



## Review article

## Liposome-polymer complex for drug delivery system and vaccine stabilization

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## ABSTRACT

Liposomes have been used extensively as micro- and nanocarriers for hydrophobic or hydrophilic molecules. However, conventional liposomes are biodegradable and quickly eliminated, making it difficult to be used for delivery in specific routes, such as the oral and systemic routes. One way to overcome this problem is through complexation with polymers, which is referred to as a liposome complex. The use of polymers can increase the stability of liposome with regard to pH, chemicals, enzymes, and the immune system. In some cases, specific polymers can condition the properties of liposomes to be explicitly used in drug delivery, such as targeted delivery and controlled release. These properties are influenced by the type of polymer, crosslinker, interaction, and bond in the complexation process. Therefore, it is crucial to study and review these parameters for the development of more optimal forms and properties of the liposome complex. This article discusses the use of natural and synthetic polymers, ways of interaction between polymers and liposomes (on the surface, incorporation in lamellar chains, and within liposomes), types of bonds, evaluation standards, and their effects on the stability and pharmacokinetic profile of the liposome complex, drugs, and vaccines. This article concludes that both natural and synthetic polymers can be used in modifying the structure and physicochemical properties of liposomes to specify their use in targeted delivery, controlled release, and stabilizing drugs and vaccines.

## 1. Introduction

A liposome is a drug delivery system composed of phospholipids and forms a bilayer membrane in an aqueous system. Liposomes have been used extensively as a drug delivery system to increase the solubility and permeability of hydrophilic or hydrophobic drugs. However, conventional liposomes are easily degraded by pH, enzymes, and the immune system in a biological environment. Therefore, its application in the provision of certain pathways will release the drug immediately, being unable to maintain the stability of the active substance. The conventional liposome quickly decomposes at acidic pH, so the delayed-release profile in the intestine cannot be obtained. The liposome can also be degraded by lipase in the digestive system [1, 2]. In systemic delivery, liposome can be recognized by the reticuloendothelial system (RES). This can decrease its bioavailability in systemic circulation [3, 4].

One solution to overcome this problem is through modification of the structure of the liposome by using a polymer. The complexation of the liposome-polymer is called a liposome complex. It is known that complexing liposomes using polymers can improve the physicochemical properties of liposome, so they can be explicitly used for targeted delivery and controlled release of drugs. The liposome complex system becomes more stable and sturdy because it is better at protecting active substances from chemicals, enzymes, the immune system, and even photolysis reactions [1, 5, 6, 7, 8, 9, 10]. Surface modification of liposomes can be used to avoid bonding or adsorption of liposomes on some proteins during circulation. Besides, surface modification is intended for targeted drug delivery using ligands, receptors, or polymers that can stimulate the destruction of liposomes through pH, enzymes, or specific bonds on the cell matrix to initiate endocytosis and drug release [11]. Likewise, the incorporation of polymers in the bilayer chain or inside the

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liposome can strengthen the structure of the liposome and regulate drug release in a controlled manner. The right complexation method can even deliver multiple drugs with different release profiles for synergistic purposes [12].

The interaction form is strongly influenced by the type and structure of the polymer, crosslinker, and initial form of phospholipids during the complexation process. The type of bond also affects the stability and properties of drug release from the liposome complex. Standard evaluations also need to be done to observe patterns of interaction and their effects on the physicochemical properties of liposome complex. Therefore, it is essential to study these parameters for the development of a more optimal controlled release and targeted drug delivery system using liposomes. This article explores the types of natural and synthetic polymers, crosslinkers, lipids, interactions, and bonds occurring in the complexation of liposome complex. Its uses and standard evaluations are also reviewed.

## 2. Definition and principles of lipoplex

Liposome complexation is a method of modifying the structure of a liposome to improve its physicochemical properties, stability, and pharmacokinetic profile. Liposomes that have been complexed are called liposome complex. One of the complex-forming agents that can be used is polymer. Each type of polymer will produce different interactions and complex shapes with liposomes. The interaction is also influenced by the initial form of phospholipids (ring form or not). Generally, polymers mixed with a proliposome material will create a fusing interaction on the lamellar chain or inside of the liposome. This reaction typically does not require a catalyst or activator [13]. Whereas, the polymer added after the formation of the liposome bilayer membrane will form interactions on the surface [14, 15, 16]. Some types of interactions between polymers and liposomes that can occur are physical adsorption or chemical reactions, such as electrostatic interactions, hydrogen bonds, disulfide

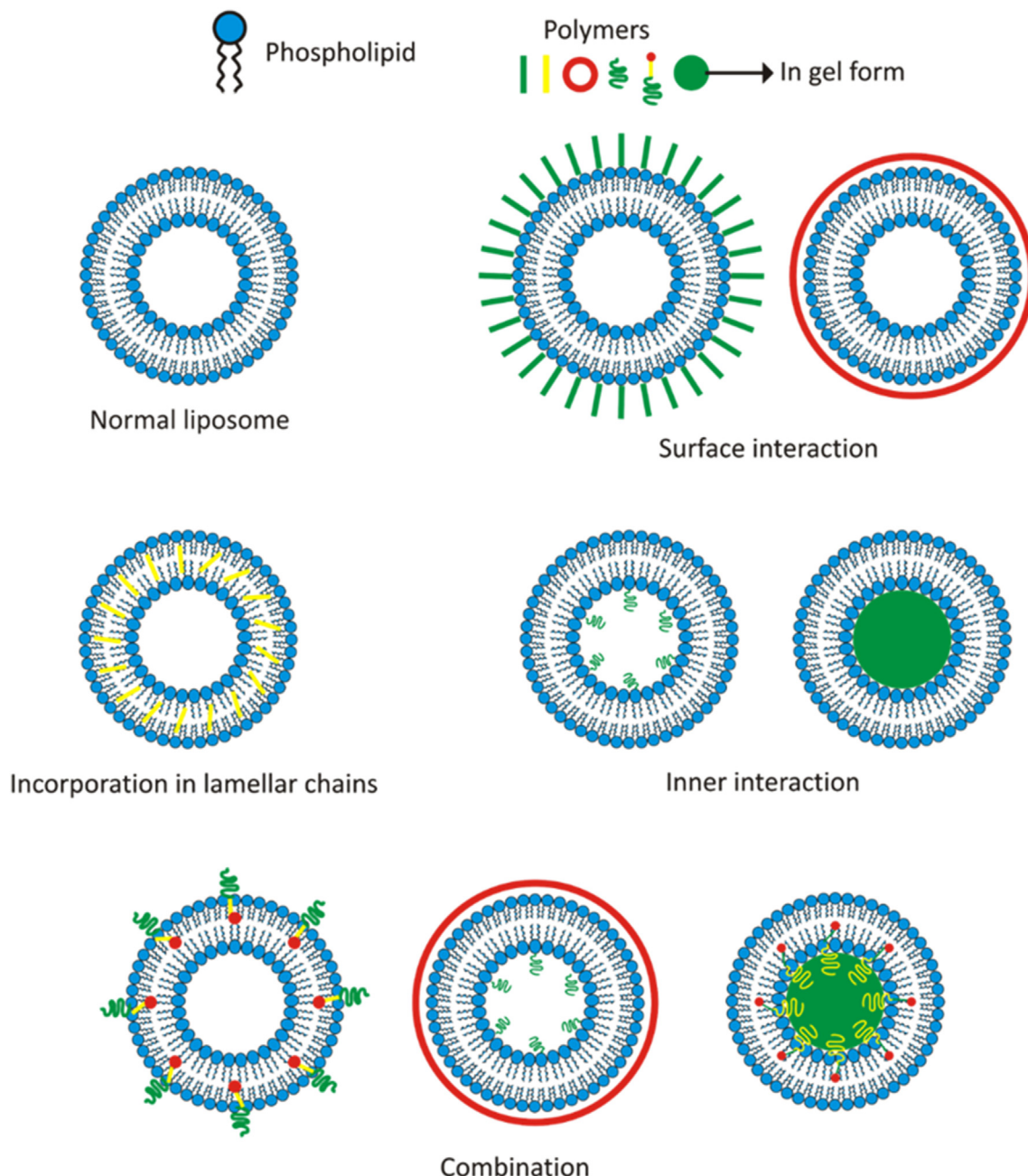


Figure 1. The possible interaction of liposome-polymer.

bonds, covalent bonds, and others [1, 17, 18, 19, 20, 21, 22, 23, 24, 25]. It is said that covalent bonds can increase the stability of liposome 10-fold better than electrostatic bonds [26]. Weak bonds, such as hydrogen, dipole, and disulfide, slowly release liposomes in a matter of hours to a couple of days [1, 17, 27]. However, if the bonds formed are covalent or ionic, more energy or bioreagents (enzymes) are needed to break the bonds [28, 29]. Controlled release of the liposomes resulting from covalent bonds can take more than 20 days [30]. It all depends on the type of polymer and phospholipid used.

In Figure 1, it can be seen that there are four interaction types between the polymer and liposome, in which the forms are influenced by the type of polymer and phospholipid used, the initial form of the phospholipid, and the mixing method. The following explanation is related to the types of interactions and what influences them.

### 2.1. Surface interaction

This interaction is the most common occurrence in the use of polymers to form matrix systems, with or without the help of a crosslinker [17]. In some polymer types, liposome can act as crosslinker, in which the polymer and liposome have opposite surface charges to form a tidier, more robust matrix. This interaction can be formed by dispersing liposomes that have developed a bilayer membrane into the polymer matrix system [17]. This reaction can be helped by preparing the surface charge on the liposome or polymer first. A catalyst, activator, or initiator is also sometimes used to assist with polymerization [17, 31, 32]. In addition to providing protection and regulating the release of liposomes, this interaction type can also affect the physicochemical properties of the polymer matrix system. In some cases, this interaction type increases (and sometimes even lowers) the quality of the system [33, 34, 35].

This type of interaction is most commonly used to regulate the release of liposomes, which is then followed by drug release from the liposomes. In some cases, drug release can be stimulated by the temperature or physiological pH of the target tissue [36]. The form of drug release produced also depends on the type of surface bond formed between the polymer and liposome. Targeted drug delivery can also use this system by introducing ligands, receptors, or DNA sequences on the surface of the liposome.

Besides, this type of interaction can also protect liposomes from chemical, enzymatic, and immune reactions, so liposomes can be stable during administration and in systemic circulation [37, 38, 39]. Indeed, multilayers formed by certain polymers and liposomes can also protect drugs that are easily degraded or photolabile [1, 5, 6, 7, 8, 9, 10].

### 2.2. Incorporation in lamellar chains

Unsaturated lipids are easily oxidized, whereas both unsaturated and saturated lipids can be hydrolyzed into lysolipids and liberate fatty acids [39, 40, 41, 42, 43]. The incorporation of polymers into lipid bilayers can strengthen bonds in the membrane of the liposome, so it becomes more sturdy and stable in chemical, enzymatic, and immune reactions [44, 45, 46]. Therefore, this interaction is widely used to improve the stability of liposome. Polymers that are incorporated in the lamellar chain are hydrophobic, so they easily bind to the aliphatic chains of phospholipids. If it does not have these properties, the polymer is usually complexed with cholesterol, poly-L-lysine, or other substances, so they can be joined to the lipid bilayer [17, 27].

This type of interaction can also provide controlled drug release from the liposome. Polymers with strong binding to the bilayer chain can reduce the release and permeability of liposome [47, 48, 49]. Usually, this interaction form is combined with surface modification to help release the drug via thermal, enzyme, or pH triggers.

### 2.3. Inner interaction

This interaction usually occurs when the matrix system of a hydrophilic polymer that has been formed is used as an aqueous medium in liposome wetting [5, 50, 51, 52]. These interactions include those that are easily made and reliable. This system can be used as a drug delivery system that is easily degraded chemically or that is photodegradable [5]. This system produces more stable liposomes because of its compact structure. This internal interaction also affects the strength of the bonds among the lipids in the lamellar chain [50].

### 2.4. Combination

Combinations of several polymer and liposome interactions can be obtained using a combination of polymers or the addition of a crosslinker. The mixture can be in the form of hydrophilic and hydrophobic polymers to streamline drug loading and give trigosable properties to the liposome [53, 54].

Each interaction type between the polymer and liposome will result in different liposome complex properties with different drug delivery and release mechanisms. Here are some of the mechanisms for delivery and release of drugs from the liposome complex.

## 3. Mechanism of drug release and delivery of lipoplex

### 3.1. Magnetic responsive

The principle of drug release from this type of liposome complex utilizes magnetic field technology. Magnetic nanoparticles (MNPs) are widely applied as drug delivery systems because their release and accumulation in specific tissues can be controlled through external magnetic fields. Drug release from MNPs has both a time- and concentration-dependent pattern [55, 56, 57, 58, 59, 60]. Magnetic nanoparticles can be encapsulated into liposomes using the thin-film hydration method [61, 62] or reverse-phase evaporation method [63, 64, 65]. Hydrophobic magnetic nanoparticles will be integrated into the liposome bilayer membrane, while hydrophilic magnetic nanoparticles will be integrated into the liposome core. The magnetic behavior of the magnetoliposome depends on the size of the magnetic nanoparticles, the larger the size, the stronger the magnetic signal [66].

### 3.2. Thermal, pH, and enzymatic responsive

Environmental pH changes can stimulate the occurrence of osmotic diffusion in the liposome complex system, causing swelling or shrinkage, and results in the disintegration of the bilayer membrane of the liposome complex. The pH-triggered liposome complex has the potential to be a targeted delivery system or delayed-release because each tissue or organ has a different physiological pH [17]. At a certain pH, the polymer can also be protonated, causing its charge to change. This phenomenon causes a decrease in electrostatic interactions, causing the lysis of liposomes [1, 67, 68, 69, 70].

Some polymers have a lower critical solution temperature (LCST). As a result, if the polymer is exposed to temperatures above the LCST value, it will become hydrophobic. This is what begins to damage the tissue between the phospholipids, causing substances in the polymer matrix to leak out [47].

The sensitivity of the liposome complex to certain enzymes is obtained through complexation with particular DNA sequences recognized by certain enzymes. If the liposome complex meets the appropriate enzyme, the DNA sequence will be cut off by the enzyme, damaging the bond between DNA and liposome, resulting in the breakage of the structure of the liposome and release of the drug [71, 72, 73, 74].

### 3.3. Tumor targeted delivery

This system can be made by adjusting the surface of the liposome with a polymer. Polymers sensitive to certain pH areas, such as the tumor matrix (pH 6.5), can be carriers and controllers of liposome release [75]. One of them is methacryloyl sulfadimethoxine, which will change to its ionic form when contacted at pH 6.5 [76]. This change in charge is known to change the surface properties of the liposome to one of hydrophobicity, which leads to changes in the structure of the liposome and initiates drug release in a controlled manner. The hydrophobic surface of the liposome also induces aggregate formation on the surface of tumor cells and prevents liposomes from returning to the bloodstream [77, 78]. This results in increased permeation and retention (EPR effect) of liposomes in tumor cells [79, 80, 81, 82]. An illustration of tumor-targeting drug delivery can be seen in Figure 2.

In the figure above, it can be seen that the modification of liposome for tumor-targeting drug delivery requires several polymers or crosslinker combinations with their respective roles. Hydrophilic polymers covering liposomes can reduce recognition by the reticuloendothelial system (RES), thereby prolonging circulation time in the bloodstream [3, 4]. A co-polymer or crosslinker that is pH-sensitive can also be used to prevent drug leakage and stimulate the breakdown of polymer-coated liposomes in the pH of the extracellular matrix of the tumor. Biotin can be used as a crosslinker and can also increase the cell uptake of liposomes [83]. After the liposome has been inserted into the cell, the liposome will be lysed by the influence of a strong positive charge initiating rapid drug release in endosomes [53, 54].

### 3.4. Controlled release

The type of surface bond between the polymer and liposome plays an essential role in controlling the release of liposome and drug. Non-covalent binding is reversible. Thus, changes occurring in the system due to dissolution, ionization, swelling, or surface erosion can cause the liposome to move out of the matrix [84]. The delayed-release profile of liposomes depends on the strength of the bonds formed between the liposome complex and the polymer. The principle of delayed-release is the same as the principle of enzymatic-responsive or pH-triggered release [2, 85, 86].

Drug delivery and release with the liposome complex system are supported by the physicochemical characteristics of the polymer and its preparation method. Therefore, by understanding this, we can determine the appropriate polymer and preparation method to produce the specific form of drug delivery and release. Here are some polymers that are commonly used in liposome complexation.

### 4. In vivo delivery of liposomes and liposomal complexes

This system can be made by adjusting the surface of the liposome with a polymer. Polymers sensitive to certain pH areas, such as the tumor matrix (pH 6.5), can be carriers and controllers of liposome release [75]. One of them is methacryloyl sulfadimethoxine, which will change to its ionic form when contacted at pH 6.5 [76]. This change in charge is known to change the surface properties of the liposome to one of hydrophobicity, which leads to changes in the structure of the liposome and initiates drug release in a controlled manner. The hydrophobic surface of the liposome also induces aggregate formation on the surface of tumor cells and prevents liposomes from returning to the bloodstream [77, 78]. This result in increased permeation and retention (EPR effect) of liposomes in tumor cells [79, 80, 81, 82, 83]. An illustration of tumor-targeting drug delivery can be seen in Figure 2.

In the figure above, it can be seen that the modification of liposome for tumor extracellular matrix (ECM) targeting drug delivery requires several polymers or crosslinker combinations with their respective roles. For example, hydrophilic polymers covering liposomes can reduce recognition by the reticuloendothelial system (RES), thereby prolonging circulation time in the bloodstream [3, 4]. A pH-sensitive copolymer or crosslinker (biotin-PEG-biotin) that is located on the interface layers of the liposomal complex can be used to prevent drug leakage, protein adsorption and stimulate the breakdown of polymer-coated liposomes in the tumor ECM. When this liposome reaches tumor ECM (pH range 6.5–6.8), the surface exposed crosslinker is dissociated by break down of hydrogen bond and uptake into tumor cells by endocytic pathway [83]. After the insertion into tumor cells, the liposome will be lysed by the influence of a strong positive charge initiating rapid drug release in endosomes and simultaneously enhancing the accumulation of anti-cancer drugs only on tumor microenvironment without affecting the normal healthy cells leading to improve therapeutic regimens [53, 54].

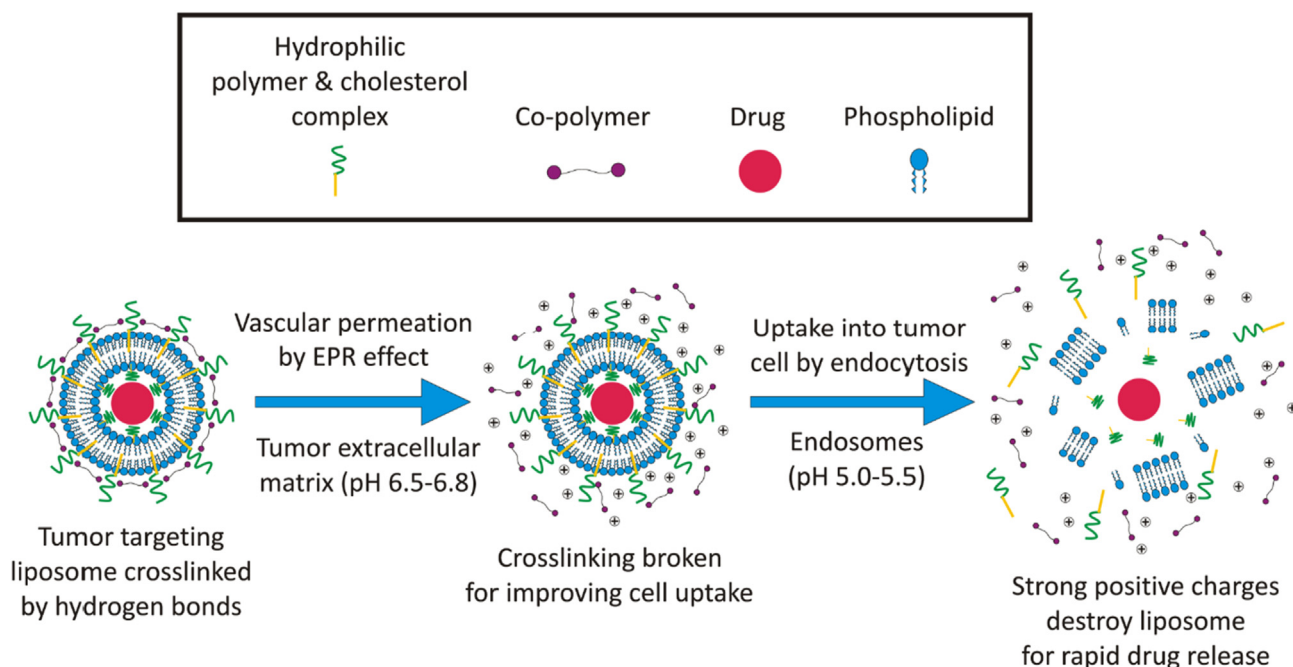


Figure 2. Illustration of tumor-targeting delivery and release mechanism.



## 5. Polymers used in lipoplex

As mentioned earlier, each polymer will form a different interaction with the liposome and will undoubtedly produce different system properties as well. The system properties can be adjusted according to their application. Table 1 describes various polymers that have been used in complexation with liposomes and their applications.

### 5.1. Alginate

Alginate is an easy polymer to complex through JetCutting [106, 107, 108, 109, 110]. Alginate-liposome complexation is aimed at controlling drug release and giving liposomes thermo-sensitive properties [89, 91]. Drug release from the liposome complex system can take up to 2 weeks. This system can also be stable for up to 2 years at a storage temperature of 4 °C [90]. Lipoplex alginate can also be complexed with magnetic nanoparticles so that delivery and release can be controlled using magnetic resonance [88]. In the delivery of proteins (serum albumin), the efficiency of encapsulation can reach 95% with a sustained-release profile [90].

### 5.2. Chitosan

Chitosan has been used extensively as a basis for efficient drug delivery. Chitosan has bioadhesive, biodegradable, biocompatible, renewable, antioxidant, antitumor, and excellent wound healing properties [111, 112, 113]. Chitosan is also one of the most widely used polymers in coating liposomes to increase their stability and pharmacokinetic profile

[114, 115, 116, 117]. This modification changes the system properties to be more sensitive to certain enzymes, prebiotics, or delayed-release in the intestine or colon. The complexation between chitosan and liposomes is called a chitosome. Interactions between chitosan and liposomes occur on the surface by forming non-covalent bonds, such as electrostatic, hydrophobic, or reversible hydrogen bonds [2, 85]. It was reported that the complexation between chitosan, P90H, and P50 resulted in a system with a particle size of 180 nm and entrapment efficiency of 91%. The system can maintain the structure of the liposome and control the release of active substances at an alkaline pH (intestinal) through the diffusion process [2].

Research conducted by Efimova et al. showed that chitosomes (liposome complex) could be used as a multidrug delivery system. The multidrug delivery system is formed at pH 5 and still maintains its structure and absorption efficiency when the system condition is changed to pH 7 [12]. The combination of drugs in one carrier and protector can undoubtedly be a more efficient therapy [118, 119, 120, 121].

Xanthan gum can also be complexed with chitosan for modification of liposome release by increasing vehicle stability and providing mucoadhesive properties [122, 123]. Complexation of chitosan-xanthan gum-liposome forms a viscoelastic gel system. However, the increase in xanthan gum levels causes its delivery using a nebulizer to be interrupted and retained. The ratio of chitosan-xanthan gum polymer to nebulization liposome delivery is 1:0.5 [94]. Chitosan-xanthan gum (0.5:0.8 w/w)-liposome can also be prepared through the process of freeze and spray drying, resulting in the release of Fickian diffusion properties for the active substance in the colon [95].

**Table 1.** Polymers used in liposome complexation.

No.	Polymer	Liposome	API	Application	Ref(s).
1.	Aldehyde modified xanthan gum	PE	-	Cell carrier and tissue engineering	[87]
2.	Alginate	DPPC, DSPC, DSPE-PEG2000, and MSPC	Bupisome	Magnetic-responsive, controlled release, protein delivery	[88, 89, 90, 91]
3.	Anti-miR-191	Soy-lecithin and cholesterol	Anti-miR-191	Breast cancer therapy	[92]
4.	Atelocollagen	DSPG and cholesterol	Doxorubicin	Magnetic drug targeting	[93]
5.	Chitosan	P90H, P50, and cholesterol; PC and cardiolipin;	Quercetin and cisplatin	Intestinal drug delivery, multidrug delivery	[2, 12]
6.	Chitosan-xanthan gum	P50 and P90H; Soy-lecithin and cholesterol	Rifampicin and C-phycocyanin	Pulmonary delivery, colon targeted	[94, 95]
7.	Cyclodextrin	Egg yolk	Barnidipine	Photodegradable drug delivery	[5, 50, 51, 52]
8.	Gelatin and methacrylic anhydride	Soy-lecithin and cholesterol	Deferoxamine and paclitaxel	Controlled release and bone regeneration	[96]
9.	Hyaluronic acid	Egg phosphatidylcholine and egg L- $\alpha$ -phosphatidylglycerol; DPPE	Corticoid, prednisolone	Sustained-release, targeted delivery	[11, 97, 98, 99]
10.	Lactoferrin	Soy-lecithin and cholesterol	7,8-Dihydroxyflavone	Sustained release	[1]
11.	mPEG-P(HPMA-g-His)-cholesterol	DPPC	Doxorubicin	Intracellular drug delivery and cancer targeting	[53, 54]
12.	PEG-SH and PEG-Mal	Egg yolk and cholesterol	-	Phototriggered targeted drug delivery	[100]
13.	PTMP and cholesterol-bearing pullulan (CHP)	DMPC	-	Multidrug delivery and controlled release	[30]
14.	pGL4.50 [luc2/CMV/Hygro] vector	DOTAP, DSPC, and cholesterol	pDNA	Colon targeted	[101]
15.	Polyacrylamide and DNA	DOPC and cholesteryl-tetraethyleneglycol (TEG)-modified	DiIC18(5) and calcein	Controlled release and endonuclease-responsive	[102]
16.	Poly (acrylic acid)	Soy-lecithin and cholesterol; Soy-lecithin and dansyl-PE	Calcein; Doxorubicin	Controlled release; Selective tumor targeted	[17, 27, 103]
17.	Polyethylenimine	DPPC, DOTAP, DPPG, and cholesterol	DNA/siRNA	Gene delivery	[104]
18.	Silk fibroin, alginate, and chitosan	Egg yolk	Calcein	pH-triggered release	[15]
19.	Stearoyl-PEG-PSD	Soy-lecithin and cholesterol	Gemcitabine	Selective tumor targeting and controlled release	[77]
20.	Transferrin	DOTAP, phosphatidylcholine, DSPE-mPEG2000, cholesterol	Erianin	Suppress the growth of liver cancer	[105]

### 5.3. Collagen

Collagen has been used extensively as a gene delivery system by forming stable complexes with plasmid DNA (pDNA) or small interfering RNA (siRNA). Collagen is biodegradable, biocompatible, and has minimal immunogenicity [124, 125, 126, 127]. Atelocollagen (10  $\mu\text{g}/\text{mL}$ ) has been reported to protect magnetic anionic liposome (MAL) from immune reactions and increase cellular uptake *in vitro* and *in vivo*. Compared with magnetic cationic liposome (MCL), MAL showed both concentration- and time-dependent cellular uptake without any cytotoxic effects *in vitro* on RAW264 cells. However, the use of atelocollagen at concentrations (more than 20  $\mu\text{g}/\text{mL}$ ) will form aggregates at 37 °C and inhibit cellular uptake due to large particle size [93].

### 5.4. Cyclodextrin

Cyclodextrin and its derivatives are widely used as complexing polymers in liposomes through inner interaction because of their solubility in aqueous solutions, chemical stability, and good bioavailability [51]. In addition to being able to carry hydrophilic drugs, it can also carry lipophilic drugs [128]. Aside to chemical degradation, the liposome complex from cyclodextrin has been reported to protect photodegradable active substances [5]. Prolonged release can also be obtained from this liposome complex system with a drug release time of more than 25 h [50]. Lipoplex is also known to increase the efficacy and solubility of antibiotics 700 times better than the single form of the drug [52].

### 5.5. Gelatin

Gelatin is a hydrophilic polymer with amphoteric and non-immunogenic properties [129]. Various modifications of gelatin are intended to improve its physicochemical properties for medical use [130, 131, 132]. One of its modifications is the complexation of gelatin with methacrylamide (GelMa), showing higher elasticity, adherent cells, and excellent mechanical properties [132, 133, 134]. Complexation of GelMa and liposome results in multidrug delivery with controlled release. This liposome complex can significantly promote both osteogenesis and angiogenesis [96].

### 5.6. Hyaluronic acid

Hyaluronic acid (HA) is a mucoadhesive, biodegradable, and biocompatible polymer. It can be found in the bodies of humans and animals. HA dissolves in water at neutral pH, it is negatively charged, and it is a robust hydrophilic polymer [135, 136, 137]. The complexation of HA-liposomes is known to be used for parenteral drug delivery. Lipoplex injection in guinea pig ears provides sustained release of corticoids for up to 30 days [98]. However, PEGylated liposomes show better immobilization in the network matrix of HA than conventional liposomes [97] and liposomes without PEGylated penetrate into mucus better in the presence of HA [99].

HA is also known to bind to CD44 cell receptors, selectively modulating autoimmune responses. With this capability, complexation using liposomes can be a delivery target for several drugs for autoimmune diseases, such as rheumatoid arthritis [138, 139, 140]. It has been reported that this liposome complex increases the cellular uptake of prednisolone. This process is mediated by caveolae- and clathrin-dependent endocytosis. The study showed that the release of prednisolone *in vitro* occurred at an acidic pH [11]. With the pH-sensitive properties of several HA derivatives, targeted delivery, and controlled release of the liposome complex system for anticancer drugs can be developed [141].

### 5.7. Lactoferrin

Lactoferrin is a protein that is positively charged at physiological pH and can coat liposomes through electrostatic interactions on both surfaces. Lactoferrin-liposome complexation has been used as a brain-specific targeting ligand and has been used successfully in increasing its accumulation in the brain [142]. The role of lactoferrin with a decrease in surface tension can be more effective when crosslinked with glycosylate. The modification of lactoferrin enhances its flow characteristics, emulsibility, and thermostability [143]. The lactoferrin and liposome complexes are also able to prevent the degradation of active substances from the effects of light and heat. In addition, liposome degradation by lipolytic enzymes in the digestive tract can be restricted by the lactoferrin complex [1, 46]. However, the lactoferrin charge can turn negative when the pH of the system is alkaline (>8.2) or above its isoelectric point. This can reduce the strength of electrostatic interactions in liposome, which can end in changes in particle size and lysis. The liposome complex from lactoferrin can also release drugs in a controlled manner and is suitable for hydrophobic drug delivery [1].

### 5.8. Transferrin

Transferrin (Tf) is widely known as a metal-binding glycoprotein, is one such example of a multi-tasking protein of approximately 76.5–79 kDa in size whose main function is to absorb  $\text{Fe}^{3+}$  ions via transferrin receptor (TfR). It has been detected in various body fluids like plasma, bile, cerebrospinal, amniotic, lymph and breast milk also [144, 145]. Tf is another receptor-ligand pair that has been extensively utilized for cancer targeting since cancer cells have a higher affinity of TfR expression on their surface than normal cells, therefore, it is a potential target for treating various cancers like brain, breast, liver, lung, ovarian and prostate cancer. After binding with its corresponding receptor, the Tf-TfR complex is internalized and transported to endosomes via receptor-mediated endocytosis [146, 147, 148]. It is being realized that surface of the nanoparticles functionalized with Tf, creating an excellent tool for favoring the accumulation of active moieties at the targeted sites while reducing the off-target effect on normal healthy cells. By taking the advantage of the transferrin receptor overexpression on the cancerous cell surface, transferrin-conjugated nanoparticles mainly liposomes or lipoidal vesicles have been designed to maximize the therapeutic benefits [149, 150]. For example, erianin loaded liposomes treated with Tf exhibited excellent anticancer activity both *in vitro* and *in vivo* than free erianin as well as free erianin loaded liposomes. These functionalized liposomes enhanced the tumor targeting efficacy by improving the solubility of erianin, inducing cellular apoptosis, displaying immunoregulatory actions and subsequently internalized by TfR-mediated endocytosis as a result increased the cellular uptake of erianin in cancer [105]. It has been reported that Tf conjugated plumbagin liposomes are bearing negative surface charges as lower than that of free liposomes, which is most likely due to the anionic nature of Tf, as it can easily bind to  $\text{Fe}^{3+}$  ions with a higher affinity. These negative surface charges would minimize the risk of electrostatic interactions between the liposomes and the negatively charged serum proteins as well as cell membranes, thereby resulting in prolonged blood circulation and reduce nonspecific uptake of liposomes by the healthy cells [151]. Further, PEGylated liposomes modified with TfR monoclonal antibodies (OX26) are able to magnificently increase the transport of plasmid DNA across the blood-brain barrier, promote cell transfection and exhibited the most significant therapeutic efficacy against the brain glioma cells, because ligand OX26 plays a potential role in delivering the lipoplexes across the blood-brain barrier [152, 153]. Therefore, Tf-liposome complexes act as a major role in the development of next-generation therapeutics for cancers and other inflammatory diseases.

### 5.9. Poly(acrylic acid)

Poly(acrylic acid) (PAA) is widely used as a matrix system for controlling drug release because it is known to have sensitive to pH changes. PAA can interact with lipid bilayers if previously complexed with cholesterol. This complexation shows the difference in size and potential zeta of the liposome at low and high pH. These changes confirm the occurrence of liposome swelling or shrinkage, which is very significant and can lead to the disintegration of the liposome complex bilayer membrane [17]. The liposome complex formed can also maintain a drug release profile for 3 months of storage and is stable when rehydrated. Cell viability testing also shows that this liposome complex has no toxicity toward HEP-2 cells, and their delivery can be transcellular. The particle size of the cholesterol-PAA-liposome was smaller and remained constant for 3 months of storage compared to the PAA-liposome. The efficiency of cholesterol-PAA-liposome absorption is also 1.8 times better. However, its interaction with cells decreases compared to PAA-liposomes without cholesterol [27].

### 5.10. Polyacrylamide

Polyacrylamide hydrogel has excellent viscoelastic properties, so it can be applied by injection. With its ability to form complexes with genetic material, polyacrylamide is usually used as a matrix and carrier with a stimuli-responsive system. Genetic material can also interact with lipid bilayers through complexation with cholesterol. Cholesterol can support the complexation between polyacrylamide and liposomes. This liposome complex can release drugs in a controlled manner through stimulation by endonuclease enzymes and increased cellular uptake. This liposome complex supports the ability of liposome to coat hydrophilic or hydrophobic molecules and deliver them by injection into the systemic circulation. This enzyme-triggered drug release occurs constantly for up to 11 h. The tidiness of the polyacrylamide matrix obtained by cross-linking with DNA causes an even distribution of liposomes compared to the form of the liposomes themselves [102].

### 5.11. Polyethylene glycol

Polyethylene glycol (PEG) is a widely used polymer, since it can increase the longevity of liposomes both in vitro and in vivo [34, 40, 154, 155]. Some modifications of PEG, such as the addition of acrylic or methacrylic monomers, can improve the thermal- and pH-sensitive properties of liposomes [17, 53, 54, 156]. It was also reported that the transformation of hydrophilic to hydrophobic or vice versa occurs at pH 7.0, when PEG is complexed with stearyl and methacryloyl sulfadimethoxine (SDM). Stearyl-PEG-poly(SDM) polymers will dissolve above pH 7.0 and will form aggregates below pH 7.0 [77]. Through ionization of SDM at pH 6.5 (pH of tumor matrix), the surface of the liposome will turn hydrophobic and start aggregating on the surface of tumor cells. It prevents liposomes from returning to the bloodstream, making their toxic effect on tumor cells even more substantial. Stearyl-PEG-poly(SDM) polymers can also maintain the size of the liposome at blood pH 7.4, so it can provide an EPR effect on tumor cells in which the size requirement is approximately 400–600 nm [77, 81]. The addition of thiol and maleimide groups to PEG can build a targeted delivery system through photo-triggered complexation. This liposome complex can increase cellular uptake [100].

### 5.12. Polyethylenimine

Polyethylenimine (PEI) is widely used in gene delivery because of its high gene transfer efficacy [157, 158]. PEI is water-soluble with protonated amine groups in every third position [159, 160]. With cationic charge densities, PEI can form non-covalent complexes with DNA and facilitate siRNA to induce RNAi [161, 162, 163]. The release of the PEI-nucleic acid complex from the endosome occurs through the proton

sponge effect, leading to osmotic swelling, so it does not require an endosomolytic agent [164]. However, it is said that PEI can form complexes with anionic molecules in the body. To overcome this problem, PEI can be complexed with hydrophobic compounds, such as cholesterol, to form a liposome complex. This liposome complex is reported to increase the biological activity of nucleic acids and reduce their toxicity [104].

### 5.13. Pullulan

Pullulan with the help of cholesterol can form stable nanogels (30 nm) in aqueous solution. These nanogels have beneficial properties, such as their large loading capacity for bioactive molecules (i.e., proteins and DNA/RNA) [165, 166]. With the addition of methacryloyl or acryloyl groups, this pullulan nanogel can be a control block for the macrogel structure of other polymers. Besides, it can also interact on the surface of the liposome to form a system that can deliver multiple drugs with different times and amounts of release because the number of bonds that must be broken at each layer also varies. This trait is very advantageous for multidrug delivery requiring release at different speeds and amounts, such as a combination of growth factors for tissue regeneration [167, 168]. Total liposome release in this system can reach more than 20 days, and the entire nanogel release can reach up to 40 days. The pattern and length of release also vary with pH variations. The system is more resistant to an acidic environment and more quickly disintegrated in an alkaline environment [30].

### 5.14. Silk protein

Silk protein can be used as a liposome surface-modifying agent for stimuli-release purposes. Silk protein imbues the pH-responsive nature of liposomes at pH 5.5–6.0, with rapid drug release. This delivery system is intended for water-soluble drugs [15].

### 5.15. Xanthan gum

Xanthan gum is a hydrophilic polymer with a 3D network structure through physical and chemical crosslinking that has been widely used in biomedical applications [169]. The matrix structure of this type of polymer can be adjusted for the delivery of cells, drugs, and tissue engineering [87]. Research conducted by Ma et al. showed that aldehyde-xanthan gum and liposome complexation was formed through a covalent bond. The hydrogel is quickly formed within 5 min at room temperature. The system can be stimulated physically (heat), chemically (pH variations), and biologically (histidine exposure). Besides, the system can be biodegraded to form papain, which is the result of digestion of xanthan gum backbones by enzymes. This system also shows excellent self-healing abilities. It was also said that this system could coat cells and last for a long time, so it can be used in cell culture, tissue engineering, and as a cell carrier [87].

## 6. Supporting agents used in lipoplex

In some conditions, the polymer and liposome cannot interact directly to form complexes because there is no supporting bond between the two. Some materials such as catalysts, activators, or initiators are used to help complex the polymer and liposome. There are those that provide surface charge, and some that modify the hydrophobic and hydrophilic properties of both.

Research conducted by Simoes showed that without stearylamine, PAA could not be incorporated into the liposome bilayer [17]. Stearylamine (5% molar ratio) can also regulate the surface charge of the liposome and increase its transepithelial permeation [27, 170, 171]. However, it is said that the use of stearylamine has the potential to increase the cytotoxic effects of cationic liposomes on lymphocyte cells and macrophages [172]. Research conducted by Shivani Sharma showed that

complexing liposomes and stearylamine increased the efficacy of anti-miR-191. This liposome complex can increase the apoptosis of cancer cells and prevent their migration. This liposome complex also enhances the chemosensitivity of cancer cells to anticancer drugs, such as doxorubicin or cisplatin in the free form [92].

In addition to stearylamine, cholesterol has also been widely used in increasing the stability of liposomes and helping to incorporate polymers into lipid bilayers [17, 27]. The study conducted by Chen et al. demonstrated that cholesterol could maintain particle size in the liposome complex in pH variations [1, 2, 3, 4, 5, 6, 7, 8, 9, 10]. Poly-L-lysine is known to interact with lipid bilayers and form stronger electrostatic bonds than poly-L-arginine [49]. It can cause the movement and permeability of liposome to decrease [47].

## 7. Evaluations

The evaluation standard discussed in this article focuses more on the analysis of interactions and physicochemical characteristics of liposome and its pharmacokinetic profile. Commonly, the quality aspects of dosage forms, such as hydrogels, creams, pastes, etc., are not included in the discussion.

### 7.1. Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) is used in the elucidation of structures based on carbon or hydrogen atoms and their connectivity. One of the purposes of using NMR in liposome complex analysis is to see the complexation of polymers and cholesterol or other crosslinkers to support the interaction of polymers to the liposome bilayer chain [17, 54, 87].

### 7.2. Infrared spectroscopy

Infrared (IR) spectroscopy aims at finding out how complex interactions between polymer and liposome are based on the unique peaks formed [53, 54, 87]. The peak shows the types of bonds that are likely to develop in the formation of the liposome complex. Besides, IR spectroscopy can be used as one of the reinforcements for the alleged occurrence of coatings by liposomes. The typical peak loss of an active substance can be assumed that the active material is coated in a liposome [1].

### 7.3. Drop-coating deposition Raman spectroscopy

Drop-coating deposition Raman (DCDR) spectroscopy can be used to see the interactions inside the liposome complex. The homogeneity or distribution of its complexities can also be observed [173]. Conventional Raman spectroscopy has several disadvantages, such as requiring large samples (mL), high sample concentrations (0.01–0.1 M), and the complex biomolecular structure in solution. In DCDR spectroscopy, this problem can be overcome. The number of samples that can be measured reaches a microliter size. The optimal lipid levels for analysis using DCDR spectroscopy are around 0.3–0.5 mg/mL. Lower lipid levels can still be measured, but the formation of closed liposome rings does not always occur [174, 175]. The principle of DCDR spectroscopy is the deposition and drying of samples placed on a hydrophobic plate. A hydrophobic surface allows the coffee-ring effect, making the liquid in the sample droplet to evaporate and causing the material contained in it to form a ring [173]. The properties of liposomes and proteins do not change during the drying process on the hydrophobic surface [176, 177].

### 7.4. Fluorescence quenching

Fluorescence in the liposomes occurs after the free active substances are removed ( $F_i$ ), and it is compared with fluorescence from the active substances after coating the liposome complex without removing the free active substances ( $F_f$ ). If the percentage of quenching was large, then the

degree of liposome formation was also assumed to be large, as the amounts of free active substances read were small. This indicates that the active substance is coated in the bilayer membrane of the liposome formed resulting a low fluorescence quenching. This method is only work for the fluorescent active substances. The following equation is used in determining the quenching of fluorescence [15]:

$$\% \text{ Quenching} = \left(1 - \frac{F_i}{F_f}\right) \times 100 \quad (1)$$

### 7.5. Particle size, polydispersity index, and zeta potential

Measurement of particle size, polydispersity index (PDI), and zeta potential are fundamental in the development of liposomes. The liposome itself has been known to be a nanosized drug delivery system. Changes in the size of the particle and zeta potential can be used as benchmarks in the stability of the liposome complex against variations in pH and temperature and during storage. Particle size is also related to the permeation transport mechanism. Nanoparticle permeation can pass through the endocytosis pathway, such as the caveolae (particle size <500 nm) and clathrin (particle size <300 nm) pathways [178].

### 7.6. Differential scanning calorimetry

Differential scanning calorimetry (DSC) is used to study how polymers influence the thermal behavior of liposomes and drugs.  $\Delta H$  and  $T_c$  values reflect the aliphatic chain interactions of lipids, polymers, and drugs. An increase in  $\Delta H$  value indicates the effect of the polymer or drug on the membrane fluidity of the liposome [1, 94].

### 7.7. X-ray diffractometry

X-ray diffraction (XRD) is one of the most extensively used techniques for the characterization of liposome based nanoformulations. Generally, XRD analysis imparts information regarding the crystalline or molecular structure, purity, degree of crystallinity, particle size, microencapsulation process, lattice parameters, nature of the phase and qualitative identification of various compounds that exist on the liposomal structure. Analysis of these liposomal nanoformulations are largely depended on the formation of diffraction patterns. Each material used in the development of liposomal formulation has a distinct diffraction peak which can define and identify it by comparing the diffracted peaks with the reference database available from the International Centre for Diffraction Data (ICDD). Further, the diffracted patterns also explain whether the sample materials used in the liposomes are pure or contain some impurities [171, 172, 173, 174].

### 7.8. Thermal- and pH-sensitivity

This test is carried out to see whether drug release from the liposome can be triggered by changes in temperature or pH. In this test, the temperature or pH of the sample is directly adjusted. Changes in the size of particle and zeta potential can be used as references that the sample is sensitive to changes in temperature or pH [17, 179, 180].

### 7.9. Entrapment efficiency

Entrapment efficiency testing begins with centrifugation of the sample, allowing it to form two phases (natant and supernatant). Natant is a liposome complex that looks more turbid when it settles, while the supernatant is a surface phase that looks clearer and is considered to contain free active substances. The supernatant is then taken and the levels are measured using an instrument that is suitable for the free active substances. Measurement of the free active substances in the supernatant ( $C_{free}$ ) is carried out using a linear equation from the standard curve. Then, it is compared with the theoretical levels of active substances in the



liposome complex ( $C_t$ ) [175]. The percentage of entrapment efficiency (% EE) is calculated using the following equation:

$$\% \text{ EE} = \left( \frac{C_t - C_{\text{free}}}{C_t} \right) \times 100 \quad (2)$$

### 7.10. Drug loading capacity

Determination of drug loading capacity also follows the similar protocol as discussed in the testing of entrapment efficiency. Here, the only difference is that the obtained free active substances are compared with the total amount of obtained liposome complex ( $T_{LC}$ ) [176]. The percentage of drug loading is determined using the following equation:

$$\% \text{ DL} = \left( \frac{C_t - C_{\text{free}}}{T_{LC}} \right) \times 100 \quad (3)$$

### 7.11. Biodegradation test

This test is carried out to see whether or not a liposome complex can be degraded by an enzyme. The enzymes used are tailored to the target tissue, route of drug administration, or the type of complexing polymer used. Morikraz (serine proteinase, collagenase, and metalloproteinase) is the most common enzyme used [12]. The liposome complex is degraded by these enzymes under conditions of tissue pH and physiological temperature of administration.

### 7.12. In vitro drug release

Drug release testing is usually done by conditioning the liposome complex system for lysis. The material that can be used to induce lysis of the liposome complex is Triton X-100 (20  $\mu\text{L}$ ). The mixture is placed in a dialysis bag and stirred at 37 °C (100 rpm). Drug quantification can be achieved using spectrophotometry, fluorometry, chromatography, and other methods. The percentage of drug released from the liposome complex can be calculated using the cumulative drug level ( $C_c$ ) at each sampling time compared to the theoretical levels of the active substances ( $C_i$ ) [17]. The following equation can be used [1]:

$$\% \text{ Drug Release} = \frac{C_c}{C_i} \times 100 \quad (4)$$

### 7.13. In vitro release kinetics

After conducting the drug release study, it is very crucial to investigate the release mechanism of liposome complexes. For this, the data obtained from the *in vitro* drug release study is fitted into various kinetic models like zero order (cumulative amount of drug released vs. time), first order (log cumulative percentage of drug remaining vs. time), Higuchi (cumulative percentage drug release vs. square root of time), Korsmeyer-Peppas (log cumulative percentage drug release vs. log time) model and comparing their correlational coefficient ( $R^2$ ) with one another, the best fit model is picked up. This data is further demonstrated the release mechanism or kinetics of liposome complex encapsulated agents in the existence of biological constituents [177, 178].

### 7.14. Cytotoxicity assay

Generally, this test is carried out on Caco-2 cells. The type of cell used is adapted to the target tissue of the therapy. The test cells is cultured ( $3 \times 10^4$  cells/mL) in the culture medium, namely 10% phosphate buffer, 1% non-essential amino acid, 1% antibiotic fluid, and 88% DMEM. Then, they are incubated for 24 h. Furthermore, the test sample (liposome complex) containing the drug in various concentrations is inoculated into culture cells and left in contact for 24 h in an incubator. Cell Counting Kit-8 solution is added to each group, and they are incubated at 37 °C with

5% CO<sub>2</sub> and 95% O<sub>2</sub> for 2 h. Furthermore, the absorbance (A) is measured, and it is used to calculate the percentage of cell viability using the following equation [1, 17, 27]:

$$\% \text{ Cell viability} = \frac{A_T - A_B}{A_C - A_B} \times 100 \quad (5)$$

where  $A_T$  is the absorbance-treated cell,  $A_B$  is the blank absorbance (without cells), and  $A_C$  is the control absorbance (untreated cell) [1].

### 7.15. Permeation transport test

Permeation transport testing is carried out using the principle of two compartments. Each compartment is limited by a culture membrane. The cell line that is commonly used is Caco-2 [1]. In the donor and receiver compartments, each is given a buffer medium and allowed a few minutes ( $\pm 30$  min) to wet the surface of the membrane. Furthermore, the buffer can be removed from the two compartments. The liposome complex samples that is dissolved in the medium can be inserted into the donor compartment. Meanwhile, the receiver compartment is given only a buffer medium. After a set amount of time, sampling is done by taking several volumes from the receiver compartment and replacing them with buffer media in the same volume. The permeability coefficient (P) can be calculated using the following equation [1]:

$$P \left( \text{cm/s} \right) = \frac{dQ}{dt} \times \frac{1}{AC_0} \quad (6)$$

where  $dQ/dt$  is the transport rate of the receiver compartment ( $\mu\text{M/s}$ ),  $A$  is the surface area of the membrane, and  $C_0$  is the initial concentration of the liposome-coated active substance in the donor compartment [1]. The illustration of tools from the transport permeation test can be seen in Figure 3.

### 7.16. Stability test

The stability test of the liposome complex is usually adjusted to the intended use and route of administration. It is related to the pharmacodynamics of the liposome complex to variations in pH, ionic, and enzymatic concentrations, which can damage the liposome complex. Stability tests of the liposome complex may be carried out by exposure to electrolytes (NaCl) and pH variations in the liposome [1, 181]. For drug delivery to the target release in the intestine or colon, the drug must be resistant to the influence of physiological pH on the gastrointestinal (GI) tract. Therefore, electrostatic repulsion of the liposome complex must be strong against any attractive interactions [1]. In addition, the effect of lipolytic enzymes also needs to be observed. Liposomes can naturally be degraded by trypsin and pancreatic lipolytic enzymes in the digestive system. So, the effectiveness of protection from liposome complexation can be quantified through this test [1].

## 8. Biological interaction of lipoplexes with plasma proteins

As the most crucial biological fluid i.e., blood contains more than 1000 types of blood proteins also termed as plasma proteins which serve many functions like transport of vitamins, minerals, hormones and functioning the immune systems. Despite the promising body functions offered by these plasma proteins, their interaction with therapeutic moieties like drug and nanocarriers mainly liposome-polymer complexes are very important concerns for its translation from basic research to biomedical applications [182, 183]. The binding of plasma proteins on the lipoplex surface occurs immediately upon their introduction into a physiological environment and is the first barrier of defense, which could lead to physical changes in the formulation, such as aggregation and charge neutralization, non-specific biodistribution, trigger elimination by phagocytic cells and biochemical activation of defense cascades, as a

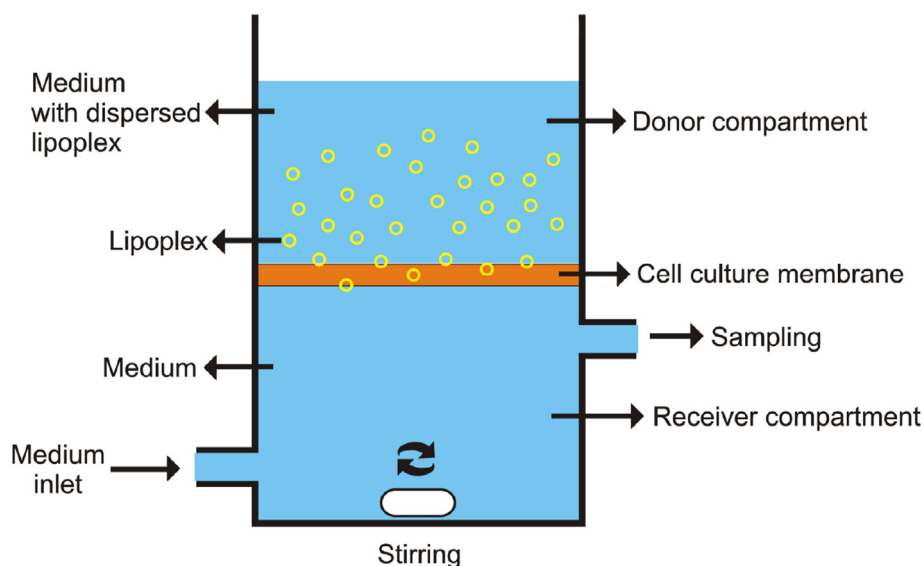


Figure 3. Instrumental illustration of transport permeation testing.

result, decrease the therapeutic efficacy and increase the undesirable toxicities [184]. Further, this interaction is being affected by many other properties of lipoplexes, including their composition, size, shape, surface charges, surface area, surface defects and surface characteristics [185, 186, 187]. To prevent the lipoplex-plasma proteins interactions, the above mentioned factors are critically analyzed for designing the effective lipoplex complexes. The effect of plasma proteins may have special implications for nanomaterials, due to the increased significance of surface effects for these tiny particles. As particles become smaller, their surface areas shrink much more slowly than their volumes, causing nanoscale materials to have far greater surface-to-volume ratios than larger particles. A larger surface-to-volume ratio also intimates more proteins to bind a nanoparticle (relative to its mass) than a particle of larger in size [188]. Even for larger particles, protein binding is established as one of the most important factors influencing biodistribution. Lipoplexes with larger in size or hydrophobic in nature are easily taken up by macrophages more efficiently than their smaller counterparts possessing the same composition and surface properties [189, 190, 191]. Therefore, physicochemical properties of lipoplexes can be manipulated either to avoid the immune recognition when the uptake is unwanted i.e., during drug delivery for cancer treatment and to specifically attract the immune cells when particle uptake is desirable i.e., antigen delivery for vaccine applications [192]. To initiate the above strategies, the surface of the lipoplexes is often covered with various hydrophilic polymers including polyethylene glycol (PEG), poloxamer, dextran, polyethyleneoxide, polyvinyl alcohol, etc. to avoid lipoplexes recognition by the immune cells and subsequently prolong the systemic circulation [193, 194]. Noteworthy, the surface charge of lipoplexes is directly influenced by the nano-bio interaction. For example, the positively charged particles tend to be rapidly covered with plasma proteins with anionic charges like fibrinogen and albumin shows a higher affinity to cationic liposomes with a high membrane charge density [195, 196, 197, 198]. To prevent the adsorption of plasma proteins, cationic liposomes are often covered with polymers that produce a negative charge. PEG is one of the most widely used polymer to decrease the plasma protein adsorption on lipoplex surfaces. The masking of the lipoplexes surface charges by PEG chains as well as the negative charges originated from the phosphate group in the DSPE moiety bearing the PEG chain contributes to the higher negative zeta potential in PEGylated liposomes. This negatively charged surface and hydrophilicity are presented by the lipoplexes repel the anionic albumin away from their surface [199, 200]. However, the plasma proteins comprise different charges and sizes of protein

molecules. Thus, the existence of a negative surface as in the case of the lipoplexes will drive the adsorption of positively charged proteins such as lysozyme present in the plasma [201]. To minimize this attraction, negatively charged liposome surface is further modified with the cationic polymer mainly chitosan. Since chitosan is a positively charged biopolymer, it can easily interact with the negatively charged liposome by electrostatic interaction and finally deliver DNA vaccine via oral route to the distal part of the intestine by protecting from the enzymatic degradation. Because the delivery of protein, peptide and plasmid DNA via the oral route remains relatively challenging due to their instability, degradation and low permeability in the intestinal mucosa. Interestingly, by using these positively charged lipoplexes i.e., combining the benefits of chitosan and liposome can extend the stability and uptake of the DNA vaccine by the Microfold cells (Peyer's patches in the small intestine) which are crucially significant for oral vaccine delivery [116]. Hence, the engineered lipoplexes can be designed in this way that is easily preventing the plasma proteins from binding to lipoplex surfaces. However, at present, there is not enough knowledge on the mechanisms that dictate the interaction of lipoplexes with plasma proteins or recognition by immune cells or as well as stability of these complexes in the biological counterparts. In depth understanding of such interactions can be supervised towards designing the biocompatible lipoplexes with controlled surface properties in a biological environment.

## 9. Vaccine delivery and stabilization by liposome-polymer complexes

Vaccine delivery is one of the most paramount medical interventions that help to eradicate the number of diseases like measles, diphtheria, tetanus, influenza, polio, hepatitis, mumps, pox, and finally COVID 19 too. While at the time of designing a vaccine, must needs to define the antigen, adjuvant, manufacturing process and also the delivery strategy to evoke a robust immune response [202, 203, 204]. Throughout history, most of the vaccines have been formulated using live attenuated organisms, killed whole organisms and inactivated toxins mainly toxoids. Live vaccines have the benefits of generating both humoral or cellular immunity and often require only a single dose, while killed and inactivated vaccines initiate a weaker immune response and usually require multiple doses [205]. Recently, recombinant DNA methodologies are used to develop DNA and subunit vaccines as well as conjugate vaccines in which a weak antigen is linked to a stronger immunogen such as a protein or membrane complex or capsular polysaccharides and can induce strong

long-term cellular immune responses [206, 207, 208]. Although DNA and subunit vaccines have supremacy over conventional vaccines, but these vaccines suffer from a comparatively lower immunogenicity and intrinsic instability and are prone to degradation by nucleases. Despite the outstanding progress in vaccine development, improvements are carefully needed due to concerns about their weak immunogenicity, in vivo instability, toxicity, and subsequently require multiple-dose administration [209, 210, 211].

To overcome such issues, nanotechnology based platforms have recently been utilized in vaccine development as well as antigen delivery. These new technologies have progressively been explored to enhance the therapeutic efficacy, humoral or cellular immune responses and can offer significant advantages over conventional vaccine formulations in terms of higher specificity, higher stability, higher loading capacity, capability for sustained or controlled release [212]. Noteworthy, as nanoparticles composed materials are designed at the molecular, atomic and macromolecular levels, they are usually tiny-sized particles with distinct physicochemical properties like composition, size, surface properties, shape, molecular weight [213]. Being nanosized, these particles can directly interact with the diseased tissues or cells with improved efficiency, protect the encapsulated antigens from the hostile in vivo environment and facilitate more uptake of the active moieties by phagocytic cells, mucosa-associated lymphoid tissue and gut-associated lymphoid tissue leading to efficient antigen recognition and presentation [214, 215, 216]. Several vaccine nanocarriers have been engineered and investigated for their applicability in the delivery of antigens and adjuvants to the immune cells as well as mimics of viral structures in an effort to provide a protective immune response [217]. Finally, nanocarriers can combine their primary function as antigen delivery system with other features such as immunostimulation or immunomodulation that are conjointly induced both humoral and cell-mediated immune responses [218]. To produce above or specific immune response like Th1 or Th2 or CD4+ T cell response, the nanocarriers are must be loaded with specific immunostimulatory adjuvants such as CpG oligodeoxynucleotide. Unfortunately, although single functional nanocarriers loaded with antigens may be taken up by the immune cells and insufficient adjuvant activity may result in restricted immunogenicity [219, 220].

Therefore, it is necessary to develop a new multifunctional nano-vehicle systems that can increase both antigen presenting efficiency as well as better adjuvant activity. At presently, liposomal-polymer derivatives, cationic liposomes, and liposome protein complexes mainly lipoplex have been identified as novel adjuvants and vaccine delivery platforms due to some of their distinct potentialities over non-functional nanocarriers as well as traditional carrier proteins on immunogenic stimulation: firstly, it can be converted a non-immunogenic substance to an immunogenic one; secondly, surface charge, size, and composition of liposomal derivatives could be easily adjusted to cope with the need of different antigens; thirdly, it can enhance the antigen presentation to antigen presenting cells mostly dendritic cells [221, 222, 223, 224]. Depending on the above chemical properties and benefits, liposomal derivatives having the potentiality to encapsulate the water-soluble antigens such as proteins, peptides, nucleic acids, carbohydrates or haptens within the inner aqueous space of the lipoidal core, whereas the lipophilic compounds mostly antigens, adjuvants, lipopeptides, linker molecules are embedded into the lipid bilayer [225]. In fact, the first vaccine candidate launched into clinical trials was made of liposomes and mRNA encoding an influenza virus nucleoprotein. This vaccine was designed in 1993 for specific antigens in a shorter period of time compared with other vaccine platforms, such as recombinant proteins and inactivated vaccines and also having the ability to induce virus-specific cytotoxic T-cell responses in mice, since then the lipid nanoparticle based mRNA vaccine formulations have emerged as a potent vaccine delivery platform [226]. It has been also reported that antigens or antibodies can be linked to the surface of the liposomal derivatives or lipoplex either by adsorption or stable chemical linking techniques to enhance the better target specificity. A number of surface-functionalized liposomal carrier systems,

known as immunoliposomes, have been shown to navigate themselves to pathological cells due to the specific interaction among antibodies that has been attached to the surface of the liposomes and antigen epitopes or small particles that are displayed by the targeted pathogenic cells. Such types of immunoliposomal carriers having the tendency to mimicking the apoptotic cells as a result efficiently internalized by macrophages [227, 228, 229, 230]. For example, recombinant B subunit of cholera toxin (rCTB) was covalently coupled to the outer surface of the small unilamellar liposomes for targeted delivery of encapsulated saliva-binding region (SBR) of *Streptococcus mutans* antigen I/II (AgI/II) protein to Peyer's patches (aggregated lymphoid nodules) and enhanced the secretory IgA responses in mice. The results revealed that antibody responses in mice immunized with rCTB-coupled liposomes preserved at high levels for at least 6 months by a single oral dose and significantly enhances their robustness and stability as an antigen delivery platform. This oral immunization approach should be suitable for the designing of vaccines against oral, intestinal, and also sexually transmitted diseases [231]. Similarly, antigen molecules like the lipid binding proteins and non-lipid binding proteins have been assembled with liposomes to help improve their immunogenicity and evoke antibody production in immunized entities. In a study, human liver fatty acid binding protein 1 (hI-FABP1) was assembled with homogeneous unilamellar cationic liposomes and characterized as a potential vaccine delivery vehicle in diverse thermal conditions. Under room temperature, the protein is mainly assembled to the liposomes via electrostatic interactions and under elevated temperature, the externally accessible proteins are embedded in the lipid bilayer structure similar to common biological membranes, which is further indicated that the thermal stability of a target protein is very crucial for successful production of desired lipoplexes as a suitable delivery carrier for vaccines [232]. While in another study, non-lipid binding protein or hapten carrier protein mainly bovine serum albumin (BSA) was assembled with cationic liposomes and the efficacy of the lipoplex vaccine was examined in mice and compared with that of Nicotine-BSA conjugate (Nic-BSA). The results illustrated that the lipoplex vaccine with adjuvant (Alum or Aluminum salt) was able to evoke the highest serum nicotine-specific antibody (NicAb) titers of  $11169 \pm 2112$ , which is undoubtedly higher than that steered by either the lipoplex vaccine without Alum or Nic-BSA with Alum. The outstanding immunostimulatory effect of this nano-lipoplex may promote an innovative approach to upgrade the immunogenic capability of current nicotine vaccines or other vaccines utilizing small addictive compounds to attain high titers of effective antibodies [233]. Apart from the cationic liposomes and liposome protein complexes, liposome-based polymeric derivatives or liposomes modified with pH-sensitive polymers are another important contemporary approach which is acting as a novel adjuvant for enhancing the antibodies production, delivery of antigen to the cytosol of antigen-presenting cells and also promotes isotype switching [234]. It has been reported that cationic lipopolymer adjuvant (liposome-PEG-polyethyleneimine complex or LPPC) robustly adsorbs immunomodulators or antigens onto its surface to boost up the immune responses and simultaneously enhances the uptake capability, cytokine release, surface marker expression, and the presentation of antigens on the mouse phagocytic cells. In the case of Freund's adjuvant, this liposome-polymer complexes conversely activate the Th1 immunity across antigens in vivo, while in contrast to immunomodulators like CpG oligodeoxynucleotides, this CPPC efficaciously improves the IgA or IgG2A immune responses even in hosts that have generated high IgG1 serum titers and Th2 immunities. These results are sufficiently demonstrated that LPPC adjuvant not only enhances the antigens immunogenicity but also modulates the host immunity to produce an appropriate antibody isotype switching in combination with immunomodulators and subsequently enhances the stability of vaccine antigens [235].

Basically, nanoparticle based adjuvants contribute to shaping and enhancing the vaccine immune response via various modes of action. The vaccine formulations containing the liposomal cationic adjuvant formulation (CAF01) systems and squalene based oil/water emulsions, which

evoked a significant primary antigen-specific CD4<sup>+</sup> T cell response for 7 days after immunization. The effector function of activated CD4<sup>+</sup> T cells was distorting toward a Th1/Th17 response by CAF01, while a Th1/Th2 response was directly elicited by o/w squalene. Previously reported that the CAF01 can gear up the both Th1 and Th17 responses and provide long-lived, robust Th1 memory. Conjointly mice immunized with only o/w squalene or CpG adjuvants leading to generate early H56-specific IgG response. These tested adjuvants produced the germinal center reaction with different magnitude through the T follicular helper cells (Tfh) and the results indicated that immunological activity of different adjuvants can be characterized by profiling early immunization biomarkers after primary immunization. Indeed, understanding the modes of action of adjuvant molecules loaded in liposomal derivatives will allow the development of novel vaccination strategies based on a rational design of heterologous prime-boost liposomal nanoformulations [236, 237, 238, 239]. However, the development of novel vaccine delivery systems and adjuvants has been facilitated by nanotechnology resulted in some concern regarding their safety, toxicity, optimal combination of antigens or immunomodulators, and storage conditions are needed to be critically examined in the preclinical stages before clinical use.

### 10. Clinical trials of liposome complexes in vaccine delivery

Several clinical trials of vaccines using liposomes as delivery or adjuvant therapy have been reported. These tests were still in phases I-II and were aimed at testing the effectiveness and safety of the melanoma, chlamydia, HIV, porcine circovirus, *plasmodium falciparum*, and hepatitis B vaccines [240, 241, 242, 243, 244, 245]. Gargett, T., et al. successfully demonstrated that a dendritic cell-targeted liposomal vaccine (Lipovaxin-MM) for melanoma, is safe and practical for use in future clinical trials. Lipovaxin-MM was adequately tolerated and did not cause any clinically relevant harm. Lipovaxin-MM did not appear to be immunogenic [241]. When liposomes were utilized, the peptide vaccination was the most effective in significantly higher HBeAg seroconversion rate [240], considerably ( $p < 0.05$ ) lowering viremia while raising the amounts of neutralizing antibodies and interferon- $\gamma$  secreting cells. This shows that the peptide vaccine was able to activate both humoral and cellular immune responses in the presence of liposomes [243]. Adjuvant liposome treatment results in faster seroconversion, higher IgG titres, a better mucosal antibody profile, and a more consistent cell-mediated immune response profile as compared to aluminum hydroxide [245]. A clinically safe liposomal adjuvant can direct antibody effector activity toward higher phagocytic function in comparison to alum, and this ability provides protection against severe mucosal SHIV infection in humans [244]. Based on these reports, it can be concluded that the use of liposomes as a delivery system or vaccine adjuvant therapy can reduce the toxicity and immune response to vaccines and increase their effectiveness.

### 11. Author's perspective

In modifying the liposome to adjust the delivery and controlled release of drugs, it is necessary to understand the characteristics and structure of the polymer to be used. Each type of polymer produces different liposome complex characteristics for specific uses. The development of the liposome complex can be very extensive through modification of the structure of natural polymers or new synthetic polymers. However, the aspect that needs to be considered is its relation to biodegradability and biocompatibility with the body, so it can be used safely and effectively.

### 12. Conclusion

Various natural and synthetic polymers produce different liposome complex characteristics. These characteristics also provide different functions in the regulation of drug release and delivery by liposome

complex. These different properties are influenced by the form of interactions and bonds between the polymer and liposome. Additional setup and excipient methods also influence the complexity of the interaction. The interaction form and its influence on the characteristics of the liposome complex can be studied through a variety of methods and instruments that are appropriate. Based on the results of reviews from several libraries, it can be concluded that the liposome complex can be an optimal system for targeted delivery, controlled release, and stabilization of drugs and vaccines.

### Declarations

#### Author contribution statement

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