repeated dosing of PEGylated liposomes is still unclear, but this may be a great hurdle for clinical approval and application of PEGylated liposomes. This will be particularly challenging for surface-functionalized liposomes as most of these liposomes use PEG. ABC phenomenon is affected by PEG density, lipid dose, and dosing intervals (Ishida and Kiwada 2008; Sercombe et al. 2015). Such factors need to be carefully optimized to prevent this ABC phenomenon.

6.9.3 *High Serum Protein Binding*

Liposomes show a higher affinity to the serum protein that may result in the masking of ligands to the receptors. It may also affect the liposome biodistribution and pharmacokinetic properties lowering the targeting and drug delivery efficiency of the liposomes (Sercombe et al. 2015).

6.9.4 Masking of Surface Ligands by Polymers

Polymers like PEG are used for enhancing the circulation time of liposomes, but they might have a limitation in actively targeted liposomes. PEG and other polymers may mask the ligand by steric hindrance and may prevent the interaction of the ligand with the receptor. This will greatly affect the target-specific delivery of liposomes. This limitation can be overcome by careful selection of the length of PEG.

6.9.5 Difficulty in the Accurate Characterization of Surface-Functionalized Liposomes

There are well-defined methods for the characterization of conventional liposomes, but when it comes to surface-functionalized liposomes, there are limited guidelines and characterization methods. This makes it very difficult for accurate quantification of ligands and drugs in the functionalized liposomes. Proper technique to quantify the ligand attached is limited. Highly reliable biochemical and biophysical methods for quantification of the peptide and proteins attached on the surface of liposomes are still lacking. Hence batch-to-batch variation in production is very hard to address.

6.9.6 Large-Scale Production of Surface-Functionalized Liposomes

Production of conventional liposomes that are FDA approved has been well characterized, and large-scale production is relatively smooth due to their simple composition. However, the surface-functionalized liposomes with ligands such as peptides and antibodies face challenges in characterization, and hence industrial-scale production is an uphill task. These surface-engineered liposomes are studied on a small scale in a laboratory setting, and industrial scaling of these liposomes might have a very high batch-to-batch variation due to the difficulty in accurately quantifying. Thus, the physiochemical, pharmacokinetic, and biological properties of the peptide-functionalized liposomes may be compromised during industrial scaling (Wagner and Vorauer-Uhl 2011).

6.9.7 Stringent Storage Conditions

Liposomes require very strict storage conditions as high temperature leads to instability, and freezing them may result in ice crystal formation, which compromises the structural integrity of the formulation. Hence, liposomes need to be stored in a refrigerator at all times.

6.9.8 Aggregation of Liposomes

The high density of the ligands such as peptides, antibodies may lead to the aggregation of the liposomes resulting in loss of membrane integrity of liposomes. Proper optimization and study of the optimum ligand density, particle size and composition, and cell type to target may help in the prevention of aggregation (Barenholz 2001).

6.9.9 High Cost

Surface-functionalized liposomes require high costs for development. Unlike conventional liposomes, surface engineering of liposomes by ligands requires expertise and various optimization process, which results in higher cost.

6.9.10 Recent Examples of Liposome-Mediated Peptide Drug Delivery in Clinical Trials

Until now, some peptides containing liposomal formulations are in clinical trials, for example, Mepact® containing Mifamurtide which is a synthesized derivative of muramyl dipeptide (MDP), the smallest naturally occurring immunological stimulatory component of mycobacterium species' cell walls. The formulation exhibits similar immunostimulatory properties to natural MDP but with a longer plasma half-life. This formulation was approved in Europe in March 2009 (Kager et al. 2010). Xemys, an immunodominant MBP peptide encapsulated in mannosylated liposomes, is in a clinical trial to treat multiple sclerosis (MS) (Lomakin et al. 2016). MUC-1 is a cell surface glycoprotein that is significantly expressed in lung cancer (Turner 2008; Gandhi et al. 2018). A mucin-1 peptide-based vaccine was formulated with a synthetic lipopeptide and liposome (Tecemotide). The vaccine was designed to induce a cellular immune response to cancer cells that express MUC-1. There are attempts to deliver insulin via different routes of administration using insulin-liposomal formulations (El-Wakeel and Dawoud 2019; Degim et al. 2006; Akimoto et al. 2019; Huang and Wang 2006).

6.10 Summary

Liposomes serve as an attractive platform for drug delivery. Peptide-based liposomes can successfully target the tumor cells and can lower the off-target effects of chemotherapeutics. Toxicity to normal cells by anticancer therapeutics has been dramatically improved by the use of peptide-based liposomes. Therapeutic

peptides can be incorporated inside the liposomes to enhance stability and better tumor accumulation. Apart from that, development of peptide-functionalized liposome to specifically target the desired site has several advantages. Different peptide-functionalized liposomal formulations are being developed for various diseases and for diagnostic applications. Peptide-based liposomes have increased the dimensions of nanomedicine for clinical applications. Surface functionalization of liposomes by peptides is a relatively new technology that requires further study to generate clinically approved therapeutics.

With the ever-growing field of nanotechnology and the success of Covid-19 mRNA vaccines encapsulating the mRNA in lipid capsules developed by Pfizer-BioNtech and Moderna, liposomes as drug delivery agents are getting wide attention. Covid-19 mRNA vaccines were developed using liposomal technology to deliver the mRNA. This overwhelming success of the vaccines further bolsters liposomes as an effective drug delivery agent for susceptible drugs like proteins, peptides, mRNA, DNA, and viral vectors. Peptide-based liposomes for delivery of peptides and for specific targeting are being developed. Various challenges of peptide-based liposomes like difficulty in characterization and quantification of attached ligands, stability issues, masking of ligands, and accelerated blood clearances should be addressed. The development of a different biophysical and biochemical method for accurate quantification and reduction of the batch-to-batch variation for industrial production of surface-functionalized liposomes would help these liposomes to be clinically approved. Further research, including in vitro, pre-clinical, and clinical trials of the peptide-based liposomes, would certainly forward the field of nanomedicine.

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