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CURRENT IMPROVEMENTS IN LIPOSOME TECHNOLOGY

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ABSTRACT

The hydrophilic core in a lipid bilayer membrane of liposomes, an artificial colloidal vesicular structure, has continued to be recognized as an important nano-sized drug delivery system with attractive properties, including ease of preparation, high biocompatibility, excellent entrapment capacity, and safety. These spherical vesicles can carry both hydrophilic and lipophilic drugs since they are made of amphiphilic molecules like cholesterol and sterols. Over the past few decades, significant effort has been made to create drug delivery systems based on liposomes. Systemic drug delivery through the use of liposomes has been widely considered a desirable goal. To address the persistent need for novel drugs in clinical settings, current developments have been explored to enhance the liposomal-based drug delivery system. This includes the implementation of a more consistent preparation technique and an expanded application to innovative modalities such as nucleic acid therapies, CRISPR/Cas9 therapies, and immunotherapies. Additionally, this review presents concise details regarding the classification, composition, and characterization of liposomes, recent techniques employed in liposome preparation, the excipients utilized in liposomal formulations in various novel studies, and the routes of administration employed to deliver liposomes to targeted areas of disease. The objective of this study is to provide an updated analysis of liposomal delivery research and to emphasize potential nanotechnological strategies for the future.

KEYWORDS

Liposomes, Recent Advancement, Structure, Classification, Method of preparation, Nano drug delivery

1. INTRODUCTION

The liposome is a highly suitable and secure mode of drug delivery, characterized by lipid bilayers that closely resemble the plasma membrane of the cell. It is possible for them to integrate a diverse range of pharmaceutical candidates into their hydrophilic and hydrophobic compartments. It is evident that liposomes have the capability to encapsulate gases, including nitric oxide, as stated in the US patent US007976743B2 (2011). This method can be utilized for the treatment of pulmonary hypertension. Numerous extensive methodologies have been elucidated in the recent period. The techniques encompass the time-honored extrusion method [1,2], electroformation [3,4], freeze-drying [5,6], hydration or swelling [7,8], double emulsions (specifically water-in-oil-in-water, or W/O/W) [9,10], and bubbling [11]. Nevertheless, these methods provide only a restricted level of process control and reproducibility. Consequently, they frequently lead to inconsistency and ineffective utilization of the materials in clinical

translations. The ongoing advancement of liposome technology has facilitated the utilization of numerous liposome-based preparations in contemporary times, with many products undergoing diverse clinical trials (Fig 1). Ever since the inception of the initial liposome product, Doxil®, which was developed by Gabizon and Barenholz [13], numerous other anti-cancer formulations have been effectively formulated, such as DaunoXome® [14], Depocyt® [15], Myocet® [16], Mepact® [17], Marqibo® [18], and Onivyde™ [19]. Furthermore, the utilization of liposomal application extends beyond the scope of anti-cancer therapies. It serves as a valuable means for administering anti-fungal agents such as Abelcet® [20], Ambisome® [21], and Amphotec® [22]. Additionally, liposomal application proves effective in delivering pain relief agents like DepoDur™ [23] and Exparel® [24], as well as oral anti-viral agents such as Epaxal® [25] and Inflexal® V [26]. The scope of potential applications for liposomes is continuously expanding. This is demonstrated by the multitude of liposomal formulations currently undergoing clinical trials, as depicted in Fig 1. The current liposomal formulations being evaluated in clinical trials are expected to provide significant advantages to a diverse patient population.

The characteristics of liposomes can be regulated by the selection of lipids employed during their formulation. According to a recent study, porphyrin phospholipids were utilized in a liposome formulation that has the ability to release its contents upon exposure to near infrared irradiation [27]. The fatty acid elastin-like polypeptide was utilized in the preparation of a temperature-triggered liposome, wherein temperature serves as an external stimulus [28]. Temperature-sensitive liposomes have clinical advantages, including the expansion of intravenous drug administration, enhanced drug accumulation due to their in vivo stability, and improved drug release from the liposomes [28].

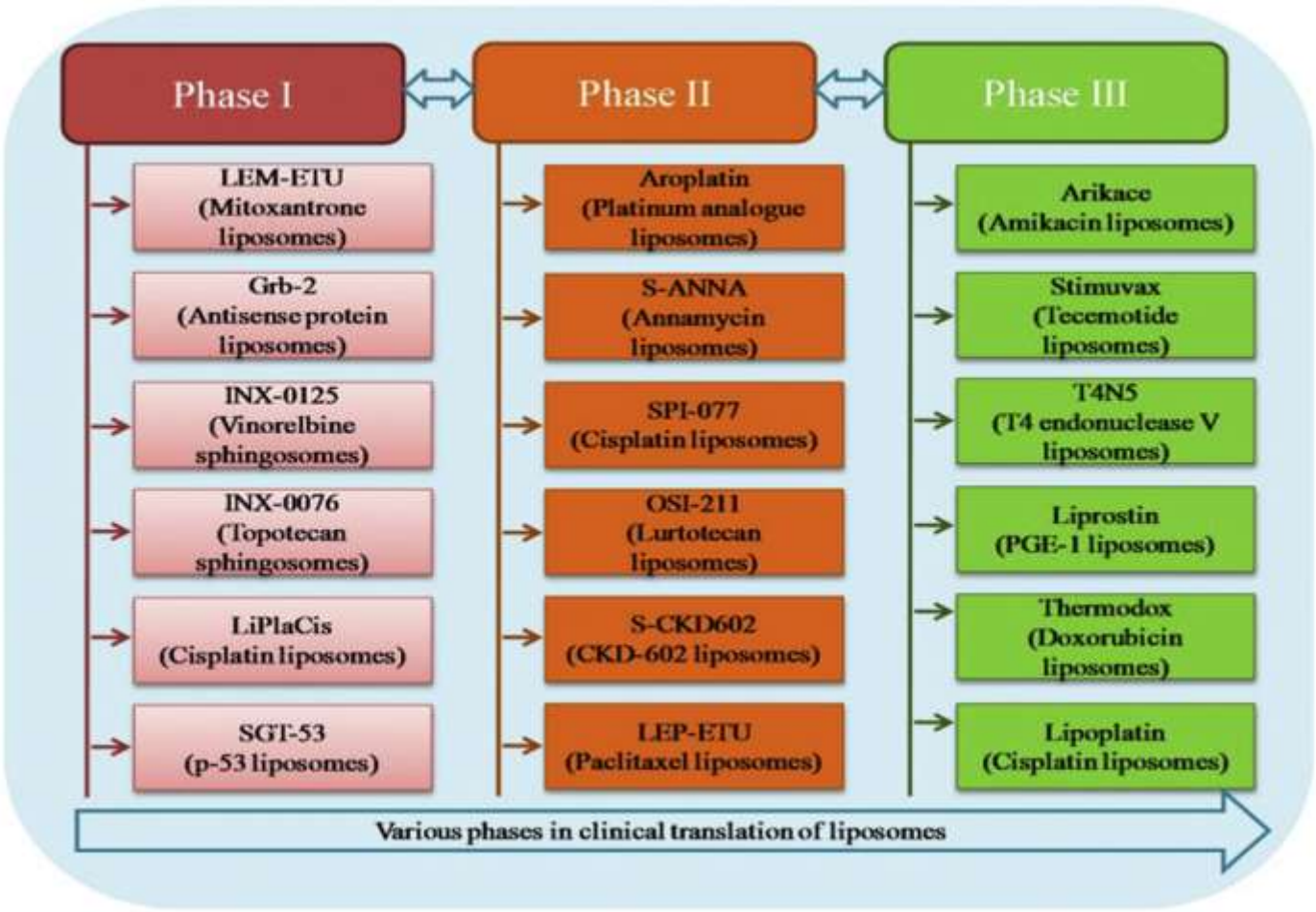

Fig. 1. Liposomal formulations present in clinical trials [12].

2. Recent Approaches to Liposome Production

Techniques such as Electroformation, hydration, and extrusion are widely recognized as established bulk methods, extensively employed by numerous research groups across various applications. The process of electroformation and hydration entails the creation of a lipid layer on an electrode. Subsequently, an electric field is employed in the presence of an aqueous solution to detach the lipids, allowing for their self-assembly into vesicles [3]. The presence of an electric field poses a potential disadvantage in this technique, as it may cause degradation of proteins that are sensitive to electric fields. An alternative method, which is akin to electroformation, is hydration, wherein vesicles are formed without the use of an electric field. Additionally, extrusion techniques involve the passage of the prepared vesicle solution through a polycarbonate membrane to regulate the size and lamellarity of the vesicles [2,29]. Further methods that have been recently developed will be expounded upon in the forthcoming sections. The production of vesicles using bulk methods results in vesicles that are non-uniform in both their lamellarity and size due to poorly controlled mechanical and chemical conditions during vesicle formation. However, novel production approaches offer advantages such as the ability to control both the size and lamellarity of the vesicles formed, as well as greater reproducibility compared to conventional bulk methods. Additionally, microfluidic systems have the advantage of being able to remove the organic phase from

the final vesicles, unlike bulk methods where there is a high possibility of the organic layer being entrapped in the lipid bilayer. Despite these advantages, there are some disadvantages to using microfluidic methods compared to bulk methods. These include the low volume of the manufacturing process and the cumbersome setup of some of the methods. While microfluidic approaches are generally robust, they require very low quantities of solutions.

2.1 Liposomes integrated onto microchips

Microfluidic technologies offer a high level of control over the manufacturing process and address issues related to reproducibility. Microfluidics refers to the precise manipulation of fluids within a confined volume, typically in microliter or picoliter quantities, with dimensions on a sub-millimeter scale and low Reynolds numbers. This reduction in reaction volumes can lead to cost savings in reagents and enhance throughput and analytical performance [30]. The presence of residual solvents in the bilayer can also modify the mechanical and physical properties of the membrane [31–35]. Another important characteristic of the membrane is its lamellarity, which refers to the number of bilayers present. The manufacturing techniques employed also influence the size and size distribution of the liposomes being prepared. Most applications require a monodisperse population of vesicles [36]. The stability, encapsulation efficiency, membrane curvature, and transport rates across the membrane are all influenced by the size distribution. Any variation in size results in variability in these parameters [36–39]. The main advantage of this method lies in its ability to generate monodisperse vesicles. Apart from membrane composition and size, the stability of the vesicles is also influenced by factors such as osmolarity, salinity, pH, and temperature [40–42]. Instability of the vesicles can lead to processes such as budding, coalescence, aggregation, or lysis [43]. Tan et al. reported the formation of unilamellar vesicles through the emulsion transfer process, which exhibit remarkable stability for a duration exceeding 26 days [44]. In order to produce long-lasting vesicles, a greater level of process control is required, which can be effectively achieved through the utilization of microfluidic systems. These systems enable precise control over various factors including vesicle size, temperature, osmolarity, pH, salinity, and fluid mechanical forces. Usability is a crucial factor to consider when comparing various microfluidic technologies. The setup and operation of microfluidic tools can be challenging and cumbersome, and therefore, the ease of use often determines their usage. Several bulk methods can be employed as microfluidic approaches in liposome preparation, including double emulsion templating [45], pulsed jetting [46], transient membrane ejection [9], ice droplet hydration [47], droplet emulsion transfer [48], and hydrodynamic focusing [49]. A microfluidic method with excellent encapsulation efficiency and monodisperse unilamellar liposomes was reported using a hydrodynamic pinch-off mechanism [50]. Staggered herringbone based microfluidic chip for synthesizing the nanolipomer nanoparticles is shown in Fig 2.

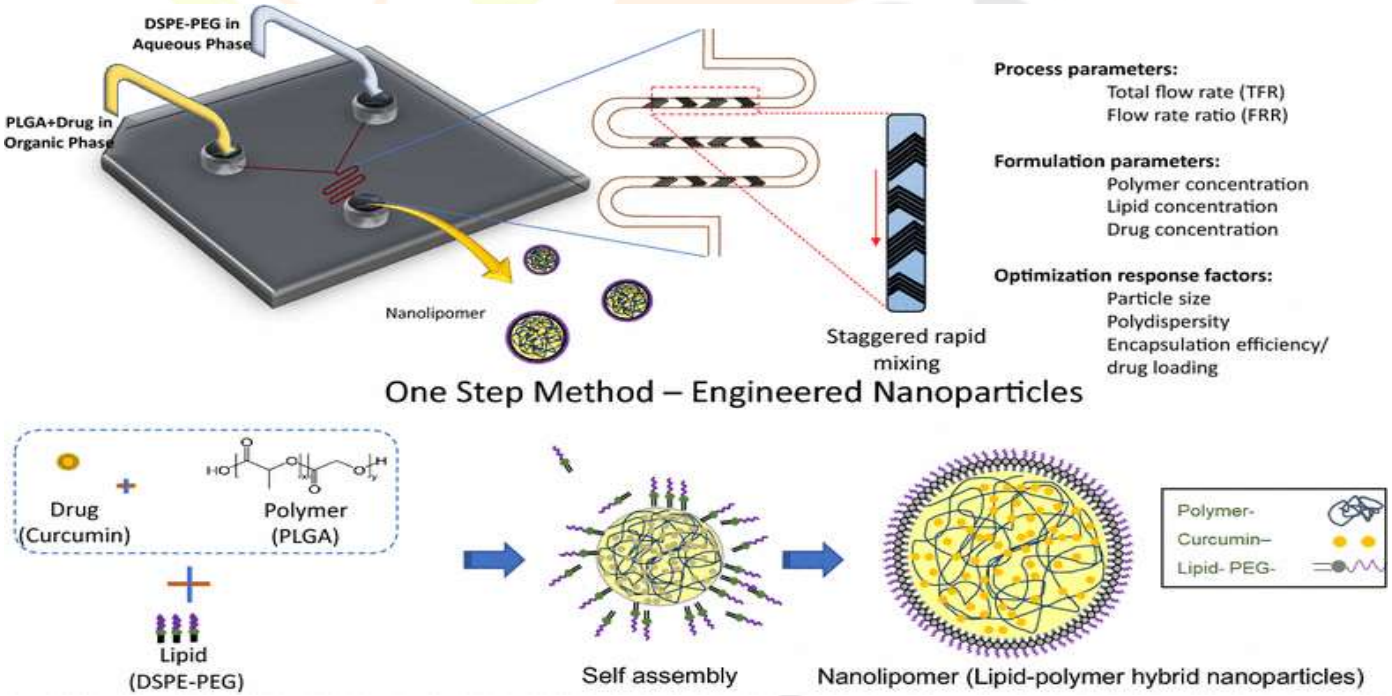

Fig. 2 Schematic diagram of synthesis process. Staggered herringbone based microfluidic chip for synthesizing the nanolipomer nanoparticles. The PLGA + drug core is formed by nano-precipitation in the microfluidic chip and introduced from one inlet while the DSPE-PEG lipid coating is injected from the opposite port. The final nanolipomer is collected in the third port after rapid mixing has occurred [49]

2.2 Flow focusing

The technique demonstrated by Jahn et al. [51] involves a central stream of solution containing phospholipids in alcohol, surrounded by aqueous solution on either side. When these three flows are merged into a microchannel, the alcohol diffuses into the aqueous layer, diluting it past a critical concentration, causing the lipids to self-assemble and form liposomes. The vesicles

formed using this method are monodisperse and have a diameter of around 50-150 nm, depending on the flow rates. Lin et al. [52] described a microfluidic tweezing technique, which is comparable to flow focusing, used to develop membrane tubes adapted for the formation of vesicles from tubes [53]. The vesicles resulting from this method are polydisperse. The flow focusing method can be used in a continuous process to prepare vesicles [54,55]. It allows for the preparation of organic and aqueous solutions stocked and connected to a microfluidic device to enable continuous manufacturing of vesicles. Although Jahn et al. do not discuss encapsulation efficiency much, it should be noted that since the formation of vesicles is dependent on a diffusive process from the organic layer into the aqueous layer, it is possible that some of the material escapes into its surroundings before the vesicles are formed. Additionally, there is a chance that liposomes formed from this method could have alcohol entrapped in them by partitioning into the membrane, leading to stability issues [56].

2.3 Pulsed jetting

This technique is an intriguing method that bears resemblance to the act of blowing soap bubbles through a loop. By utilizing a micro-nozzle, an aqueous solution is propelled from small jets into a planar lipid membrane. The momentum of the aqueous solution propels it further and causes the lipid membrane to constrict, resulting in the formation of a vesicle [57]. The initial description of this method was provided by Funakoshi et al. [58]. The encapsulation efficiency achieved through this method is notably high. However, one limitation lies in the encapsulation of large molecules, such as proteins, as it remains uncertain whether they can withstand the shear stress. Another significant issue associated with this technique is the presence of residual solvent in the membrane from the phospholipid solution [58]. Through the use of Raman spectroscopy, Kirchner et al. discovered that decane, utilized in the formation of the membrane, can be detected in the final vesicles at thicknesses of up to tens of nanometers [59]. Additionally, two more drawbacks of this method are its cumbersome setup process and its sensitivity to operating conditions and materials. Properly positioning the micro-nozzle or micropipette proves to be a highly challenging task. During the bilayer formation step, the manual repositioning of the micronozzle is required each time the instrument is used. The parameters of viscosity, temperature, and membrane composition play a critical role in determining how the membrane is deformed by the fluid jet. Consequently, these parameters often necessitate changes in the solutions used each time the apparatus is employed. Pulse jetted flow method illustrated in Fig 3.

1.1 Double Emulsion Template

This method entails the evaporation of the oil phase from a lipid-stabilized water-in-oil-in-water emulsion using a combination of organic solvents, such as toluene and chloroform [60,61]. As the oil phase is eliminated, the external and internal oil-water phases merge to create a lipid bilayer, resulting in the formation of a liposome. Shum et al. [60] initially demonstrated this high-throughput technique, which yields vesicles with high encapsulation efficiencies and monodisperse characteristics. However, a significant challenge encountered in this method is the incomplete evaporation of the solvent during the evaporation step. Lorenceau et al. showed that it is possible to remove the oil phase without employing organic solvents and utilized this approach to generate polymerosomes that resemble vesicles with deblock copolymer membranes [62]. Additionally, Tan et al. employed a similar method but utilized a microfluidic device and oleic acid to produce the lipid-stabilized water-in-oil emulsion, which was

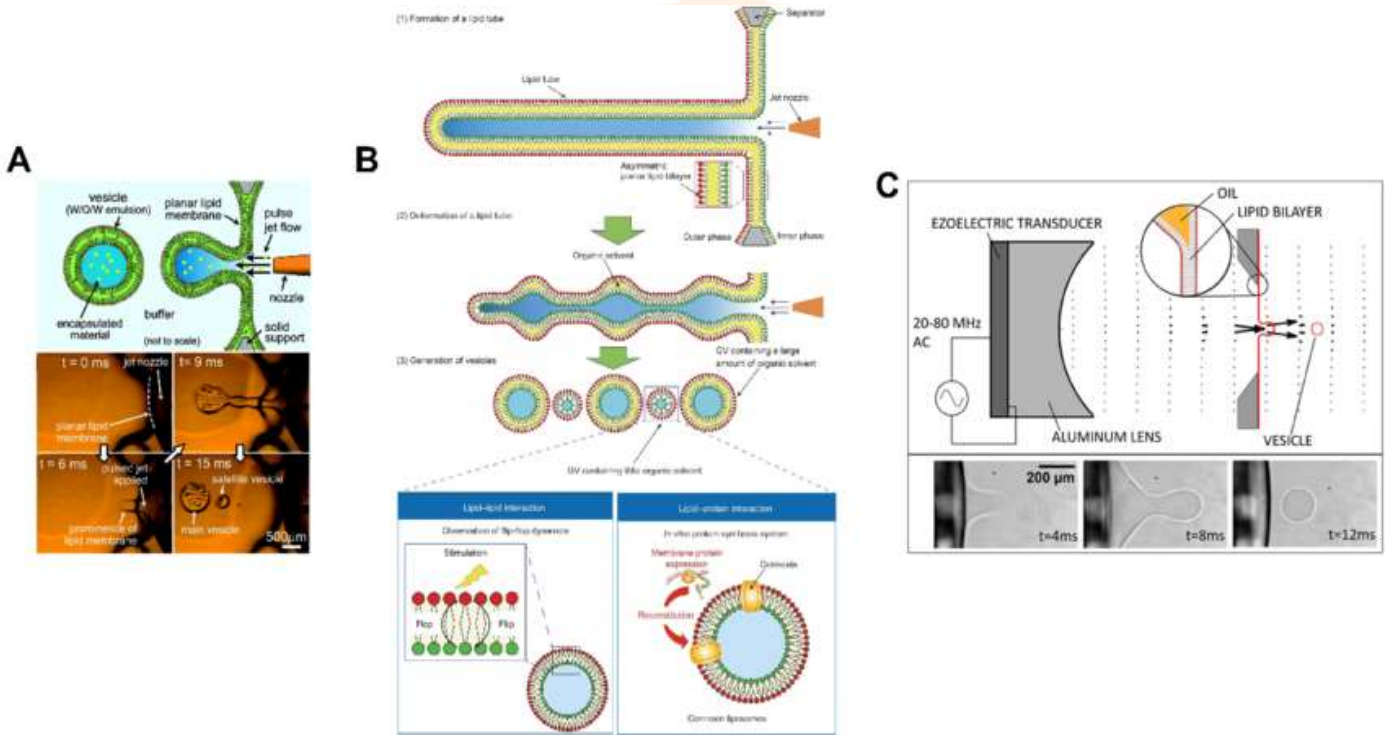

Fig 3. Examples of pulsed jet flow method. (A) Conceptual diagram of the "blowing vesicle" method. (B) Formation of a lipid microtube from the asymmetric planar lipid bilayer by the jet flow method. (C) Schematic of the acoustic jetting method [59]

subsequently transferred to an aqueous solution containing ethanol and water [63]. The oleic acid dissolves in ethanol and induces the self-assembly of phospholipids into a lipid bilayer, resulting in the formation of unilamellar and monodisperse vesicles. The

parameters, such as size and stability, can be easily controlled. The vesicles produced using this straightforward method have been shown to remain stable for over 26 days. Subsequently, the same research group conducted the entire process within a microfluidic device [64]. Additionally, the utilization of ethanol for the removal of oleic acid renders it more biocompatible compared to previous methodologies employed for double emulsion. Moreover, Deshpande et al. have elucidated a technique, referred to as Octanol assisted liposome assembly (OLA), which bears resemblance to the double emulsion technique. OLA facilitates the formation of unilamellar, monodisperse, and cell-sized liposomes (as depicted in Figure 3). This technique employs 1-octanol as a lipid carrier, which is biocompatible and facilitates an effortless solvent extraction process. A double emulsion droplet swiftly undergoes a transformation into an intermediate state, wherein an aqueous volume is encompassed by a lipid bilayer with a solvent pocket attached to its side. As illustrated in Fig 4, the lipid bilayer seamlessly seals the entire interface, effectively separating the 1-octanol pocket from the assembled liposome. The one-step formation of droplet emulsion using 1-octanol proves to be an efficient and user-friendly technique when compared to alternative methods [65].

1.2 Droplet emulsion transfer

Pautot et al. presented this technique on a macroscale. A water-in-oil emulsion was stabilized using phospholipids and subsequently transferred the droplets to an aqueous medium. As the droplets passed the interface between the oil and aqueous phases, they acquired a second lipid layer. The vesicles formed through this method are characterized by a unilamellar bilayer structure [10]. Norieaux et al. employed the droplet emulsion method to prepare vesicles encapsulated for gene expression [66]. The emulsification process was conducted through vortexing, resulting in vesicles that exhibit non-uniform size distribution. The emulsification technique was greatly enhanced through the utilization of a microfluidic device in order to generate a droplet emulsion. Subsequently, this emulsion was introduced into a pre-existing oil-water interface on a larger scale [67–69]. The problem of polydispersity was resolved by employing a widely accepted microfluidic droplet formation technique. Despite employing various approaches, the method of transferring the emulsion was unable to completely eliminate residual oil matter [70].

1.3 Hydrodynamic focusing

In contrast to the aforementioned methods that lead to the formation of large vesicular systems or microtubules, the hydrodynamic focusing method described here produces a nanoscale vesicular system that holds potential for clinical applications. It is important to note that the previously mentioned methods cannot be strictly classified as microfluidic systems. The microfluidic hydrodynamic focusing (MHF) technique involves a typical microfluidic system with a low Reynolds number and diffusion-dominated mass transfer. MHF utilizes microfluidic devices with either a 3D angular coaxial geometry or a cross flow geometry [71]. In this technique, the central channel of the device carries an alcohol solution containing lipids, which is surrounded by coaxial flows of an aqueous phase consisting of either water or buffers. The lipid solution is hydrodynamically focused into narrow sheets with a cross-flow geometry or a circular cross-section in the case of 3D annular coaxial chips. The size of the focused stream can be controlled by parameters such as the volumetric flow rate ratio (FRR) and total flow rate (TFR) [72]. By employing this MHF technique, monodispersed liposomes can be produced, and their size can be controlled by adjusting the FRR or TFR. Jahn et al. proposed that the micrometer length scale of the sample stream allows for precise and reproducible chemical and mechanical conditions across the stream width, in contrast to traditional bulk-phase preparation techniques [73]. M. Guimarães Sá Correia et al. investigated the effects of different FRR and TFR on the size of liposomes and observed that lower FRR and TFR resulted in larger particle sizes. Specifically, in their study, particles formed at a flow rate of 20 ml min−1 were smaller than those formed at 6 ml min−1 [74]. This system offers the opportunity for easy scalability to an industrial level and can be utilized for the production of clinically relevant batches [56].

1.1 Other methods

Ota et al. established the phenomenon of transient membrane ejection through the use of microfluidic technology [48]. This process involves the creation of a lipid bilayer, which is then disrupted to form vesicles. A laser is utilized to heat up the aqueous solution on one side of the membrane, causing the solution to inflate and form a bubble that eventually breaks off into a liposome. The primary advantage of this method is its complete integration with the microfluidic platform, resulting in the production of monodisperse unilamellar vesicles. Additionally, the size of the vesicles can be adjusted due to its integration with the microfluidic platform. However, a major drawback of this method is the depletion of the membrane used to form vesicles, which can lead to intermittent oil phases separating batches of liposome samples. Despite this, the encapsulation efficiencies are high due to the separation of both the internal and external aqueous phases by the lipid bilayer membrane. Another method, described by Sugiura et al. [47], is Ice droplet hydration. This process involves the stabilization of a monodisperse water-in-oil emulsion through the use of span-80 and stearylamine. The encapsulation efficiencies are high with this method due to the separation of both the internal and external aqueous phases by the lipid bilayer. However, this method results in low encapsulation efficiencies and unilamellar vesicles after extrusion [75], making it a cumbersome process. In contrast to conventional bulk methods that necessitate post-processing steps, MHF possesses the capability to generate liposomes that exhibit uniformity in size and lamellarity to an exceptional degree [76]. This microfluidic technology offers continuous production and the potential for scaling up through microreactor

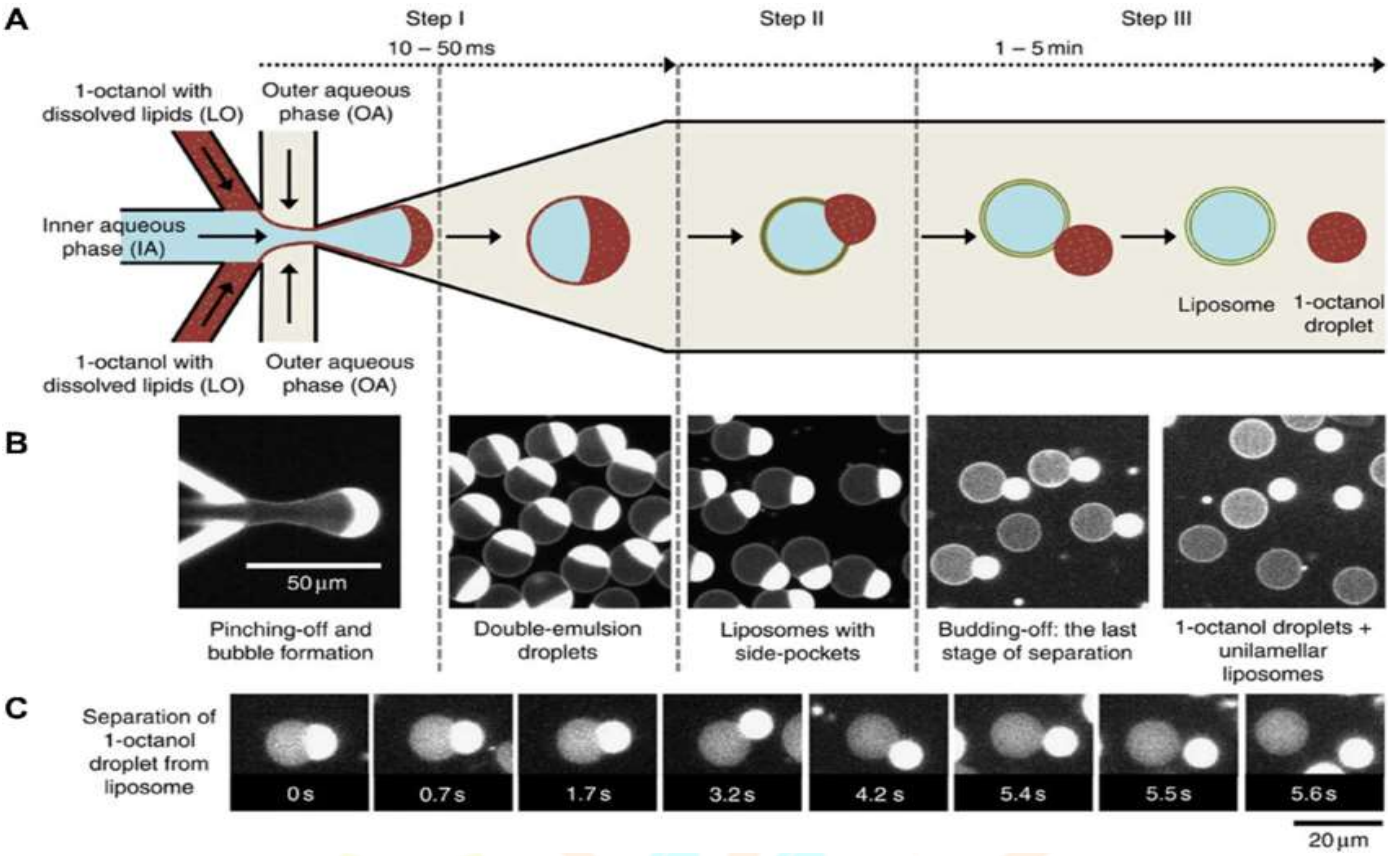

Fig. 4 Schematic of octanol-assisted liposome assembly (OLA). (A) Schematic representation showing the working principle of on-chip production of liposomes using OLA. (B) Corresponding fluorescence images showing each of the steps described above. (C) Temporal-resolution sequences showing the separation of the 1-octanol droplet from the liposome. The first frame of the sequence in c was obtained about 1 min after the double-emulsion droplet formed [65].

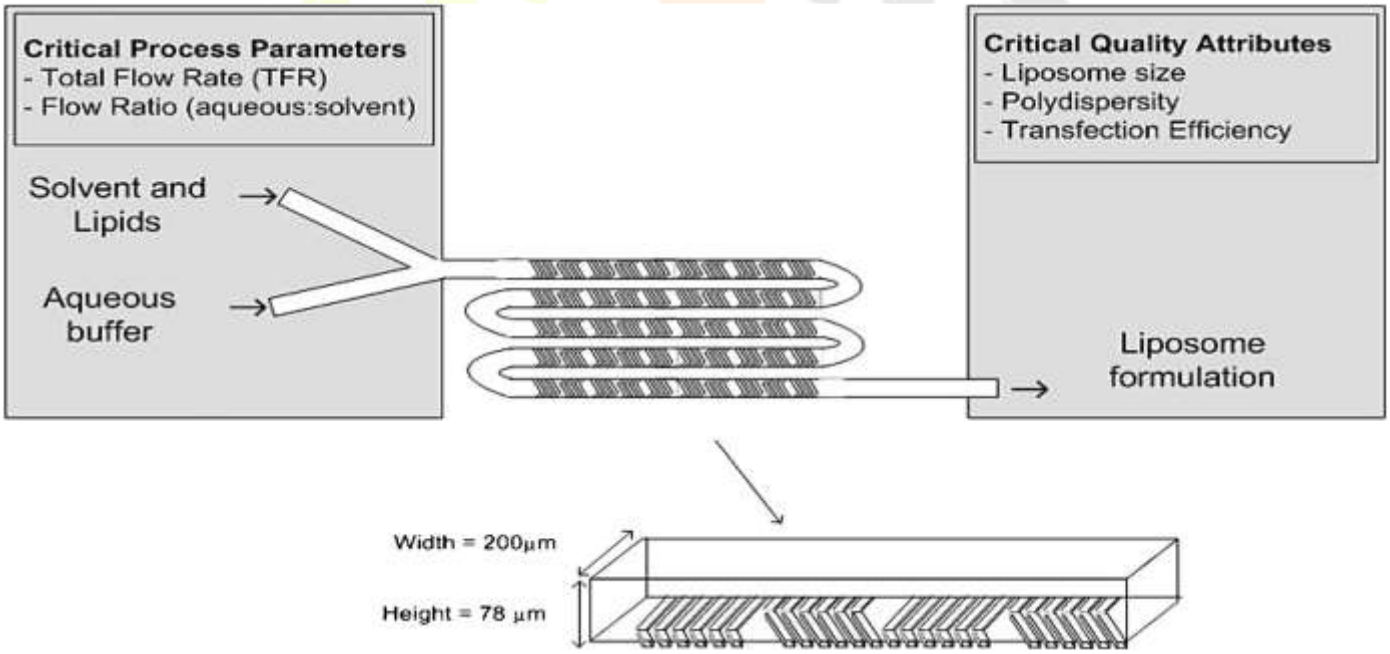

Fig. 5. Staggered Herringbone Micromixer (SHM) schematic. Solutions containing lipids and aqueous buffers are injected separately and chaotic advection is aided by the grooves on the channel floor [78]

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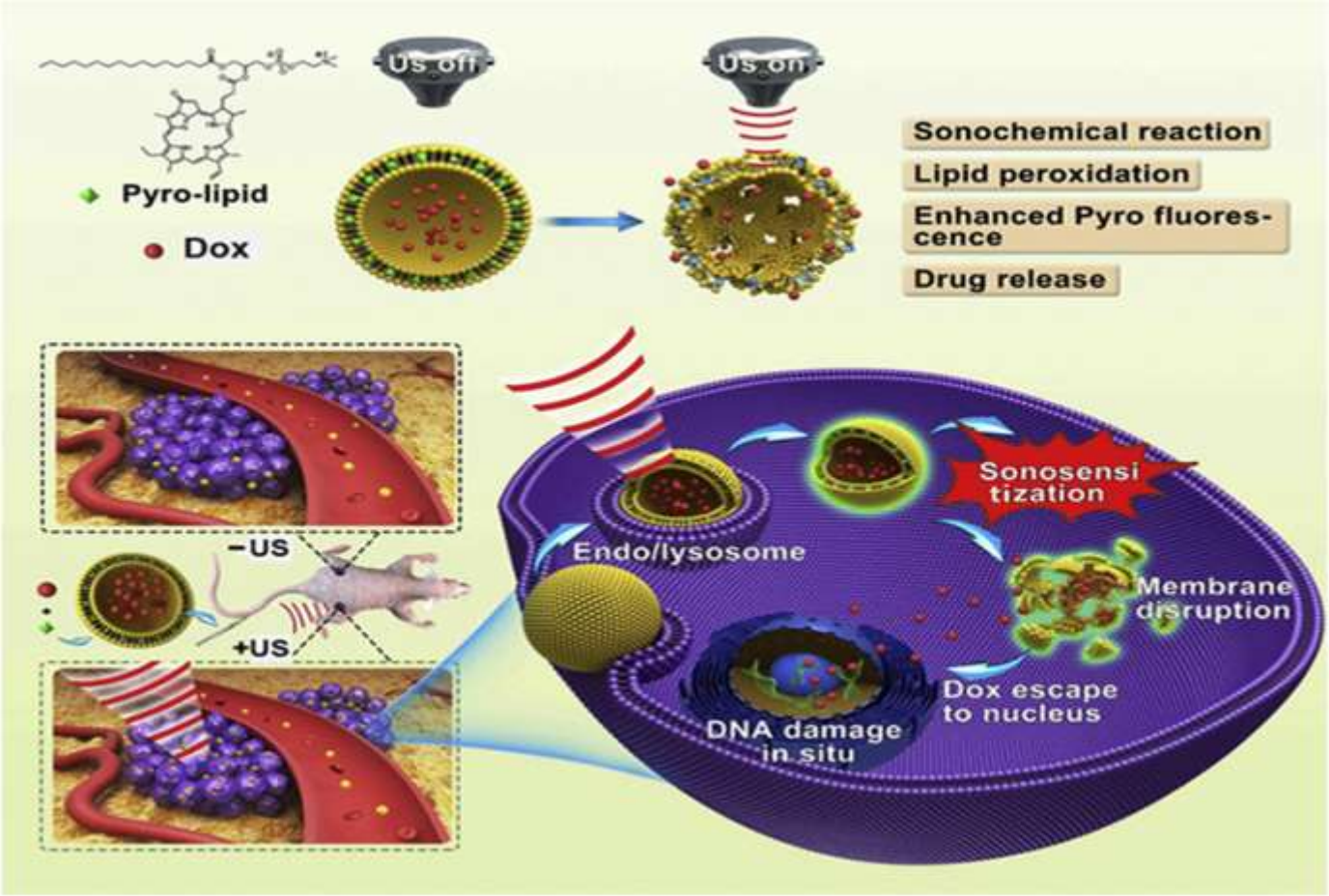

Fig. 6. Ultrasound activatable Dox-loaded porphyrin-phospholipid-liposomes for anti-tumor treatment [79].

2. Types of payload in liposome-based drug delivery system

2.1 Liposomes for nucleic acid delivery

In recent times, significant efforts have been dedicated to the advancement of delivery platforms for pharmacological biologics with large molecular weight, particularly for nucleic acid molecules. The pharmacokinetic properties of nucleic acid therapeutics, such as plasmid DNA, messenger RNA, small interfering RNA, microRNA, and other types of antisense oligonucleotides, hinder the achievement of effective therapeutic outcomes. The highly negatively charged backbones of these molecules greatly restrict their interaction with cells and their ability to be transfected into target cells. Consequently, the delivery of nucleic acid molecules using non-viral vectors has emerged as a widely recognized strategy for the successful administration of nucleic acid therapy (see Fig 7). Liposomal nucleic acid delivery systems have garnered attention from both academic and industrial sectors for their potential in this field. Cationic liposomes are composed of cationic lipids that are electrostatically bound to anionic nucleic acids, resulting in the formation of stable lipoplexes that facilitate their uptake into cells through endocytosis. The cationic lipids serve as the core functional component of the liposome, neutralizing the anionic nucleic acid molecules and enabling their delivery across the cellular membrane. Additionally, the cationic charges facilitate the interaction and escape of nucleic acid-loaded liposomes to the cytoplasm. Various novel synthetic cationic lipids have been utilized in the preparation of liposomes, resulting in improved biocompatibility, nucleic acid loading capacity, and transfection efficiency, as illustrated in Fig 8.

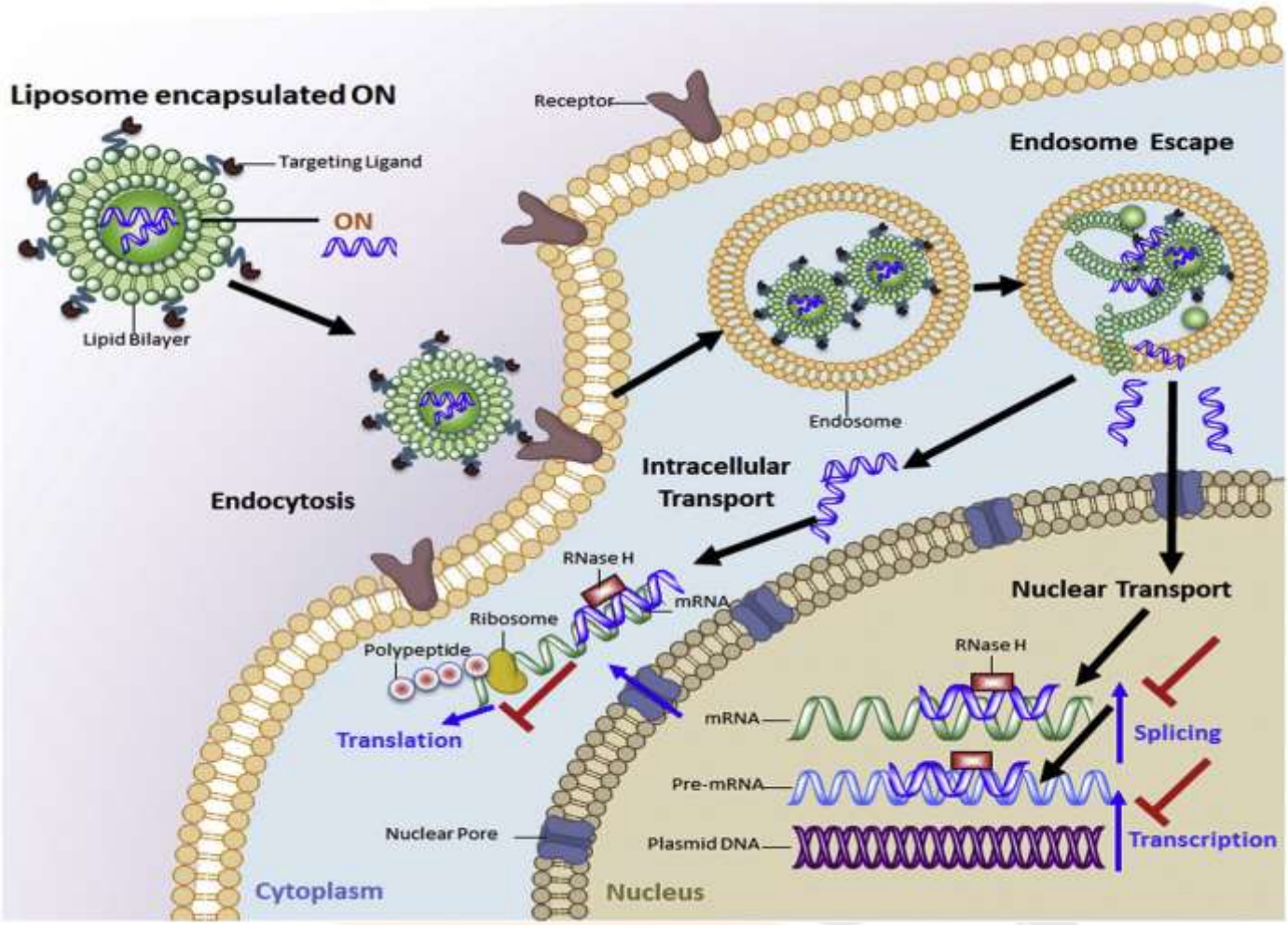

Fig. 7. Schematic illustration of the intracellular trafficking of liposome-based nanoparticles for delivery of nucleic acid/oligonucleotide molecules to cells [80].

Helper lipids, which are typically electrically neutral, have been commonly utilized in the formulation of cationic liposomes for the delivery of nucleic acids. One specific type of these helper lipids is fusogenic lipid, such as dioleoylphosphatidylethanolamine (DOPE), which has been extensively employed in liposomal formulations to facilitate the destabilization of the cellular bilayer and assist in the escape of the nucleic acid molecules complexed within the liposomes from the endosomes into the cytoplasm. The substitution of DOPE with DOPC significantly impeded the transfection efficiency of cationic liposomes [81]. Another essential helper lipid is cholesterol, which, when incorporated between the phospholipids, enhances the rigidity of the liposomes. In a novel approach, cationic cholesterol, such as 3β-{N-(N′,N′-dimethylaminoethane) carbamoyl} cholesterol (DC-Chol), has been investigated for its complexation with nucleic acids in liposome delivery systems, instead of using cationic phospholipids [82,83]. It should be noted that in the design of liposomes for nucleic acid delivery, the use of cationic lipids can destabilize the anionic cellular membrane, leading to undesirable cytotoxicity. Therefore, the incorporation of PEGylated neutral lipid chains has become a common strategy employed to shield the exposed cationic charges in liposomal formulations for nucleic acid delivery [84].

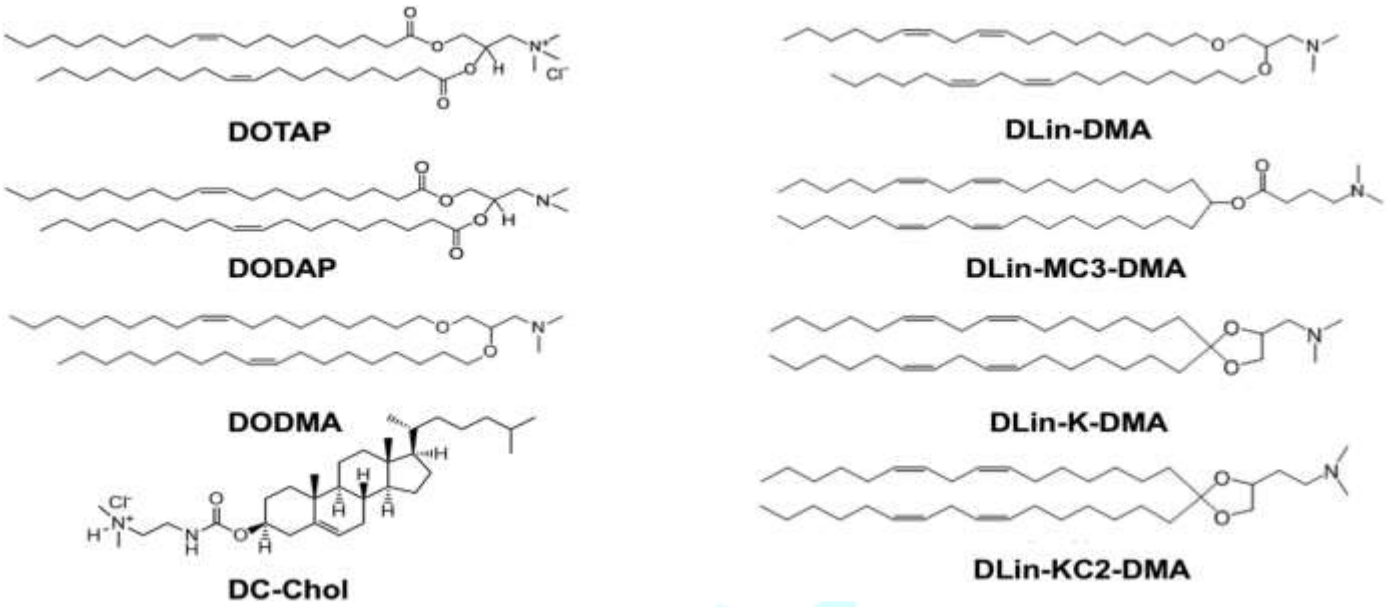

Fig. 8. Chemical structures of recently used cationic lipids for liposome-based nucleic acid delivery [81].

2.1.1 siRNA

The delivery of siRNA using liposomes has been extensively studied in RNA interference (RNAi) technology and has garnered significant attention over the past few decades. Recent studies have focused on investigating the impact of excipient and surface properties on the efficiency of intracellular delivery of siRNA using liposomes. Cationic lipids, such as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-3-dimethylammonium-propane (DODAP), 1,2-dioleyloxy-3-dimethylaminopropane (DODMA), and 2-di-Ooctadecenyl-3-trimethylammonium propane (DOTMA), have been combined with DOPE/Cholesterol/PEG at various mole ratios by different research groups for siRNA delivery (see Figure 7). A widely used liposomal formulation for the delivery of siRNA is DC-Chol and DOPE at a 1:1 molar ratio. This formulation has been found to effectively deliver siRNA systemically and result in significant inhibition of SK-OV-3 ovarian tumor growth [89]. However, another study has suggested that DC-Chol/DOPE liposomes may be more toxic than DOTAP/Chol liposomes at equivalent molar ratios [85].

Anionic and neutral liposomes have the potential to be effective platforms for delivering siRNA. A phase I clinical trial (NCT01591356) is currently underway to assess the safety and tolerability of EphA2 siRNA delivered via intravenous administration using 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC)-based neutral liposomes in patients with advanced or recurrent malignancies. Anionic liposomes can also be combined with a small molecular weight cationic polymer to enhance their delivery capabilities. For instance, DOPE/DPPC/Chol liposomes were utilized to encapsulate 22 kDa polyethylenimine (PEI)-complexed siRNA as a potential delivery system for ovarian tumors [90]. Another study involved the delivery of siRNA to both in vitro and in vivo ovarian tumor models, where egg PC/Chol liposomes were used to complex siRNA through a surface-conjugated 600 Da PEI [91].

2.1.2 microRNA

The delivery of microRNA (miR), which shares a similar structure to siRNA, has emerged as a focal point of recent investigations due to its significant impact on disease progression and prognosis. The successful delivery of miR through synthetic cationic lipids-based liposomes has the ability to inhibit the translation of target mRNA and regulate the levels of other functional genes, thereby demonstrating their potential as therapeutic agents [92]. For instance, vascular endothelial growth factor (VEGF-A) has been associated with the metastasis and recurrence of thyroid carcinoma [93]. VEGF-A is also recognized as one of the key factors in controlling tumor angiogenesis. The delivery of miR-34b-5p via DOTAP/Chol/DOPE liposomes to thyroid carcinoma effectively suppressed the expression of VEGF-A both in vivo and in vitro [94]. Intravenous administration of miR-34b-5b liposomes resulted in a 2.5-fold decrease in VEGF-A expression and a reduction in tumor size in a xenograft BHT-101 thyroid carcinoma model.

A liposome formulation based on DOTAP was utilized to deliver microRNA for the treatment of osteoporosis. The formulation was modified with eight repeating sequences of aspartate (D-Asp8), which have a preference for binding to the bone resorption surface. The DOTAP/Chol/DOPE liposomes were targeted to osteoclasts and used to deliver antagomiR-148a. The liposomal delivery of antogomiR-148a resulted in approximately 80% downregulation of miR-148a levels. This led to a reduction in bone resorption and an improvement in bone microarchitecture in osteoporotic mice, without any observed liver or kidney toxicity.

A further intriguing investigation into the delivery of microRNA focused on the regeneration of the eye and brain (Fig. 9) [97]. Cationic lipids with symmetrical di-octadecyl tails and asymmetrical ocatdecyl/oleyl tails were synthesized and formulated into cationic liposomes, either individually or in a 1:1 molar ratio with cholesterol. All three liposomes effectively formed complexes with anti-miR-124 and successfully delivered them into live schmidtea mediterranea planarians. These formulations of cationic liposomes demonstrated a high degree of fusion with planarian cell membranes and a strong suppression of miR-124 functions [97].

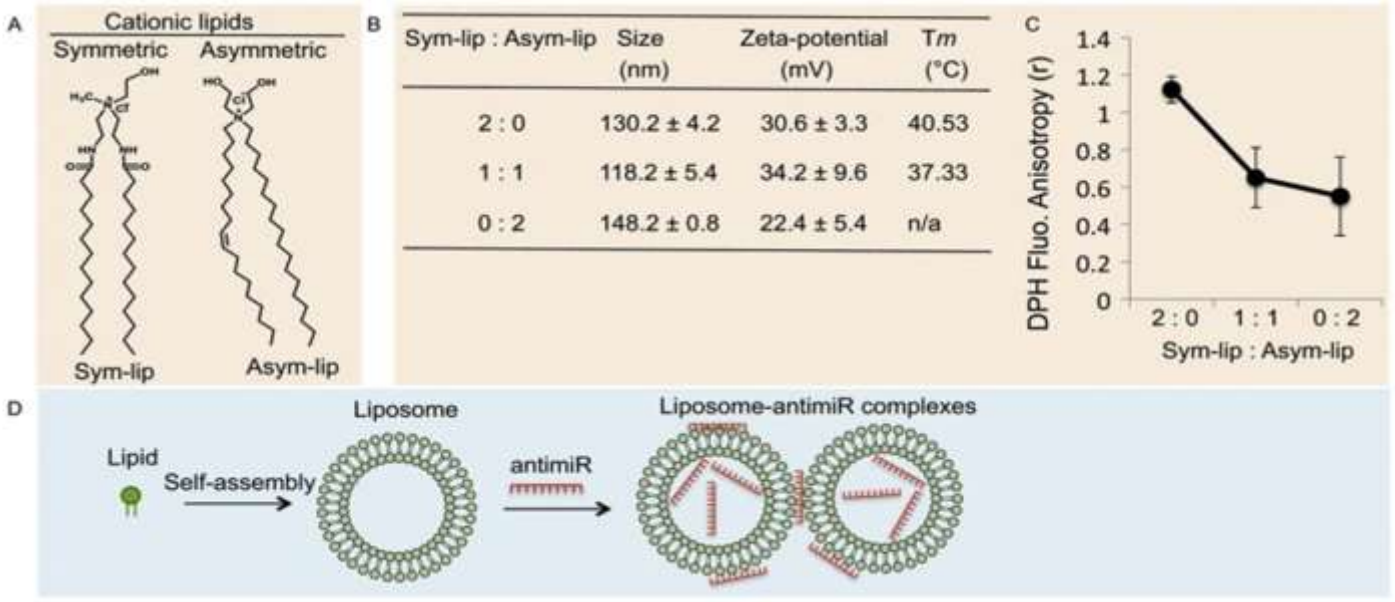

Fig. 9. (A) Chemical structures of the synthetic symmetric and asymmetric cationic lipids. (B) Characterization of liposomes consist of various ratios of symmetric and asymmetric cationic lipids. (C) Membrane fluidity analysis by the diphenylhexatriene (DPH) fluorescence anisotropy measurement in three types of liposomes. Error bars indicate S.D. (D) Schematic illustration of self-assembled liposomes and anti-miRs-loaded lipoplex formation [97].

A recent study utilized a nontraditional liposome formulation to investigate the effects of miRNA-124-loaded bubble liposomes on blood flow in an experimental hindlimb ischemia model. The results of the study demonstrated that these bubble liposomes, formulated with 1,2-distearoyl-sn-glycero-phosphatidylcholine (DSPC), 1,2-distearoyl-3-dimethylammonium-propane (DSDAP), and PEG2000, and pressurized with perfluropropane gas in a bath sonicator, were able to promote angiogenesis and improve blood flow. These cationic lipid-shelled nanobubbles have the potential to serve as effective carriers for targeted delivery into tissues. Furthermore, the intravascular injection of miR-126-loaded bubble liposomes resulted in the induction of angiogenic factors and further improvement in blood flow.

2.1.3 mRNA

Messenger RNA (mRNA), which encodes various therapeutic proteins, has been extensively studied for its potential applications in cancer immunotherapy [99], infectious disease vaccines [100], and protein replacement therapy [101]. Additionally, certain liposome formulations have been utilized for the delivery of mRNA, along with small interfering RNA (siRNA). In a notable example, a classic formulation known as DOTAP:Chol, with a 1:1 molar ratio, was employed to deliver mRNA encoding survivin-T34A in both in vitro and in vivo C26 colon cancer models [102]. The process involved an initial incubation with protamine sulfate solution (1:1, w/w) for 10 minutes, followed by a subsequent 15-minute incubation with liposomes at room temperature. The survivin-T34A mRNA was then loaded into the DOTAP liposome at a ratio of 1:1:1 (w/w/w) for liposome:protamine:mRNA. The administration of survivin-T34A mRNA-loaded nanoparticles, whether locally or systemically, resulted in a highly effective antitumor response with minimal toxicity.

In a separate instance, cationic liposomes based on di-linoleylmethyl-4-dimethylaminobutyrate (D-Lin-MC3-DMA) were developed for the purpose of delivering mRNA that encodes the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is an anion channel that plays a crucial role in chloride efflux from secretory epithelial cells [103]. The nanoparticles were administered through the nostrils for inhalation into the lungs of female BALB/c mice. The findings indicated that exposure to the liposomes loaded with CFTR mRNA for two consecutive days resulted in the restoration of approximately one third of normal chloride efflux, with effects lasting for at least 2 weeks. This level of protein restoration was considered sufficient for disease improvement [104].

It is important to note that mRNA differs in chemical and structural properties from siRNA/microRNA. The increased molecular length and charge density of mRNA necessitate significant modifications to the liposome delivery formulations that have been developed for siRNA/microRNA delivery. The complexation of highly charged anionic mRNA with cationic liposomes can lead to uncontrolled aggregation. Therefore, techniques such as microfluidics, PEGylation, and adjustment of the lipid/mRNA ratios are often necessary to prepare liposomes loaded with mRNA that have a uniform size distribution [105]. Kauffman et al. optimized a previously used siRNA liposome formulation for mRNA delivery using an in vivo C57BL/6 mice model [106]. The liposome formulation for siRNA delivery consisted of C12–200 cationic lipid: DSPC: Cholesterol: PEG at a ratio of 50:10:38.5:1.5, while the optimal formulation for mRNA delivery is C12–200 cationic lipid: DSPC: Cholesterol: PEG at a ratio of 35:16:46.5:2.5. The mole ratio between cationic lipid and mRNA was also adjusted from 5:1 to 10:1. These changes in the composition of the formulations significantly increased the effectiveness of the liposome in delivering mRNA

2.1.4 pDNA

The delivery of plasmid DNA (pDNA) presents certain common obstacles, such as the need to achieve a substantial loading capacity and prevent aggregation, which arise from the high anionic charge and molecular weight of pDNA. Furthermore, unlike mRNA, pDNA must reach the nucleus rather than solely the cytoplasm in order to facilitate protein translation. Additionally, the delivery of pDNA carries the potential risk of integrating into the genome and causing mutations [107].

Previous research has demonstrated that the inclusion of DOPE in cationic liposomes has resulted in improved in vivo intracellular trafficking in target cells [108]. As a result, most cationic liposomes used for pDNA delivery now contain DOPE. Furthermore, the method used to prepare DNA-complexed liposomes can have a significant impact on transfection efficiency and cytotoxicity. Cationic DC-Chol and DOTAP, together with DOPC and DOPE at a ratio of 1:1:1:1, were prepared using either a self-assembling method or microfluidic mixing. Self-assembled liposomes consist of multilayers, while liposomes prepared using microfluidic mixing techniques possess fewer lipid bilayers, larger numbers, but contain fewer DNA molecules individually. Consequently, when delivering the same amount of pDNA, liposomes prepared using microfluidic mixing led to a higher number of transfection events but lower transfection intensity per cell [109].

Another group has also reported a similar effect of microfluidic preparation. [110] They conducted a comparison of the performance of a combination library of cationic and helper lipids in transfection both in vitro and in vivo. The substitution of heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino) butanoate (DLin-MC3-DMA) in liposomes with 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-(1,3)-dioxolane (DLin-KC2-DMA) resulted in a higher transfection efficiency. Specifically, the transfection potency order was DLin-KC2-DMA > DLin-MC3-DMA > 1,2-dilinoleyloxy-3-dimethylaminopropane (DLinDMA) > 1,2-dilinoleoyl-3-dimethylaminopropane (DLinDAP). Additionally, the replacement of saturated PC by unsaturated PC and PE in the liposome formulation of pDNA could also enhance the transfection efficiency. The necessity of helper lipids was supported by the fact that little or no transfection was observed for formulations in the absence of helper lipids.

The examination of versatile membrane-tethered DNA has also been conducted. DNA molecules that have been modified with a cholesterol anchor through a flexible tetra (ethylene glycol) (TEG) linker at the 5′ terminus of the strands was conjugated [111]. The cholesterol moieties effectively anchored the DNA into the bilayers of liposomes composed of DOPE and DOPC. Through quantitative analysis, it was determined that the cholesterol DNA densely packed onto the membranes, with a maximum density of one strand per 0.02 to 0.04 nm2, depending on the lipid composition. Interestingly, double strand DNA exhibited a higher affinity for the liposomal membrane compared to single strand DNA of the same length, despite having double the negative charge. This study is anticipated to facilitate the development of biomimetic DNA versions of natural membrane nanopores and cytoskeleton for nanobiotechnology research [111].

2.2 Liposomes for CRISPR/Cas9 delivery

The CRISPR technology, known as clustered regularly-interspaced short palindromic repeats, has brought about a significant revolution in the field of molecular biology as an innovative tool for genomic editing [112]. CRISPR/Cas9 therapeutics have been developed to target and downregulate specific genes of interest, with the aim of treating various genetic diseases and cancers. However, the administration of CRISPR/Cas9 therapeutics has typically involved the use of Cas9 protein with sgRNA, Ca9 mRNA with sgRNA, or CRISPR/Cas9 plasmid [113]. Unfortunately, each of these approaches has suffered from poor stability and susceptibility to enzymatic digestion in the serum. Additionally, the negatively charged plasmid molecules have limited cellular uptake due to electrostatic repulsion by negatively charged cell membranes. Nevertheless, there is potential for liposome-based delivery systems to overcome these challenges [114].

Due to their high cellular uptake and efficient endosomal escape, cationic liposomes have been identified as a potential delivery platform for CRISPR/Cas9. For instance, a recent study demonstrated that DOTAP-based liposomes were able to deliver Cas9 and sgRNA plasmid, resulting in a 39% gene-editing efficiency in knocking out a GFP reporter in the HEK293 cell line [115]. Another study employed DOTAP-based liposomes to deliver CRISPR/Cas9 plasmid for the treatment of mucopolysaccharidosis type I (MPS I). The liposomes were complexed with CRISPR/Cas9 plasmid using microfluidic mixing. Genetic editing performance was evaluated in both an in vitro human fibroblast model and in an in vivo murine model [116]. In both studies, PEGylated DOTAP-liposomes provided serum stability, higher endosomal escape, and transfection efficiency, supporting their promise for therapeutic application.

A hydrogel nanoparticle, known as a DOTAP-liposome-templated hydrogel nanoparticle (LHNP), was developed for the targeted delivery of CRISPR/Cas9 to inhibit the polo-like kinase 1 (PLK1) gene in tumors [117]. The LHNP's core consisted of a hydrogel formed by crosslinking cyclodextrin-polyethylenimine (PEI, 25 kDa) with adamantine-PEI (25 kDa) to encapsulate the Cas9 protein. The shell, composed of DOTAP, effectively delivered the sgRNA. To enhance the accumulation and inhibit tumor growth of xenograft U87 tumors in mice, the LHNP was further modified with internalizing RGD (Arg-Gly-Asp) [117]. Another cancer-targeting moiety, R8-dGR, which binds to integrin αvβ3 and neuropilin-1, has been attached to the surface of DOTAP liposomes for the specific delivery of CRISPR/Cas9 plasmid targeting hypoxia-inducible factor-1 alpha (HIF-1α) to cancer cells [118]. The inhibition of HIF-1α was expected to prolong survival time by suppressing metastasis. These R8-dGR-modified cationic liposomes were co-loaded with both paclitaxel and CRISPR/Cas9 plasmid to achieve a combinational therapeutic efficacy [118].

A high level of genome editing efficiency in vitro often cannot be replicated in vivo [119]. Li et al. [113] have summarized the challenges and obstacles of CRISPR/Cas9-mediated genome editing using non-viral delivery systems. Firstly, the properties of CRISPR/Cas9 plasmids or proteins that make them susceptible to recognition and clearance by the reticuloendothelial system (RES)

hinder efficient encapsulation and stable blood circulation [120]. The second obstacle is the activation of the host immune response by the elements of the CRISPR/Cas9 system, which originated from bacteria. Although the inclusion of PEG can help evade the RES system and reduce the immune response, recent reports have indicated the generation of specific antibodies following the administration of PEGylated liposomes [121,122]. These antibodies specific to the formulation raise concerns about the efficacy and safety of PEGylated nanoparticle therapeutics [123]. Another obstacle is the inadequate accumulation of CRISPR/Cas9-loaded non-viral vectors in the targeted tissues. Cationic liposomes are known to enhance cellular association in vitro and facilitate intracellular endosomal escape, which are crucial for the function of CRISPR/Cas9. However, some studies have suggested that the cationic charges may lead to poor tumor penetration, non-specific accumulation, and increased uptake by macrophages [124]. On the other hand, neutral liposomes allow for deeper tissue penetration but sacrifice cellular uptake. For instance, a neutral liposome composed of lecithin, cholesterol, and 1,2-dioleoyl-sn-glycerol-3-((N-(5-amino-1-carboxylpentyl)iminodiacetic acid)succinyl)-(nickel salt) (DOGS-NTANi) was used to deliver the complex of recombinant Cas9 protein and sgRNA [125]. In order to enhance the encapsulation efficiency, a positively charged PEI 2 kDa was employed as a fusion material to counterbalance the negative charge of the Cas9-sgRNA complex. The sgRNA sequence was specifically designed to target the dipeptidyl peptidase-4 gene (DPP-4) with the aim of modulating the function of glucagon-like peptide 1 in the liver for the treatment of type II diabetes. Blood analysis indicated normal liver and kidney function, likely due to reduced drug dispensation. In general, both cationic and neutral liposomes demonstrated potential as effective delivery platforms, although further development is still necessary. Anionic liposomes were not preferred formulations due to their diminished transfection efficiency.

2.3 Gas filled Liposomes

Nitric oxide (NO), in addition to its vasodilatory effects, possesses antiatherogenic properties attributed to its anti-inflammatory effects, reduced plaque formation, and inhibition of platelet aggregation and adhesion. However, the systemic delivery of NO may result in unintended side effects due to its off-target effects. Huang et al. have filed a US patent to further investigate the potential of preparing liposomes containing NO. Gas-containing liposomes were created by combining liposomes with gas under pressure and subsequently subjecting the gas-liposome dispersion to freeze-thaw cycles. In certain cases, a second gas was also encapsulated to regulate the release rate of NO from the liposome. The amount of gas encapsulated is directly proportional to the pressure of NO. According to Henry's law, the uptake of gas by the liposomes is directly proportional to the amount of gas present in the solution. The freezing step in the preparation serves two purposes: to enhance the local concentration of dissolved gas and to form small pockets of bulk gas phase. This invention describes liposomes capable of encapsulating both NO and CO, which are actively targeted due to their diverse bioactivities. Subsequently, ultrasound imaging was employed to track the particles following in vivo administration. The gas filled liposome is illustrated in Fig 10.

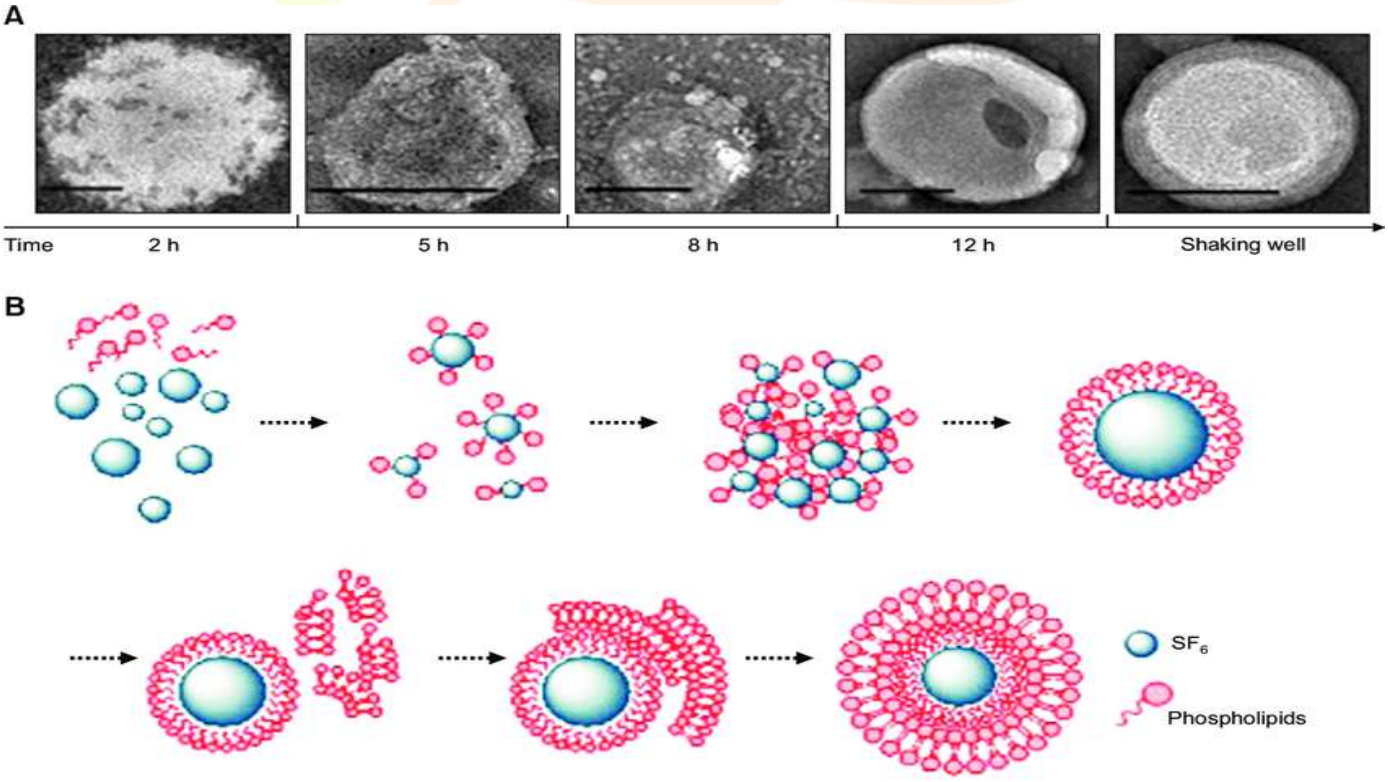

Fig 10. Gas-filled liposomes. (A) Transmission electron micrographs at different reaction time points. Bars=100 nm. (B) Schematic diagram of the hypothetical formation [124].

2.4 Liposome for immunotherapy

Immunotherapy is a powerful tool utilized in the battle against cancer, offering a wide range of possibilities. In order for it to be effective, the appropriate antigen must be delivered to antigen presenting cells and released at the appropriate locations. Both of these conditions must be met in order to induce cross-presentation and activate immunocompetent cells. Liposomes are

excellent candidates for use as antigen-presenting carriers due to their ease of functionalization [127]. Liposome-based treatment used in immunotherapy is shown in Fig 11.

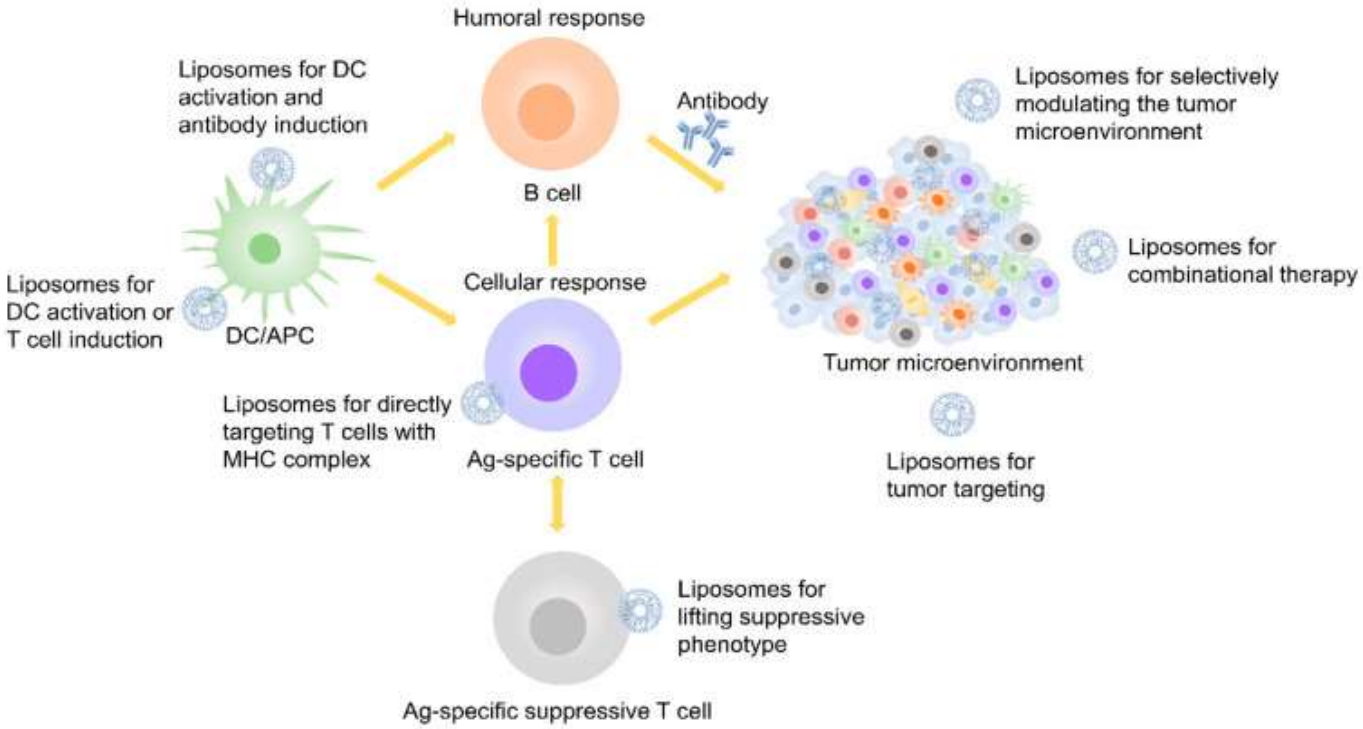

Fig 11 Liposome-based treatment used in immunotherapy [127].

The surface modification of liposomes with fusogenic or pH-sensitive components allows for the delivery of antigens to the cytoplasm, followed by cross-presentation via a "cytosolic pathway". Conversely, antigen escape in early endosomes leads to cross-presentation via the "vacuolar pathway" after recognition of surface receptors in antigen presenting cells [128] (Fig. 12). To enhance the immune-inducing properties of liposomes, adjuvants such as synthetic cationic lipids, Toll-like receptor agonists, or bioactive polysaccharides are incorporated. Systems that can reverse immunosuppression in the tumor microenvironment are also valuable tools for increasing the antitumor resistance of antigen-presenting delivery systems.

Furthermore, a comprehensive understanding of the immune-inducing mechanisms and the molecular basis of tumor immunosuppressive environments is crucial for the successful design of liposome-based antigen delivery systems.

2.4.1 Stimulation of interferon genes

The immune system possesses the capability to eradicate cancer cells within the body. The spontaneous immune response to cancer plays a significant role in impeding the growth of tumors in cancer patients, as the infiltration of pre-existing T cells into the tumor microenvironment proves effective. This infiltration of T cells is particularly advantageous for patients undergoing anti-tumor immunotherapy utilizing specific antibodies targeting T cell-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) [130]. Consequently, it appears probable that the activation of endogenous immune responses enhances cancer treatment. Although the precise mechanism by which the host initiates spontaneous cancer detection remains largely unknown, a definitive answer to this question is forthcoming. The primary mechanism through which the innate immune system detects cancerous cells is via the interferon gene pathway host simulator (STING) [131]. Recently, researchers from Harvard University demonstrated that the liposomal form of STING agonists (cGMP) can augment anti-tumor resistance. They employed positively charged liposomes to demonstrate the anti-tumor efficacy against lung metastases of melanoma. Liposomes have been utilized for the delivery of STING ligands and vaccines to the lymph nodes. The combination of these two components revealed that PEGylated liposomes containing cGMP were capable of reducing the size of melanoma tumors [132,133] (Fig. 13).

The STING technology was employed to administer aerosolized liposomal-cGAMP for the purpose of managing lung metastases in mice with lung metastases, as documented in reference [134]. STING detects the presence of cytosolic, cyclic dinucleotide (2′3’-cGAMP), which is produced by the cyclic-GMP-AMP (cGAMP) synthase (cGAS). This detection leads to the activation of the tank binding kinase 1 (TBK1)-interferon regulator factor 3 (IRF3) pathway. As a result of this pathway activation, activated antigen presenting cells (APCs) produce type I interferons (IFNs). The ultimate outcome of this activation is the priming of CD8+ T cells against tumor-specific antigens [130,131]. Based on this approach, the first commercial DNA immune stimulator product, ZelNate®, was introduced as a veterinary product in 2015. ZelNate® is used as a support in the therapy of bovine respiratory disease caused by Mannheimia haemolytica [135]. The ZEL product utilizes cGAS and STING to activate the IRF3 pathway. ZEL liposomes consist of cationic DOTIM and cholesterol, and pMB75.6 plasmid DNA entrapped inside. It is well-known that when plasmid DNA is complexed with, or encapsulated in positively charged lipid particles that involve endocytosis, residence in endosomes, and escape into the cytosol in the endosomal compartment, ZEL gains access to a cytosolic pool of DNA, followed by activation of specific pathways [136].

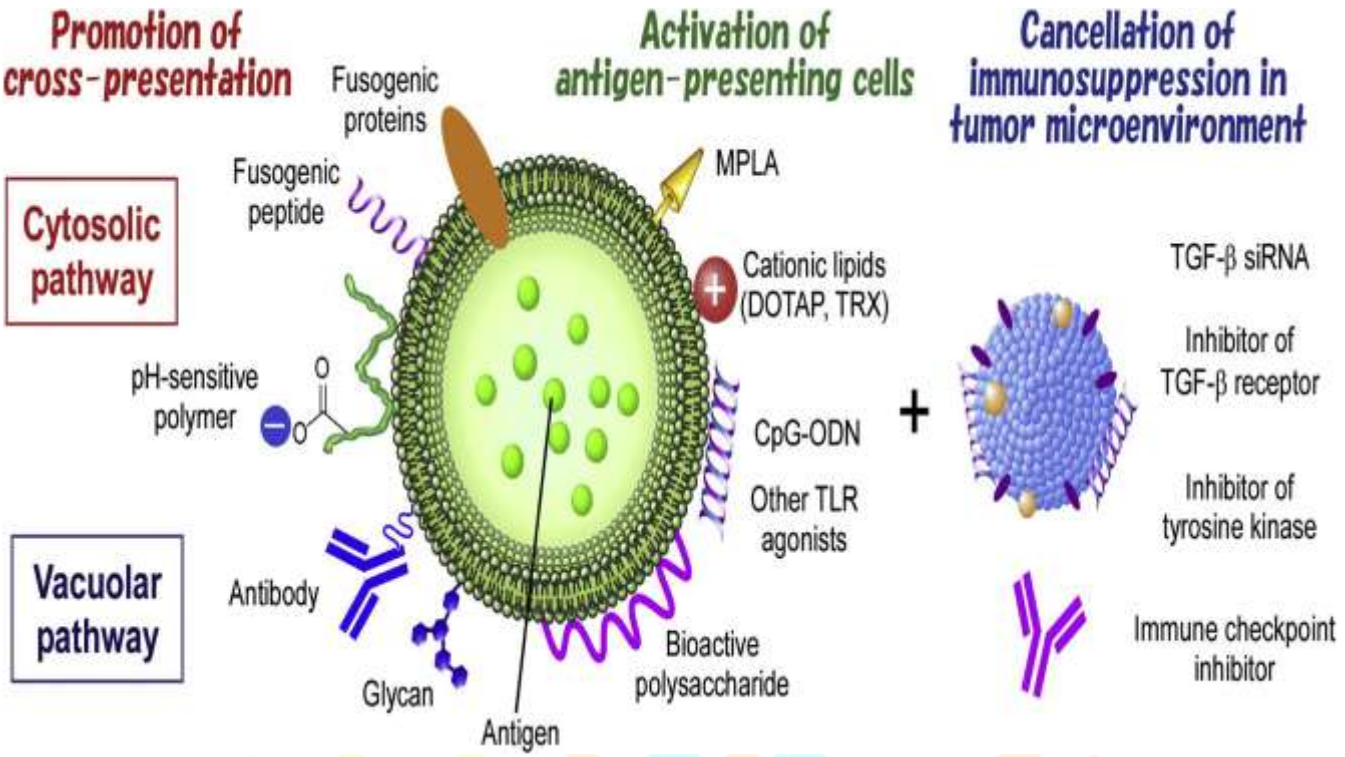

Fig. 12. Scheme of liposome-based antigen-presenting carriers for immunotherapy of cancers. [129].

2.4.2 Cancer vaccines

Therapeutic cancer vaccines have been developed with the aim of stimulating the immune system to combat cancer cells. However, the body's natural immune response against tumors is often ineffective. Furthermore, the specific activation of antigen-presenting cells (such as dendritic cells) by oncological vaccines poses technical challenges. These activated antigen-presenting cells play a crucial role in presenting antigens to effector cells (such as T cells), which then identify and eliminate tumor cells. It is worth noting that currently, the only oncological vaccine approved by the US Food and Drug Administration (FDA) for use against prostate cancer is Sipuleucel-T [137].

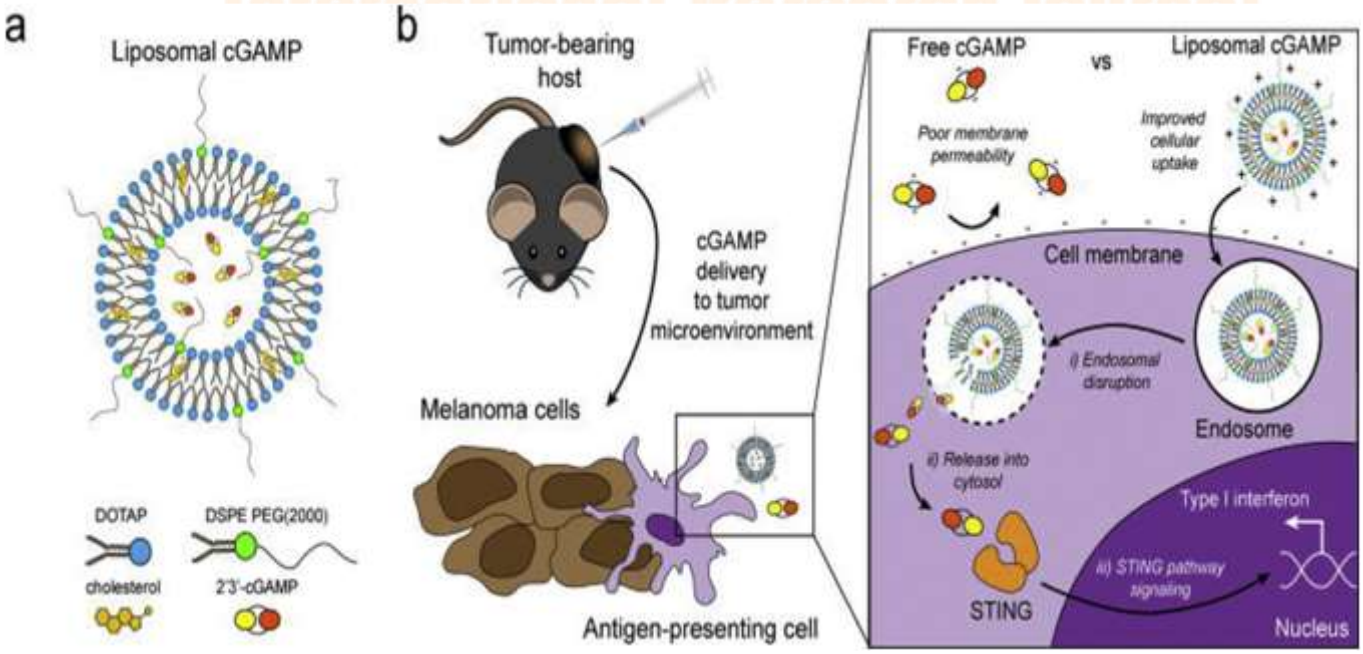

Fig. 13. Scheme of STING signal path activation. In the first stage, IFN type I and proinflammatory cytokines are produced, which then activate natural killer cells. Their activation leads to the penetration of NK cells into cancer cells and, as a result, their death. [132].

However, the administration of this vaccine necessitates the separation of the dendritic cells and their subsequent stimulation in vitro prior to re-administration. An alternative approach to activate dendritic cells involves the utilization of nanoparticles that are labelled with specific antibodies for these cells. Encouraging outcomes of oncological vaccines developed by the esteemed Jan Gutenberg University in Mainz, Germany, have been published in the prestigious scientific journal, Nature. The researchers have devised a straightforward method to activate dendritic cells without the need for their isolation from the body or the labelling of nanoparticles with antibodies. They employed liposomes, which are composed of commonly used lipids DOTMA and DOPE, with a specific particle size range of 200-400nm. These liposomes contained various RNA fragments that encode the tumor antigen and effectively stimulated the body's immune response. Upon intravenous administration, these liposomes, owing to their slightly negative charge, exhibited a superior and distinct affinity for the spleen and other lymph nodes where antigen-presenting cells (APC cells) are predominantly located. The efficacy of the anti-tumor vaccine was evaluated through tests conducted on murine models of lung cancer. As a result of vaccination, the mice exhibited a sustained activation of the specific immune response against tumor antigens, as well as a reduction in tumor volume. Furthermore, these vaccines have successfully passed safety tests conducted on murine and macaque models [138].

3. Analytical development in liposome

3.1 Characterization methods for size and zeta potential

The characterization of liposomes is of utmost importance as it is necessary to monitor physical and chemical parameters to ensure that the preparation of liposomes is reproducible and has the desired function. One of the most important parameters to monitor is size, as it can affect the functions of liposomes [139]. The physical stability of liposomes can be evaluated by analyzing their size distribution and polydispersity. A variety of methods, including electron microscopy, fluorescence microscopy, atomic force microscopy, field flow fractionation, dynamic light scattering, nanoparticle tracking analysis, flow cytometry, size exclusion chromatography, scanning ion occlusion sensing, centrifugal sedimentation, and differential scanning calorimetry, can be employed for the size analysis of liposomes. Furthermore, relatively new technologies for size and zeta measurement are also discussed.

Scanning electron microscopy is a commonly employed technique for the measurement of liposome size. These methods enable visualization of the liposomes and provide information on their lamellarity and morphology. Cryo-transmission electron microscopy (Cryo-TEM) is a specialized variant of transmission electron microscopy that allows for the visualization of liposomes in a frozen state. The liposome grid is placed in liquid nitrogen, which rapidly freezes the liposomes without causing crystal formation, thereby preserving their integrity. This technique enables the individual visualization of each liposome, providing detailed information on their size. However, it is unable to measure the hydrodynamic radii of PEGylated liposomes, which is crucial for size determination [140]. Additionally, this method can be considered laborious. Another method, freeze-fracture transmission electron microscopy [141], involves shock-freezing the sample on a carbon film grid in melting nitrogen. The sample is then inserted into a cryo transfer holder for imaging. This method offers the advantage of observing the sample without any structural disruption [139]. However, it requires a large amount of sample [40].

Field Flow Fractionation (FFF) is a gentle technique utilized for the separation of macromolecules and nanoparticles, such as liposomes [142]. The separation process takes place within a narrow, ribbon-like channel, where external electric fields are applied perpendicular to the carrier flow, facilitating the separation. The carrier flow moves from the channel's inlet to the detector, creating a parabolic flow profile within the channel. This perpendicular flow influences the liposomes, compelling them to migrate towards the membrane through their Brownian motion. Smaller liposomes, possessing higher diffusion coefficients and being further away from the channel wall, elute first. FFF is particularly useful for analysing highly polydisperse solutions with varying particle sizes, and its retention behaviour can be predicted. However, there are certain limitations associated with FFF, including the complexity involved in constructing the channel, the need for measuring spherical particles accurately, and the requirement for background correction of the UV detector [142].

The determination of the surface charge of liposomes is achieved through the measurement of their zeta potential. The charge of the liposomes can be either positive, negative or neutral, depending on their composition. The zeta potential plays a crucial role in maintaining the stability of the liposomal suspension by preventing aggregation. This is due to the fact that particles carrying charge repel each other. Liposomes with a zeta potential higher than 30 mV and less than -30 mV are considered stable. Laser doppler velocimetry is one of the techniques used for the rapid detection of the charge on the liposome, in addition to light scattering methods. In light scattering methods, the application of an electric field causes the movement of the liposomes due to their charge, resulting in a doppler shift in frequency that can be detected by the fluctuations in the detected light.

3.2 Quantification of liposomal drug release in plasma

Liposomal formulations of various drug candidates have been extensively developed for the treatment of different diseases in clinical settings. The significant alterations in drug pharmacokinetics and biodistribution distinguish liposomal drugs as distinct entities from non-encapsulated free drugs [143]. Upon intravenous administration into the bloodstream, drug molecules delivered by liposomes can be categorized into three distinct forms: liposome-entrapped drug, protein-bound drug, and free drug. The disposition of these three forms is closely linked to their pharmacokinetics, biodistribution, toxicity, and therapeutic effects. In 2018, the Food and Drug Administration of the United States (FDA) issued the Liposome Drug Products Guidance for Industry, which specifically highlights the importance of including both the free and total drug concentrations in plasma when assessing pharmacokinetic measures or parameters, as appropriate. Therefore, pharmacokinetic studies that solely analyze the total drug plasma concentration

for conventional dosage forms are not suitable for evaluating liposomal drugs. It is crucial for analytical development to monitor the pharmacokinetic profiles of both liposome-associated and non-liposomal drugs in plasma after administration [144].

The isolation of free and liposomal drugs in plasma has been conducted using various techniques, including ultrafiltration, ion-exchange chromatography, size-exclusion chromatography, solid-phase extraction (SPE), and capillary electrophoresis. However, it is important to note that all of these methods have certain limitations. For instance, ion-exchange chromatography has shown poor reproducibility and low recovery rates. Gel chromatography, on the other hand, requires high sample dilution and results in slow separation. Ultrafiltration is limited by the adsorption of drugs on devices, while SPE may encounter potential drug release issues during the separation process. Therefore, an ideal separation method should be fast, simple, and capable of high-throughput analysis to prevent drug leakage and ensure a high recovery efficiency. It is worth mentioning that the separation of protein-bound drugs poses an even greater challenge, as protein binding alters the properties of the drug and further restricts the application of separation methods such as ultrafiltration, ion-exchange chromatography, and SPE.

Several recent studies have successfully optimized the separation procedures by utilizing solid-phase extraction (SPE) for separation and liquid chromatography-tandem mass spectrometry (LC-MS/MS) for detection. Xie et al. have developed a modified SPE method to determine the concentration of non-liposomal and liposomal doxorubicin in plasma. They compared the results with an ultrafiltration technique [144]. By employing Oasis® hydrophilic-lipophilic-balanced (HLB) reverse-phase sorbents and pre-coating the sorbent with plasma, they demonstrated effective separation with excellent specificity and high sensitivity. The LC-MS/MS technique was used to quantify the concentration of free doxorubicin in plasma after separation. The lower limit of quantification (LLOQ) for the separation method was determined to be 3.13 ng/ml for non-liposomal doxorubicin and 0.156 µg/ml for liposomal doxorubicin. This SPE method based on HLB sorbents was also successfully employed to characterize the pharmacokinetic profiles of Doxil® after intravenous administration in canines.

In a separate investigation, a comparable solid-phase extraction (SPE) technique was employed in conjunction with liquid chromatography-tandem mass spectrometry (LC-MS/MS) detection to examine the levels of free and liposomal Amphotericin B (AMB) in rat plasma [150]. Free AMB exhibits greater lipophilicity than doxorubicin, resulting in increased protein-binding. To mitigate drug-protein interactions, rat plasma containing liposomal AMB was pretreated with 0.1% NH4OH in this SPE method. The HLB reverse phase cartridge was utilized, and the lower limit of quantification (LLOQ) for free AMB and liposomal AMB was reported as 0.2 µg/ml and 0.5 µg/ml, respectively. All separation efficiency data were precise within 5% in rat plasma spiked with 0.5–80 µg/ml of liposomal AMB concentration.

In summary, recent studies have developed an efficient and user-friendly solid-phase extraction (SPE) method for separating free drug and liposome-encapsulated drug in plasma. Successful separation requires pretreatment of the column with plasma to minimize sorbent adsorption. Depending on the lipophilicity and protein-binding affinity of the target drug molecules, it is necessary to consider the amount of protein-bound drug. To reduce the percentage of protein-bound drug without interfering with LC-MS/MS analysis, plasma samples can be incubated with a certain amount of NH4OH or formic acid. Additionally, to prevent liposomal breakdown during SPE separation resulting from the low osmotic pressure encountered in the cartridges, it is necessary to precondition plasma samples with a 5% glucose solution and pretreat the SPE sorbent with a 5% glucose solution.

4. Administration routes of liposome

The primary challenge faced by many treatments is not the absence of an efficient medication, but rather the difficulty in effectively administering it to the targeted pathological site. One of the main reasons for this is that drug molecules often encounter obstacles within the body, such as the blood-brain barrier, skin, mucous membranes in the small intestine, nose, and mouth. These natural barriers serve to filter out foreign substances before they can enter the body, which significantly affects most biologics, including recombinant proteins, antibodies, and gene therapies. Another reason is the immune response triggered by the host's body to eliminate therapeutic substances. Therefore, the most effective delivery system should aid in protecting drug candidates from the immune response and facilitating their passage through these physical barriers.

4.1 Oral administration

Liposomes have been utilized for parenteral drug delivery since their discovery. However, the oral administration of liposomes still presents challenges due to their low stability, weak penetration properties, and difficulties in bulk production. Currently, many of these issues have been resolved through the modification of the bilayer composition and the attachment of polymers or ligands to the surface. These modifications enhance the durability and permeability of liposomes, making them a valuable tool for oral administration [151]. Conventional liposomes are not resistant to the harmful effects of gastric acid, gall, and lipases in the gastrointestinal tract, which decrease the concentration of intact liposomes and promote cargo release. Additionally, conventional liposomes have poor permeability across enteric epithelia due to their large size and the presence of various barriers. Two primary pathways for liposome-based oral drug delivery have been proposed. In the first pathway, the drug is released in the gastrointestinal lumen or through the conversion of liposomes into mixed micelles, followed by the penetration of the drug through the intestinal epithelia [151]. The second pathway involves the uptake of liposomes by M cells located in the follicle-associated epithelium (FAE) of Peyer's patches [152]. Schematic presentation of enhanced oral absorption of biotin-decorated liposomes via ligand-mediated endocytosis following active targeting to intestinal epithelia is illustrated in Fig 14.

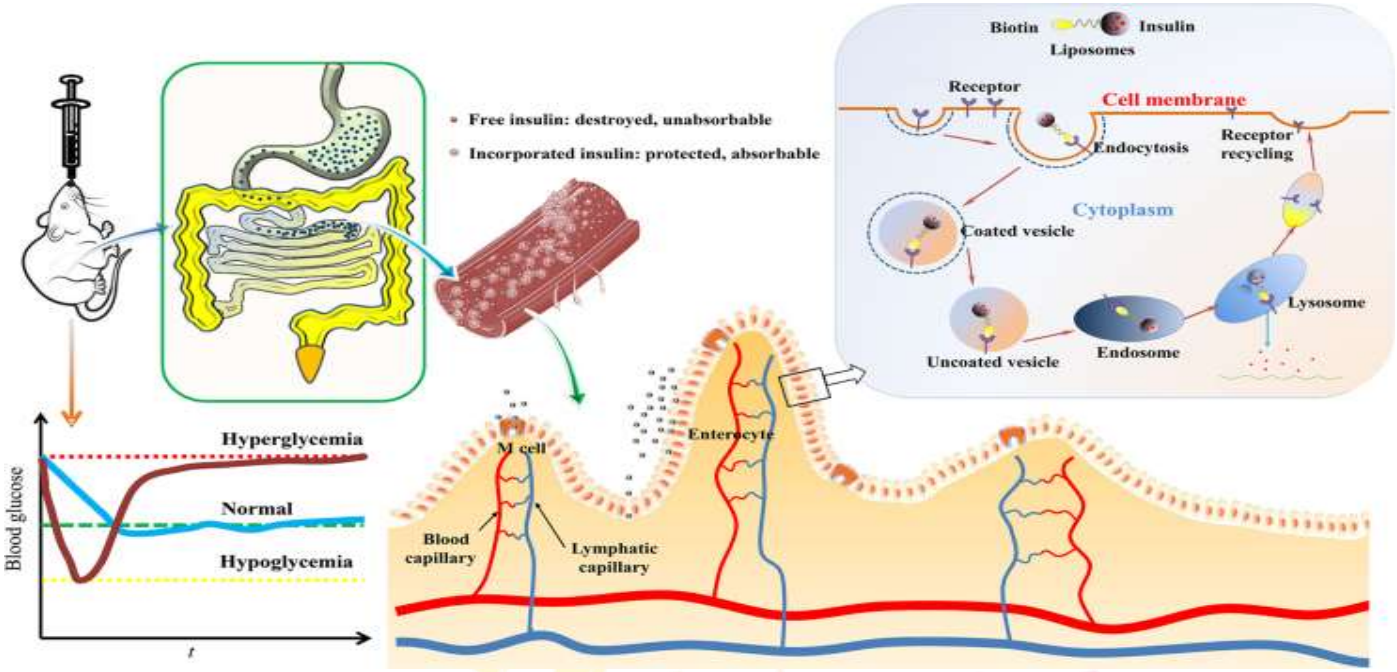

Fig 14. Schematic presentation of enhanced oral absorption of biotin-decorated liposomes via ligand-mediated endocytosis following active targeting to intestinal epithelia [152].

Several strategies have been employed to enhance the efficacy of liposomes as an oral drug delivery system. For instance, the addition of sterylamine to the bilayer composition alters the charge on liposomes, thereby inhibiting the digestion of cargo by trypsin. This was demonstrated in a study involving insulin [153]. Another commonly utilized approach is the surface coating of liposomes with polysaccharides such as pectin [154], chitosan [155], O-palmitoylpullutan [156], or polymers like Eudragit S 100 [157] to stabilize them. The use of chitosan, its derivatives, or other polysaccharides also enhances the mucoadhesion of liposomes, thereby prolonging their residence in the gastrointestinal tract. This extended contact with intestinal epithelia subsequently improves the oral absorption of liposomes or their cargo [158]. Additionally, the glycosylation of lipids and proteins present in cell membranes of the digestive tract has been employed to overcome the low permeability of traditional liposomes. This approach leverages the affinity of lectins for glycans [159]. Lectin-modified liposomes can also be taken up by M cells due to specific glycosylation patterns present in these cells. An advantage of this approach is that M cells are less protected by mucus, making them more accessible for liposomes [160]. A novel approach that combines pH-responsive gold nanoparticles with liposomes has been developed for the gastric delivery of antimicrobial agents. Chitosan-modified gold nanoparticles are linked to the external surface of anionic liposomes, ensuring their stability at gastric pH. However, at neutral pH, the gold nanoparticles detach from the liposomes, leading to rapid fusion of doxycycline-charged liposomes with bacterial membranes. Compared to free doxycycline, liposomes achieve a more effective antibacterial effect [161].

The study pertaining to liposomal oral delivery has experienced significant growth; however, there remains a need for substantial advancements in order to effectively develop and market these products for clinical application. The primary challenge in formulating successful oral liposomes lies in the limited comprehension of the mechanisms governing their absorption within the gastrointestinal tract.

4.2 Transdermal administration

The three primary obstacles to local and transdermal delivery are the skin itself, including the cuticle and dermis, the skin surface, and the hypodermic tissue with its systemic circulation. Disinfectants, insect repellents, and cosmetics are primarily applied to the skin surface. When the illness condition is located in the organ itself, such as tumors, inflammatory disorders, and microbial infections, targeting the different stratum of the skin remains crucial. The local approach provides direct access to targets located within several microns below the skin surface, resulting in prolonged effects and higher effectiveness, reducing the therapeutic dose and risk of side effects compared to parenteral administration. Transdermal administration, in which the subcutaneous tissue or systemic circulation is the primary focus, is an alternative to systemic and oral routes of administration. The transdermal route offers numerous advantages over the oral route, including avoiding degradation by a gastrointestinal environment, avoiding first-pass systemic metabolism, and high patient compliance. However, transdermal delivery of hydrophilic and high molecular weight drugs has its limitations. The first limitation is permeability through the stratum corneum (SC), which consists largely of lipids and represents a penetration barrier for hydrophilic molecules. The second limitation is the low skin partition coefficient (<< 1), which leads to non-absorption of drugs by the skin [162,163]. The utilization of nanotechnology has been employed to facilitate the delivery of substances through the skin, enabling topical administration, enhancing drug penetration, and protecting the drug from degradation. Liposomes were initially utilized as carriers for transdermal delivery starting in 1980. However, traditional liposomes typically remain on the skin's surface due to their limited ability to penetrate [164,165] In 1992, deformable liposomes, also referred to as elastic or transferosomes, were introduced [166]. These liposomes possess self-adaptive properties and high flexibility, allowing them to effectively penetrate the barrier of the stratum corneum in comparison to other nanoparticles of similar size.

Elastic liposomes have been utilized as topical carriers for zinc phthalocyanine (ZnPc) and the nitrosyl ruthenium complex {Ru (NH.NHq) (tpy)NO}3+ (RuNO) as a photosensitizer. These liposomes were composed of dioleylphosphocholine (DOPC) as the phospholipid and Tween 20 as an edge activator [167]. Transferosomes have also been employed to transport nonsteroidal anti-inflammatory drugs (NSAIDs) such as diclofenac and ketoprofen (Transfenac® and Diractin®, respectively) [168]. Other anti-inflammatory drugs that have been delivered by elastic liposomes include piroxicam [169] and lornoxicam [170]. Ultra-deformable liposomes have also been used to deliver acne treatments, such as transferosomes made up of phosphatidylcholine and Tween 80 with tretinoin [171]. Elastic liposomes have also been utilized for the topical delivery of anti-infective drugs. Neomycin sulfate [172], clotrimazole [173], and metronidazole [174] can be efficiently entrapped in this type of liposome and released at the target site. Most of these drugs can be administered using transferosomes to increase the efficacy of the drug for localized action, such as insulin [175], curcumin [176], methotrexate [177], and loratadine [178]. The use of elastic liposomes helps to address drug-associated physicochemical and clinical issues, such as low aqueous solubility, skin irritation, gastrointestinal after-effects (namely vomiting, nausea, and diarrhoea), and drug accumulation in bone marrow when orally administered [179]. Despite the potential of ultra-deformable liposomes to address numerous issues related to topical drug delivery, there are several challenges that must be overcome. These challenges include ensuring the storage stability of the liposomes, maintaining their stability after application to the skin, preserving their intact structure within the deeper layers of the skin, and developing suitable lipid compositions that balance both flexibility and stability requirements. Liposomal transdermal delivery is shown in Fig 15.

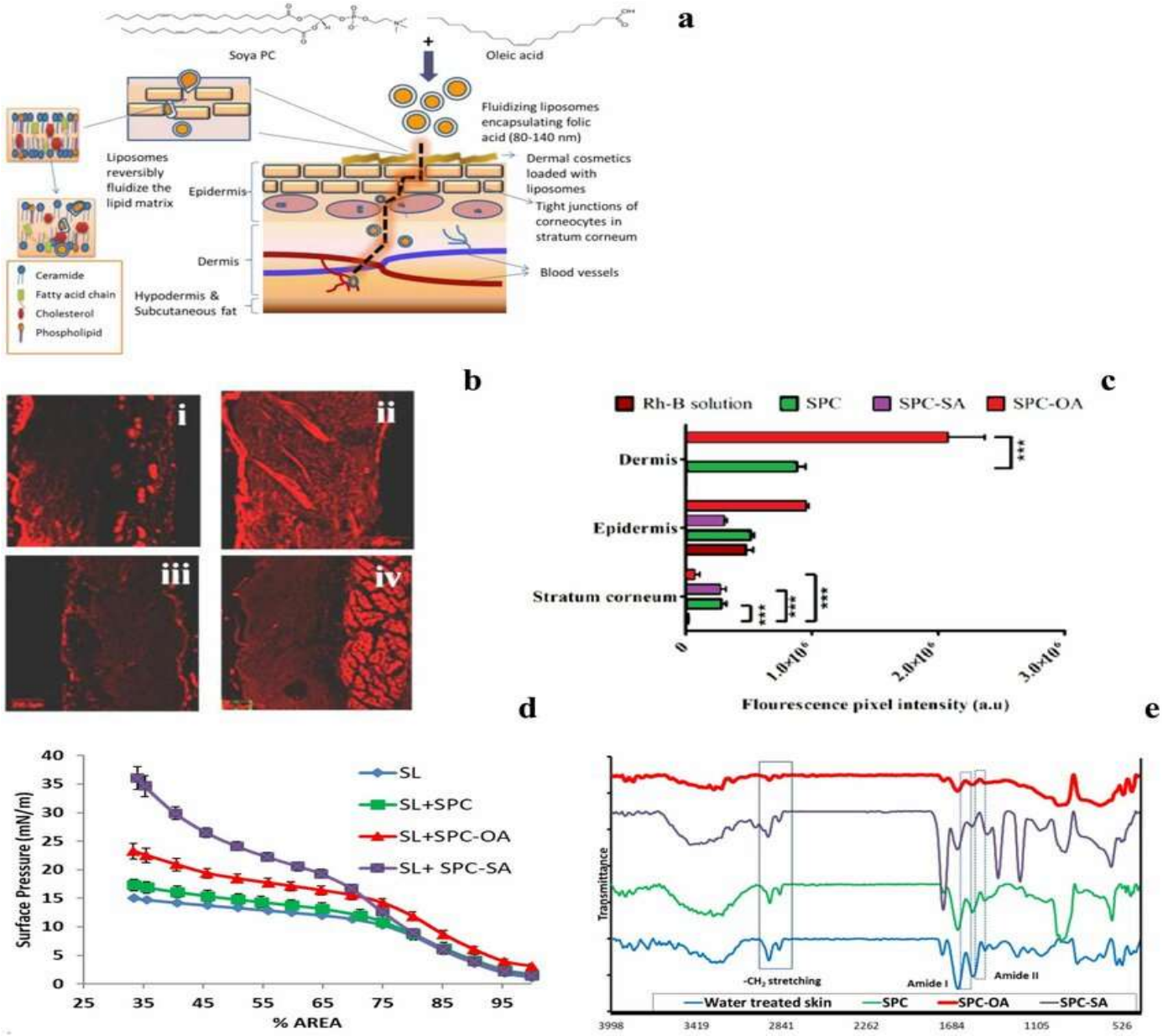

Fig 15. Liposomes for Transdermal delivery. (a) Schematic representation of Fluidization of stratum corneum by folate loaded liposomes incorporated in cosmetic base. (b) Confocal laser microscopy of rat skin treated with Rhodamine-B:i-Rh-B solution(control), ii-SPC, iii-SPC-SA, iv SPC-OA. SPC, SPC-SA, SPC-OA liposomes encapsulating Rhodamine-B, Scale bar = 200 µm, SPC shows follicular penetration, SPC-SA does not show any penetration in dermis, SPC-OA shows highest penetration beyond dermis. (c) Fluorescence intensity graph showing depth of penetration of Rh-B loaded liposomes ***P < 0.001 by student t-test. SPC-SA shows no dermis penetration, SPC-OA shows maximum penetration into the dermis. (d) Surface pressure-area isotherm of SL, SPC, SPC-OA and SPC-SA mixture at 37 °C. (e) ATR-FTIR spectra of blank rat skin (untreated) and post treatment with SPC, SPC-SA and SPC-OA liposomes representing molecular shifts in lipid chains of stratum corneum, kinked structure of oleic acid can efficiently disrupt the ordered packing temporarily and diffuse across the lipid bilayers of stratum corneum. (b-d) n = 3. Mean ± SD. [179].

4.3 Delivery across the blood brain barrier

The brain is an extremely delicate and sensitive organ, which is why it is protected from the bloodstream by a dynamic physical and biological barrier that plays a crucial role in regulating its internal environment. This barrier, known as the blood-brain barrier (BBB), serves to safeguard nerve cells against various harmful factors such as viruses, bacteria, and toxins. Additionally, it restricts the passage of most foreign substances, including anti-cancer drugs, antibiotics, and other treatments for neurodegenerative diseases, from the blood to the brain [180]. The unique structure and properties of the BBB prevent 98% of small particles and 100% of large particles from reaching the brain [181]. The blood-brain barrier is composed of a single layer of polarized squamous cells that line the inner surfaces of the brain's capillaries. These cells are constructed from glycosaminoglycans linked to proteins and cell membrane lipids. Within the barrier, there are numerous membrane receptors, enzymes, and specific proteins that facilitate the transport of glucose and amino acids from the blood to the brain. The tight connections between these cells create an additional barrier that prevents the diffusion of ions and molecules into the intercellular space. However, the presence of pores allows for the selective passage of many compounds. Liposomes transport through blood brain barrier is illustrated in Fig 16.

4.3.1 Glioma treatment with liposomal preparations

Glioblastoma multiforme (GBM), also known as an "octopus tumor" due to its ability to spread cancer cells to adjacent tissue, is an inoperable and therapy-resistant disease that inevitably leads to the death of the patient, usually within 15 months of onset. In the United States alone, GBM kills approximately 15,000 people each year. One of the primary challenges in treating GBM is the blood-brain barrier, a network of blood vessels that allows nutrients to reach the brain but blocks access to other substances. Therefore, there is a need for efficient methods of transporting drugs across this barrier. In this context, liposomes offer an ideal and safe drug-carrier system due to their lipid bilayer, which resembles the plasma membrane of the cell. Additionally, liposomes can incorporate both hydrophilic and hydrophobic substances, making them a versatile drug delivery option. The penetration of the blood-brain barrier (BBB) can be facilitated through the injection of layered liposomes that contain epirubicin in the aqueous layer, tamoxifen in the lipid bilayer, and transferrin attached to the polyethylene glycol (PEG) chain. The selection of these liposome components was based on the fact that transferrin receptors are located on the BBB and on the surface of glioma cells, and that tamoxifen acts on P-glycoproteins belonging to multi-drug resistance proteins. The amount of cytostatics that penetrated the BBB from this multi-component liposome was 52.2%, which is twice as high as that of free epirubicin. In a mouse model, intravenous administration of layered liposomes with 92% cytostatics, 34 mg tamoxifen / mmol lipids, and 123 mg transferrin / mmol lipids at a dose of 5 mg / kg resulted in an increase in median survival up to day 23, while for free epirubicin it was only 15 days [183]. A similar approach to enhance BBB penetration utilizes liposomes with CB5005 peptide, which is used as a cell membrane penetration enhancer and NF-κB inhibitor. The PEG-peptide conjugate was used to make doxorubicin-bearing PEGylated liposomes. In vivo research has shown that these liposomes can cross the BBB and reach tumor cells [184].

The utilization of hyperthermia as a supplementary treatment for glioma, in conjunction with systemic chemotherapy, is now considered a promising approach. There is evidence indicating that several chemotherapeutic agents exhibit enhanced antitumor activity when exposed to appropriate levels of heat (40-44 °C). Hyperthermia can be induced for clinical purposes through the use of an alternating magnetic field in combination with magnetic nanoparticles, such as thermosensitive magnetoliposomes containing doxorubicin and iron oxide in the form of magnetic nanoparticles. These magnetoliposomes can be rapidly heated to 43 °C, which is the phase transition temperature of the lipid used, within a matter of minutes. During this process, doxorubicin is released in a sustained manner. In vivo experiments have demonstrated that magnetic drug targeting yields a potent anti-glioma effect, leading to complete remission of the tumor. This drug delivery strategy holds the potential to offer clinical advantages when compared to currently available methods [185]

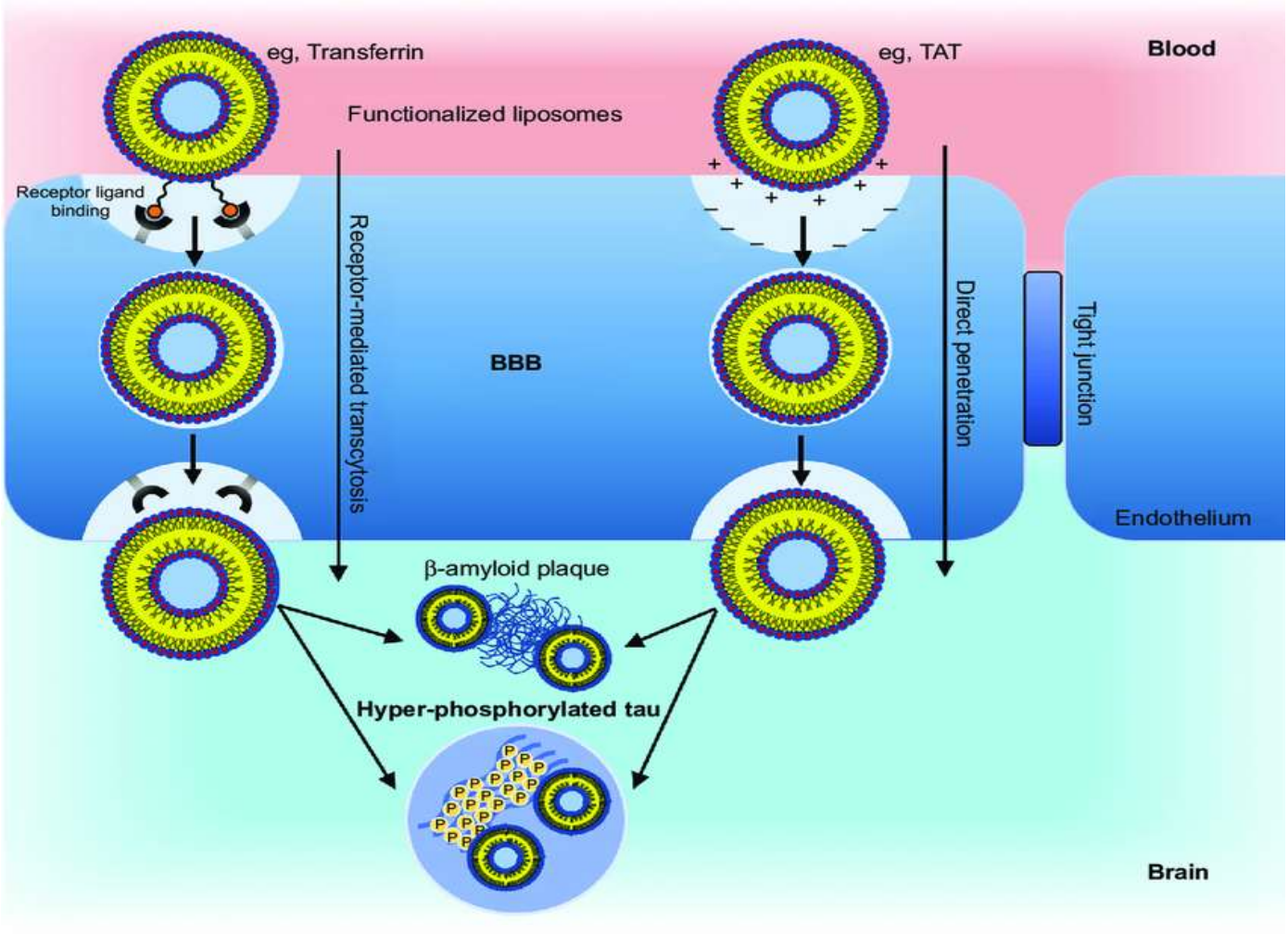

Fig 16 Promising liposomal BBB transport mechanisms [180].

4.3.2 Liposomal drugs to treat the Alzheimer's disease

The main issue in the management of Alzheimer's disease lies in the challenge of effectively transporting active substances through the blood-brain barrier. Numerous approaches have been devised to address this concern. Among them is the direct administration of medications to the brain. Nevertheless, it is only through the utilization of nanotechnology that significant advancements have been made in the development of methods for precise delivery of treatments using carriers such as liposomes or polymer nanoparticles [186].

One therapeutic approach in the treatment of Alzheimer's disease has involved reducing the presence of amyloid β (Aβ) in the peripheral blood. This intervention aims to prevent the accumulation of Aβ in the small arteries and capillaries of the brain, thereby inhibiting the formation of senile plaques. The impact of liposomes containing phosphatidic acid (PA-LIP) and cardiolipin (CL-LIP) on the levels of β-amyloid in plasma, blood, and the brain was investigated. It was discovered that structures containing PA or CL can directly or indirectly influence the distribution of Aβ and modify brain metabolism. Both types of liposomes facilitated the reduction of β-amyloid levels in the peripheral blood. This effect was attributed to the rapid interaction between phosphatidic acid and Aβ, leading to its subsequent elimination by liver and spleen macrophages. Additionally, both phosphatidic acid and released cardiolipin bound to amyloid β, resulting in the removal of the resulting complex from the brain to the peripheral blood. This therapeutic outcome serves as a significant illustration of the application of liposomes in the treatment of Alzheimer's disease [187].

An additional promising approach for the treatment of Alzheimer's disease involved the development of multifunctional liposomes known as mApoE-PA-LIP, which were tested on mice. The objective of the study was to enhance the liposomes' ability to cross the blood-brain barrier and hinder the formation and breakdown of large Aβ aggregates into smaller fragments. The liposomes' surface contained protein receptors (mApoE) that bound to specific sites on brain endothelial cells, enabling them to traverse the blood-brain barrier. The mApoE-PA-LIP liposomes successfully reached the brain without damage and accumulated in the hippocampus. They effectively reduced the presence of amyloid plaques in the brain by binding phosphatidic acid to Aβ and preventing its aggregation into senile plaques. However, it is important to note that treatment with mApoE-PA-LIP did not eliminate the underlying causes of Aβ overproduction, but rather slowed down the process of neurodegeneration in transgenic mice [188].

The blood-brain barrier (BBB) serves as a protective barrier against the entry of toxins and drug molecules. However, certain arrangements within the BBB allow for the transport of necessary substances like glucose, which is essential for proper brain functioning. These substances are able to cross the BBB through a receptor-mediated pathway. This characteristic can be utilized to successfully deliver drugs to the brain. To achieve this, liposomes composed of cholesterol and soybean phospholipid were loaded

with coumarin 6 and modified with glucose by incorporating PEG-glucose conjugates of varying chain lengths into the lipid bilayer. The results of this study demonstrated that liposomes with a medium-chain PEG were found in higher concentrations in the brain compared to peripheral organs. Conversely, liposomes prepared with short-chain PEG showed higher accumulation in peripheral organs rather than the brain itself. In the latter case, glucose was not present on the surface of the liposome due to the insufficient length of the PEG chain, rendering the liposomes unable to cross the BBB via the glucose transporter. On the other hand, longer chain PEG exhibited some elasticity, causing a fold of PEG on the surface of the liposomes, which reduced their ability to interact with glucose receptors and subsequently cross the BBB. These findings highlight the importance of not only selecting the appropriate receptor but also ensuring its exposure on the surface of the drug carrier. Various other strategies have been employed to target the BBB, including the use of modified liposomes. One such example is G-Technology®, a well-established, patented, and FDA-approved technology that has been proven to enhance brain targeting. This approach involves PEGylation of liposomes combined with glutathione, which is able to cross the BBB via a sodium-dependent transporter.

The intranasal route of administration provides an alternative method for directly delivering a drug to the brain, bypassing the blood-brain barrier (BBB). This approach avoids the initial metabolism and enzymatic degradation of liposomes, while also offering a larger surface area for drug absorption and direct delivery to the brain via the olfactory pathway. However, there is a need for improvements in the absorption of hydrophilic molecules and the ability to target specific drugs. Liposomes containing EPC69, DMPC-PEG2000, cholesterol, and H102 peptide have been effectively utilized in the treatment of Alzheimer's disease. H102 is a novel molecule that inhibits the formation of β-amyloid plaque by disrupting the β-sheets responsible for amyloid β accumulation. The liposomal formulation of this peptide, when administered intranasally, has demonstrated prolonged drug release and increased accumulation in brain tissue in vivo [191].

4.3.3 Liposomal drugs to treat the Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative disorder that affects approximately 1–2 individuals per 1000 globally. The primary cause of PD is the degeneration of dopaminergic neurons (DA) located in the substantia nigra (SN) of the midbrain. It has long been believed that PD occurs as a result of oxidative stress, which leads to the formation of unstable free radicals that contribute to the death of nerve cells. While various therapeutic strategies are available for the treatment of Parkinson's disease, their effectiveness is limited. The challenges faced in treating PD, two particularly problematic ones include the delivery of therapeutic agents across the blood-brain barrier to targeted brain tissue and the adverse effects associated with long-term administration. Extensive research is currently being conducted on various drug delivery systems, such as micelles, dendrimers, liposomes, niosomes, and other nanoparticles, for the purposes of diagnosis and therapy [192]. One approach to treating Parkinson's disease involves the delivery of dopamine HCl encapsulated in charged liposomes. This approach offers the advantages of effectively delivering the therapeutic cargo to the brain through passive means and protecting the drug against degradation in animal models [193]. Another approach utilizes liposomal tyrosinase delivery directly to the tumor, taking advantage of tyrosinase's ability to increase dopamine levels in brain tissue by providing L-tyrosine. Liposomes containing glutathione have also been developed for the treatment of PD, with the goal of maintaining proper glutathione levels in mesencephalic neuronal cells to protect them [194]. Recently, L-DOPA, a classic anti-Parkinson's drug, has been encapsulated in chlorotoxin-modified liposomes. Chlorotoxin has been used as a targeting moiety in stealth liposomes to efficiently deliver the therapeutic cargo in animal models [195].

Glial cell line-derived neurotrophic factor (GDNF) has also been utilized in the therapy of Parkinson's disease. The administration of liposome-encapsulated GDNF through the intranasal route has demonstrated neurotrophic effects in the substantia nigra and has provided protection against neuronal degradation in the specific animal model of this disease [196]. Another successful approach for the treatment of Parkinson's disease involves gene therapy, wherein the cDNA and gDNA of the tyrosine hydroxylase gene are delivered in combination with GDNF. The intravenous administration of these Trojan horse liposomes has proven to be an effective strategy for delivering the treatment to the brain, as they actively target the rat transferrin receptor [197]. Similarly, PEGylated liposomes containing DNA have been developed for transvacuolar gene therapy of Parkinson's disease. Furthermore, monoclonal antibodies specific to blood-brain barrier receptors, such as insulin and transferrin receptors, have been attached to these liposomes to achieve active targeting of brain tissue. This approach has successfully restored tyrosine hydroxylase activity in the brains of rats [198]. Recent advancements in the treatment of Parkinson's disease involve combining multiple strategies into a single treatment. An example of this approach is the combination of ultrasounds and liposomes containing GDNF and nuclear receptor-related factor 1 (Nurr1), along with microbubbles. The liposomes coupled with microbubbles (PLs-GDNF + Nurr1-MBs) have been shown to increase the number of dopaminergic neurons in the brains of rats. Therefore, the delivery of PLs-GDNF + Nurr1-MBs into the brain through focused ultrasound appears to be a promising strategy for the treatment of Parkinson's disease [199].

5. Scaling up of liposome production

At present, the production of liposomes on a laboratory scale does not pose a significant challenge. Nevertheless, there are only a limited number of techniques available for mass production. Despite their availability, the utilization of these techniques is constrained by various limitations pertaining to the encapsulation of delicate molecules, as they are susceptible to mechanical and/or chemical stress [200]. The characteristics of liposomes exhibit considerable variation depending on the lipid composition employed. However, regardless of the composition, liposomes can be prepared using the same method. The standard procedure for liposome preparation involves the hydration of a lipid film and subsequent sizing to achieve a uniform population of vesicles.

Numerous variations of this technique have been developed. However, despite the various modifications possible, it is important to consider that lipids associated with a negative or positive charge will form smaller vehicles with fewer bilayers. Additionally, the characteristics of the aqueous phase, as well as the power and energy input of agitation, will influence the final product. Most of the liposome preparation methods developed in the 1980s and 1990s paid little attention to the large-scale production of liposomes. Currently, the focus of formulation advancement is primarily on mass manufacturing. Strict control of the product is required by pharmaceutical regulations, including features such as sterility, purity, and quality. Until now, no general, large-scale liposome production method has been successfully established, mostly due to technical difficulties in fine-tuning liposomes with various properties and applications. The choice of the production method often depends on liposome composition, size, size distribution, and the drug release pattern. All of these factors together determine the pharmacokinetic demonstration of adsorption, distribution, metabolism, and elimination (ADME) [200,201].

Up until now, the methods used to scale up liposome production have adhered to established techniques such as alcohol injection or crossflow technique. These methods involve precipitating dissolved lipids into an antisolvent, typically an aqueous solution. This process takes advantage of the local solubility of the lipids, which changes during the process and leads to the spontaneous formation of liposomes. The location of the Active Pharmaceutical Ingredient (API) within the liposome can vary depending on its characteristics, either encapsulated within the liposome core or within the lipid bilayer. The principles behind the injection and crossflow methods make them suitable for conversion into continuous production methods. Both methods rely on maintaining a continuous flow of feed streams to produce liposomes. These types of liposome production methods show great promise in transitioning to a continuous process, primarily due to the ease of maintaining sterile conditions. However, despite their potential, there are several challenges that need to be addressed before these methods can be approved by the pharmaceutical industry. These challenges include improving the formulation, constructing the system, and selecting appropriate materials to ensure the maintenance of sterility [201].

6. Summary

Liposomes are considered to be highly desirable drug delivery systems due to their versatility and wide range of applications. They are capable of accommodating various excipient compositions and chemical modifications, and have been safely administered through multiple routes to deliver different types of drug modalities. Since their discovery, the technology of liposome production has undergone significant modifications through the development of novel lipid components and preparation procedures. Despite the existence of several FDA-approved liposome-based therapeutics on the market, with more under active development, the clinical needs of liposome-based therapeutics have not yet been fully met. Production hurdles include reproducibility between batches, low entrapment in some drug candidates, effective sterilization methods, on-shelf stability, and most importantly, scale-up for clinical use. The production of multifunctional liposomes remains a challenge for the industry, making medical-purpose liposomes expensive. However, classic methods such as transmembrane gradients and new methods such as microfluidization and spray drying have improved encapsulation efficiency and reproducibility, with significantly effective size control. Recent technological progress provides hope that justifies further development of liposomes and nanomedicine in general as drug delivery systems.

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