BILOSOME: A BILE SALT BASED NOVEL CARRIER SYSTEM GAINING INTEREST IN PHARMACEUTICAL RESEARCH

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ABSTRACT

The human body has long provided pharmaceutical science with biomaterials of interesting applications. Bile salts (BSs) are biomaterials reminiscent of traditional surfactants with peculiar structure and self-assembled topologies. Most of the new drugs, biological therapeutics (proteins/peptides) and vaccines have poor performance after oral administration due to poor solubility or degradation in the gastrointestinal tract (GIT). Though, vesicular carriers exemplified by liposomes or niosomes can protect the entrapped agent to a certain extent from degradation. Nevertheless, the harsh GIT environment i.e., low pH, the presence of bile salts and enzymes limits their capabilities by destabilizing them. In response to that, more resistant bile salts-containing vesicles (BS-vesicles) were developed by the inclusion of bile salts into lipid bilayers constructs. Tremendous research in the last decade has made bilosomes potential carrier system. Bilosomes with its name derived from bile salts (which is one of its major constituents), is a ‘niosome-like’ colloidal carrier. Here, we focus on different aspects of bile salt based drug delivery systems including their composition, developmental techniques, characterization, comparative advantages of BS-integrated nanomedicines over conventional nanocarriers, stability, transitional modifications and scale-up – emphasizing their biomedical potential in oral immunization against various diseases and delivery of peptide drugs. Bile acid-based amphiphiles, in the form of mixed micelles, bilosomes, drug conjugates and hybrid lipid-polymer nanoparticles are critically discussed as delivery systems for anticancer drugs, antimicrobial agents and therapeutic peptides/proteins, including vaccines. Current pitfalls, future perspectives, and opinions have also been outlined. Bile acid-based nanoparticles are a growing research area therefore, multifaceted pharmaceutical and biomedical applications of bile salts are to be expected in the near future.

Key words: Bile acids, bilosomes, vaccines, protein and peptides, bile salt containing liposomes, M-cell

1. INTRODUCTION

Human body homeostasis is maintained by numerous endogenous substrates characterized by unique properties that help them to perform specific functions. For years, bile acids were recognized as biosurfactants with crucial roles in endogenous organotropism. They are facial amphiphiles synthesized in the liver and stored in the gall bladder, and exist as ionized bile salts (BSs) under physiological conditions. The unique molecular structure and interfacial properties of these amphiphilic steroidal compounds have made them an intriguing subject for pure academic research. Bile acids are facial amphiphiles which renders them peculiar surface active and interfacial properties, attaining higher surface tension values in aqueous solution than classical surfactants. The adsorption dynamics is a very fast process but despite their strong affinity for the interface, bile acids do not reach high surface pressures due to less efficient interfacial packing compared to conventional amphiphiles. The values for the molecular areas suggest that bile acids adopt an almost flat conformation at interfaces, with the rigid steroidal backbone parallel to...
the interface to allow the contact of the hydroxyl groups with the aqueous environment, which correlates with their facial amphiphilic structure. As a consequence of their facial amphiphilic nature and the rigid steroid backbone, bile acids exhibit a complex self-assembly behavior, namely a stepwise aggregation process over a broad concentration range that differs from conventional surfactants. Their extraordinary emulsifying and solubilizing properties have led to their utilization as delivery systems for medicines and cosmetics as well. In the pharmaceutical field, BSs have been exploited to improve hydrophilicity of water-insoluble active pharmaceutical ingredients (such as amphotericin B, resveratrol, and oxaprozin) mainly by the wetting effect. Furthermore, BSs have been employed as permeation enhancers in topical dosage forms including buccal, ocular, nasal, and transdermal routes of administration. Besides, the serendipitous discovery of BS gelation tendency led to their use as low-molecular-weight cationic hydrogelators. A recent era in pharmaceutical research encompassed fabrication of BS-integrated nanocarriers to overcome major obstacles of drug delivery. To improve drug−receptor interactions, many new designed potent drug molecules based on contemporary drug discovery programs possess high degree of lipophilicity that unfortunately causes poor solubility in GI fluids accompanied by low and variable bioavailability. Different strategies that include encapsulating therapeutic agents or vaccines into nano-colloidal delivery systems (polymeric or lipid-based nanoparticles, nanoemulsions, nanostructured lipid carriers, mixed micelles and vesicular structures) have been adopted by researchers to cope with impediments associated with delivering these challenging agents to specific target tissues. Among different classes of colloidal systems, vesicular carriers have gained particular attention in delivering poorly soluble drugs and proteins/peptides. Vesicular carriers comprise of unilamellar or multilamellar spherical structures formed of lipid molecules gathered into bilayers orientation and capable of encapsulating drug molecules. Conventional vesicular systems exemplified by liposomes and niosomes have demonstrated an appealing potential in augmenting the oral bioavailability of therapeutic agents and immunogenic response of vaccines. Nevertheless, the efficacy of conventional vesicles has been compromised by their instability in the GIT, which necessitated modification in their bilayers constructs to improve their in vivo resistance. Different research efforts conducted over the past decade has demonstrated the effectiveness of including bile salts into vesicular systems bilayers in improving their in vivo resistance and performance after oral administration. Accordingly, the aim of this review is to provide a comprehensive overview of the fundamentals of bile salts-containing vesicles (BS-vesicles) with a focus on their successful applications in delivery of drugs, therapeutic proteins/peptides and vaccines.

<table>
<thead>
<tr>
<th>Characterization parameter</th>
<th>Liposomes</th>
<th>Niosomes</th>
<th>Bilosomes</th>
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<tbody>
<tr>
<td>Composition</td>
<td>Phospholipids With cholesterol and charge inducer</td>
<td>Nonionic Surfactant with cholesterol and charge inducer</td>
<td>Nonionic Surfactant and bile salt and charge inducer</td>
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<td>Chemical stability</td>
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<td>Does Not undergo oxidative degradation</td>
<td>Does not undergo oxidative degradation</td>
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<td>Stability in simulated gastric fluid</td>
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<td>Unstable</td>
<td>Stable</td>
</tr>
<tr>
<td>Stability in simulated intestinal fluid</td>
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<td>Unstable</td>
<td>Stable</td>
</tr>
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<td>Antigen dose</td>
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<td>Relatively high</td>
<td>Relatively low</td>
</tr>
<tr>
<td>Storage stability</td>
<td>Required Liquid nitrogen for storage</td>
<td>Special Conditions not required</td>
<td>Special Conditions not required</td>
</tr>
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</table>

2. BILE SALT INTEGRATED DELIVERY SYSTEMS

Recently, BSs have been exploited as a core for versatile nanosystems. From a review of the literature, major categories of BS-integrated nanocarriers can be classified as follows. Figure 1 summarizes the four major BS-integrated nanosystems.

2.1. Size-tunable cholate nanocarriers

Recently, a unique class of amphiphilic copolymers was introduced into the field of cancer targeting and gene delivery based on BS core. Such polymers are composed of a hydrophilic linear hydrophilic biodegradable branch such as PEG [polyethylene glycol], dextran, pullan, chitosan, and PLGA [poly (lactic-co-glycolic acid)] and a flexible two-arm linear oligomer of cholic acids as a hydrophobic core-forming block. Owing to hydrophilicity of the surface, such amphiphilic structures would spontaneously form self-nanoaggregates in water promoted by intra- and/or intermolecular association between hydrophobic segments to minimize interfacial free energy.
Furthermore, the resultant nanocarriers exhibit tunable properties owing to the rigidity of the steroidal polycyclic backbone and the amphiphilic properties of bile acids. Consequently, biodegradable nanocarriers based on bile acids are of captivating potential in the field of drug delivery.

2.2. Bile salt-phospholipid mixed micelles

Bile acids can form mixed micelles when combined with polar lipids, conventional surfactants or amphiphilic drugs, usually with lower CMC and better solubilization capacity than the individual components due to synergistic interactions. Additionally, mixed micellar systems can be prepared without the aid of organic solvents and are applied in pharmaceutical formulations to improve the bioavailability of poorly water soluble drugs. Moreover, phospholipids such as lecithin buffer the detergent properties of bile salts and attenuate their cytotoxic effects due to mixed micelle formation. A commercial formulation for intravenous administration of diazepam, a lipophilic benzodiazepine, employs mixed micelles of glycocholic acid and soy lecithin as solubilising agent. The antifungal drug amphotericin B, a hydrophobic polyene antibiotic, is commercialized as micellar dispersion with sodium deoxycholate for parenteral administration. The formulation takes advantage of the facial amphiphilic nature of both the drug and the bile acid, which promotes their association.

2.3. Bile acid-polymer nanocarriers

Polymeric nanoparticles represent a new generation of therapeutic delivery platforms with several advantages over lipid-based carriers, such as micelles or liposomes, due to their smaller size and higher cell-penetrating capacity, showing enhanced circulation time and preferential accumulation at the target site via the enhanced permeability and retention effect. In this context, lipid-polymer hybrid amphiphiles obtained by conjugation of bile acids with hydrophilic and biodegradable polymers constitute promising drug delivery systems. These hybrid amphiphiles can self-assemble in aqueous environments to form nano-sized micelles with a unique core-shell structure, made of a hydrophobic bile salt core stabilized by a hydrophilic corona. The size of both core and corona-forming blocks is relevant for drug loading, and higher entrapment efficiency of hydrophobic drugs is associated with larger cores, whereas the increase in polymer length of the hydrophilic corona-forming block contributes to increasing the CMC. Some of these systems formed micelles with sizes between 10–100 nm, which has been reported as the optimum size for passive tumor targeting with minimum liver uptake. The solubilization efficiency of PEGylated bile acids was found to depend on the nature of the bile acid and on the length and number of grafted PEG chains. Self-emulsifying drug delivery systems (SEDDSs) based on PEGylated bile acids and oleic acid enhanced the solubilization and absorption of itraconazole, a poorly water-soluble antifungal agent, providing a controlled release system with significant improvement of itraconazole bioavailability in rats.

2.4. Bile acid-drug conjugates

Bile acid-drug conjugates act as Trojan horses taking advantage of the organotropism of bile acids in the enterohepatic circulation for specific drug targeting to the liver or to improve metabolic stability and enhance intestinal absorption of poorly water soluble drugs making use of the bile acid transport systems. Conjugation of therapeutic peptides, proteins and oligonucleotides to bile acids increases metabolic stability and improves intestinal absorption and systemic bioavailability of the macromolecules. Recombinant human insulin has been covalently attached to deoxycholic acid to obtain orally active insulin analogues. Conjugation of cytotoxic agents with bile acids can also improve the pharmacokinetic properties of the drug while targeting it to the liver. For example, conjugation of cytarabine with cholic acid showed potent antitumor activities on cytarabine-sensitive HL60 cells, excellent targeting to the liver, good absorption and longer half-life in vivo compared to cytarabine alone.
2.5. Bilosomes

Bilosomes, first described by Conacher et al.\textsuperscript{37} are closed bilayer structures of non-ionic amphiphiles closely correlated to non-ionic surfactant vesicles (niosomes) but incorporating bile salts. Figure 2 shows composition with key features of a typical bilosome and protection of bilosomes with their contents offered by bile salts against the harsh environment of the gut. Several research groups have demonstrated the potential of utilizing bilosomes in facilitating successful oral vaccine delivery\textsuperscript{38-41}.

![Figure 2](image.png)

Figure 2: a) Composition and key features of a typical bilosome. (b) Protection of bilosomes and their contents offered by bile salts against the harsh environment of the gut.\textsuperscript{45}

2.5.1. Oral drug candidates

Amenability of bilosomes for enhancing oral availability was not confined to hydrophilic drugs entrapped in hydrophilic core.\textsuperscript{42} Few hydrophobic actives have been successful candidates for bilosomes as well where the drug would be entrapped in the phospholipid (PL) bilayer of the vesicles. In this context, Chen et al.\textsuperscript{23} have investigated the oral bioavailability of fenofibrate-loaded bilosomes and conventional liposomes compared with the micronized form of the drug. Bioavailability from bilosomes was significantly higher than that from liposomes and the micronized form. Guan et al.\textsuperscript{43} loaded Cyclosporine in bilosomes in comparison with conventional liposomes and marketed a microemulsion product (Sandimmune® and Neoral® [Novartis Pharmaceuticals Corporation, East Hanover, NJ, USA]). Among others, bilosomes exhibited the lowest in vitro drug release but the highest oral bioavailability.

2.5.2. Oral Immunization

Oral immunization is always preferred over conventional immunization achieved through the parenteral route because of the associated high production costs associated with the need for a sterile environment, pain at the site of administration, uneasiness to the patient and risk of disease transmission. Moreover, oral immunization is considered superior because it induces mucosal immune response (i.e. induction of sIgA), which is the predominant entry site for most of the infectious pathogens.

**Bilosomes in oral immunization with model antigen**

Conacher et al. reported oral immunization using BSA \textsuperscript{37}. The orally administered bilosomal formulation containing BSA induced high antibody titers against it, which were found to be equivalent to those generated after systemic immunization. Singh et al. formulated BSA-loaded and CTB-conjugated bilosomes to enhance their affinity toward M cells of Peyer’s patches\textsuperscript{44}.

**Bilosomes in oral immunization against influenza**

Conacher et al. reported oral immunization using an influenza subunit vaccine and demonstrated that orally
administered formulation induced high antibody titers and cell-mediated response as compared with systemic immunization. T helper type 1 and type 2 responses were found to be induced 37. Mann et al. optimized bilosomes containing A/Panama influenza hemagglutinin and demonstrated that bilosome-entrapped influenza HA not only induced significant specific systemic antibody production but also mucosal IgA. Whereas parenterally administered inactivated influenza vaccines resulted in protection from homologous viral infection by induction of serum anti-IA IgG antibodies, they were relatively inefficient in protection against variant strains arising during the influenza season 45.

**Bilosomes in oral immunization against hepatitis B**

Arora et al. reported oral immunization against hepatitis B virus using mannosylated bilosomes. Immune response was found to be significantly higher along with enhanced sIgA level at all local and distal mucosal sites as compared with bilosomes alone, whereas parenteral vaccine was unsuccessful at providing any considerable cell-mediated response 46. Shukla et al. reported oral delivery of recombinant HBsAg using bilosomes 40.

**Bilosomes in oral immunization against tetanus**

Mann et al. reported the significant systemic and mucosal immunity on oral immunization with Tetanus toxoid-loaded bilosomes 45. The Tetanus toxoid entrapped in bilosomes was found capable of inducing Th2 response characterized by systemic IgG1. A clear dose-dependency was observed with specific Tetanus toxoid IgG1 antibody titers, which was induced only with a higher concentration of Tetanus toxoid (200 mg/dose), not with the lower one (40 mg/dose). In addition to antibody production, only the Tetanus toxoid entrapped in bilosomes resulted in sIgA antibodies. The endpoint antibody titers were superior to oral administration of the un-entrapped antigen but comparable to parenterally delivered Tetanus toxoid. Only Th2 and IgA responses were induced with orally delivered, entrapped antigen 47.

**Bilosomes in oral immunization against diphtheria**

Katere and coworkers developed nano-bilosomes containing Diphtheria toxoid prepared by thin film hydration that was characterized in vitro for their shape, size, percent antigen entrapment and stability and in vivo for anti-Diphtheria toxoid IgG and anti-Diphtheria toxoid sIgA response in serum and in various body secretions, following oral immunization with different doses of Diphtheria toxoid entrapped in nano-bilosomes 41. The high-dose-loaded nano-bilosomes (Diphtheria toxoid NB3, 2 Lf) produced comparable anti-Diphtheria toxoid IgG levels in serum to those induced by IM-administered alum-adsorbed Diphtheria toxoid (0.5 Lf). All nano-bilosomal formulations elicited a measurable anti-Diphtheria toxoid sIgA response in mucosal secretions, whereas IM-administered alum-adsorbed Diphtheria toxoid (0.5 Lf) was devoid of this response. The orally administered nano-bilosomal Diphtheria toxoid formulation produced comparable serum antibody titers to IM administered alum-adsorbed Diphtheria toxoid at a fourfold higher dose and without the induction of tolerance.

**2.6. Nonoral bilosomes**

In a paradoxical effect for oral bilosomes where BS content strengthens the wall against digestion, BS-modified liposomes have been utilized via other routes as permeation enhancers due to elasticity. In this context, transdermal transferosomes have been investigated in numerous research articles for enhancing percutaneous drug absorption. Transferosomes are ultraformable liposomes that contain different edge activators including BSs 48. Transferosomes containing BSs demonstrated efficacy in improving transdermal permeation of cosmetics and pharmaceutical molecules when used in concentrations lower than 0.2% 49. Sodium cholate demonstrated efficacy as edge activator as equal as Tween-80 and Span-80 in improving transdermal permeation of estradiol 50. The application of bilosomes in corneal penetration is a novel field that has not so far been fully investigated. Dai et al. 51 have considered elaboration and corneal permeation of bilosomes as novel ocular delivery systems for tacrolimus. A previous study has indicated that liposomes loaded with tacrolimus can facilitate penetration of the drug across the cornea into the aqueous humor 51. However, transcorneal permeation from liposomal suspension was still too small to achieve a therapeutic effect. Realizing poor corneal permeation of drugs and delivery systems, the authors investigated ocular tacrolimus bilosomes containing different types of BSs as permeation enhancers.

**2.7. Probilosomes**

Nanocarrier preconcentrates can spontaneously form the corresponding liquid-state nanomedicine upon ingestion. Because they are deprived of water, self-nanocarriers possess higher physical stability compared with liquid nanocarriers. Proliposomes are dry, free-flowing granular products that instantaneously form multilamellar liposomal dispersion upon ingestion. It was reported that oral delivery of liposomes could be improved by enhancing their ability to retain their integrity at the site of absorption, which could be achieved by formulating them into proliposomes 52-53. In this context, Song et al. have developed proliposomes using STDC (2.5%, w/w) for the oral delivery of salmon calcitonin. They reported a 7.1-fold increase in the calcitonin bioavailability from proliposomes. They assumed that proliposomes are superior in protecting calcitonin against possible degradation by gastric fluid. Furthermore, entrapment efficiency and permeation of proliposomes were higher compared with the proliposomes that could be attributed to the formation of a lipophilic ion pair between the drug and BS 54.

**2.8. Surface-engineered bilosomes**

Surface modification of bilosomes has been recently proposed in a few research articles attempting to increase stability and targeting efficiency of the nanovesicles. In the field of oral immunization, surface-modified vesicles were reported by anchoring a suitable ligand for a variety of receptors (such as mannosyl,
galactosyl, folic acid and fibronectin) preferentially and abundantly present on the cell surface of antigen-presenting cells in mucosal linings. In this context, Jain et al. 57 developed novel glucomannan-modified (GM) bilosomes for eliciting an immune response following the oral administration of tetanus toxoid. The authors compared three types of vesicular systems, niosomes, bilosomes, and GM-bilosomes. All vesicular systems exhibited comparable in vitro quality attributes (particle size, zeta potential, and entrapment efficiency). Nevertheless, GM-bilosomes showed a higher immunological response parallel to maintained chemical and conformation stability of the tetanus toxoid entrapped. Results revealed significant immunological superiority of GM-bilosomes to conventional bilosomes and superiority of both formulations to niosomes, oral and IM peptides. The authors attributed this improvement to the polymeric nature of GM that increases the surface functionality by increasing mannose molecules density on the surface, which can enhance the uptake of mannosylated bilosomes by the antigen-presenting cells. Furthermore, the polymeric nature of GM can also provide stability against digestive enzymes. The ability of glucamannosylated bilosomes to improve targeting efficiency for oral immunization with bovine serum albumin has also been reported 57.

3. PREPARATION OF BS-VESICLES
Based on the reviewed literature, BS-vesicles were prepared using different methods are schematically illustrated in Figure 3. Table 2 shows examples of various bilosomes prepared by different methods.

![Figure 3: Representation of different methods adopted for preparation of bile salts-containing vesicles. (A) Reverse phase evaporation method, (B) Thin film hydration method and (C) Hot homogenization method.](image)

4. IN VITRO CHARACTERIZATIONS AND VARIABLES INFLUENCING BS-VESICLES
The in vitro characterizations of BS-vesicles included determination of the vesicle size, polydispersity index (PI), zeta potential, vesicles morphology, entrapment efficiency, in vitro release and cellular uptake studies. A brief description of the characterization techniques and variables influencing some properties of BS-vesicles are provided hereafter. Table 3 shows different methods for BS-vesicles characterization.

4.1. Particle size, PI, Morphology, Zeta potential and Entrapment efficiency
Particle size of BS-vesicles exerts substantial impact on their in vitro and in vivo performances. Regarding the influence of bile salts content, substantial decrease in particle size and PI of BS-vesicles was associated with the increase in bile salt contents due to the enhanced flexibility and decrease in surface tension of the vesicles. However, further increase in bile salts content caused vesicles enlargement bound with increase in PI due to the increase in medium viscosity. 
Table 2: Formulation details and preparation methods of bile salt-containing liposomes (BS-liposomes).

<table>
<thead>
<tr>
<th>Vesicular carrier</th>
<th>Composition</th>
<th>Antigen</th>
<th>Preparation method</th>
<th>Vesicle size reduction</th>
<th>Ref</th>
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</thead>
<tbody>
<tr>
<td>BS-liposomes</td>
<td>SPC:STC (5:1)</td>
<td>Tacrolimus/ Poorly water solubility drug</td>
<td>Thin film hydration method</td>
<td>Ultrasonication</td>
<td>31</td>
</tr>
<tr>
<td>BS-liposomes</td>
<td>SPC:SGC (4:1)</td>
<td>Porcine insulin/ Protein drug</td>
<td>Reverse phase evaporative method</td>
<td>High-pressure homogenization</td>
<td>39</td>
</tr>
<tr>
<td>Bilosomes</td>
<td>MPG:CH:DCP (5:4:1) 100 mM of SDC</td>
<td>Gonadotropin-releasing hormone immunogen</td>
<td>Hot homogenization method</td>
<td>Homogenization</td>
<td>60</td>
</tr>
<tr>
<td>BS-liposomes</td>
<td>SPC:SDC (4:1)</td>
<td>Fenofibrate/ Poorly water soluble drug</td>
<td>Thin film hydration method</td>
<td>Sonication followed by high-pressure homogenization</td>
<td>27</td>
</tr>
<tr>
<td>BS-liposomes</td>
<td>SPC:SDC (3:1)</td>
<td>Cyclosporine A/ poorly water soluble drug</td>
<td>Thin film hydration method</td>
<td>High-pressure homogenization</td>
<td>45</td>
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<tr>
<td>BS-liposomes</td>
<td>SPC:bile salts (SGC/STC/SDC) (4:1)</td>
<td>Recombinant human insulin/ Protein drug</td>
<td>Reverse phase evaporation method</td>
<td>High-pressure homogenization</td>
<td>44</td>
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<tr>
<td>Bilosomes</td>
<td>STS:CH:DCP (7:3:1), 100 mg of SDC</td>
<td>Diphtheria toxoid</td>
<td>Thin film hydration method</td>
<td>Extrusion through 200 nm pore membrane</td>
<td>41</td>
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<tr>
<td>Bilosomes</td>
<td>STS:CH:DCP (7:3:1), 100 mg of SDC</td>
<td>Hepatitis B Antigen</td>
<td>Thin film hydration method</td>
<td>Extrusion through 200 nm pore membrane</td>
<td>40</td>
</tr>
<tr>
<td>Bilosomes</td>
<td>MPG:CH:DCP (5:4:1), 100 mg of SDC</td>
<td>BSA and measles peptide</td>
<td>Thin film hydration method</td>
<td>37</td>
<td></td>
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<tr>
<td>CTB-bilosomes</td>
<td>STS:CH:modified DPPE (7:3:1); 100 mg of SDC, Conjugation of CTB to bilosomes</td>
<td>Hepatitis B surface Antigen</td>
<td>Thin film hydration method</td>
<td>Extrusion through 200 nm pore membrane</td>
<td>38</td>
</tr>
<tr>
<td>GM-bilosomes</td>
<td>SMO:CH:SDC (2:1:0:1) GM-OCM-DSP (10% w/w of total lipid content)</td>
<td>Tetanus toxoid</td>
<td>Thin film hydration method</td>
<td>Sonication</td>
<td>27</td>
</tr>
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</table>

The pH of the hydration medium also influences the particle size of the vesicles due to the ionization of bile salts at higher pH which in turn increases its surface activity. Likewise, different processing variables influenced the vesicles particle size. The multilamellar micron-size BS-vesicles produced by thin film dispersion method were converted into small unilamellar nano-sized vesicles using extrusion through membrane, ultrasonication or homogenization. The preparation method also influences the vesicles particle size. Mann et al. reported that homogenization method produced single population of small vesicles; however, thin film method produced a mixture of large vesicles combined with smaller ones. It is worth mentioning that the uptake of bilosomes by Peyer’s patches and mesenteric lymph tissue is somehow dependent on the vesicles size. Bilosomes with size between 5 and 10 mm are retained within the Peyer’s patches offering mucosal immunoglobulin A (IgA) response, whereas particles less than 5 mm are conveyed into the efferent lymphatics inside macrophages inducing predominantly circulating antibody response. Likewise, particle size of BS-liposomes influences their in vivo cellular uptake via endocytosis. Niu et al. reported that SGC-liposomes with particle size of 80–400 nm showed more cellular uptake in comparison to their counterpart with size of 2000 nm. Endocytosis is an energy dependent process, and the greater extent of uptake shown for smaller particle is related to the less energy required during the endocytosis process. Transmission electron microscopy was used to ascertain the formation of BS-vesicles and visualize their morphology. Zeta potential is the measure of overall charges acquired by particles in a particular medium. In general, vesicles with surface charge are more stable against accumulation than uncharged ones. Opposing conventional liposomes that showed a tendency to agglomerate, BS-liposomes were negatively charged due the presence of bile salts that stimulated the zeta potential and hindered the aggregation of the vesicles. Regarding in vivo performance, it is reported that negatively charged vesicles are favorably taken up by the Peyer’s patches.
increases the drug solubility in the dispersion medium, thereby compromising the entrapment efficiency. Furthermore, the fluidizing effect of bile salts on the lipid bilayers causes leakage of the entrapped drug. The PC content in the vesicles as well contributes to the EE. Significant increase in the amount of cyclosporine A and hexamethylmelamine entrapped was bound with the increase of SPC content, which was attributable to the fact that higher lipid content propose more space for drug molecules. The hydration pH affects the EE% as it affects the bile salt dissociation, which in turn affects the particle size and ability of the bile salts to hold the drug molecules.

4.2. In vitro release and cellular uptake studies

Regarding BS-vesicles, the in vitro release study was evaluated by dynamic dialysis method to circumvent the leakage of vesicles into the external release medium. In vitro release studies are essential indicators for in vivo performance of a drug delivery system. In dynamic dialysis study, compared to conventional liposomes, BS-liposomes released more drug due to the enhance lipid bilayer fluidity that allowed drug leakage from the vesicles.

5. IN VIVO PERFORMANCE OF BILE SALT VESICLES (BS-VESICLES)

5.1. Improvement in oral drug bioavailability

Based on the published literature, incorporation of drugs or proteins into BS-liposomes substantially enhanced their bioavailability and in vivo efficacy. It is reported that orally administrated SDC-liposomes loaded with fenofibrate exhibited 1.57-fold increase in bioavailability compared to conventional liposomes in beagle dogs. The observed enhancement was related to the uptake of the vesicles through M-cells in the Peyer’s patch and facilitated transmembrane absorption of fenofibrate due to the ultra-deformability of BS-liposomes. Likewise, the superiority of BS-liposomes in enhancing the oral bioavailability of cyclosporine A relative to the marketed microemulsion formulation (Sandimmune Neoral) and conventional liposomes was confirmed by. Facilitated absorption of intact BS-liposomes vesicles rather than enhancing drug solubility and release rate was the main reason for the increase in cyclosporine A bioavailability.

5.2. Enhancement of vaccine immunogenicity

It is reported that orally administrated bilosomes induced cell-mediated responses against synthetic peptides and high antibody titers against protein antigens comparable to those engendered succeeding systemic immunization. The potential of utilizing SDC-bilosomes loaded with either HBsAg or Diphtheria toxoid in providing transmucosal immunization was investigated by Shukla et al. In both studies, orally administrated bilosomes loaded with high dose of antigen produced systemic immunoglobulin G (IgG) response in mice comparable to those induced by intramuscular administrated antigens. In addition, bilosomes elicited measurable secretory IgA in mucosal secretions that were not induced by IM administrated antigens. Similarly, the combinatorial bilosomes formulation containing high dose of HBsAg and tetanus toxoid, prepared by Shukla et al, produced anti-HBsAg-IgG and anti-Tetanus toxoid-IgG levels mice serum similar to IM administered HBsAg and Tetanus toxoid. Figure 4 shows in vivo performance of orally administrated bilosomes depicting confocal laser scanning microscopy (CLSM) of the intestine and immunological study and measurement of specific IgA and IgG.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Methodology</th>
<th>References</th>
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<tbody>
<tr>
<td>Particle size</td>
<td>Dynamic light scattering instrument</td>
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<tr>
<td></td>
<td>Laser diffraction particle size analyzer</td>
<td>54,61</td>
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<tr>
<td>Polydispersity index</td>
<td>Dynamic light scattering instrument</td>
<td>57</td>
</tr>
<tr>
<td>Zeta potential</td>
<td>Dynamic light scattering instrument</td>
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<td>Electrophoretic mobility (EPM) measurements</td>
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<tr>
<td>Morphology</td>
<td>Transmission electron microscopy (TEM)</td>
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<tr>
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<td>Cryogenic-transmission electron microscopy (Cryo-TEM)</td>
<td>23</td>
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<td>Scanning electron microscopy (SEM)</td>
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<td>Freeze fracture electron microscopy (FFEM)</td>
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<tr>
<td>Separation of free drug from drug-loaded vesicles</td>
<td>Molecular exclusion chromatography</td>
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<td>Ultracentrifugation</td>
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<tr>
<td>Entrapment efficiency percent</td>
<td>High performance liquid chromatography (HPLC)</td>
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</tr>
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<td></td>
<td>UV spectrophotometer</td>
<td>43,51</td>
</tr>
<tr>
<td></td>
<td>Bicinchoninic acid (BCA) method</td>
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<tr>
<td></td>
<td>Micro bichoninic acid (MicroBCA) method</td>
<td>41,57</td>
</tr>
<tr>
<td></td>
<td>Modified ninhydrin assay</td>
<td>37,43,47</td>
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<tr>
<td>In vitro release</td>
<td>Dynamic dialysis method</td>
<td>2,23,43,51</td>
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<tr>
<td>Cellular uptake</td>
<td>Human colon adenocarcinoma cell line (Caco-2 cells)</td>
<td>24,54</td>
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<tr>
<td></td>
<td>Spontaneously derived human corneal epithelial cells</td>
<td>51</td>
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<tr>
<td></td>
<td>Murine macrophage cell line</td>
<td>57</td>
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Table 3: Major methods for BS-vesicles characterization
6. STABILITY CONSIDERATIONS OF BS-VESICLES

Stability studies (whether in-process, in simulated fluids or during storage) are of vital prominence for successful development of pharmaceutical carrier systems. Decomposition of entrapped therapeutic agents may lead to decrease in their potency, whereas in the case of immunological preparations, denaturation of antigens may cause inadequate immune response making the subject prone to diseases.

6.1. In processing stability

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Different research groups utilized sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to examine the chemical stability of entrapped peptides or antigens in BS-vesicles after being exposed to the preparation stress conditions. SDS-PAGE is a widely used procedure to isolated proteins according to their electrophoretic mobility. SDS, as an anionic surfactant, was added to protein specimens to linearize proteins and impart negative charge thus fractionate them according to estimated size through electrophoresis. In all the published studies, symmetrical position of bands between the pure as well as extracted antigens/proteins along with the absence of additional bands were evident confirming that the preparation method, and the quantity of bile salts included did induce any irreversible accumulation or decomposition of the entrapped agents.

6.2. Storage stability

Stability studies were carried out to explore the leaching of the entrapped agent from the vesicles during storage. Shukla et al examined the amount of Diphtheria toxoid retained in the bilosomes after storage at refrigerated (5±3°C) and room temperature (25±2°C) at 70% relative humidity. After one-month storage, around 94% of the antigen remained in bilosomes stored at room temperature, whereas more than 98% of the antigen was found in samples stored at refrigerated conditions. The stability of the formulations was credited to the negative charge induced by DCP on the bilosomes surface that caused electrostatic repulsion preventing fusion and aggregation of vesicles upon storage.

6.3. Stability in simulated biological milieu

It is crucial to interpret the stability of vesicular carriers and encapsulated peptides and proteins when confronted by gastric pH, bile salts and GI enzymes as the intact fraction of the vesicles predominantly influences the associated in vivo response. The superiority of bilosomes to niosomes, affirmed in terms of their ability to protect the loaded antigen in simulated biological fluids, was attributed to repulsion between the bile salts in the vesicle bilayer and the external bile salts in the solution. In this domain, Conacher et al reported that niosomes lost all of their initially entrapped BSA content in bile salt concentration of 20 mM in contrast to bilosomes that retained 85% of its initial BSA, thereby, confirming the stabilizing role of bile salts in vesicle constructs. In accordance with the aforementioned result, Shukla et al reported that bilosomes loaded with either HBsAg or Diphtheria toxoid retained considerable amount of entrapped antigen during stability studies conducted in simulated GIF and bile salts solutions (5mM and 20mM concentration). Following a comparable approach, Wilkhu et al examined the changes in antigen load of bilosomes-incubated GIF. The initial antigen loaded (32%) was retained in gastric media; however, it decreased to around 8.5–9.5% when the bilosomes were simultaneously transferred to intestinal medium due to the degradation of antigen located on the surface of bilosomes.

Figure 4: In vivo performance of orally administrated bilosomes. (A) Confocal laser scanning microscopy (CLSM) of the intestine and (B) Immunological study and measurement of specific IgA and IgG.
Different authors have compared the stability of BS-liposomes in simulated GI media in comparison to conventional liposomes. Hu et al reported that SGC liposomes retained most of the insulin load as compared with conventional liposomes in either simulated GI fluids (including pancreatin or pepsin) or in ex vivo GI fluids obtained from rats. The protective effect was credited to the enzyme inhibiting ability of SGC and its associated membrane stabilization aptitude. The influence of incorporating different bile salts (SGC, STC, SDC) on the integrity of BS-liposomes against different protease enzymes (pepsin, trypsin and a-chymotrypsin) was tested by Niu et al who further affirmed the superiority of SGC in formulating BS-liposomes due to its enzyme-inhibiting ability compared to the other investigated bile salts.

7. CYTOTOXICITY OF BS-VESICLES

Bile salts are reported to cause irritation and toxicity when used as penetration enhancers because their enhancement effect is somehow bound to damaging the intestinal epithelial barrier. However, some researchers have shown that PL can reduce the noxiousness of bile salts. In this domain, Niu et al evaluated the cytotoxicity of BS-liposomes in concentration range of 0.25–6.25 mmol/L using cell growth inhibition assay employing Caco-2 cell monolayers. Although, there was a slight reduction in cell viability as the liposomes concentration increased, non-significant difference was observed among various bile salts (SGC, STC and SDC) used or between different concentration after incubation for 4 h. The obtained findings implied that BS-liposomes exhibited non-significant toxicity toward Caco-2 cells at the investigated concentrations. Similarly, Dai et al evaluated the toxicity of BS-liposomes prepared using SGC, STC and SDC in comparison to conventional liposomes on the viability of corneal epithelial cells. After incubation for 12 h, at low lipid concentration (54 mg/mL), the viability of the cells were more than 85% for all liposomal formulations; however, at high lipid concentration (46 mg/mL), both SGC and STC showed low toxicity affirming their suitability for ocular drug delivery opposite to SDC-liposomes that had greater toxicity to corneal cells.

8. TRANSPORTATION OF BILOSOMES TO PEYER’S PATCHES (M CELLS)

The M cells of Peyer’s patches can transport macromolecules, particles and microorganisms directly into intestinal Peyer’s patches. The apical surface of M cells differs from intestinal absorptive cells because they lack a brush border. M cells also contain a large number of endocytic vesicles to uptake and transport lumen contents across the epithelial layer. A unique feature of M cells is the presence of an intraepithelial pocket in which transcytosed particles and macromolecules are delivered. The pocket contains lymphocytes and a few macrophages, which interact with the transported antigen or microorganisms. Thus, the transport of soluble and particulate antigen by M cells is an important primary step in producing a mucosal immune response. Additionally, the cellular processes of the M cells, which extend into the underlying lymphoid tissue, provide potential contacts with resident lymphocytes and dendritic cells. The M cells can also secrete IL-1, which indicates that M cells could provide co-stimulatory signals, such as cytokines and cell surface molecules, to T cells and B cells in the microenvironment of Peyer’s patches. Figure 5 shows schematic representation of the uptake of bile salts liposomes by intestinal epithelium after oral administration.

Figure 5: Schematic representation of the uptake of bile salts liposomes by intestinal epithelium after oral administration. (A) Uptake of the vesicles by M-cells in the Payer’s patch, (B) Transport of the vesicles through the paracellular pathway and (C) Transcellular uptake of vesicles through the enterocytes.
CLSM studies of transportation of bilosomes to GALT

The CLSM studies by Shukla et al. revealed that, after administration of R-123-loaded bilosomes, the localized fluorescence in the GALT region was found to be much higher as compared with the unentrapped R-123 on oral administration 41. This study confirmed the efficient uptake of bilosomes by the GALT and demonstrated the capability of bilosomes for transporting the vaccine antigens to the Peyer’s patches containing M cells. Bilosomes protected the vaccine antigen during its passage through the hostile GIT 45,47. The CLSM study assured the nano-bilosomes mediated effective uptake of vaccine antigens by GALT on oral administration, which eventually escorted the comprehensive immune response.

9. TRANSLATIONAL MODIFICATIONS AND INDUSTRIAL SCALE-UP OF BILOSOMES

In translational research, from laboratory bench to industrial production, it is necessary to optimize the manufacturing process to improve efficiency and simplify production, giving a more economical end-product. Bennett et al. tested two simplified production methods along with two different storage methods (lyophilized and non lyophilized) as well as looking at the effect of buffer pH 67. The formulations were assessed in a murine system for immunogenicity, alongside characterization in terms of size and antigen entrapment, with the stability of these aspects assessed with respect to time. Lyophilized and non lyophilized 3-step formulations induced significant IgG1, IgG2a and IgA titers, with the lyophilized version showing stable size and antigen entrapment for up to 9 months.

10. CURRENT RESTRAINS AND FUTURE PERSPECTIVES

A close examination of the literature accentuates two major restraints during bilosomal development. Poor in vitro/in vivo correlation is a common drawback with a lack of in vitro method that mimics the actual conditions. Regular in vitro release methods lack the biological constituents significantly affecting vesicular digestion (BSs and enzymes). Using ex vivo permeation models and regular cell lines to assess vesicular permeability would lack this factor as well. A proposed solution would involve employment of the in vitro digestion-Caco-2 cell model 58. This would encompass digestive enzymes and BS content in addition to amenability for assessment of depot liposomal formulations. Although bilosomes proved to be a successful carrier for cationic water-soluble actives, sufficient loading of anionic active is still an obstacle 56. Taking into consideration negative charge and hydrophilicity of BSs, incorporation of cationic active would hold the BS in the bilayer to exert its membrane-stabilizing effect. Nevertheless, incorporation of anionic hydrophilic drugs would be accompanied by low entrapment efficiency and migration of both hydrophilic BSs and active to external phase 7.

Based on the reviewed literature, BS-liposomes, not only were they capable of enhancing the bioavailability of poorly water soluble drugs in animal models but also they demonstrated the ability to protect the entrapped peptides and proteins after oral administration. Moreover, after the first breakthrough of utilizing bilosomes for oral vaccines delivering by Conacher et al. in 2001, investigations by different research groups in BALB/c mice confirmed the usefulness of oral bilosomes entrapping antigens in alerting both mucosal and systemic immune responses 37. In addition, surface engineered bilosomes (CTB-bilosomes and GM-liposomes) prepared via anchoring ligands to the bilosomal surface demonstrated the ability of targeting the antigens into specific immune cells. The availability and low cost of bile acids, which can be easily derivatized, turns these chiral templates into attractive building blocks for the design of novel drugs and drug carrier systems 59.

Realizing the importance of vaccines and their delivery challenges, the 21st century has witnessed an enormous amount of research work in the domain of vaccine development. The development of a needle-free, painless, patient-compliant and orally active vaccine suitable for mass vaccination campaigns in the developing countries is warranted 25. Accomplishing this objective, however, has been constrained by the fact that purified protein antigens usually induce systemic nonresponsiveness rather than active immunity by orally administered vaccine antigens. Moreover, acid degradation in the stomach and poor permeability across the gastrointestinal mucosa further limit the uptake of antigens by M cells, which is an essential step for an immune response. Therefore, the development of an effective oral delivery system for mucosal vaccines is a significant challenge for the immunologists. In this regard, various lipid-based delivery systems including bilosomes have been increasingly researched and developed for oral immunization. However, more research is needed to elucidate the mechanisms of selective transport of antigens to the intestinal lymphatics by bilosomes, specifically processes at the cellular level including digestion, uptake and intracellular metabolism. Additionally, delivery of a broader range of candidate antigens having different physicochemical characteristics and instability in the GIT must be tested. Further advanced study of different aspects of bilosomes for lymphatic delivery is required not only to overcome these issues but also to develop more-efficient and smart bilosomal vaccine delivery systems for better immunization against different fatal diseases. Use of this biocompatible, stable, and highly specific carrier for vaccination in the near future would contribute to a global countermeasure against infectious diseases, and would greatly benefit in the eradication of the same 70.

The current challenge that faces the researchers is applying the gained knowledge to carry out safe trials in human subjects to systematically monitor all parameters of the elicited immune response and elucidate the exact immune mechanism after oral administration of bilosomes 55. Besides, the precise mechanisms of facilitated absorption and the interaction between the
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6. Rajput A. Membranolytic bile acids. Journal of Drug Delivery & Therapeutics. Recent advances with liposomes as pharmaceutical carriers. An efficient and adequate approach for large industrial scale production that ensures high and reproducible levels of protein-peptide entrapment should be addressed. Furthermore, the long-term storage stability of BS-vesicles should be scrutinized in details, which is still yet far from being accomplished due to the limited researches that address long-term stability.


BS-liposomes and biological environment have not yet been explained due to the complexity of the GI physiology. Nevertheless, it is of remarkable significance to explicate the exact underlying mechanisms of improved absorption of macromolecules by BS-liposomes to serve as basis for their optimization. Further studies should also include the assessment of the influence of the ingested food on the in vivo performance of BS-vesicles.


