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Review

Oral delivery of glutathione: antioxidant function, barriers and strategies

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ABSTRACT

Glutathione (GSH) is a tripeptide with potent antioxidant activity, which is involved in numerous basic biological processes and has been used for interventions in various degenerative diseases. However, oral delivery of GSH remains challenging, similarly to that of other protein and peptide drugs, because the physicochemical barriers in the gastrointestinal (GI) tract lead to low oral bioavailability. Although several approaches have been explored to improve delivery, such as co-administration with penetration enhancers and enzymatic inhibitors, or encapsulation into nanoparticles, microemulsions and liposomes, appropriate formulations with clinical therapeutic effects remain to be developed. This review discusses approaches explored to developing an oral GSH delivery system that could provide protection against proteolytic degradation in the GI tract and enhance molecular absorption across the epithelial membrane. This system may be beneficial for the design and development of an oral formulation of GSH in the future.

Keywords: glutathione, GSH, protein, peptide, physical barriers, biological barriers, oral delivery, oral bioavailability, proteolytic degradation, formulation

1. INTRODUCTION

Glutathione (GSH) is a tripeptide containing the amino acids glutamic acid, cysteine and glycine [1]. Endogenous GSH is a potent antioxidant involved in many basic biological processes, including protein and DNA synthesis, cell proliferation, and oxidation/reduction signaling [2]. In the past decade, GSH has been used for various medical interventions in degenerative diseases such as Alzheimer's disease and Parkinson's disease [3-7].

Peptides, such as GSH, are chemical compounds composed of 2–50 amino acids linked by peptide bonds [8, 9]. Endogenous peptides are involved in many physiological processes, acting as hormones, neurotransmitters, growth factors, ion-channel ligands or anti-infective agents [10, 11]. Their unique pharmacological profiles and intrinsic properties have made peptides excellent drug candidates that are better tolerated and have lower toxicity than traditional "small molecule" drugs (<500 Da). Their highly selective receptor-binding properties ensure good clinical efficacy [12, 13].

In recent years, interest in pharmaceutical research and formulation development of peptide therapeutics has increased. By 2018, 7000 naturally occurring peptides had been identified. More than 60 peptide drugs had been approved by authorities across the United States of America, Japan and Europe. In addition, approximately 150 peptides were in clinical trials, and more than 500 were in preclinical trials at the time [11, 14]. Over the years, peptide-related patents have shown financial potential in the pharmaceutical industry and have led to remarkable profits. For example, Lupron™ Depot, a synthesized peptide drug used primarily to treat endometriosis and prostate cancer, achieved global sales of US $2.3 billion for Abbott Laboratories in 2011 [11, 15]. Moreover, the global peptide drug market has been predicted to grow further, at a rate of 9–10% per annum [16].

In principle, peptides could have great value in medicinal applications; however their use has been severely restricted by physical and chemical barriers in the gastrointestinal (GI) tract after oral administration, thus resulting in low oral bioavailability [17]. The GI tract serves as a physical barrier including the intestinal epithelial membranes, tight junctions, unstirred water layer and efflux systems, which restrict peptide transport across

the intestinal epithelium. Chemical barriers include the extremely acidic environment in the stomach and various GI-tract proteases, which catalyze hydrolytic or enzymatic degradation of the peptide drug. These barriers have made peptides unsuitable for administration as conventional oral formulations [18-20].

Most peptide drugs are therefore currently marketed as parenteral injections. Unfortunately, injections' invasive nature, and the associated pain and potential tissue damage have made these formulations uncommonly chosen for patient use. Therefore, strategies for developing peptide formulations with enhanced oral bioavailability have received attention among scientists worldwide, who are exploring novel groundbreaking approaches to deliver peptide drugs in the most convenient and patient-friendly manner. Currently only 13 oral-form peptide drugs (tablets, capsules or solution) have been approved by the US Food and Drug Administration (FDA; **Table 1**) [21].

In the past two decades, numerous articles have reported novel technologies for oral peptide delivery. Strategies to increase oral bioavailability have included adding enzymatic inhibitors and/or penetration enhancers in the formulation, or chemical modification of peptides to form analogs and pro-drugs. Many innovative techniques using nanocarrier systems have also been evaluated, including polymeric nanoparticles, solid lipid nanoparticles, liposomes and niosomes [22-24].

As a peptide, GSH faces the same challenges as all other peptides in oral-formulation development. Although some studies have suggested promising potential for enhancing GSH oral bioavailability through different strategies [22, 23, 25], further evaluation remains essential. In this review, we highlight the physiological roles and molecular properties of GSH; the enzymatic and physical barriers of GSH uptake and transport across intestinal epithelial membranes in the GI tract; and the strategies used to enhance oral bioavailability of GSH and other peptide therapeutics (including using enzymatic inhibitors, permeation enhancers, chemical modification and formulation approaches).

2. RATIONALE FOR THERAPEUTIC GSH

Oxidative stress is a biological imbalance between the plasma concentration of reactive oxygen species (ROS) and the systemic ability to scavenge ROS and repair the resulting damage to proteins, lipids and DNA [26]. ROS (also referred to as free radicals) [27] are highly reactive, and include the superoxide radical $\cdot O_2^-$, the peroxide O_2^{2-}, the hydroxyl radical ·OH and nitric oxide ·NO [28]. The outer shell of these molecules has one or more unpaired electrons, thus making ROS highly unstable and prone to reacting with various organic substances, including lipids, proteins and DNA [29, 30].

Releasing free radicals is a mechanism used by the human immune system to destroy the structures of invading pathogenic microorganisms [29]. However, chronic accumulation of free radicals *in vivo* can be harmful by causing oxidative stress, which has been demonstrated to be responsible for the development of degenerative diseases [31]. Two pathways result in

Table 1 | Oral formulations of peptide drugs approved by the FDA

Brand	Form	Company	Name	Peptide sequence	Absorption	Indications
Tekturna	Tablets	Physicians Total Care, Inc.	Aliskiren	N. A.	2.50%	Hypertension, renal impairment and hepatic impairment
Amturnide	Tablets (aliskiren/ amlodipine/ hydrochlorothiazide)	Novartis Pharmaceuticals Corporation				
Tekamlo	Tablets (aliskiren/amlodipine)					
Tekturna HCT	Film-coated tablets					
Tekturna	Tablets					
Pertzye	Delayed-release capsules	Digestive care US, Inc.	Pancreatic lipase	Pancreatic alpha amylase	N. A.	Exocrine pancreatic insufficiency Allergic reaction
Pancrecarb	Delayed-release capsules	Digestive care US, Inc.				
Ultrase	Capsules	Axcan Pharma				
Zenpep	Delayed-release capsules	Aptalis Pharma US, Inc.				
Utresal	Delayed-release capsules					
Viokace	Tablets					
Ragwitek	Tablets	Merck Sharp & Dohme	Ragweed Pollen Extract	N. A.	N. A.	
Sucraid	Solution	QOL Medical, LLC	Sacrosidase	N. A.	N. A.	Congenital sucrose-isomaltase deficiency

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high concentrations of free radicals in the human body: the first is the accumulation of free radicals that are produced endogenously via normal cell metabolism, whereas the second is build-up through environmental factors such as pollution, cigarette smoking, radiation or medication use [32].

The human body has defense mechanisms to counteract oxidative stress by either endogenously producing antioxidants such as GSH or exogenously acquiring them through food and/or supplements. During the process, antioxidants act as free-radical removers that neutralize oxidants/free radicals, thereby protecting cells, repairing cellular damage, promoting enhanced immune function and decreasing the risk of subsequent diseases [33]. As an antioxidant, the reduced form of GSH is readily oxidized into GSH disulfide by free radicals and/or reactive oxidative species, owing to its cysteine residue. The intracellular balance of both forms of GSH determines the antioxidative state and capacity of cells [34].

3. GSH

3.1 Cellular structure and discovery of GSH

GSH (*N*-(*N*-L-γ-glutamyl-L-cysteinyl) glycine) is a tripeptide composed of γ-glutamic acid, cysteine and glycine [35, 36] (**Figure 1**). It was first discovered by Rey-Paihade in 1888 from extracts of yeast and many animal tissues including skeletal muscle, liver, intestine, brain and fresh egg white [37]. In 1929, Pirie and Pinhey reported the molecular structure of GSH as a tripeptide, as confirmed by Harington and Mead in 1935 after successful chemical synthesis based on *N*-carbobenzoxycysteine and glycine ethyl ester [37]. Since then, the molecular structure of GSH has been well established: a γ-carboxyl peptide bond links the carboxyl group of the glutamate side chain with cysteine, and a normal peptide linkage bonds cysteine's carboxyl group to glycine [35, 36]. Although exogenous GSH can come from many sources, endogenous GSH is produced mainly in the liver during normal cellular metabolism, and is abundant in the cytoplasm, nuclei and mitochondria in all living cells [32, 37-39].

Endogenous GSH is synthesized via two steps: the first step is the formation of γ-glutamylcysteine from glutamate and cysteine, catalyzed by glutamate-cysteine ligase. The second step is the formation of GSH from the reaction between γ-glutamylcysteine generated in the first step and glycine, catalyzed by GSH synthetase [40]. GSH is hydrophilic and is quickly degraded *in vivo*, with an elimination half-life of 10 min in the human body [35] and 2–3 h in rat liver [41]; consequently, GSH has extremely low bioavailability via the oral route. Cellular GSH is degraded via hydrolysis catalyzed by γ-glutamyl-transpeptidase, which breaks the peptide bond linking glutamate and cysteine, thus generating glutamate and cysteinylglycine, which are further degraded into cysteine and glycine by dipeptidases [41].

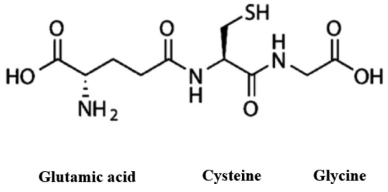

Figure 1 | Molecular structure of GSH.

3.2 Roles of GSH in preventing degenerative diseases

GSH has been well studied and accepted as a potent antioxidant participating in numerous basic cellular processes, such as protein synthesis, DNA synthesis and repair, cell proliferation and redox signaling [42]. Additionally, it plays a major role in detoxifying various electrophilic compounds such as heavy metals [43, 44].

Naturally, glutathione exists in two forms in living cells: the thiol-reduced form (L-GSH) and the disulfide-oxidized form (GSSG). In healthy cells, L-GSH is the predominant form, accounting for >98% of total GSH [45, 46], and is stored mostly in the cytosol (80–85%) and mitochondria (10–15%), whereas a small amount is stored in the endoplasmic reticulum [45]. The ratio of GSSG to L-GSH in cells represents the oxidative stress level [45]: the higher the ratio of GSSG to L-GSH in cells, the greater the oxidative stress.

The antioxidant function of GSH is generally accepted to be associated with its scavenging activity toward free radicals that accumulate during oxidative stress [47, 48], thereby protecting living cells by neutralizing excessive ROS from oxidative damage [1]. Deficiency in GSH can cause excess oxidative stress and cellular dysfunction, thus leading to various degenerative and chronic diseases including cancers, cardiovascular diseases, neurodegenerative diseases (Parkinson's disease and Alzheimer's disease) and glaucoma [3-5].

Studies have demonstrated that most human degenerative diseases, as well as the general human aging process, involve deleterious free radical reactions, which are typically caused by ROS [49, 50]. For example, the cardiovascular condition atherosclerosis involves the build-up of fatty deposits on the endothelium of blood vessels whose structure has been damaged by ROS [49, 50]. In cancerous diseases, the first mutagenic event is typically caused by ROS reactions. Interestingly, oxidative processes also help metastasized cancer cells attach to tissues [49, 50]. Finally, the eye has a high concentration of unsaturated lipids, and, owing to its poor clearance mechanisms, is defenseless against oxidative damage [49, 50], thus leading to various age-related eye diseases.

Unfortunately, most of these disorders have no cure. Consequently, preventive strategies are applied, such as health supplements, including GSH, which can slow degenerative processes.

3.3 Mechanism of GSH as an antioxidant

The mechanism of GSH as an antioxidant can be explained by cellular oxidation and reduction (redox) reactions between the sulfhydryl group of the molecule and GSH-related enzymes [38]. GSH contains a functional sulfhydryl group (also known as a thiol group) on its cysteine moiety, consisting of sulfur bonded to a hydrogen atom. GSH's primary antioxidative role is to maintain the redox state of sulfhydryl groups of important proteins by forming a disulfide bridge, which protects the structures of those important proteins. (**Figure 2**).

GSH protects the body against oxidative stress both directly and indirectly. In direct protection, GSH, in a process to catalyzed by glutathione peroxidase, scavenges ROS such as hydrogen peroxide (H_2O_2) by donating an electron, thus forming GSSG and water [41]. Indirectly, GSH is involved in producing other critical cellular antioxidants, such as vitamins C or E, as the electron source [2, 51].

3.4 Evidence of GSH's medicinal function

In the past two decades, interest in studying GSH as a therapeutic agent has increased. Many clinical trials and *in vitro* studies have been performed to evaluate the clinical value of GSH by using various administration routes (intravenous, nasal, pulmonary and oral). Some encouraging results have been reported. For example, Cascinu et al. performed a randomized double-blind placebo-controlled trial of GSH injection [50, 52] in 50 patients with advanced gastric cancer who were receiving weekly cisplatin treatment. In the treatment group, GSH was given intravenously at a dose of 1.5 mg/m^2 in normal saline solution immediately before cisplatin administration. By the 9th week of the study, no patients treated with GSH showed signs of neuropathy, whereas 16 of 18 patients in the control group did. By the 15th week, only 4 of 24 patients in the GSH group had developed neurotoxic symptoms.

Cascinu conducted a similar study in 52 patients who received a GSH infusion at 1,500 mg/m^2 over 15 min before treatment with oxaliplatin or saline [53, 54]. Clinical and electrophysiologic assessment was performed at baseline and after 4, 8 and 12 cycles of treatment. At the 4th cycle, 7 of 26 patients showed clinical signs of neuropathy (grade 1 or 2) in the GSH group, compared with 11 of 26 in the placebo group. After eight cycles, 9 of 21 patients in the GSH group experienced grade 1 or 2 neuropathy, compared with 15 of 19 in the placebo group. In terms of grade 3 or 4 neurotoxicity, zero cases were observed in the GSH group, compared with five in the placebo group. Only 18 patients completed 12 cycles of treatment for various reasons, among whom only 3 of 10 patients in the GSH group developed neuropathy (grade 2 to 4), compared with 8 of 8 in the placebo group. Therefore, both studies concluded that GSH may aid in preventing drug-induced neuropathy in platinum treatment without affecting the drug's chemotherapeutic activity (both cisplatin and oxaliplatin) [53, 54].

Although various routes have been studied for GSH-containing formulations, e.g., injections as anticancer agents [53, 55] and eye drops for glaucoma treatment [56], an oral formulation of GSH has long been the most desirable administration route, owing to the low cost of production and excellent patient compliance. Recent studies have suggested that oral administration of GSH may enhance both blood and tissue GSH levels in rats and also lead to GSH restoration in the intestinal mucosa under oxidative stress conditions [40, 57]. Oral GSH supplements have been suggested to provide a therapeutic strategy for the treatment of diseases caused by abnormalities in ROS levels in tissues.

3.5 Importance of oral formulation of GSH

Although no single GSH-containing preparation has been approved by the FDA as a therapeutic agent, many GSH supplements have already been available on

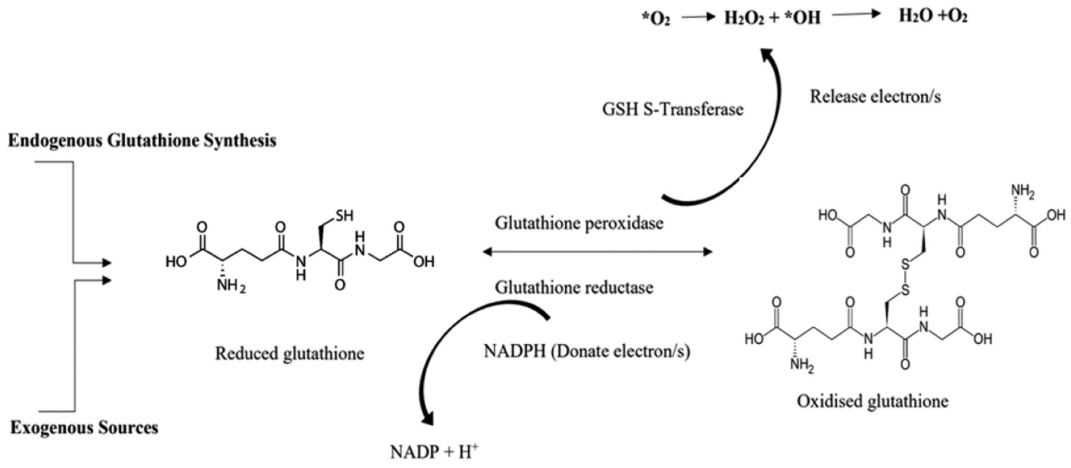

Figure 2 | Antioxidant mechanism of GSH.

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the current market in different forms, such as injections [58], lozenges [59], oral sprays [60], oral liquids [61] and oral capsules [62]. Moreover, several studies have been performed to evaluate the therapeutic potential of GSH in different formulations, such as injections (for prevention of drug-induced neurotoxicity as discussed previously), eye drops (for glaucoma treatment) [56], dermal preparations [63] and oral formulations. GSH supplements may potentially provide a therapeutic strategy for diseases caused by abnormalities in tissue ROS levels.

Among all formulations being studied, oral formulations have long been the most desirable strategy for researchers, because of their low cost of production, excellent patient compliance, convenience of storage and transport, and good shelf life. However, the primary challenge in oral formulation of GSH is its extremely low bioavailability, owing to the physical and enzymatic GI barriers. Therefore, this review focuses on investigative strategies that may improve GSH oral bioavailability by using various pharmaceutical modifications.

4. BARRIERS TO ORAL DELIVERY OF PEPTIDES

Orally administered peptides face several barriers in the GI tract. The GI tract's predominant functions are to digest food; absorb essential nutrients, electrolytes, and fluids; and excrete waste. Simultaneously, the GI tract serves as a physicochemical barrier protecting the human body from systemic invasion of toxins, antigens and pathogens [64].

To be absorbed into the blood stream, intact drug molecules, including peptides and proteins, must diffuse either between or through the intestinal epithelial cells. This process is hampered by physical and biochemical barriers in GI tract [18]. The epithelial membranes in the GI tract act as physical barriers that selectively allow the transportation of drug molecules. The phospholipid bilayer structure of the epithelial membrane restricts the transcellular transport of hydrophilic macromolecules (e.g., peptides and protein drugs), whereas the tight junctions are responsible for limiting paracellular transport [64]. Because absorption is a slow process that does not occur readily, peptide and protein drugs remain vulnerable in the GI tract, and their enzymatic degradation occurs at multiple sites along the GI tract, including the brush border, lumen and intracellular environment.

4.1 Physical barriers

The physical barriers to oral delivery comprise the cell lining itself in the GI tract, which includes the intestinal cell membranes and tight junctions between neighboring cells, as well as the unstirred water layer and efflux systems, which play important roles in regulating drug absorption [64].

4.1.1 Intestinal epithelial cell membrane. The anatomic structures of the intestine have been well described in the literature [65]; here, only the functional details associated with barriers to drug transportation and absorption are discussed. The intestinal wall primarily comprises a monolayer of column-like epithelial cells, with goblet cells, enterocytes, endocrine cells and Paneth cells interspersed in the architecture [65, 66]. Drug transportation and absorption after oral administration may depend on the physiochemical properties of the bioactive molecules, including their size, charge, lipophilicity, hydrogen-bonding potential and solution conformation, which are constrained by Lipinski's rule of five [67].

The phospholipid bilayer structure of epithelial cell membranes allows cells to have semi-permeable properties, thus enabling lipophilic drug molecules to be absorbed transcellularly via passive diffusion (**Figure 3B**). However, hydrophilic or highly charged molecules and macromolecules, such as peptides and protein drugs, in principle, face great difficulties in transcellular absorption unless they are recognized and transported via a carrier-mediated pathway or endocytosis (**Figure 3C** and **3E**, respectively). Although the size of molecules is recognized as the fundamental limitation to the oral absorption of peptide and protein drugs, some successes in the development of oral dosage forms of polypeptides have been achieved, for instance, cyclosporin A and desmopressin [68].

4.1.2 Unstirred water layer. The unstirred water layer is an aqueous boundary layer that covers the entire intestinal wall, and consists of water, mucus and glycocalyx. It arises from incomplete mixing of luminal contents.

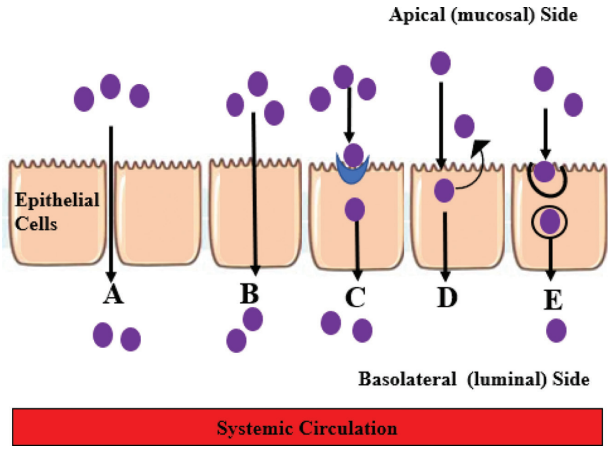

Figure 3 | Pathways of intestinal absorption, with example molecules that use this absorption method.
(a) Paracellular transportation, e.g., thyrotropin-releasing hormone [69]; (b) transcellular passive transportation, e.g., estradiol or testosterone; (c) carrier-mediated transcellular transport, e.g., amino acids, penicillins or ACE inhibitors [19]; (d) transcellular transportation modified by an efflux pathway; (e) transcellular vesicular transportation (including endocytosis or receptor-mediated transcytosis), e.g., cyclosporin [19].

This unstirred water layer acts as an essential physical barrier to drug absorption, and its thickness it is controlled by the rate of mucus secretion and the rate of layer shedding. While this water layer is continually being turned over, drug molecules must move upstream through this structure to reach the epithelial surface [70]. Additionally, complexation/binding interactions between the diffusing drug molecules and mucins are involved in the barrier function [71].

4.1.3 Tight junctions. Tight junctions are dense, hydrophobic intercellular structures that facilitate the paracellular pathway of GI drug absorption [71]. From the apical to the basolateral epithelial membrane, junctional complexes are divided into three layers: apical tight junctions (zonula occludens), underlying adherens junctions (zonula adherens) and basal desmosomes (macula adherens) [72]. Tight junctions form a continuous intercellular barrier among adjacent epithelial cells, thus creating a selective channel for solute movement across the epithelial membrane. The selectivity of tight junctions is regulated predominantly by claudins, a family of transmembrane proteins. They continuously seal the spaces between neighboring epithelial cells on the apical side and hence create a physical barrier for drug absorption [73].

Tight junctions primarily regulate the absorption of hydrophilic molecules across the epithelial membrane. Transport efficiency through this paracellular pathway is determined by the molecular size and polarity of the substances absorbed [74]. Tight junctions permit the intercellular diffusion of small hydrophilic molecules (e.g., ions, nutrients, and certain small drugs) while preventing large hydrophilic molecules (e.g., peptide and protein drugs) from passing through [17]. Tight junctions are generally considered dynamic structures that can be affected by certain chemicals such as Ca^{2+} chelators, surfactants and cationic polymers, thus increasing their permeability [17]. With the absence of peptideolytic and proteolytic activities in the paracellular transportation, the formulation design of peptide and protein drugs for oral applications via this route has drawn increasing attention from scientists.

4.1.4 Efflux systems. Efflux systems are also considered an essential part of the physical barrier in the GI tract. Efflux systems consist of a protein transporter functioning via an intracellular pathway, and are responsible for the poor oral bioavailability of certain compounds, particularly peptides and proteins [75-77] (**Figure 3D**). P-glycoprotein (P-gp), one efflux system, is located on the apical side of the epithelial cell membrane, where it actively pumps drug molecules from inside the epithelial cells back into the intestinal lumen [78]. P-gp was first discovered in cancer cells [79], and it has since been found in high levels in healthy tissues, such as cells of the intestine, liver, kidney, blood-brain barrier and placenta [79, 80].

4.2 Biochemical barriers

The biochemical barriers to oral peptide drug delivery systems include the acidic gastric environment and the presence of various metabolizing enzymes and luminal bacteria [81]. The pH of intestinal fluid varies considerably along the GI tract; consequently, the mechanism of pH-dependent hydrolysis varies in different parts of the intestine. The enzymatic barrier is the primary obstacle to oral peptide delivery. Enzymes catalyzing proteolysis or peptidolysis are located at specific sites of the GI tract. For example, pepsin is located in the stomach, and elastase, carboxypeptidases A and B, chymotrypsin and trypsin are located in the intestines, after secretion by the pancreas. Owing to the wide distribution of digestive enzymes, enzymatic degradation can occur at multiple sites throughout the GI tract. Meanwhile, degradation can also occur at multiple linkages of the peptide backbone [23]. Microorganisms in the colon secrete enzymes responsible for reactions including decarboxylation, deglucuronidation, amide hydrolysis and dihydroxylation, and the reduction of double bonds and esters [81].

Under the specific conditions of the GI tract, protein molecules are broken into polypeptides, and polypeptides are further broken into smaller units, such as bi- or tri-peptides, and/or single amino acids via peptidolysis, before being transported across the GI-tract membrane into the bloodstream [82-84]. These smaller units are the essential components that facilitate several crucial biological processes including DNA synthesis. Unfortunately, the same mechanisms pose challenges to the oral delivery of peptide drugs, because of their chemical and structural similarities to ingested proteins.

5. PROSPECTIVE PHARMACEUTICAL STRATEGIES FOR IMPROVING GSH BIOAVAILABILITY

The physicochemical properties of GSH as a peptide drug have severely restricted the clinical development of oral dosage forms, owing to the limited membrane permeability and proneness to enzymatic degradation, primarily in the jejunum [85]. Studies have suggested that the thiol group of GSH is susceptible to γ-glutamyltranspeptidase in the jejunum and is oxidized to GSSH [85], thus resulting in loss of its antioxidant activity. Therefore, strategies focusing on improving GSH's physicochemical profiles and stability in the GI tract could potentially lead to a breakthrough in formulation development, enabling enhanced oral bioavailability. These strategies include chemical modifications, formulation approaches and nanocarrier technologies.

5.1 Chemical modification strategies

Chemical modification is an approach to modify the native structure of a peptide or a protein drug to enhance its stability and absorption across the epithelial membrane [86, 87].

Application of prodrugs is one such strategy. A prodrug is defined as a biologically inactive derivative that is

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metabolized in the body and converted into a pharmacologically active drug. A prodrug protects the parent drug from enzymatic and/or chemical degradation in the GI tract, thus increasing its permeability across the biological membrane and the subsequent restoration of its pharmacological activity by systemic enzymatic cleavage before it reaches its site of action [23, 88]. For example, one study has reported that a prodrug of GSH (L-cysteine-glutathione mixed disulfide), compared with a saline control, has better bioavailability in mice after oral administration, thus protecting mice against the hepatic toxicity of acetaminophen.

Application of an analog is another effective method for improving a parent drug's therapeutic effect. *S*-allyl glutathione (SAG) is an analog of GSH obtained through modifying the thiol group of GSH with an allyl group [89]. The *S*-allyl group has been demonstrated to have an anticancer effect by inhibiting topoisomerase activity, thus resulting in cell cycle arrest and cell death [90, 91]. One study has investigated the anticancer effects of SAG by using SAG-containing selenium nanoparticles. [89]. After a 12-h treatment with the formulation, SAG was released from the nanoparticles effectively into a hepatocarcinoma cell line in both acidic (pH 5.3) and neutral (pH 7.4) conditions, with release rates of 72% and 67%, respectively. Moreover, the SAG antiproliferation effect was improved by selenium nanoparticles: the required concentration of SAG to achieve an anticancer effect was lower than that of SAG alone *in vitro*.

5.2 Absorption enhancers

Absorption enhancers are a group of functional additives incorporated into formulations to improve the permeability of drugs across biological membranes. This approach has long been investigated and applied in the development of oral formulations for protein and peptide drugs [92, 93]. Their mechanisms include chemically opening tight junctions, decreasing mucous viscosity and changing intestinal membrane fluidity [93, 94]. Commonly used absorption enhancers and candidate drugs whose absorption has been enhanced are listed in **Table 2**.

Table 2 | Commonly used absorption enhancers

Absorption enhancers	Drug absorption enhanced
Chitosan	Cyclosporine A [95]
Citric acid	Insulin [96, 97]
Cyclodextrins	Limaprost [98]
Glycerides	DuP 532 [99]
Lauroyl carnitine chloride	Insulin [100]
Sodium lauryl sulfate (SLS/sodium dodecyl sulfate)	Cefazolin [101]
Sodium N-[8-(2-hydroxybenzoyl) aminocaprylate]	Semaglutide [102]

Chitosan, a nontoxic biocompatible polymer, is a commonly used absorption enhancer for peptide drug formulations [103]. A study conducted by Liu et al. has illustrated that chitosan enhances the permeation and absorption of cyclosporine A, an immunosuppressive agent, across the intestinal membrane *in vivo* in rats [95]. To overcome the limitation of the solubility of chitosan in a neutral pH environment (as in intestinal tract), the use of chitosan derivatives has led to more effective intestinal absorption enhancement. For example, trimethyl chitosan chloride considerably increases the intestinal permeability of peptide analogs. Chitosan and its derivatives reversibly widen tight junctions, thus enhancing the biological penetration of peptide drugs [95]. Surfactants and detergents are another group of absorption enhancers that reversibly disrupt the phospholipid structure of the membrane, and consequently open tight junctions. Examples include dodecyl sulfate; sodium caprate; and long-chain acylcarnitine, fatty acids and glycerides [17].

Interestingly, GSH has been used as an absorption enhancer in some studies. One study has reported that GSH significantly enhances the permeability of sodium fluorescein across the intestinal epithelium, owing to the disruption of membrane integrity. That study has reported a significant increase in the permeability of guinea pig mucosa to sodium fluorescein *in vitro* with increasing concentrations of GSH from 0.1% to 0.4%, compared with control medium without GSH [104]. Permeation enhancement has also been observed for GSH used in combination with polycarbophil cysteine. These results have been further confirmed by another study using sodium caprate, a widely recognized absorption enhancer, for comparison [105].

Despite the favorable function of absorption enhancement, important disadvantages of these absorption enhancers have also been reported: these compounds may themselves penetrate biological membranes and cause systemic toxicity. In addition, the disruption of the epithelial membrane structure might potentially have prolonged effects and compromise biological functions [106, 107].

5.3 Enzymatic inhibitors

Oral peptide drugs are degraded by various proteases in the GI tract, such as trypsin, chymotrypsin, peptidases, and other proteolytic enzymes. Enzymatic inhibitors are molecules that bind these enzymes and decrease their activity [23]. In a promising approach, concomitant administration of enzyme inhibitors has been found to restrict the metabolism of proteins and peptides, thus increasing the availability of intact peptide drug molecules for absorption across the intestinal membrane [93].

Aprotinin (a small protein with a molecular weight of 6500 Da) is a competitive enzyme inhibitor of several serine proteases, such as trypsin and chymotrypsin [108]. It has been used as an enzyme inhibitor in various studies investigating protein and peptide drug absorption across

the intestinal membrane. One study has revealed that insulin-containing microemulsions concomitantly orally administered with aprotinin, compared with those without aprotinin, significantly decrease plasma glucose levels between 90–120 min after administration in both non-diabetic and diabetic rat models [109]. Pechenkin et al. have investigated the effects of several protease inhibitors (aprotinin, soybean derived Bowman-Birk inhibitor and Kunitz soybean trypsin inhibitor) on oral delivery of insulin. Insulin is well protected from proteolytic degradation (triggered by trypsin and chymotrypsin) when encapsulated with these enzyme inhibitors, as compared with unadulterated insulin solutions, *in vitro* [110].

Bacitracin, a cyclic polypeptide antibiotic with a molecular weight of 1422.7 Da, is another enzyme inhibitor that effectively inhibits various proteases, including trypsin, pepsin and aminopeptidase [111]. An *in vitro* study has reported that bacitracin, camostat mesilate and sodium glycocholate decrease insulin degradation in rat large intestinal homogenate [112]. Although no published studies have described the use of an enzymatic inhibitor with GSH, the process would be expected to follow the same approaches applied to other oral peptide drugs.

The limitations of using enzyme inhibitors in peptide drug delivery include systemic toxicity, digestive disorders and pancreatic islet cell hyperplasia [113], which must be carefully considered for formulation development.

5.4 Formulation approaches

The properties of chemical materials change when their particle sizes approach atomic size, because the increase in the ratio of surface area to volume may cause nanoscale particles to exhibit optical, physical and chemical properties significantly different from those of larger particles [114]. Nano-sized carriers offer many advantages in protein and peptide delivery, including high physical and chemical stability, high drug loading capacity, capability of incorporation of both hydrophilic and hydrophobic drugs, and enhanced bioavailability with sustained-release properties [22]. In addition, nano-carriers can be designed as formulations with various administration routes, e.g., oral, nasal, dermal, pulmonary and parenteral routes [22].

Numerous forms of nanocarriers have been widely studied. This review discusses microemulsions, nanoparticles, liposomes, niosomes and proniosomes. The transport mechanisms of these various formulation approaches over the barriers are illustrated in **Figure 4**. The mechanisms, advantages and limitations of these strategies are also summarized in **Table 3**.

5.4.1 Microemulsions. A microemulsion is defined as a dispersion of oil, water and surfactant (with co-surfactant). It is a spontaneously forming liquid mixture that is transparent, optically isotropic and thermodynamically stable, with droplet sizes ranging from 10 to 200 nm [122]. Three types of microemulsions exist, according to the internal and external phase: oil-in-water, water-in-oil and bicontinuous [23] (**Figure 5**).

Compared with colloidal systems, such as suspensions, microemulsions have several advantages as drug carriers, such as improved drug solubility, longer shelf life, enhanced bioavailability and ease of preparation [123]. Therefore, formulation designs using microemulsions

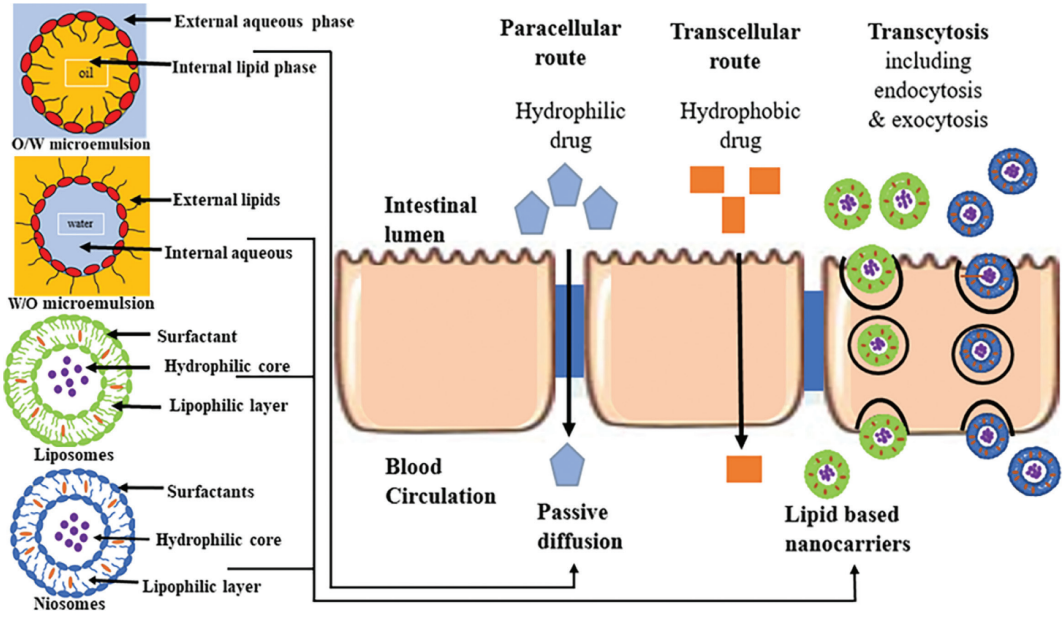

Figure 4 | Nanocarriers and their transport mechanisms across intestinal barriers.

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Table 3 | Mechanisms, advantages and limitations of various nanocarriers

Nanocarriers	Mechanism	Advantages	Limitations	Applications	Refs.
Microemulsion	Combination of passive diffusion and/or active transport and/or endocytosis	Thermodynamically stable; spontaneous formation; improved drug solubility; long shelf life; ability to load large quantities of both hydrophilic and hydrophobic drugs; enhanced bioavailability	Toxicity due to high surfactant concentration	Oral administration of insulin in rats; cyclosporin oral formulation for treatment of rheumatoid arthritis and psoriasis in humans	[115, 116]
Nanoparticle	Transcytosis including endocytosis and exocytosis transport	Long circulation half-life; low hepatic filtration; enhanced stability; enhanced bioavailability	Cytotoxicity due to altered regulation function of endothelial cells	Oral formulation of GSH for therapeutic effects on intestinal diseases caused by oxidative stress	[26, 117]
Liposome	Phosphate lipid-based transcytosis pathway	Biocompatibility; biodegradability; non-immunogenicity; ability to entrap both hydrophilic and hydrophobic drugs; protection of drug from Gi tract; enhanced drug cellular uptake and transport; high tolerability; controlled or sustained drug release	High manufacturing cost; high time consumption; physical instability; inability to sterilize; low drug entrapment; batch reproducibility; leaking of entrapped medicine	Oral GSH supplement for increasing GSH levels in the human body	[40, 118]
Niosome	Surfactant-mediated transcytosis pathway	All advantages of liposomes; ease of scale-up production; low cost for preparation; better physical stability than liposomes	Physical instability; inability to sterilize; low drug entrapment; leaking of entrapped medicine	GSH-loaded niosome oral formulation for hepatic protection and enhanced hepatic cell uptake and GSH bioavailability	[119, 120]
Pro-niosome	Transfer into niosomes by hydration and surfactant-mediated transcytosis transport	All advantages of niosomes; better physical and chemical stability than niosomes; prolonged shelf life; better approach in dosing design	Requirement for compounding before use; limited relevant studies available	Oral form of vinpocetine with improved oral absorption	[121]

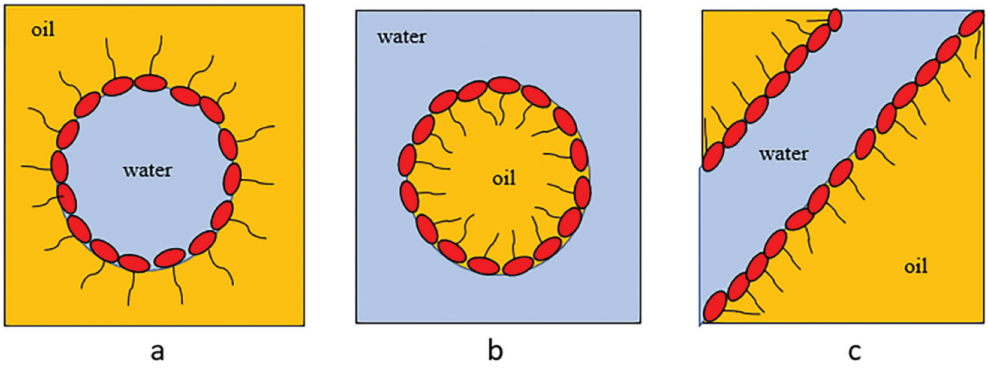

Figure 5 | Structure of microemulsion droplets.
(a) water-in-oil; (b) oil-in-water; (c) bicontinuous.

for oral peptide and protein drug delivery have received great interest.

For example, Çilek et al. have developed a lecithin-based microemulsion formulation of recombinant human insulin with aprotinin for oral administration, aiming to examine the hypoglycemic effects in non-diabetic and streptozotocin-induced diabetic rats [115]. After oral administration, the insulin-containing microemulsion (with or without aprotinin), compared with unformulated oral insulin solution, has been found to decrease plasma glucose levels by approximately 30%, with effects lasting for approximately 90 min.

In a study conducted by Wen et al., microemulsions applied as a GSH delivery system, compared with a colloidal emulsion system and GSH alone, have been found to achieve sustained-release profiles of GSH. *In vitro* profiles from the study indicated that the microemulsion might have provided sustained release of GSH after oral administration, thus suggesting the promise of this oral delivery system with enhanced GSH bioavailability [124].

A microemulsion oral solution of cyclosporin, Neoral, has been approved by the FDA. This product is used to prevent organ rejection after transplantation (of the liver, kidneys and heart) and for treatment of rheumatoid arthritis and psoriasis [116]. Research has demonstrated that microemulsions may serve as promising drug carriers for oral protein and peptide drug delivery, and therefore can be considered in designing oral formulations for GSH.

Despite being promising delivery systems, microemulsions may be concerning because of their potential toxicity due to the high surfactant concentration. This toxicity must be addressed in designing oral delivery systems [125].

5.4.2 Nanoparticles. Nanoparticles have been extensively studied as peptide and protein drug delivery systems in the past decade. They are defined as colloidal particles (consisting of biodegradable or nonbiodegradable polymers) [126]. The advantages of using nanoparticles as peptide drug delivery systems include their long circulation half-life *in vivo* and low hepatic filtration, thus enhancing stability and bioavailability [126]. The small size of nanoparticles allows for higher cellular uptake of peptide drug molecules, thus improving drug absorption across biological membranes. Furthermore, the use of nanoparticles as drug carriers may result in fast drug release because of the increased surface area corresponding to the small particle size [127]. However, limitations of such formulations have been reported, including cytotoxicity due to altered regulatory function of endothelial cells [128]. These challenges must be overcome before nanoparticles are applied as peptide drug carriers [129].

Many studies have evaluated the potential of using nanoparticles as delivery systems for oral GSH formulations. To examine GSH's therapeutic effects on intestinal diseases caused by oxidative stress, Bertoni et al. have developed solid lipid nano-scaled particles (250–355 μm) loaded with GSH [26]. They have found that the encapsulation capacity of GSH is as high as of 20% w/w, and GSH's physicochemical properties are effectively retained during the process. Moreover, varying the composition of the formulation can modulate the release of GSH: the more hydrophobic the lipid contained in the particles, the longer the GSH release time in intestinal fluids. The authors have concluded that these GSH containing formulations co-administered with another antioxidant (catalase) show excellent radical scavenging activity by decreasing intracellular ROS levels, and display superior antioxidant activities to rescue H_2O_2 oxidation *in vitro* [26].

Another study by Alobaidy has investigated the effects of chitosan-formulated nanoparticles on the oral bioavailability of GSH. In that study, GSH-loaded nanoparticles have shown a rapid and prolonged release profile of GSH after oral administration comparable to the profile of subcutaneously administered GSH *in vivo* in rats. The effect is dose-dependent, and the plasma concentration of GSH in rats is proportional to the GSH dose uploaded in the nanoparticles [117]. A separate study has reported that the release of GSH from nanoparticles (composed of basil seed gum loaded with GSH) is pH dependent. *In vitro* studies have indicated faster and more comprehensive GSH release in pH 6.8 (mimicking the intestinal environment) than pH 1.2 (mimicking the stomach environment) [130].

5.4.3 Liposomes. Liposomes are spherical particles composed of an aqueous core surrounded by one or more phospholipid bilayers, generally with sizes ranging from 20 nm to 10 μm [23, 131, 132]. Liposomes can entrap both hydrophilic drugs (in the aqueous core) and hydrophobic drugs (in the lipid bilayers) (**Figure 6a**) [133, 134]. Liposomes can be categorized into multilamellar vesicles and unilamellar vesicles, which can be further classified into small unilamellar vesicles and large unilamellar vesicles. A unilamellar liposome has a single phospholipid

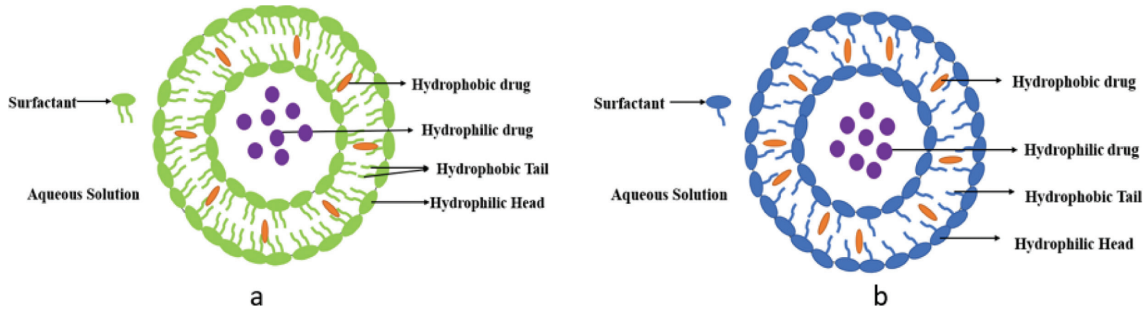

Figure 6 | (a) Structure of a unilamellar liposome, showing the loading locations of hydrophilic and hydrophobic drugs; (b) structure of a niosome, showing the loading locations of hydrophilic and hydrophobic drugs.

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bilayer, whereas a multilamellar vesicle has an onion-type structure [135].

The use of liposomes for oral peptide and protein drug delivery has been investigated for many years, owing to their unique advantages, including favorable biocompatibility, protection of drug molecules from the harsh environment of the GI tract, and enhanced cellular uptake and transport. However, disadvantages such as high manufacturing cost, formulation instability and time consumption remain challenging during formulation development [134, 136, 137].

Many studies have investigated liposomes for the oral delivery of GSH. A clinical study of 12 healthy adults has revealed that oral administration of liposomal GSH supplements significantly increases GSH levels in the body. Compared with those at baseline, the GSH levels increased by 40% in whole blood, 25% in erythrocytes, 28% in plasma (1 week after administration) and 100% in peripheral blood mononuclear cells (2 weeks after administration). Elevated immune function markers and decreased oxidative stress were also observed [40]. Another study has examined the effects of a proliposome formulation on the oral bioavailability of GSH and formulation stability. The structure of GSH was found to be maintained in the proliposome formulation. Compared with commercially available capsules and pure GSH, the proliposomes prepared in this study displayed a more than one-fold increase in the oral bioavailability of GSH in rats. Moreover, no significant changes in particle size and zeta-potential of the formulation were observed. Hence, the authors concluded that proliposome formulations might be applied as a novel delivery system for oral administration of GSH with enhanced oral bioavailability and stability [118].

5.4.4 Niosomes. Niosomes are nano-structured vesicles with a size ranging from 10 nm to 3 μm [138], produced from surfactants and cholesterol in an aqueous medium (**Figure 6b**) [22, 139]. Their structures are similar to those of liposomes, as small or large unilamellar or multilamellar vesicles, and they are prepared through similar production procedures [140, 141].

As drug delivery systems, niosomes can accommodate both hydrophilic and hydrophobic drugs, and generate sufficient surface areas to facilitate targeted drug delivery to the site of therapeutic action, thereby increasing drug efficacy and decreasing adverse effects. In addition to possessing all the advantages of liposomes [142], niosomes have unique features to overcome the limitations associated with liposomes, such as difficulties in scaling up production, the high cost of organic materials used for preparation and low physical stability [22]. Niosomes have been extensively studied as drug delivery systems, and their applications have been widely used in various pharmaceutical fields such as topical, oral, parental and transdermal application [22].

Owing to their biodegradability, biocompatibility, non-immunogenicity and low cost with respect to those of liposomes, niosomes have increasingly drawn attention as nanocarriers in GSH oral delivery [142]. For example, a recently published study has evaluated GSH-loaded niosomes' hepatic protection, hepatic cell uptake and GSH bioavailability [120]. The study has reported that after oral administration, GSH-containing niosomes, compared with the pure GSH solution, significantly restored rat liver damage (induced by CCl_4 administered intraperitoneally; $P < 0.05$). The GSH content in liver tissues was 15.90 μg/g for the GSH-containing niosome group and at 9.91 μg/g for the GSH solution group, whereas the baseline in damaged liver was 8.15 μg/g. Stability studies indicated no significant change in particle size, zeta-potential, polydispersity index and encapsulation efficiency after storage for 4 weeks at room temperature or 4°C. This formulation exhibited GSH-mediated protective effects against stomach environment (pH 1.2) with 35.5% drug release at pH 1.2 compared with 45% at pH 6.8 (mimic small intestine) after 6 h incubation *in vitro*. This pH-sensitive drug release profile of GSH-containing niosomes has been demonstrated by another study indicating this nanocarrier's non-toxic effect to cells *in vitro*, even at high concentrations of GSH (400 μg/mL) [119]. The observed anti-cancer effects and sustained protein alteration effects observed in this study lasted for 48 h [119]. Therefore, niosomes may serve as future drug carriers for oral GSH delivery for therapeutic purposes.

5.4.5 Proniosomes. A proniosome is a dry, free-flowing granular product that is hydrated after contacting aqueous media, thus forming a niosome dispersion immediately before use [143]. Proniosomes provide all the advantages of niosomes, such as better chemical stability and lower preparation cost than liposomes. Additionally, pronisomes exhibit better physical stability than niosomes because of their dry nature. however, niosome suspensions face problems that must be addressed during storage, including aggregation and fusion of vesicles, and leaking and hydrolysis of entrapped drug molecules. [144-146]. Consequently, with their prolonged shelf lives, proniosome formulations may provide convenience in transportation, storage and distribution for large-scale pharmaceutical production. Furthermore, owing to their dry state, proniosomes could be further processed into granules, tablets or capsules, thus providing a better approach in unit dosing design than the liquid dosage form of niosomes [147].

Studies have investigated proniosome preparation and the physicochemical characteristics, including particle size/distribution analysis and drug release profiles [147, 148]. Compared with those of conventional niosomes, niosome dispersions derived from proniosomes are easier to prepare without requiring long agitation times. In addition, proniosome dispersions tend to display better profiles in particle size uniformity. Meanwhile, their drug entrapment efficiency and *in vitro* drug release profiles remain unchanged [144-146].

Another study has evaluated the effects of proniosmes on the oral absorption of vinpocetine, a poorly

water-soluble drug. Drug-entrapped niosomes have significantly higher permeability in *in vitro* study using segments from different intestinal regions of rats than unformulated vinpocetine suspension. Similar increases in absorption have been observed *in vivo* after oral administration of niosome formulations to rabbits. The proniosomes have displayed sustained physical and chemical stability. Data showed that there were no significant changes in terms of particle size and drug entrapment efficiency compared to the ones of 6 months ago [121]. Therefore, proniosomes may be an ideal nanocarrier to deliver protein and peptide drugs with low bioavailability and poor stability in the GI tract.

6. CONCLUSION

The main challenge in the oral delivery of protein and peptide drugs is their enzymatic degradation in the GI tract, which is the main cause of their low oral bioavailability. To address this problem, many approaches have been studied by scientists, including chemical intervention, absorption enhancers, enzymatic inhibitors and formulation strategies, e.g., microemulsions, nanoparticles, liposomes and niosomes. In general, every strategy has its own advantages and limitations as oral drug carriers. Therefore, the best approach for a sufficient oral delivery system for protein and peptide drugs as well as GSH would be a comprehensive formulation combined with multiple strategies depending on the physicochemical characteristics of the drug molecules. On the basis of this review, niosomes might be the ideal drug carrier for GSH oral delivery, owing to their the unique advantages and cost-effectiveness. However, further study is needed to examine the feasibility of this approach in terms of the pharmacokinetic and pharmacodynamic profiles.

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CONFLICTS OF INTEREST

The authors declare they have no actual or potential competing interests.

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