Soluble but Still Lost: Bilosomes and the Intestinal Problem of Apigenin

Anjali Gupta[1], Anoop Kumar Singh[2]
[1]*Department of Pharmaceutics, S. N. College of Pharmacy, Babupur, Lucknow Road, Jaunpur – 222132, Uttar Pradesh, India. Affiliated to Dr. A. P. J. Abdul Kalam Technical University, Lucknow.*
[2]*Professor, Department of Pharmaceutics, S. N. College of Pharmacy, Babupur, Lucknow Road, Jaunpur – 222132, Uttar Pradesh, India.*

***Abstract*—Apigenin is classified in the Biopharmaceutics Classification System as a class II compound: poorly soluble but highly permeable. On that classification alone, a solubilisation strategy should be sufficient, and permeability should not be the limiting step. The pharmacokinetic record says otherwise. When eleven healthy volunteers consumed 2 g of blanched parsley per kilogram body weight, delivering 65.8 ± 15.5 µmol of apigenin, plasma concentrations peaked at 127 ± 81 nmol L^{-1} after 7.2 ± 1.3 h and fell below a 2.3 nmol L^{-1} detection limit within 28 h. In rats given a single oral radiolabelled dose, 51.0% of the radioactivity was recovered in urine and 12.0% in faeces, with 1.2% in blood and 24.8% still in the body after ten days. The resolution of the apparent contradiction is that BCS permeability measures how much crosses the apical membrane, not how much reaches the portal blood unchanged. Recent work partitioning pre-systemic loss between organs classified apigenin as a compound of low hepatic extraction (E_h 0.0167–0.0389) but moderate-to-high gut extraction (E_G 0.626–0.979), placing the intestine ahead of the liver as the site of first-pass elimination. The enterocyte is therefore both the gateway and the principal site of loss, and a delivery system must do more than dissolve the drug: it must change how the drug crosses that cell. Bilosomes bile-salt-stabilised vesicles first described for oral immunisation in 2001 are a rational response, because the bile salt is simultaneously a structural stabiliser against gastrointestinal bile and enzymes, an edge activator conferring deformability, an epithelial permeation enhancer, and a determinant of surface charge favouring uptake by M cells of the Peyer's patches and entry to the lymphatic route. This review assembles the pharmacokinetic evidence, examines each of those four roles separately, surveys reported apigenin and flavonoid bilosomes, sets out the formulation variables and their systematic optimisation, and argues that the response which should govern the design is the fraction of apigenin crossing intact rather than the vesicle size.**

***Index Terms*—Apigenin; bilosomes; bile salts; sodium deoxycholate; intestinal permeation; oral bioavailability; glucuronidation; Quality by Design; Box–Behnken design**

I. INTRODUCTION

Apigenin, 4′,5,7-trihydroxyflavone, is among the most studied dietary flavones and among the least successfully delivered. It occurs in parsley, celery, chamomile, grapefruit and a wide range of common foods, and it has documented anti-inflammatory, antioxidant, antiproliferative and neuroprotective activity across a large preclinical literature [1,2,3]. Its appeal as a candidate rest on an unusual combination: broad activity together with very low intrinsic toxicity in normal cells [1,4].

What has not followed is clinical translation, and the reason is pharmacokinetic. Human exposure after dietary intake is in the low nanomolar range [5], while the concentrations at which apigenin acts on its molecular targets in vitro are micromolar. Two to three orders of magnitude separate what can be achieved in a person from what is needed, and no amount of additional target biology closes that gap.

The formulation literature has responded, largely by attacking solubility. Solid dispersions, nanocrystals, cyclodextrin complexes, polymeric micelles, polymeric nanoparticles, self-nanoemulsifying systems and lipid nanocarriers have all been applied to apigenin and most improve exposure [6,7,8,9]. The improvements are real but modest relative to the deficit, and the pattern is informative: if solubility were the only barrier, dissolving the compound efficiently should produce more than a three- or four-fold gain.

There is a specific reason to expect that it would not, and it is the starting point of this review. Apigenin is classified as a BCS class II compound low solubility, high permeability [10]. By that classification permeability is not the limiting step. Yet a formulation that solubilises apigenin perfectly still delivers it into an enterocyte whose conjugating enzymes remove most of it before it reaches the portal blood. High permeability into the cell is not the same as high delivery through it, and the distinction is what this review is built around.

Bilosomes vesicles in which bile salts are incorporated into the bilayer are a rational response, because the bile salt is not a single-purpose excipient. It stabilises the vesicle against the bile and enzymes that destroy conventional liposomes in the gut, it fluidises the bilayer and confers the deformability associated with enhanced permeation, it acts in its own right as an epithelial permeation enhancer, and it imparts a surface charge that favours uptake by the M cells of the Peyer's patches and entry to the intestinal lymphatics [11,12,13]. This review examines each of those claims against the evidence, surveys what reported apigenin vesicles have achieved, and sets out what such a formulation should measure if it is to demonstrate the mechanism it invokes.

II. APIGENIN: THE MOLECULE AND THE PROBLEM

2.1 Activity, and the concentrations it requires

Apigenin acts on an unusually wide set of targets. It induces cell-cycle arrest and apoptosis in several tumour cell types while sparing normal cells, suppresses insulin-like growth factor receptor signalling, inhibits protein kinases including epidermal growth factor receptor and src tyrosine kinase, and suppresses angiogenesis through degradation of hypoxia-inducible factor 1α [1,4,14,15]. Comprehensive target identification has since mapped a wider interaction profile than any single-pathway account suggested [16].

The point relevant to formulation is that these effects are observed at micromolar concentrations in cell culture, and that no human trial of apigenin supplementation for disease prevention had been conducted at the time of the major reviews of its chemopreventive potential [1,4]. The gap between in vitro activity and achievable human exposure is the reason the delivery problem matters, and it is a quantitative gap rather than a qualitative doubt about the pharmacology.

2.2 What the pharmacokinetic data show

Table 1 collects the principal studies.

The human data are the most directly relevant and the most sobering. Eleven healthy subjects, after a flavone-free diet, consumed a single oral bolus of 2 g of blanched parsley per kilogram body weight, corresponding to 65.8 ± 15.5 μmol of apigenin. Plasma concentrations rose in every participant, reached a mean maximum of 127 ± 81 nmol L^{-1} after 7.2 ± 1.3 h with wide inter-individual variation, and fell below the 2.3 nmol L^{-1} detection limit within 28 h. The authors concluded that a small portion of dietary apigenin reaches the circulation [5]. A peak of 127 nmol L^{-1} is roughly 0.13 μM, against in vitro activity typically requiring tens of micromolar.

The rat mass-balance study is the most complete account of where the dose goes. After a single oral administration of radiolabelled apigenin, 51.0% of the radioactivity was recovered in urine, 12.0% in faeces, 1.2% in blood, 0.4% in kidneys, 9.4% in intestine, 1.2% in liver and 24.8% in the rest of the body within ten days. Clearance was estimated at 2 mL h^{-1} and the terminal half-life was long. Urinary products were substantially conjugated, and sex differences appeared in their nature: immature male and female rats, like mature females, excreted a higher proportion of the mono-glucuronoconjugate than the mono-sulfoconjugate, 10.0–31.6% against 2.0–3.6% [17]. The authors characterised the overall picture as slow absorption, slow metabolism and a slow elimination phase. Figure 1A plots this distribution, and the shape of it is the argument: only 1.2% of the dose is in blood, while the single largest identified compartment is urinary conjugate.

Metabolic studies locate the loss. Phase I oxidation gives luteolin and scutellarein, while glucuronidation and sulfation constitute the dominant phase II routes, and both occur in the intestine as well as the liver [18]. Apigenin induces UDP-glucuronosyltransferase 1A1 in intestinal as well as hepatic cell lines, so the compound participates in regulating the enzyme system that eliminates it [3].

Glycoside forms behave differently enough to be instructive. Systemic exposure after oral administration of apigenin-7-O-glucoside was

reported to be markedly higher than after the aglycone, by 2.62-fold for peak concentration and 14.3-fold for area under the curve, which indicates that the presentation of the molecule at the intestinal surface not the molecule itself governs how much is absorbed. Reported figures for absolute oral bioavailability vary considerably between laboratories, from well under one per cent to several per cent, reflecting differences in dose, assay sensitivity, whether free or total apigenin was measured and whether an intravenous arm was included. The variation is itself worth noting rather than smoothing over: a review that quotes a single figure for apigenin bioavailability is quoting one study's conditions.

Table 1. Pharmacokinetic and disposition studies of apigenin. Across species and doses, circulating concentrations remain far below the micromolar range at which apigenin acts in vitro, and most of the absorbed dose appears conjugated.

Study and dose	Principal finding	Reported values	Ref.
Eleven healthy volunteers (5 women, 6 men); single oral bolus of 2 g blanched parsley per kg body weight = 65.8 ± 15.5 µmol apigenin	Plasma concentration rose in all participants and returned below the detection limit within 28 h; a small portion of dietary apigenin reaches the human circulation	C_{max} 127 ± 81 nmol L^{-1} at 7.2 ± 1.3 h; wide inter-individual variation; detection limit 2.3 nmol L^{-1}	[5]
Male and female rats; single oral dose of radiolabelled apigenin, mass balance over 10 days	Slow absorption, slow metabolism and a slow elimination phase; urinary products largely conjugated, with sex differences in conjugate type	Recovery: 51.0% urine, 12.0% faeces, 9.4% intestine, 1.2% blood, 1.2% liver, 0.4% kidneys, 24.8% remaining in body. Clearance ≈ 2 mL h^{-1}. Mono-glucuronoconjugate 10.0–31.6% vs mono-sulfoconjugate 2.0–3.6% of urinary output	[17]
Rat intestinal permeability and intrinsic dissolution rate; four intestinal segments	Apigenin assigned to BCS class II (low solubility, high permeability); absorbed throughout the intestine with the duodenum as the main site	Passive and active carrier-mediated saturable transport in duodenum and jejunum; concentration-independent, primarily passive transport in ileum and colon	[10]
Rat hepatic and intestinal S9 fractions with well-stirred and Q_Gut modelling; apigenin and apigenin-7-O-glucuronide	The intestine contributes more than the liver to pre-systemic elimination of both compounds	Hepatic extraction E_h 0.0167–0.0389 (low); gut extraction E_G 0.626–0.979 (moderate to high)	[19]
Rats; apigenin-loaded self-nanoemulsifying systems (droplet size ≈ 10–20 nm) vs apigenin coarse powder	Improved transcellular absorption across Caco-2 monolayers and improved oral exposure; benchmark for a purely solubilising strategy	Relative oral bioavailability increased 3.8-fold and 3.3-fold for the two formulations; acute oral toxicity LD_{50} > 2000 mg kg^{-1} (OECD 425)	[6]

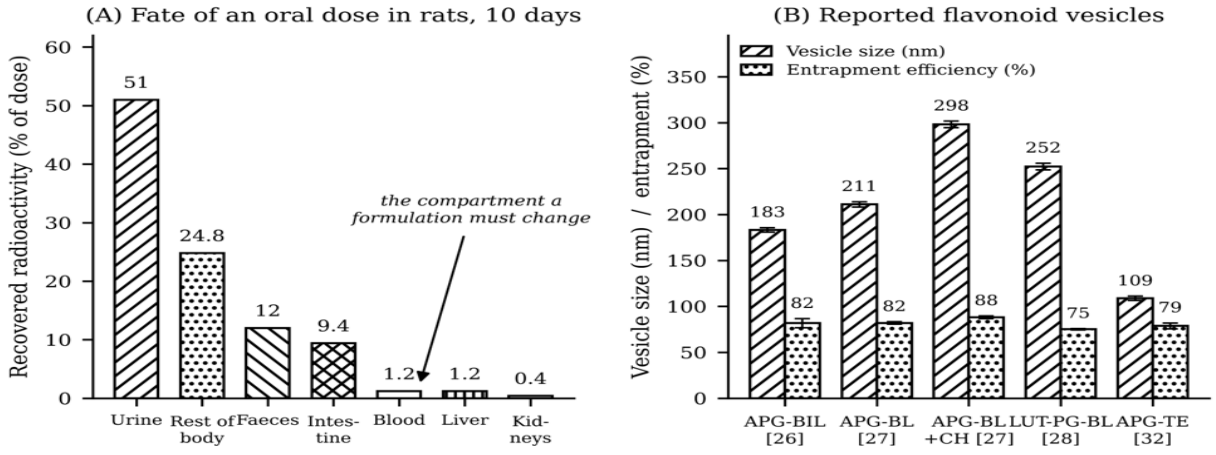


Fig. 1. (A) Fate of a single oral dose of radiolabelled apigenin in rats over ten days, as recovered radioactivity: urine 51.0%, faeces 12.0%, intestine 9.4%, blood 1.2%, liver 1.2%, kidneys 0.4%, with 24.8% remaining in the rest of the body [17]. The bar for blood is the quantity a formulation must change, and it is the smallest identified compartment. (B) Vesicle size and entrapment efficiency of reported flavonoid bilosomes and related vesicles: apigenin bilosomes optimised by Box–Behnken design [26], apigenin bilosomes before and after chitosan coating [27], luteolin pegylated bilosomes [28] and apigenin transethosomes [32]. Values come from different laboratories and methods and are indicative rather than strictly comparable.

2.3 The classification paradox

Apigenin's BCS assignment was established experimentally using intrinsic dissolution rate and rat intestinal permeability, placing it in class II low solubility, high permeability. The same study found that apigenin is absorbed throughout the intestine with the main absorption site at the duodenum, and that transport involves both passive and active carrier-mediated saturable mechanisms in duodenum and jejunum but concentration-independent, primarily passive transport in ileum and colon [10].

Reported aqueous solubility values differ by nearly an order of magnitude between sources, from around 2 µg mL^{-1} to about 15.6 µg mL^{-1} at ambient temperature depending on method and medium [7,10]. Either way the compound is practically insoluble in water, and either way the classification stands.

This creates an apparent contradiction. If permeability is high, why does so little reach the circulation, and why should a project aim at enhanced intestinal permeation rather than at solubility alone?

The resolution is that BCS permeability describes flux across the apical membrane, not delivery of intact drug to the portal blood. A molecule can enter the enterocyte readily and still fail to leave it unchanged. Recent work separating pre-systemic loss between organs makes the point quantitatively: using well-stirred and Q_Gut model concepts, apigenin and its metabolite apigenin-7-O-glucuronide were classified in rats as compounds of low hepatic extraction, E_h between 0.0167 and 0.0389, but moderate-to-high gut extraction, E_G between 0.626 and 0.979, indicating that the intestine contributes more than the liver to pre-systemic elimination of both [19].

Three consequences follow, and they shape everything after this section.

First, the enterocyte is both gateway and principal site of loss. Getting more apigenin to the apical membrane increases the substrate presented to the conjugating enzymes behind it; the ceiling moves without disappearing, which is consistent with the modest gains reported for purely solubilising systems.

Second, because the loss is intestinal rather than hepatic, lymphatic routing has a different value here than it does for drugs lost to hepatic first pass. Entering the lymphatics bypasses the liver, but the liver was never the main problem for apigenin. What the lymphatic route offers in this case is uptake by a different cell population the M cells of the follicle-associated epithelium, whose conjugating capacity differs from that of the absorptive enterocyte rather than hepatic avoidance. The distinction is rarely made and it changes what a lymphatic-uptake experiment would be evidence for.

Third, and most consequentially for study design: the quantity that matters is intact apigenin crossing the epithelium, not total apigenin-equivalents. An assay that hydrolyses conjugates before measurement cannot distinguish a formulation that protected the drug from one that merely delivered more of it to be conjugated.

III. BILOSOMES

3.1 Three generations of vesicle

Table 2 compares the vesicular options, and the lineage is worth setting out because each generation answers a defect of the last.

Liposomes, built from phospholipid and cholesterol, were the original vesicular carrier and remain excellent by most criteria except one: they do not survive the gut. Bile salts secreted into the duodenum solubilise phospholipid bilayers, and lipases and proteases attack their contents, so orally administered liposomes release their payload prematurely [12,13].

Niosomes substituted non-ionic surfactants such as the sorbitan esters for phospholipid, gaining chemical stability, lower cost and freedom from phospholipid oxidation [20]. They are more robust, but they do not solve the bile problem, because the issue is the

surfactant action of bile on any bilayer rather than the particular lipid chosen.

Bilosomes resolve it by a counter-intuitive route: incorporating bile salt into the bilayer itself. The term was introduced in 2001 by Conacher and colleagues, who formulated peptide and protein antigens in lipid vesicles containing bile salts for oral immunisation [11]. A vesicle already containing bile salt is not destabilised by encountering more of it; the repulsion between internal and external bile salt contributes to stability rather than removing it. The approach was extended by work conjugating cholera toxin B subunit to bile-salt-stabilised vesicles to target M cells directly [21], and the field has since broadened well beyond vaccines [12,13,22].

Table 2. Vesicular carriers for oral flavonoid delivery. Each generation answers a specific defect of the one before it.

Carrier	Bilayer composition	Strengths	Limitation for oral apigenin
Liposome	Phospholipid with cholesterol	Biocompatible, well characterised, high loading of lipophilic actives	Destabilised by luminal bile salts and attacked by lipases; premature release in the gut
Niosome	Non-ionic surfactant (e.g. sorbitan ester) with cholesterol	Chemically stable, inexpensive, no phospholipid oxidation	Robust to oxidation but still destabilised by bile; no intrinsic permeation enhancement
Transfersome / transethosome	Phospholipid with an edge activator (surfactant or ethanol)	Ultra-deformable; well established for transdermal and topical flavonoid delivery	Developed for the stratum corneum rather than the gut; no gastrointestinal stabilisation
Bilosome	Non-ionic surfactant or phospholipid, cholesterol and a bile salt in the bilayer	Stable in gastrointestinal bile and enzymes; deformable; bile salt enhances epithelial permeation; negative charge favours M-cell uptake and lymphatic access	Bile salt above an optimum solubilises the bilayer into mixed micelles; permeation enhancement is non-specific and needs an integrity check
Surface-modified bilosome (e.g. chitosan-coated, pegylated)	Bilosome with a mucoadhesive or hydrophilic polymer coating	Mucoadhesion and longer residence time; pegylation adds steric stability and deformability	A cationic coat reverses the negative charge that favours M-cell uptake, trading one mechanism for another

3.2 What the bile salt actually does

Bilosome reviews commonly list the advantages of bile salts as though they were one property. They are at least four distinct mechanisms, operating at different places, and separating them matters because a formulation can be optimised for one at the expense of another. Figure 2 sets them out against the intestinal wall.

Structural stabilisation is the original function and the best established. Vesicles containing bile salt resist the gastric and intestinal environment pH, enzymes and luminal bile markedly better than conventional vesicles, and maintaining intact vesicles in the gastrointestinal tract for longer is itself what permits the later mechanisms to operate at all [12,13,21].

Membrane fluidisation and deformability is the second. Bile salts act as edge activators, lowering the energy cost of bilayer curvature so that vesicles deform rather than rupture when forced through a constriction. The effect is measurable: atomic force microscopy of phosphatidylcholine liposomes formulated with bile-salt edge activators showed altered hydrodynamic diameter, morphology and mechanical properties, with deformability and Young's modulus following the order sodium deoxycholate ≥ sodium cholate > sodium taurocholate [23]. That ranking is a formulation-relevant result rather than a generic claim, and it gives a basis for choosing between bile salts other than availability.

Permeation enhancement is the third and the most double-edged. Bile salts were among the first epithelial permeation enhancers investigated, acting non-specifically through effects on tight junctions and on the apical membrane; in vitro and ex vivo intestinal and buccal bioassays confirm the activity of sodium deoxycholate and sodium glycodeoxycholate, while the same work notes that in vitro assays tend to overestimate their toxicity relative to clinical experience in the dynamic intestinal environment [24]. Mechanistic studies in intestinal mucosal explants

show that such enhancers increase transcellular and paracellular uptake by routes that do not require endocytosis [25]. For a formulation this is genuinely useful and genuinely a safety-relevant variable, and it should be presented as both.

M-cell uptake and lymphatic transport is the fourth. The negative surface charge conferred by anionic bile salts favours uptake by M cells of the Peyer's patches, giving access to the intestinal lymphatic route [12,13]. Vesicle size matters here in a direction opposite to the usual preference for the smallest particle: larger bilosomes of the order of 6 μm showed greater accumulation in Peyer's patches than vesicles around 2 μm in an influenza challenge study [22]. A formulation optimised to minimise vesicle size, as nearly all published designs are, may be optimising away the M-cell pathway it claims as a mechanism. This is a real and under-acknowledged tension in the bilosome literature.

The four mechanisms do not point in the same direction. Maximum deformability, maximum permeation enhancement, minimum size and maximum M-cell uptake are not simultaneously achievable, and a formulation that reports only size and entrapment efficiency has not revealed which trade-off it made.

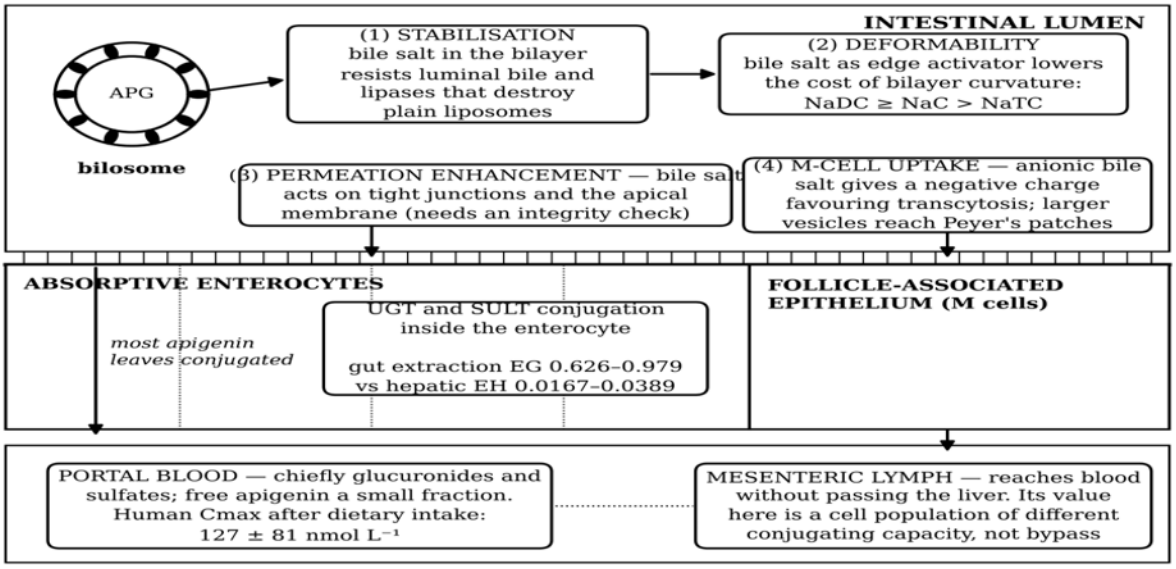


Fig. 2. The four roles of the bile salt in an apigenin bilosome, shown against the site at which each acts. In the lumen, bile salt within the bilayer resists the luminal bile and enzymes that destabilise conventional liposomes, so intact vesicles persist long enough for the remaining mechanisms to operate. At the epithelial surface, the bile salt acts as an edge activator, lowering the energy cost of bilayer curvature so vesicles deform rather than rupture, and acts independently as a permeation enhancer affecting tight junctions and the apical membrane. Within the absorptive enterocyte, UDP-glucuronosyltransferases and sulfotransferases conjugate most of the apigenin that enters, which is why gut extraction exceeds hepatic extraction and why the fraction crossing intact is the quantity of interest. At the follicle-associated epithelium, the negative surface charge conferred by anionic bile salts favours uptake by M cells of the Peyer's patches and entry to the mesenteric lymph, a route whose value here is access to a cell population of different conjugating capacity rather than hepatic bypass. Simplified; not all transport and metabolic routes are shown.

IV. APIGENIN AND RELATED FLAVONOIDS IN VESICULAR CARRIERS

The reported work is substantial and, for apigenin specifically, recent. Figure 1B plots vesicle size against entrapment efficiency for the systems described below.

The directly relevant study is that of Zafar and colleagues, who prepared apigenin-loaded oral nano-bilosomes by thin-film evaporation using cholesterol, span 60 and sodium deoxycholate, and optimised them

with a three-factor three-level Box–Behnken design using particle size, entrapment efficiency and drug release as responses. The optimised formulation had a vesicle size of 183.25 ± 2.43 nm and an entrapment efficiency of 81.67 ± 4.87%, with transmission electron microscopy showing spherical vesicles with sharp boundaries [26]. This establishes that the system is formulable and that the design approach works for it.

A second apigenin bilosome study approached surface modification. Imam and colleagues prepared apigenin bilosomes by solvent evaporation from cholesterol, sodium deoxycholate, Tween 80 and phosphatidylcholine, obtaining vesicles of 211 ± 2.87 to 433 ± 1.98 nm with polydispersity index below 0.5, zeta potential between −15 and −29 mV and encapsulation efficiency from 69.5 ± 0.93 to 81.9 ± 1.3%. Coating the selected formulation with chitosan increased vesicle size to 298 ± 3.56 nm, reversed the zeta potential to +17 mV, raised encapsulation efficiency to 88.1 ± 1.48% and improved drug release to 69.37 ± 1.34%, and the coated vesicles were additionally evaluated for mucoadhesion and permeation [27].

That charge reversal deserves comment, because it is a decision with a mechanistic cost. A cationic coating buys mucoadhesion and residence time; it also removes the negative surface charge identified above as favouring M-cell uptake. Neither paper is wrong, but the two optimisations pull in opposite directions, and a study that adopted one should say which mechanism it is choosing.

Work on close structural analogues supports the platform. Luteolin apigenin with one additional hydroxyl was formulated as pegylated bilosomes by thin-film hydration and optimised by a four-factor three-level Box–Behnken design with surfactant, cholesterol, sodium deoxycholate and PEG 2000 as factors; the optimised vesicles were 252.24 ± 3.54 nm with polydispersity index 0.24, zeta potential −32 mV and encapsulation efficiency 75.05 ± 0.65% [28]. Bilosomes of other poorly available actives have been optimised the same way, with dapagliflozin nano-bilosomes reaching 155.36 ± 2.48 nm and 86.37 ± 2.6% entrapment [29], and lycopene bilosomes evaluated as an oral platform with modulated in vivo activity [30].

Apigenin has also been placed in non-bilosomal vesicles, which gives a useful comparison. Transfersomes optimised by Box–Behnken design reached an initial entrapment efficiency of 84.24 ± 0.38% [31], and transethosomes prepared with Span 80 gave unilamellar vesicles of 108.75 ± 2.31 nm with 78.75 ± 3.14% entrapment and a flux of 4.10 ± 0.63 μg cm^{-2} h^{-1} [32]. Both were developed for topical delivery, which is the relevant caveat: deformable vesicles for skin and deformable vesicles for intestine share a design logic but not an endpoint.

Non-vesicular systems provide the benchmark for oral delivery. Apigenin solid dispersions prepared by several techniques improved dissolution and bioavailability [7], and self-nanoemulsifying systems of 10 to 20 nm droplet size increased relative oral bioavailability in rats by 3.8-fold and 3.3-fold for two formulations, with acute oral toxicity testing to OECD 425 giving an LD_{50} above 2000 mg kg^{-1} [6].

Two observations follow. The platform works: apigenin bilosomes are reproducibly formulable at nanometric size with entrapment efficiencies above 80%. And the comparator benchmark is informative a well-made solubilising system delivers roughly three- to four-fold, so a bilosome claiming mechanistic advantage over solubilisation alone should be measured against that number rather than against a plain suspension.

V. FORMULATION VARIABLES AND SYSTEMATIC OPTIMISATION

Table 3 maps the variables onto the attributes each governs.

Bile salt identity and concentration is the decision with the most mechanistic weight. The deformability ranking from atomic force microscopy sodium deoxycholate ≥ sodium cholate > sodium taurocholate [23] gives a rational basis for selection, and sodium deoxycholate is in practice the most frequently chosen for exactly this reason [26,27,28]. Concentration matters in both directions: too little and the vesicle is a niosome with incidental bile salt; too much and the bile salt solubilises the bilayer it was meant to stabilise, converting vesicles to mixed micelles and collapsing entrapment. The concentration that maximises deformability and the concentration that maximises entrapment are not the same, which is precisely why this factor belongs in a formal design rather than in a one-at-a-time screen.

Non-ionic surfactant type and amount determines bilayer packing. The sorbitan esters differ in chain length and hydrophilic–lipophilic balance, and Span 60 is widely used for vesicles of lipophilic actives because its higher phase transition temperature gives a less permeable, better-retaining bilayer at body temperature. Surfactant is also a tolerability variable, which bounds the useful range from above.

Cholesterol content controls membrane rigidity, and it works directly against the bile salt. Cholesterol inserts between surfactant chains, reducing permeability and leakage and improving entrapment, but it also stiffens the bilayer and therefore reduces the deformability the bile salt was added to create. The cholesterol-to-bile-salt relationship is the central compositional tension in a bilosome and is best handled as an interaction term rather than as two independent factors.

Drug-to-lipid ratio sets the achievable payload against the risk of unentrapped drug crystallising in the dispersion, and hydration conditions medium, temperature and time govern lamellarity and size distribution; hydration above the phase transition temperature of the surfactant is a practical requirement rather than an optional refinement. Sonication or extrusion energy reduces size with diminishing and eventually reversing returns, and for a thermolabile polyphenol prolonged sonication carries a degradation risk alongside the usual coalescence risk.

The Quality by Design framework set out in ICH Q8(R2) provides the structure: a quality target product profile, critical quality attributes derived from it, a risk assessment identifying which material attributes and process parameters are critical, and a design space within which performance is robust [33,34]. A Box–Behnken design estimates a full quadratic response surface without the extreme corner combinations of a full factorial, requiring fifteen runs for three factors including centre-point replicates [35,36], and it has already been applied successfully to apigenin bilosomes with exactly these factors [26].

Where the published designs are weakest is in their choice of responses. Vesicle size, polydispersity index, zeta potential and entrapment efficiency are conventional, easily measured, and only loosely connected to the performance being claimed. None of them distinguishes a vesicle that delivers intact apigenin across the epithelium from one that delivers the same amount to be conjugated in transit. Including apparent permeability and, better, the intact fraction as a modelled response rather than as a confirmatory test on the single optimised batch is the change that would convert a vesicle-size optimisation into a delivery optimisation. It costs more runs of a harder assay, which is why it is rarely done, and it is the difference between describing a formulation and testing a hypothesis.

Table 3. Formulation and process variables for an apigenin bilosome, and the quality attributes each governs. The last column names the trade-off that makes each a candidate design factor rather than a fixed value.

Variable	Attributes affected	Trade-off
Bile salt identity (sodium deoxycholate, cholate, taurocholate)	Deformability; permeation enhancement; zeta potential; stability in bile	Deformability follows deoxycholate ≥ cholate > taurocholate, but potency as a permeation enhancer tracks with epithelial effects that need a safety check
Bile salt concentration	Vesicle integrity; entrapment; elasticity; surface charge	Too little gives a niosome with incidental bile salt; too much solubilises the bilayer into mixed micelles and collapses entrapment
Non-ionic surfactant type and amount	Bilayer packing and permeability; vesicle size; entrapment	A higher transition temperature retains drug better but stiffens the bilayer; surfactant load is also a tolerability limit
Cholesterol content	Membrane rigidity; leakage on storage; entrapment efficiency	Works directly against the bile salt: rigidity improves retention but removes the deformability the bile salt was added to create
Drug : lipid ratio	Payload; proportion of unentrapped drug; physical stability	Higher loading risks crystallisation of free apigenin in the dispersion
Hydration medium, temperature and time	Lamellarity; size distribution; entrapment	Hydration must be above the surfactant transition temperature; prolonged heating risks degrading a thermolabile polyphenol

Variable	Attributes affected	Trade-off
Sonication or extrusion energy	Vesicle size and polydispersity; lamellarity	Smaller vesicles raise surface area but may forfeit the larger size associated with Peyer's patch uptake
Surface coating (chitosan, PEG)	Zeta potential; mucoadhesion; residence time; size	A cationic coat gains mucoadhesion and loses the anionic charge favouring M-cell uptake

VI. MEASURING INTESTINAL PERMEATION

The project is titled around intestinal permeation, and the available methods differ in what they can establish particularly given that the quantity of interest is intact drug.

The Caco-2 monolayer is the standard in vitro model, with established protocols for determining apparent permeability and a long-validated correlation with human oral absorption [37,38]. For apigenin it has a specific and often overlooked virtue: Caco-2 cells express UDP-glucuronosyltransferase and conjugate apigenin as the intestinal epithelium does [3]. The model therefore reproduces the barrier that actually limits apigenin, provided the basolateral samples are assayed for free and conjugated drug separately rather than hydrolysed to total. Bidirectional experiments also allow an efflux ratio to be calculated, which matters because the conjugates formed inside the enterocyte are themselves substrates for efflux transporters back into the lumen.

The everted intestinal sac, described by Wilson and Wiseman seventy years ago and still serviceable, retains the full intestinal wall including the mucus layer and the enzymatic machinery of the enterocyte [39]. For a bilosome it has two further advantages: the mucus layer is present, which matters for a mucoadhesive or chitosan-coated variant, and the follicle-associated epithelium can be included or excluded by choice of intestinal segment, which is the simplest way to test whether the M-cell mechanism contributes.

In situ single-pass intestinal perfusion sits between the two and is how apigenin's own BCS permeability was determined, segment by segment [10]. Using the same method for the formulation makes the comparison with the free drug direct and interpretable, which few formulation studies can claim.

Deformability deserves its own measurement, because it is invoked as the mechanism far more often than it is quantified. Extrusion through a defined pore under constant pressure gives a deformability index, and comparative values are meaningful: pegylated bilosomes with a single-chain edge activator reached 28.39 ± 5.71 against 5.88 ± 0.90 for conventional bilosomes and 14.94 ± 0.63 for transethosomes in one comparison [40]. A study that measures deformability alongside permeation can test whether the two correlate within its own formulation series, which is a stronger claim than asserting the mechanism from the literature.

In vivo, the informative design has four arms: the apigenin bilosome, a bile-salt-free niosome of otherwise identical composition, a physical mixture of apigenin with free bile salt at the same amount, and a plain apigenin suspension. The second arm isolates what the bile salt contributes to the vesicle; the third isolates what the bile salt contributes as a free permeation enhancer. Without both, an increase in exposure cannot be attributed to the bilosome as a vesicle rather than to the bile salt as an additive and that attribution is the whole claim.

Plasma should be assayed for free apigenin and, separately, for total apigenin after enzymatic hydrolysis of conjugates. The ratio between them is the direct readout of whether the formulation changed the metabolic fate of the drug or merely the amount presented for metabolism, and given that gut extraction rather than hepatic extraction dominates [19], it is the single most informative number such a study can report.

Supporting work follows the standard pattern: release data fitted to zero-order, first-order, Higuchi and Korsmeyer–Peppas models [41,42]; analytical methods validated to ICH Q2(R1) [43]; and accelerated stability testing to ICH Q1A(R2) [44] monitoring vesicle size, zeta potential and drug leakage, the last being the characteristic failure mode of any vesicular system on storage.

VII. GAPS AND OUTLOOK

Several gaps in the apigenin bilosome literature are worth naming directly, because each is addressable within an ordinary dissertation.

The intact-fraction gap. Permeation and plasma data are commonly reported as total apigenin after hydrolysis. For a compound whose principal loss is intestinal conjugation, the free-to-total ratio is the primary evidence, and reporting only the total conceals exactly the quantity the formulation is supposed to change.

The comparator gap. Most studies compare the bilosome with free drug or a plain suspension. The arms that isolate the mechanism a bile-salt-free vesicle and a physical mixture with free bile salt are rarely included, and without them the bilosome's advantage over simply adding a permeation enhancer remains untested.

The size gap. Nearly every design minimises vesicle size while simultaneously invoking M-cell uptake as a mechanism, although the evidence associates Peyer's patch accumulation with substantially larger vesicles [22]. Either the size target or the mechanistic claim should be revised, and a design that treated size as a factor with permeation as the response could settle which.

The deformability gap. Elasticity is the property most often invoked and least often measured. Where it is measured, the correlation with permeation within a formulation series is almost never reported, so the mechanism remains an assumption rather than a finding.

The bile salt selection gap. Sodium deoxycholate is used in most reported bilosomes, generally without a comparison against alternatives in the same system, even though bile salts differ measurably in the deformability they confer [23] and, in their permeation, -enhancing potency [24]. A head-to-head comparison within one formulation would be a modest experiment with a durable result.

The safety gap. Permeation enhancement by bile salts is non-specific, and a carrier whose performance depends on loosening the epithelial barrier raises a question about what else crosses with it. In vitro assays appear to overstate the toxicity relative to clinical experience [24], but understanding this is not the same as having ruled it out, and an intestinal integrity measure transepithelial electrical resistance, or a paracellular marker such as mannitol or Lucifer yellow run alongside the drug costs little and addresses it directly.

The translational gap. No apigenin bilosome appears to have been evaluated clinically, despite apigenin's low toxicity, its wide dietary exposure and the existence of human pharmacokinetic data against which a formulation could be benchmarked [5]. Given that baseline, a first-in-human pharmacokinetic comparison would be an unusually low-risk study.

VIII. CONCLUSION

The case for an apigenin bilosome does not rest on solubility, and treating it as though it does misses what is interesting about the problem. Apigenin is a class II compound, formally high in permeability, and yet dietary intake produces plasma concentrations three orders of magnitude below its in vitro active range. The reconciliation is that permeability into the enterocyte is not delivery through it: apigenin is extracted principally by the gut rather than the liver, with reported gut extraction ratios between 0.626 and 0.979 against hepatic ratios below 0.04. The enterocyte is the gateway and the loss.

Bilosomes address this with four distinct mechanisms rather than one. The bile salt stabilises the vesicle against the gastrointestinal environment that destroys conventional liposomes, acts as an edge activator conferring measurable deformability in the order sodium deoxycholate ≥ sodium cholate > sodium taurocholate, enhances epithelial permeation in its own right, and confers a surface charge favouring M-cell uptake. Reported apigenin bilosomes achieve nanometric vesicles with entrapment efficiencies above 80%, so the platform is established; what is not established is which of the four mechanisms accounts for the result.

That is where a contribution is available, and it lies in the design of the comparisons rather than in the formulation itself. Measure the intact fraction rather than total apigenin-equivalents, since that is the quantity, the mechanism predicts will change. Include a bile-salt-free vesicle and a physical mixture with free bile salt, so that the vesicle's contribution can be separated from the enhancers. Quantify deformability and test whether it correlates with permeation within the series rather than assuming it from the literature. Treat vesicle size as a factor rather than a target, given that the M-cell mechanism points the opposite way from the usual minimisation. And carry apparent permeability into the design as a modelled response instead of a final check. Each is straightforward; together they would answer the question the

formulation is built around, which is not whether an apigenin bilosome can be made but why it works.

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