Drug delivery systems incorporating bile salts: advancements since the conception of bilosomes

Abstract

This review explores the advancements in drug delivery systems that incorporate bile salts since bilosomes that were developed over twenty years ago. Bile salts, recognized for their unique amphiphilic properties, have emerged as versatile agents in enhancing solubility, stability, and bioavailability of various therapeutics. We discuss the innovative formulations developed, including micelles, liposomes, and nanoparticles, that leverage bile salts to facilitate targeted and sustained release. The review also highlights the mechanisms by which bile salts improve drug absorption, particularly for hydrophobic compounds, and examines the evolving regulatory landscape surrounding these systems. Furthermore, we address challenges faced in clinical translation and future directions for research, emphasizing the potential of bile salt-based systems in personalized medicine. Our evaluation highlights the significant role of bile salts in advancing drug delivery technologies and their promise for improving therapeutic outcomes.

Key words: Bilosomes, drug delivery systems, bile salts, nanocarriers, surfactants, immune response

1. Introduction to bilosomes

It has been over two decades since the “bilosome” was first described by researchers at the University of Strathclyde for the purpose of oral immunisation of protein-based antigens (1). Since then, these bile-salt stabilised lipid nanoparticles (LNP) have been evaluated in a diversity of applications: treatment of infectious diseases, non-infectious

diseases and conditions, with modifications for specific disease states and transition across different physiological barriers (2). In general, bilosomes have been designed to provide protection for their contents and/or to enhance penetration through membranes to enable localised delivery. The diversity and modifications have recently been comprehensively reviewed (3). Their advantage in terms of stability is also realised as following oral administration of conventional carriers such as liposomes or niosomes, bile salts present in the intestine trigger the early release of the loaded drugs before they reach their target site. The inclusion of bile salts in bilosome membranes increase the stability of these vesicles and overcome the determinantal effects of bile acids present in the intestinal environment (4, 5). Figure 1 schematically describes the main structural elements of bilosomes.

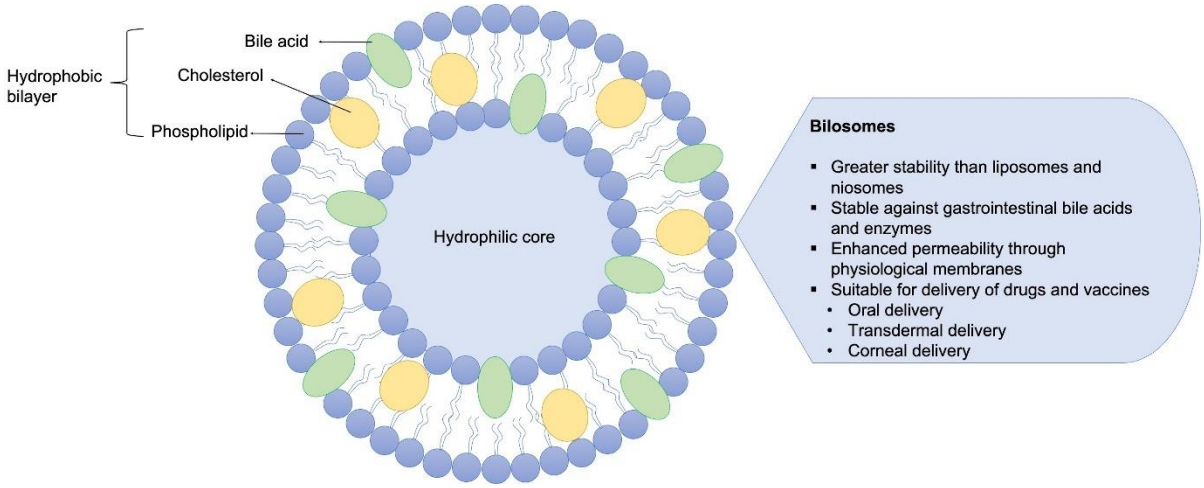


Figure 1. Schematic diagram of the bilosome structure and main features.

The aim of this review is to capture the progress that has been made with this platform technology and to examine the applications, whether experimental or commercial that have arisen from the original concept of oral administration for vaccines. Certainly, a

quick glance at the literature shows that modifications have enabled transdermal and ocular applications, thus demonstrating the versatility of these LNP.

2. Benefits of bilosomes over other vesicular systems

Bilosomes possess several advantages compared to other carrier systems. These vesicular systems are composed of non-ionic surfactant or niosomes, with the addition of bile salts. This unique structure provides higher stability compared to liposomes since the non-ionic surfactants are more stable than the phospholipids present in liposomes (6). The distinguishing advantage that bilosomes bring is related to the presence of added bile salts which provide significant stability in the presence of gastrointestinal (GI) bile salts and enzymes, enabling oral administration of these vesicles (7, 8). The mechanism by which they have high stability within GI fluids is related to the arrangement of bile salts within the membrane which cause the vesicles to repulse intestinal bile salts in the GI tract (9). Thus, they offer a viable option for oral vaccination as loaded antigens are protected from degradation in the stomach thereby eliminating the need for invasive administration by injections (10, 11).

In addition, bilosomes have adjuvant properties that allow for a small quantity of antigen to be more effective and also improve the efficacy of antigens that are weak when administered by injection (12). Bilosomes allow for the oral administration of smaller protein/peptide derivatives of pathogens and non-pathogen based antigens resulting in safer and more effective vaccination compared to traditional vaccines. In addition, oral vaccination provides the opportunity for self-administration thus lowering costs compared with nasal or immunization by injection which require trained personal for administration. Since the vesicles can be manufactured into different sizes the

intended immune response can be manipulated to achieve specific responses (eg Th1, Th2 or mixed) as desired.

Furthermore, bile salts can also increase the permeability of bilosomes by acting as penetration enhancers through the intestinal membrane resulting in improved oral bioavailability. Likewise, bile salts enable vesicles to pass through the intercellular lipids of the subcutaneous layer and then to the deeper layers of the skin in transdermal applications. Bile salts also help in the uptake through dermal blood vessels leading to systemic absorption (13). Their nanometre size also allows for effective transdermal delivery of various types of medications though enhancing their penetration and permeability through the skin (14). Sodium deoxycholate, which is a negatively charged bile salt used in bilosomes imposes a fluidizing effect on the vesicles thus enhancing the transdermal delivery (15). Due to these unique properties of intestinal stability and plasticity, bilosomes provide an effective platform for a wide range of therapeutics (16).

Their increased stability compared to other drug delivery carrier such as liposomes removes the requirement of cold-chain storage at very low temperatures. This results in efficient storage and easier transport of bilosome-based vaccines compared to other formulations. In addition, bilosomes also possess low vaccine or drug leakage during storage with higher loading capacity compared to other nanocarriers (16, 17). In fact, the unique structure of bilosomes enhance the adsorption and thus the permeation of drugs or vaccines loaded into these nanostructured lipid carriers through various biological membranes, such as the intestine, cornea, and skin (18, 19). While they do not exert toxic effects or tissue damage following mucosal administration (oral and

topical administration) (20), it is important not to use them in subcutaneous or intramuscular injections as tissue damage can occur.

3. Manufacturing bilosomes from laboratory to industrial scale-up

The original recipe for preparation of bilosomes was based on non-ionic surfactant vesicle (NISV) or niosome methodology, which had already been established for parenteral drug delivery and vaccine strategies. This involved the usual application of techniques, ranging from component selection to advanced size-reduction methodologies. Much work has been carried out into the development and optimization of NISV, particularly for poorly water-soluble drugs (21).

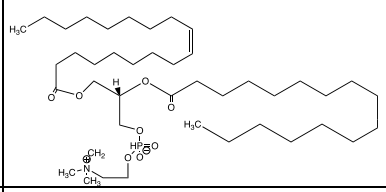
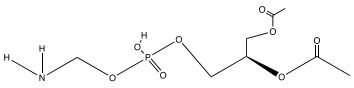
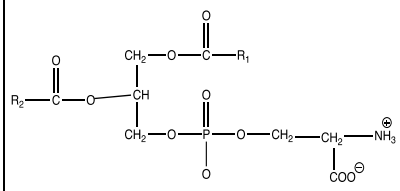
3.1 Composition of the vesicles

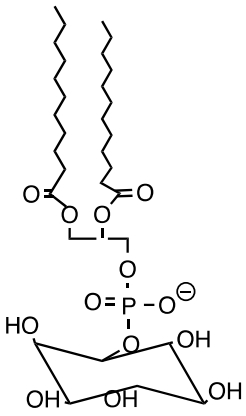
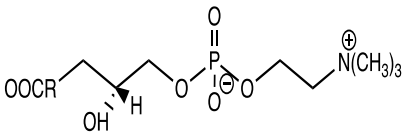
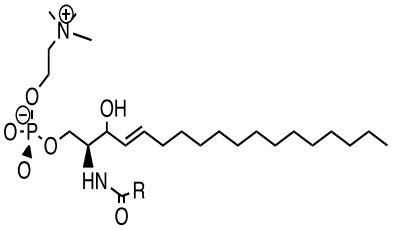
Component selection is a crucial step as it influences the subsequent properties of bilosomes, but broadly speaking phospholipids, cholesterol and surfactants make up the major components.

3.1.1 Phospholipids play a crucial role in the formation and structure of bilosomes, influencing their stability, drug encapsulation, and interaction with biological membranes. Their choice significantly affects the properties of these vesicles, and some commonly used phospholipids are summarized in Table 1.

Phospholipid composition in bilosomes can be tailored to enhance functionality and safety, aligning with encapsulation targets. This ensures optimized properties for intended applications, aiming to mimic biological membranes for effective drug delivery.

Table 1: Contribution of different components in bilosomal membranes

Phospholipid	Chemical structure	Role in bilosomal membrane	Ref
Phosphatidylcholine (PC)		Stability and offers biocompatibility. Widely used.	(22-24)
Phosphatidylethanolamine (PE)		Essential for maintaining the structural integrity. Like PC, it is sourced from natural materials.	(1, 25)
Phosphatidylserine (PS)		Enhances bilosome interactions with cells, potentially improving drug delivery efficiency. Less commonly used compared to PC and PE.	(26)

Phosphatidylinositol (PI)		Imparts a negative charge to bilosomes, reducing susceptibility to macrophage phagocytosis. Plays a role in cellular signaling pathways such as apoptosis, glucose metabolism, and cell proliferation.	(27, 28)
Lysophospholipids (e.g., Lysophosphatidylcholine, LPC)		Modify bilosome surface properties, influencing interactions with biological membranes.	(29).
Sphingomyelin (SM)		Less frequently used, reduces membrane stability with rising cholesterol levels within the bilosomal structure.	(30)

3.1.2 Surfactants play a crucial role of stabilizing the vesicles, reducing surface tension, and influencing their physicochemical properties. The selection of surfactants is vital for achieving the desired characteristics of bilosomes. Some commonly used surfactants include bile salts, amphipathic molecules (31) with biological compatibility that improve the GI stability and membrane permeability depending their structure (32). Figure 2 shows different bile salts classified into three groups depending on their conjugation with amino acids and their level of hydroxylation (33).

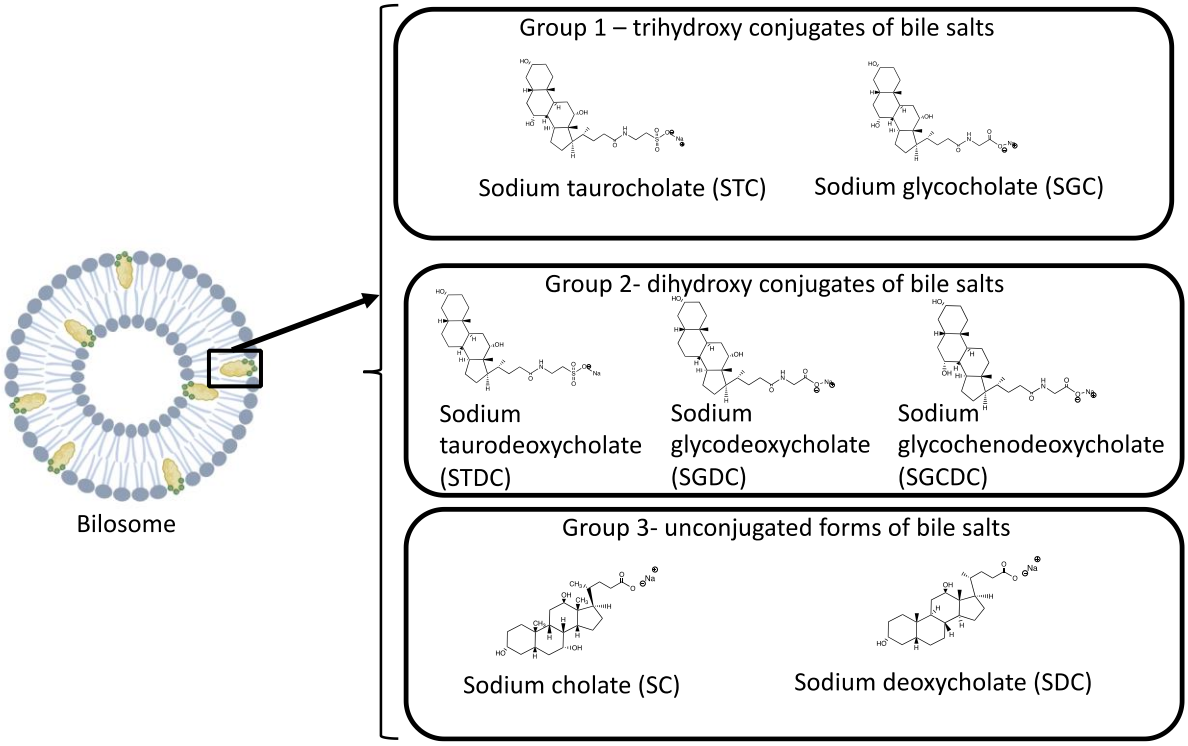


Figure 2: Classification of bile salts used as surfactants in bilosomes

Almost 60% of the bile salts in human bile can be categorised as dihydroxy, which along with the trihydroxy bile salts enhance the solubilisation of insoluble lipids such as phospholipids, monoglycerides and long chain alkyl alcohols (34). Among the various types of bile salts, SDC and SGC are the most commonly used as they exhibit high biocompatibility and are able to enhance the protease enzyme-inhibiting potential in the GI tract leading to high stability and improved permeability (16). The bile salts used in bilosome preparations are commonly food grade components which means they are non-toxic and allow their use in food preparations for oral administration (3). Other surfactants with emulsifying properties, as described in Figure 3, are used as alternative stabilizers in bilosomal structures improving drug delivery.

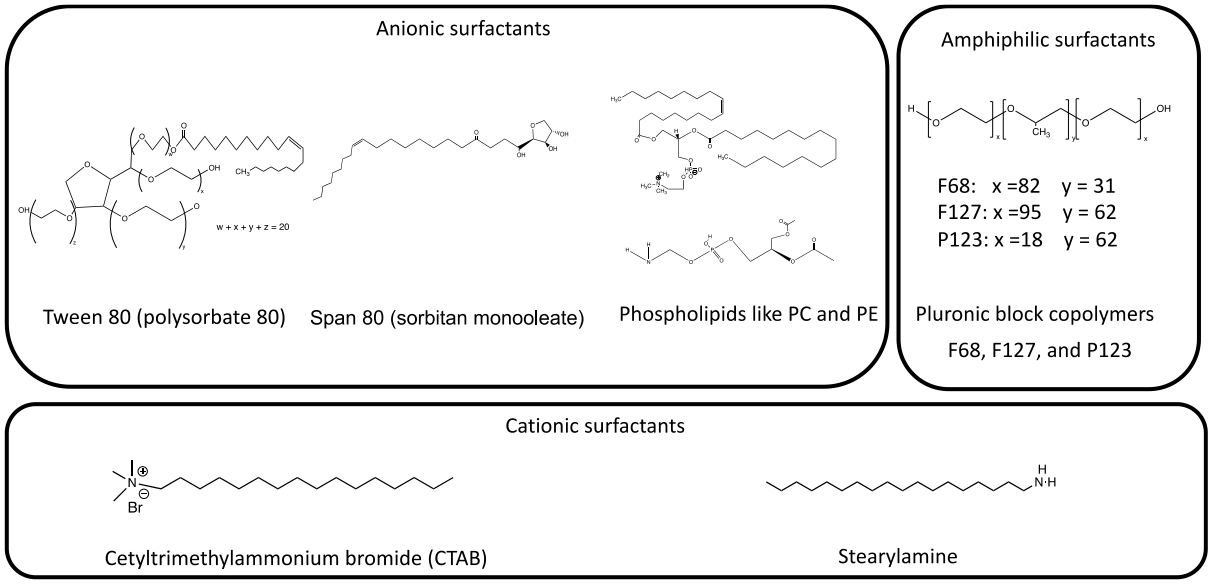


Figure 3: Other surfactants used in bilosomes: Anionic surfactants as polysorbate 80 (35, 36)., sorbitan monooleate (37). and phospholipids (PC and PE) (1, 22-24); amphiphilic surfactants as F68, F127 and P123 (38); or cationic surfactants as CTAB and stearylamine (39, 40).

3.1.3 Cholesterol, while not a traditional surfactant, this sterol is often included in bilosome production as it enhances membrane stability, reduces permeability, and influences the fluidity of the bilosomal membrane (22). The selection of surfactants depends on factors such as the nature of the entrapped payload, desired bilosome properties, and the intended route of administration. Extensive studies have been carried out to optimize surfactant combinations and concentrations for specific drug delivery applications, aiming to achieve stability, biocompatibility, and efficient drug encapsulation within bilosomes (1, 41).

3.2 Preparation methods considerations

Traditionally, production of bilosomes involves dissolving selected lipids and cholesterol in an organic solvent, such as chloroform, diethyl ether, or methanol, to create a lipid phase. However, an emerging alternative is to use lipids that dissolve each other, eliminating the need for organic solvents and avoiding potential toxicities. For example, phosphatidylcholine and cholesterol are lipids that can effectively mix without requiring organic solvents. In this method, all lipids are fully melted and homogenized by heating the lipid blend above their melting points in a controlled environment, often using a water bath.

Finally, the evaporation of the solvent, hydration, and dispersion are the steps that create differences in the methods used. Some common methods are described in Figure 4 comparing their strengths and weaknesses.

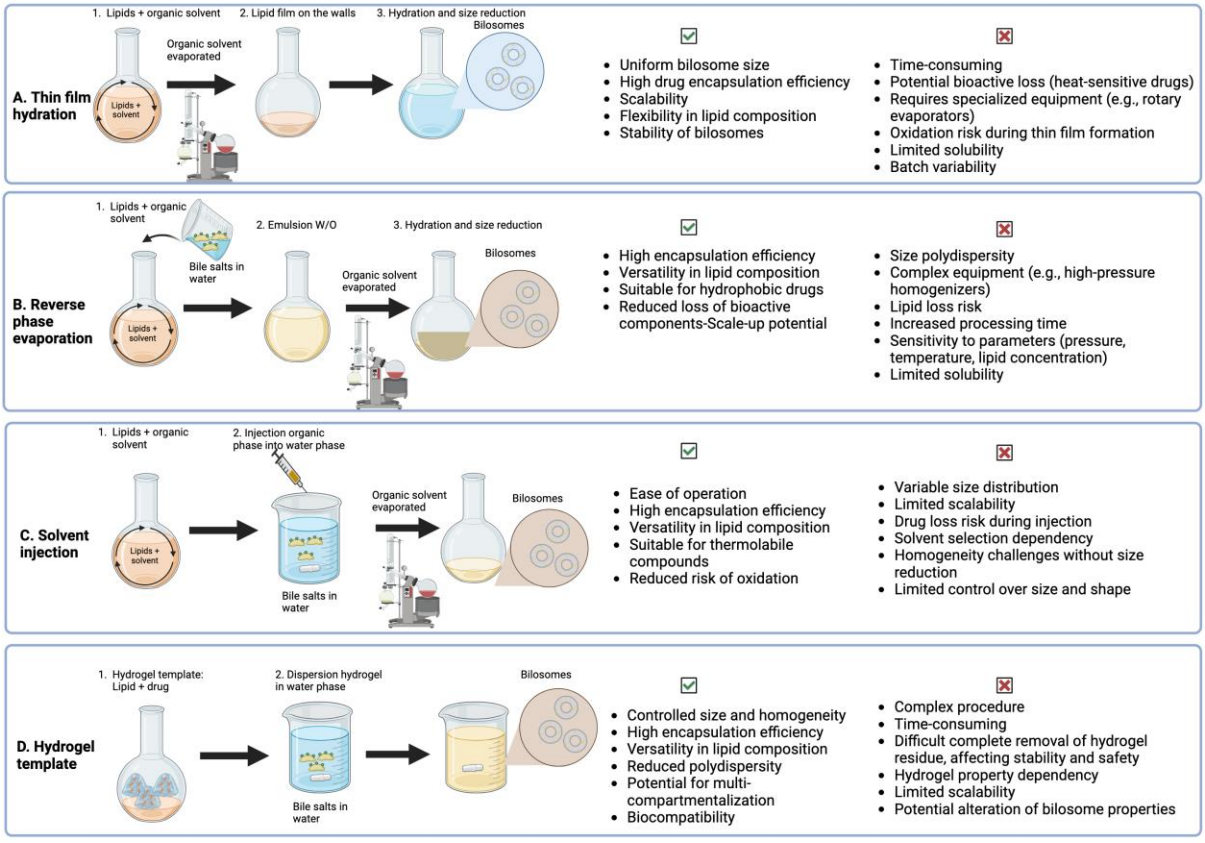


Figure 4. Comparative description of common bilosome production methods: A. Thin film hydration; B. Reverse phase evaporation; C. Solvent injection and D. Hydrogel template

Alternative methods involving melting lipids by heating in a water bath have been used for both influenza (42) and tetanus toxoid (14) antigens, demonstrating effectiveness in enhancing vaccine stability and efficacy. These methods require no organic solvents (main strength) but the temperature to melt the lipids could reach 120° C, while the minimal temperature for a total melting of the lipids is 60 °C (43). An alternative green method using a specialized microwave to melt the lipids, is faster (2 min at 140 °C under pressure with continuous stirring). One of the weaknesses of these methods is that they are not appropriate when the material used in the fabrication of the LNP or in the encapsulation is heat sensitive. For example, proteins, which are often used as antigens in vaccines, can denature or lose biological activity when exposed to high temperatures, making such heating methods unsuitable.

Microfluidic mixing offers precise control and scalability for bilosome preparation, reduced reagent consumption, rapid and efficient mixing (enhancing homogenization of the bilosome components and promoting uniform vesicle formation), and is easily automated, allowing for higher reproducibility and minimizing the need for manual intervention (44). However, researchers should be aware of challenges related to complex instrumentation, initial setup costs, sensitivity to parameters (such as flow rates, pressure, and temperature), potential clogging (especially when dealing with lipid-based formulations), handling highly viscous lipid solutions (which could potentially impact the efficiency of vesicle formation), and the need for a deeper

understanding of long-term stability. Furthermore, there are only a few companies that produce the equipment, with a need for their own disposable chips and software with a particular pattern that controls shearing forces to produce the vesicles. This potentially necessitates tie-in to a particular company for consumables. Thorough optimization and careful consideration of these factors are essential for successful bilosome production using microfluidic techniques (45-49).

3.3 Size reduction techniques

During the last two decades, several size-reduction techniques have been studied to downsize the formed vesicles and achieve a uniform size distribution as the last step defined for the preparation of bilosomes. The choice of method depends on various factors such as the physicochemical properties of the drug, desired bilosome characteristics, scalability, and the equipment available. Researchers often optimize the formulation parameters to achieve the desired properties and therapeutic efficacy of the bilosomes for specific applications. These include:

3.3.1 Extrusion involves passing the vesicular dispersion through polycarbonate membranes with defined pore sizes to obtain uniform and smaller-sized vesicles. While extrusion provides advantages, such as controlled size distribution and scalability, challenges may arise from potential lipid oxidation and the need for specialized equipment (1, 39, 50).

3.3.2 Probe-sonication involves utilizing ultrasonic waves to reduce the particle size, allowing for precise control over size. However, it can cause heat-induced degradation and may struggle to achieve a uniform size distribution, which can be influenced by the lipid composition (51-53).

3.3.3 Supercritical fluid technology involves utilizing supercritical fluids, such as supercritical carbon dioxide, to precipitate lipids and drugs, forming bilosomes. This technology offers advantages such as precise control over particle size and minimal solvent residues, yet it poses limitations due to equipment complexity and potential challenges in scaling up for industrial production (54).

3.3.4 Microfluidization involves using techniques such as high-pressure homogenization to prepare bilosomes, wherein a lipid-drug mixture is passed through a high-pressure homogenizer to form small-sized bilosomes. While microfluidization excels in size control, scalability, and reducing lipid degradation, challenges include the need for specialized equipment, process optimization, and scaling up the technology effectively (20, 55).

3.4 Manufacturing scale-up

The choice of scale-up method depends on various factors such as the physicochemical properties of the drug, desired bilosome characteristics, scalability, and the equipment available. Researchers often optimize the formulation parameters to achieve the desired properties and therapeutic efficacy of the bilosomes for specific applications. A criticism of why bilosomes have rarely progressed to commercial products is that scale-up for industrial manufacture is problematic. One of the main reasons is related to the lack of appropriate methods to produce large and reproducible quantities of LNP at different developmental stages (56).For example the thin film rehydration method is based on the mixing of individual lipid and surfactant components, resulting in easy particle formulation and active encapsulation, with high lipid working concentrations. However, scaling up from millilitre volumes to tens of litres to obtain a homogeneous and desired particle size when a lipid film is involved

is problematic. On the other hand, some protocols such as the solvent injection method or reverse-phase evaporation method, are relatively easy to perform, but they generate new challenges such as size control and removal of the organic solvent in order to reduce particle size and improve particle distribution, pore extrusion technologies using polycarbonate membrane may be carried out. However, the high energy and temperatures necessary may affect the lipid composition, the cargo and cause significant loss through membrane clogging. Recently, microfluidics has received attention from manufacturers as it can be used to produce consistent and optimised nanoparticles (57). This method can replace the lipid hydration and homogenization steps during LNP production and replace it with a single-step process where particle size is better controlled (58). Overall, fluid mixing and micromixing methods offer a huge potential to LNP scale up and open new fields for innovation in the manufacturing process (59).

Figure 5 shows several parameters and challenges for consideration during the manufacturing process. First of all, the process is subdivided into many operations and each of them possess critical attributes that will impact on the characteristics of the final product. The number of unit operations is simplified here to 4, but in practice it can increase (59).

Challenges start with the raw materials to be used in terms of acceptance for human use, suitable quality for GMP scale or the need for toxicity evaluation when combined with other materials or used in new applications. Inherent characteristics of homogenization and the mixing process need to be qualified and optimized during the process development: temperature, flow rates of dispersal and continuous phases as

well as concentration for the drug may vary case by case, depending on the process use.

The next hurdle is related to the dilution steps required to dilute organic solvents or co-solvents interfering with the particle formation and stability. Furthermore, a purification step is required to guarantee the elimination of any contaminants or impurities coming from the mixing process. Mostly. chromatography or a tangential flow filtration process or a combination of both purification steps, are needed for industrial scale-up.

Another challenge arises from the need to reduce the size of the LNP may require extrusion and high-pressure technologies to produce particles sized below 0.2 µm. This is particularly important for a sterile filtration step and to avoid contamination in the subsequent fill finish stages. If the particles cannot be sterile filtered, then the process needs to be carried out under aseptic conditions. This is a major challenge taking into consideration the many unit operations required to get the product

Last but not least, the final consideration occurs at the fill and finish stage where material compatibility and formulation attributes need to be aligned to assure drug stability in the evaluated storage conditions.

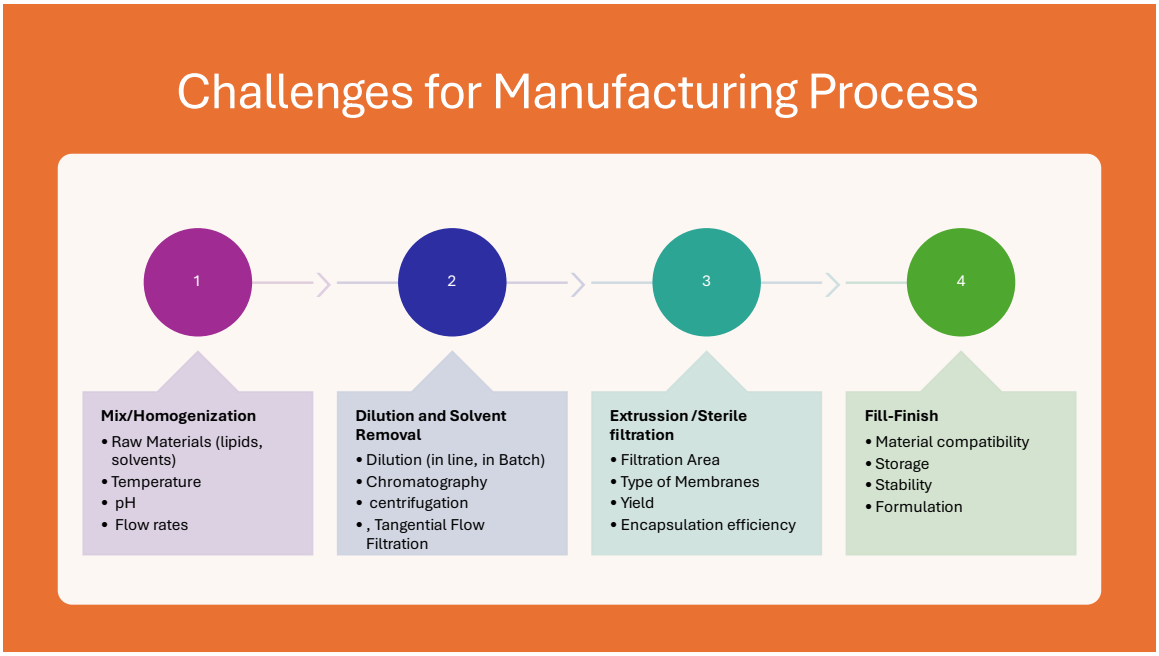


Figure 5. Challenges for the manufacturing process of LNP

4. Characterization and stability

Several characterisation techniques can be used to assess the prepared formulations. These include evaluation of their physicochemical properties, drug loading efficiency, stability, and many others. The characterization steps aim to provide stable and reproducible vesicles that can be used for industrial applications and are a requirement of regulatory authorities. A detailed description of characterization methods include:

4.1 Particle size, polydispersity index (PDI), and zeta potential measurements

The average particle size, PDI, and surface charge (zeta potential) measurements provide an indication to assess the quality of the prepared particles and their stability. This is a necessity by the regulatory authorities. These parameters are usually measured using a Zetasizer which is based on dynamic light scattering intensity and allows for the measurements to be taken while the formulation is in liquid form (60).

However, consideration then needs to be given to if the final product is going to be a dried formulation, in which case on re-suspension in liquid form for administration, the characteristics will have changed. This therefore requires further tests to evaluate stability. For example, if the final product were a vaccine for oral immunisation, to avoid cold storage a dried formulation (lyophilization) might be preferable, with re-suspension at point-of-administration. This is carried out by freeze drying the formulations under low temperature of <-40 °C and vacuum. The effect of the lyophilization process on the characteristics of the bilosomes then needs to be assessed by resuspending the bilosomes in a suitable buffer and re-evaluating the particle size, PDI, zeta potential, and drug EE%. Any significant change in any of these parameters indicates formulation problems (6).

The particle homogeneity and size distribution can be assessed from the PDI value where values close to zero indicate a monodisperse formulation and a PDI value close to 1 indicates a hyperdispersed formulation. The Zetasizer will also measure the surface charge of the vesicles by measuring the electrophoretic dispersion of charged particles in an electrical field. To evaluate the stability of the bilosomes, the size, PDI, and zeta potential should be evaluated during the storage of these formulations under various controlled temperature conditions and any change of these parameters could indicate a stability problem (61).

4.2 Determination of payload entrapment efficiency (EE %)

Determining the percentage of drug or vaccine encapsulation into bilosomes is essential. Following the preparation of for example drug-loaded bilosomes, the free unencapsulated drug is usually separated using filtration or centrifugation, then the drug loaded inside the bilosomes or the free unencapsulated drug is measured using

techniques such as UV spectrophotometry or high-performance liquid chromatography (HPLC). The percentage drug encapsulation can be calculated using the equation:

$$EE\% = Entrapped\ drug/Total\ drug\ added \times 100\%$$

or

$$EE\% = (Total\ drug\ added - unentrapped\ drug)/Total\ drug\ added \times 100\%.$$

The EE% should also be monitored during storage to assess the stability of the formulations and uncontrolled drug release during storage indicates a stability problem since the drug should be retained inside the bilosomes (1).

4.3 Stability in simulated biological fluids and drug release studies

On the other hand, controlled release in an *in vivo* situation is useful and it is important to understand how stability and/or the absorption of the loaded payload interacts in GI fluids, particularly if the bilosomes are being developed for oral administration. This can be carried out by studying the stability in simulated gastric fluid (SGF; 0.1 M HCL, 39 nM sodium taurocholate) pH 1.2, and simulated intestinal fluid (SIF; 50 mM PBS, 2.171 µM sodium deoxycholate) pH 6.8, which are used to simulate the GI environment. Bilosome formulations can be incubated at 37 °C for 2 h in SGF and 4 h in SIF that contain various concentrations of bile salts to simulate the effect of bile salts the bilosomes. The overall effect of these environments on bilosomes can be evaluated by measuring the effect on vesicle size, PDI, and the percentage of drug retained in the formulations (39).

Another important parameter that needs to be assessed is the rate of drug release from these particles as well as the mechanism of drug release. This will affect the onset and the duration of action of the loaded drug inside the bilosomes (48). The most common technique to assess this uses the release of drug during dialysis. Following the removal of the non-encapsulated drug, drug loaded bilosomes will be placed in a cellulose membrane dialysis bag with a molecular weight cut off larger than the size of the free drug and smaller than the size of the bilosomes. The sealed dialysis bag is then immersed in an excess of aqueous solvent used in the preparation of the bilosomes (receptor compartment) with continuous stirring at 37 ± 0.5°C. At specific predetermined time intervals, a sample will be withdrawn from the receptor compartment and replaced by an equivalent volume of fresh medium to maintain sink conditions. The withdrawn samples can then be analysed by UV or HPLC for drug content. This allows a release profile over time to be constructed (62). The correct kinetic model that represents the release pattern of the drug can be determined by calculating the R^2 value that represents the optimal model with the highest accuracy using the constructed graph.

Release studies can also be carried out in SGF and SIF which are used to simulate the GI environment since bilosomes are mainly intended for oral administration, mimicking the timeframe of exposure in the body (eg at least two hours in the stomach). Samples will then be taken from the SGF at predetermined intervals, and the concentration of the released drug will be measured. Then the SGF will be exchanged to SIF and samples will be taken for around six hours and the released drug concentration will be measured at each time point (63).

4.4 Morphological examination of bilosomes

Morphological examination and the surface topography of empty and drug loaded bilosome dispersions can be carried out using transmission electron microscopy, atomic force microscopy, or scanning electron microscopy. These techniques are based on placing formulation on specific grids and allowing the solvent to dry (50). After drying, the samples can be evaluated for their morphological appearance by taking photographs at a suitable magnification. This can give an indication if the prepared bilosomes are spherical or not and if the drug loading can affect the morphology of the particles by comparing the appearance of the empty and drug loaded bilosomes (64).

4.5 Thermal analysis and X-ray diffraction

The thermal behaviour of empty and drug loaded vesicles can also be assessed using Differential Scanning Calorimetry (DSC). For this analysis, the bilosomes need to be lyophilized first and then the melting point of the individual components and the whole formulation are assessed. Any chemical interactions between the components are observed as a shift in the melting point of any of the individual raw materials in the formulations (65).

X-ray diffraction analysis is also carried out lyophilized vesicles, and their corresponding individual components to evaluate any change in the crystalline structure of any of the raw materials before and after the formation of the vesicles. Changing the crystalline structure can affect the drug solubility, release, and the stability of these formulations. This test can also provide a stability indicator after storage under different conditions (66).

4.6 *Ex vivo* intestinal uptake

To confirm the uptake of bilosomes from the intestinal tissues following oral uptake, bilosomes can be formulated and loaded with fluorescent dye and then administered orally to an animal model. After around 4 hours administration, animals can be sacrificed, and the duodenal region isolated and washed with Ringer's solution. Confocal microscopy can then be used to confirm the uptake and localization of the bilosomes into the intestinal region (10). This is particularly important in understanding the mechanism of action.

5. Mechanism of action studies

Inevitably, the bulk of the work reported on bilosomes has focussed on oral immunization. Since the primary aim of these studies was to demonstrate protection of antigen, the outcome of success is deemed to be a suitable immune response at both mucosal and systemic levels. For a conventional orally delivered vaccine preparation, the low pH, proteolytic enzymes and the large mucosal surface (250-300 square meters) strongly reduces the effective dose of antigens ultimately reaching the limited number of Peyer's patches (PPs) in the gastrointestinal (GI) tract. In order to induce a successful immune response, orally delivered vaccines must reach the immune induction sites within the mucosal surface of the GI tract to correctly deliver sufficient amounts of antigen. Due to their specific characteristics oral bilosome-based vaccines have proven to facilitate targeted delivery of vaccine to the PPs in the GI tract (20, 67). PPs are largely situated as clusters in the small intestine and regarded as key induction sites of mucosal immunity. Individual PPs are dome-shaped distinct lymphoid follicles, overlaid by a follicle-associated epithelium (FAE), which contains so-called microfold cells (M-cells). M-cells are uniquely equipped in sampling macromolecules, particles and microorganisms from the luminal site of the GI tract in

order to translocate them towards the subepithelial lymphoid cells in the lamina propria (68, 69). M-cells are specialized in internalization of potential antigens since they contain few, or no microvilli and a range of specific receptors involved in phagocytoses and large numbers of endocytotic vesicles. They also contain intraepithelial pockets where sampled antigen is presented to underlying immune cells and lymphocytes (70). Figure 6 shows the cellular, humoral and mucosal responses induced by antigen interaction with M cells.

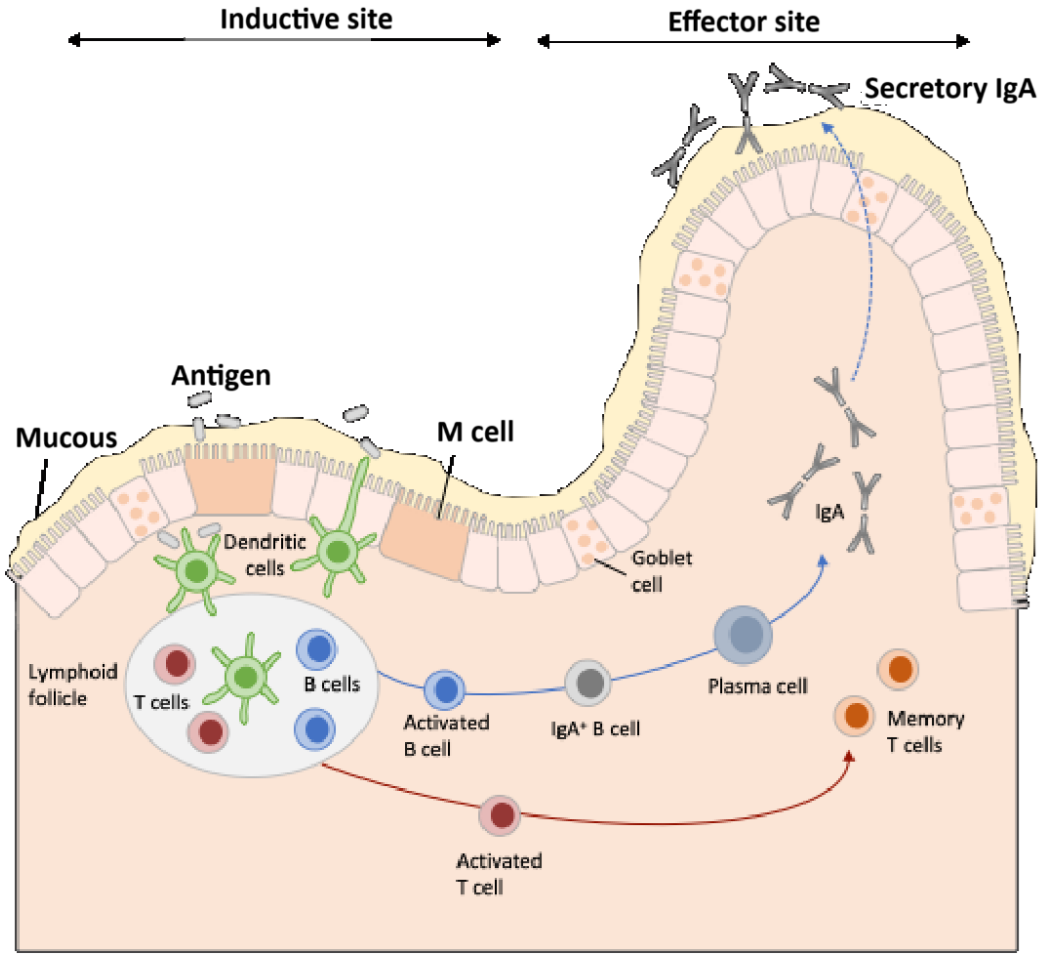


Figure 6. Immune responses triggered by antigen interaction with M cells.

Orally administered vaccine antigens sampled and transcytozed by the specialized M cells will be delivered to dense populations of subepithelial antigen-presenting dendritic cells. These antigen presenting cells will take up, process and present antigen fragments to naïve MHC-class II restricted T helper cells which are able to provide help to antigen-specific B lymphocytes and cytolytic T cells. The locally activated antigen-specific lymphocytes will migrate to mesenteric lymph nodes and distant effector sites in the lamina propria where antigen-specific B lymphocytes will differentiate into IgA antibody-producing plasma cells. The antigen-specific IgA antibodies are detectable in the systemic circulation and are also recognized by polymeric IgA receptors on the basolateral surface of the *lamina propria* and translocated as dimeric secretory IgA (sIgA) towards the lumen of the gastrointestinal tract (71, 72)**.**

Additional modification of the bilosome-vaccine can further strengthen the interaction between the loaded vaccine antigens and the mucus layer, allowing more efficient delivery to the mucosal associated immune system and improvement of the resultant immune responses. If necessary, the immune activation pathways can be improved further at different levels, for example by enhancing cellular uptake, and the targeting of APCs or lymph nodes, by decoration with mucoadhesive ligands and also by co-delivery of antigens with known adjuvants or immunostimulatory molecules into the same immune cells, which is known to be important for activating the immune system (73).

6. *In vivo* performance of bilosomes

The proven stability of vaccine antigens in bilosomes and their increased bioavailability at GI immune induction sites makes them attractive candidates for oral vaccine delivery vehicles. A variety of orally administered bilosome-based vaccine prototypes have been demonstrated to effectively induce local mucosal (IgA) and systemic (IgA and IgG) immune responses for a variety of viral vaccine preparations including: Hepatitis B surface antigen (HBsAg) (74, 75), influenza virus (76-78) and human enterovirus 71 (EDV71) (79), as well as bacterial tetanus toxoid (80) and diphtheria toxoid (81). Most studies have been performed in murine models. However, studies on humans have demonstrated that bile salts can improve mucosal absorption of biological molecules. Kecman *et al.* (2020) demonstrated that cholate species, particularly, SDC enhanced nasal adsorption of insulin and increased blood concentrations of the hormone (82).

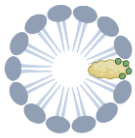
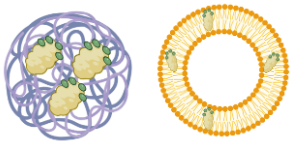
DOC has been extensively used in the manufacture of many human vaccines. For example, in the extraction of viral proteins (influenza vaccine, Fluarix, Fluarix: Package Insert - Drugs.com) or membrane vesicles (as in the case of anti-meningococcal vaccines like VAMENGOC-BC or MenB) (83). Interestingly, the residual amount of SDC in these products can be as high as 10 to 50 mcg per dose, which raises the question if this amount of surfactant may confer adjuvant or immune stimulator effects. This may be supported by recent work that has demonstrated the proinflammatory effect of deoxycolic acid by modulating interleukin-1 beta expression in a murine colitis model, although the molecular mechanism is not very clear (84).

7. Other bile-based drug delivery systems

The pre-clinical success of the bilosomes, encouraged development of other bile-based drug delivery systems since the advantages of bile components to enhance

drug solubility, stability, and bioavailability were obvious. These innovations were particularly relevant for drugs with poor aqueous solubility and so researchers have also begun to explore combinations of bile salts with other advanced drug delivery technologies, such as nanotechnology and bioconjugation, to create more effective and versatile systems for delivering a wide range of therapeutic agents(3, 10, 85-88). These innovations are presented in Table 2 and Table 3.

Table 2: Innovations in bile acid containing lipid and/or polymeric based drug delivery systems

Type of bile-based drug delivery systems	Description	Ref
Mixed micelles 	Formed of bile salts and phospholipids. Recent focus on optimizing the composition for better stability, drug loading, and controlled release.	(89, 90)
Bile acid nanoparticles and liposomes 	Bile salts as stabilizers and enhancing drug-loading capacity. Bile acids can be linked to the nanoparticles to take advantage of bile acids' natural affinity for liver cells to facilitate targeted drug delivery to hepatocytes.	(15, 91-95)

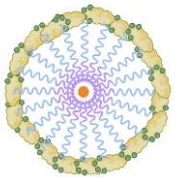
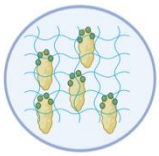
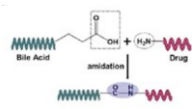
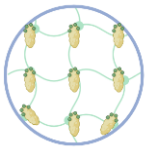
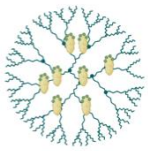
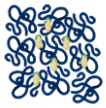
Bile acid-coated nanocarriers 	Application of a layer of bile acids onto nanocarriers, including polymeric nanoparticles or micelles, to improve their biocompatibility and targeting capabilities. Bioconjugation of drugs to surface for controlled release.	(96-100)
Bile acid-modified nanocapsules 	Hollow nanoparticles, enhanced by the addition of bile acids to improve stability and targeting efficiency. Bioconjugation of drugs to surface, facilitating controlled release.	(101, 102)

Table 3: Other innovations based on non-LNP delivery systems

Type of bile-based drug delivery systems	Description	Ref
Hydrogels 	Incorporating bile salts into hydrogels, enable site-specific drug release in the GI tract by responding to changes in pH and bile salt concentrations.	(103-105)
Drug conjugates 	Involve directly linking drugs with bile acids, creating a self-assembling drug delivery	(106-110)

	system that exploits the amphiphilic nature of bile acids.	
Functionalized nanogels 	Three-dimensional networks of crosslinked polymers that have been modified with bile acids, creating a versatile platform for drug encapsulation and controlled release; bioconjugation can be applied.	(111)
Dendrimers 	Use of highly branched macromolecules (dendrimers) incorporating bile acids, to improve targeting capabilities.	(112)
Bile acid-embedded nanofibers 	Involving natural or synthetic nanofibers, forming a fibrous system; additionally, bioconjugation of drugs can be applied.	(113)

7. Conclusion

The initial concept of the bilosome was to develop an effective, oral delivery system to target the mucosal immune system, namely oral. The need to protect protein/peptide antigens from the harsh environment of the GI tract became a reality, with the inclusion of bile salts to niosomes, which were already proving to be useful in systemic vaccination. Early research showed the versatility of the bilosomes, as demonstrated for different types of antigens and from there, poorly soluble drugs were investigated – moving away from vaccination. Their advantages have also been expanded to other

non-invasive routes of administration and the future into commercially viable products looks promising as scale-up manufacturing processes improve.

8. Future Perspective

Development of modern drug delivery systems have had a long history since the 1950s (114), with the innovation of including bile salts arriving 40 years later. However, commercialization of the latter has faced challenges, and more clinical data is required. The main hurdles have been the need for reproducible and scalable manufacturing processes for progress to advance into the clinic. In parallel, recent manufacturing scale-up innovations, such as the use of microfluidics could overcome these hurdles, enabling progress to be enhanced in the near future. These promising delivery systems could be translated into the future in a commercial product delivering various types of therapeutics taking the advantage of their efficacy and safety and the newer methods of preparation. Current data on the safety and bioavailability of bile salts and the demonstrated effect on inflammation processes, altogether addresses new applications and use of a very versatile range of bile species. Using bile salts, either from natural or synthetic source, they have shown how they improve the stability and potency of current LNPs, emulsions and hydrogels and this opens up the development of new formulations, by applying the knowledge gained from a simple concept – the addition of bile salts to niosomes to enable oral immunisation.

9. Article Highlights

The bilosome was conceptualized over two decades ago, yet despite the advances, progress into clinical applications and products has been slow. The article highlights the main attractive benefits that include:

- Improved stability: LNP reinforced with bile salts, which ensures a higher stability in the stomach and intestines when given orally, allowing them to survive effectively in the acidic environment of the stomach and the first part of the intestine.

  Better absorption: When applied via the oral route they promote the absorption of active ingredients, such as vaccines and medicines, through the intestinal wall, leading to more efficient administration. While initially developed for oral immunization and as a non-invasive alternative to injections, the advantages have enabled applications for nasal, topical, transdermal and corneal therapeutics.

However, their disadvantages include:

- Complex production: technically challenging and possibly expensive, which may limit their large-scale application.
- Limited mechanistic studies: characterization of the systems is readily facilitated, however, mechanism of action remains unknown.
- Limited safety knowledge: Relatively little is still known about the long-term effects and safety of bile salt vesicles, which requires caution in clinical use.
- Variability in response: The consistency and effectiveness of the various systems discussed varies depending on the individual and the specific application.

These disadvantages need to be overcome for the potential of this promising platform technology to be realized.

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