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REVIEW

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Oral fate and stabilization technologies of lactoferrin: a systematic review

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ABSTRACT

Lactoferrin (Lf), a bioactive protein initially found in many biological secretions including milk, is regarded as the nutritional supplement or therapeutic ligand due to its multiple functions. Research on its mode of action reveals that intact Lf or its active peptide (i.e., lactoferricin) shows an important multifunctional performance. Oral delivery is considered as the most convenient administration route for this bioactive protein. Unfortunately, Lf is sensitive to the gastrointestinal (GI) physicochemical stresses and lactoferricin is undetectable in GI digesta. This review introduces the functionality of Lf at the molecular level and its degradation behavior in GI tract is discussed in detail. Subsequently, the absorption and transport of Lf from intestine into the blood circulation, which is pivotal to its health promoting effects in various tissues, and some assisting labeling methods are discussed. Stabilization technologies aiming at preserving the structural integrity and functional properties of orally administrated Lf are summarized and compared. Altogether, this work comprehensively reviews the structure-function relationship of Lf, its oral fate and the development of stabilization technologies for the enhancement of the oral bioavailability of Lf. The existing limitations and scope for future research are also discussed.

KEYWORDS

Lactoferrin; oral; digestion; absorption; transportation; stabilization technology

Introduction

Lactoferrin (Lf), a 80 KDa glycoprotein, was first identified as a red protein in whey and then purified in 1960 from human and bovine milk (Sorensen and Sorensen 1941; Groves 1960; Johanson et al. 1960). Initially, Lf was regarded as a member of transferrin family due to its high sequence identity with serum transferrin and its reversible binding to iron (Aisen and Leibman 1972). Remarkably, Lf possesses a greater iron-binding affinity and the ability to retain the iron ions over a wide pH range owing to its specific structural properties (González-Chávez, Arévalo-Gallegos, and Rascón-Cru 2009). In recent years, extensive researches have been reported on the diverse functions of Lf, especially for intestinal health in early life (Table 1). Hence, Lf is a promising agent for using as the functional component in functional foods and in pharmaceutical applications.

Oral delivery is the most accepted and economical administration route for bioactive compounds, especially functional proteins (Tran et al. 2019). Traditionally, dietary proteins are typically digested to the amino acids or oligopeptides in the gastrointestinal (GI) tract and then absorbed through the intestinal epithelial cells mainly making use of sodium-dependent symporters and proton-motive forces to participate the anabolism and energy metabolism (Broer 2006). However, for functional protein like Lf, structural integrity is an important basis to realize its multifunctions (Wang, Timilsena, et al. 2019). Lf receptors (LfR) that have been shown to be present in many tissues and cells surface are essential for Lf to function. For example, Lf binding to LfR, expressed on the surface of epithelium and intestinal Peyer's patches, can increase the proliferation of human intestinal epithelial cells and activate the intestinal immune response (Liu et al. 2020; Kong et al. 2020). Meanwhile, it has been reported that Lf is absorbed from the gut by LfR-mediated transcytosis and then transported to blood circulation via the portal vein and/or the mesenteric lymphatics for its systemic functions (Kitagawa et al. 2003; Takeuchi, Kitagaw, and Harada 2004). Therefore, the digestion, absorption and transport of orally administrated Lf have a significant influence on its bioavailability. Correspondingly, various stabilization strategies are required for improving the stability, achieving targeted delivery, or enhancing the absorption of orally administrated Lf (Vergara et al. 2020). In addition, some activities of functional Lf protein may also be mediated through its bioactive peptide fragments (Chalamaiah, Yu, and Wu 2018). For example, the peptide lactoferricin (Lfcin), which comprises a large portion of the functional domain of the parent protein and is released from Lf through its *in vitro* proteolysis by pepsin, retains the majority of the activities of Lf (Gifford, Hunter, and Vogel 2005). However, in view of the limitations of *in vitro*

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Table 1. The new views about functions of Lf in recent reports.

Function	Scope	Mechanism	Lf form	Reference
Anticancer	• Pro-metastatic tumor micro- environment • Human colon tumor (HT29 as the model) • Six human breast cancer cell lines • Tumor-associated macrophages (TAMs)	• Lf deficiency induces a pro-metastatic tumor microenvironment, and Lf was negatively associated with tumor progression and metastasis. • Inhibiting the VEGFR2/ VEGFA/PI3K/Akt/Erk1/2 angiogenesis pathway • Using the apoptosis pathway as its mechanism to inflict cell death • Lf-containing immunocomplex as a promising cancer therapeutic agent was capable of converting TAMs into tumoricidal M1-like cells.	• Endogenous Lf • Iron-saturated Lf • Recombinant hLf • hLf	• Wei et al. 2020 • Li et al. 2019 • Iglesias-Figueroa et al. 2019 • Dong et al. 2020
Regulating lipid metabolism	• Promoting bile acid metabolism and reduces hepatic cholesterol deposition	• Inhibiting the farnesoid X receptor-mediated enterohepatic axis	• Orally administrated Lf	• Ling et al. 2019
Anti-microorganism	• Protozoa • Bacteria • Virus	• Internalized to Lf and Lfcin halting *Giardia* cell growth and prevent infective cyst production • Aggregating the pathogenic bacterium and further inhibiting their binding to intestinal cells • The mechanism of antiviral action was likely indirect and may involve induction of innate interferon responses	• Lf and Lfcin • Bovine milk free secretory concluding bLf • 8.7% iron saturated bLf	• Frontera et al. 2018 • Cakebread et al. 2017 • Oda et al. 2021
Immunomodulatory and anti-inflammatory	• Interacting with enterocyte and intestinal commensal bacteria • Acting on neonatal monocyte-derived macrophages • Acting on the polarized M1 macrophage	• Modulating the expression of immune-related gene; Binding with the surface or DNA of cells for immune system and the triggering signaling pathways • Promoting the IEC-6 viability and proliferation; Modulating proinflammatory pathways • Preventing of harmful TLR-mediated inflammatory disorders to attenuate the proinflammatory response of neonatal monocyte-derived macrophages • Lf lessens inflammation associated with M1 macrophage polarization and positively modulates the related changes of iron metabolism (iron trafficking and storage)	• Orally administrated bLf; • Orally administered bLf ; Orally administered the *Bifidobacterium longum* plus bLf; Human milk • HLf • hLf	• Kong et al. 2020; Fornai et al. 2020; Thai and Gregory 2020 • Wisgrill et al. 2018 • Cutone et al. 2017
Influencing cognition	• Contributing to the neurodegenerative disease • Improving the cognitive ability for the aging • Being vital in early neurodevelopment and cognition	• Due to the surge in high-affinity receptor density and the lack of a feedback loop for Lf, iron transported to the brain continuously, which increased brain iron to pathological levels and can contribute to neurodegeneration.	• \(Mathematical modeling) • Intragastric administration of Lf • Orally administrated bLf	• Khan, Liu, and Dutta 2020 • Zheng et al. 2020 • Chen et al. 2015

(*continued*)

Table 1. Continued.

Function	Scope	Mechanism	Lf form	Reference
		• LF reduces ROS production in the hippocampi and induced metabolomic changes in the brain. • Upregulating several canonical signaling pathways, the genes expression, transcriptional and translational levels associated with neurodevelopment and cognition		
Prebiotic function	• *Lactobacillus* genus	• Regulating the strains growing in biofilm, aggregating and adhering to biotic or abiotic surfaces	• bLf	• Bertuccini et al. 2018
Bio-marker	• Associated with re-transplantation free survival • Associated with small intestinal lesions	• Calprotectin and Lf can differentiate progressive biliary damage from non-biliary liver value alterations after liver transplantation • Fecal calprotectin and lactoferrin levels were significantly higher in patients with versus without small intestinal lesions	• Biliary Lf • Fecal Lf	• Rauber et al. 2020 • Mari et al. 2020
Promote vitamin D absorption	• Reversing vitamin D deficiency	• Stimulating the expression of vitamin D receptor	• Diet containing Lf	• Wang et al. 2019

digestion experiments, further determination on the presence of Lfcin in the *in vivo* GI digesta is necessary. To date, a systematic review focusing on the oral fate of Lf is lacking (Table 2). Herein, we present an overview on the current studies relating to the oral delivery of Lf in the GI tract and then into the blood circulation. Additionally, relevant stabilization technologies for improving the oral bioaccessibility and bioavailability of Lf are summarized. Finally, the limitations of current studies and the scope for future research are identified and addressed. This review aims to provide a comprehensive understanding on the oral fate of Lf based on the current progresses and encourage more rational and suitable technologies for the development of nutraceutical products with Lf.

Structure-function relation of Lf

Lf exists in the secretory fluids, body fluids and specific granules of polymorphonuclear leukocytes in many species. Its distribution and amount are related with an individual's physiological and pathological state and is reportedly found in the highest concentrations in human colostrum (5 g/L) (González-Chávez, Arévalo-Gallegos, and Rascón-Cru 2009; Liang et al. 2020). Recent research (Semak et al. 2019; Xu et al. 2019) has developed the transgenic goats and silkworms as biofactories for the large-scale production (16 g/L and 12.07 mg/g, respectively) of biologically active recombinant human Lf (hLf). The amino acids sequence has been determined for Lf from many species, the mature protein contains around 690–700 residues, and shares high sequence identity for mammalian species especially in the case of human Lf and chimpanzee Lf (97%) (Baker et al. 2000; Yount et al. 2007). The sequence repeat (∼40%) for the amino acid in C- and N-terminal halves indicates an evolutionary gene duplication event (Metz-Boutigue et al. 1984). In terms of the primary structure, Lf is not an ideal nutritional source of essential amino acids (EAA) but with a distinctive basic character as shown in Table 3 (Wang 2005; Baker and Baker 2009).

The deep understanding in the structure-function relation of Lf derives from following determination on its spatial structure (Anderson et al. 1987). As a whole, the component amino acids are connected to a single polypeptide chain that folds into two symmetrical lobes corresponding to the N- and C-terminal halves (N lobe and C lobe). These two lobes of Lf are connected by a flexible hinge region of a 3-turn α-helix in residues 334–344. Each lobe contains two domains having α-helix and β-pleated sheet structures, respectively (N_1/N_2 or C_1/C_2), with corresponding residue sequences 1–90 and 251–333/91–250 or 345–431 and 593–676/432–592. The internal cleft enclosed by the two domains provides binding sites for Fe^{3+} and CO_3^{2-}, which are conserved for both lobes and for Lf from different species (Sharma et al. 2013). The amino acids involving in the binding process are distributed in two domains of each lobe (Figure 1b). Using molecular dynamics simulations, Anghel, Radulescu, and Erhan (2018) revealed that Arg121 is the key

Table 2. The current reviews about Lf.

Scope	Contents/Conclusions	References
Function and biological properties of Lf	Structure-function relation, stability and applications	Wang, Timilsena, et al. 2019; Iglesiaa-Figueroa et al. 2019
N-Glycosylation structure	Glycosylation pattern that may be responsible for heterogeneity of the biological properties of Lf/ Structural changes over the whole course of lactation, including pre-dry period milk	Karav et al. 2017; Valk-Weeber, Eshuis-de Ruiter, et al. 2020
As the anti-biofilm therapeutic	The anti-biofilm ability of may attributed to many issues including binding and sequestering the iron in the environment/diffusing through the bacterial biofilm/changing the central metabolism of bacteria/ binding with bacterial membranes and then disrupting the bacterial biofilm	Ammons and Copie 2013
Effect in mucosal cervicovaginal defense	Biomarker of altered microbial homeostasis at vaginal level and being promising to be used in the restore of mucosal immune homeostasis	Valenti et al. 2018
Role of Lf in neonatal care	Lf could significantly reduce the incidence of NEC and LOS, and decrease the risk of hospital-acquired infection and infection-related mortality in premature infants without obvious adverse effects.	He, Cao, and Yu 2018; Telang 2018; Sharma, Shastri, and Sharma 2017
Lf treatment for dermatological conditions	Current clinical studies suggested Lf may be beneficial in acne, psoriasis and diabetic ulcerations	Hassoun and Sivaman 2017
Biomarker for inflammatory bowel disease	Fecal Lf is useful biomarkers, but its value in managing individual patients must be considered in specific clinical contexts	Mosli et al. 2015; Stragier and Van Assche 2013
Quantitative changes in Lf in term and preterm human milk during lactation	Lf concentration was highest during early lactation and rapidly declined to remain relatively unchanged from 1 month to 2 years of lactation.	Rai et al. 2014; Villavicencio et al. 2017
Oral delivery systems for Lf	Reviewing various pharmaceutical strategies including: PEGylation, absorption enhancers, enzymatic inhibitors and advanced drug carrier systems.	Yao et al. 2013a
Lf-based vehicles for bioactive compounds	Utilizing Lf-based nanocarriers as natural vehicles for nutraceutical delivery and release, as well as strategies for encapsulating Lf as a functional ingredient.	Liu, Zhang, et al. 2018
Technological processes for Lf	Effect of classical treatments on lactoferrin structure and activity, such as heat treatment or drying, and also of emerging technologies, like high pressure or pulsed electric field	Franco et al. 2018

amino acid residue for the stabilization of the iron ion in the N-lobe of hLf. The iron binding in all lobes induces a conformationally rigid structure and suggests a resistant property for Lf toward thermal and proteolytic denaturation (Wang et al. 2017). In addition, the sequestration of Fe^{3+} by Lf is correlated with its anti-bacterial/fungal/viral and anti-oxidant activities by inhibiting the microbial growth, biofilm generation and through iron-induced free radical production (Jegasothy et al. 2014). The iron binding properties of Lf are highly dependent on the structural integrity of Lf (Rosa et al. 2020).

In addition to the structure-function for Lf as the member of transferrin family. The unique surface properties and specific residues composition of Lf are contributed to its other multifunctions (Baker and Baker 2009). Previous researches (Adlerova, Bartoskova, and Faldyna 2008) have revealed the uneven distribution of positively charged residues in Lf, which are mainly concentrated in the N-lobe and in the inter-lobe regions (Baker and Baker 2009). The basic residues arraying along the outside of first helix of N-lobe form the most important domain of positive charge known as Lfcin (Figure 2). This Lfcin domain is involved in the diverse functions of Lf by recognizing the surface factors or the structural components of cell outer membrane (Senkovich et al. 2007; González-Chávez, Arévalo-Gallegos, and Rascón-Cru 2009). The importance of these direct interactions includes: preventing the attack of virulence factors toward the host cell; triggering signaling pathways that lead physiological to an anti-inflammatory response; and changing the properties of the contacted membrane. The targeted molecules are reported to include the surface protein A from human pathogen *Streptococcus pneumoniae*, proteoglycans of immune cells, heparan sulfate (HS) and glycosaminoglycans (GAGs), proteoglycans of immune cells, lipopolysaccharide (LPS) and teichoic acid of Gram-negative/positive bacteria, phosphatidylserine of tumor cells, and Lf receptors in various cells (Senkovich et al. 2007; González-Chávez, Arévalo-Gallegos, and Rascón-Cru 2009; Legrand 2016; Ostan et al. 2017; Mancinelli et al. 2020). Cakebread et al. (2017) reported the free secretory component containing Lf from bovine milk induced the aggregation of enteropathogenic *Escherichia coli*, which might result from the surface charge neutralization through the direct interaction with positively charged Lf. The aggregation of *Escherichia coli* inhibited their binding to intestinal cells. It is noteworthy that hydrogen bond and hydrophobic

Table 3. Amino acid sequence and the essential amino acid (EAA) composition of human Lf.

Amino acid residues		Essential amino acid (EAA) composition	
	Number	Percentage in hLf (%)	Recommended percentage by FAO (%)
Val (V)	48	19.76	14.10
Leu (L)	58	23.86	17.20
Ile (I)	16	6.60	12.90
Phe (F)	30	12.34	19.50
Trp (W)	10	4.12	3.10
Lys (K)	45	17.49	12.50
Met (M)	5	2.05	10.70
Thr (T)	31	12.77	10.00
Ala (A)	63		
Arg (R)	45		
Asn (N)	33		
Asp (D)	38		
Cys (C)	32		
His (H)	9		
Ser (S)	50		
Tyr (Y)	21		
Glu (E)	41		
Gln (Q)	28		
Gly (G)	54		
Pro (P)	30		
Total	691		

interaction are two forces mediating the interaction of Arg residues with negatively charged surfaces. Thus, the N-terminal sequence GRRRR, containing four consecutive Arg, is specific to human Lf, further increasing the effective targeting of Lf to the cell membrane (Penco et al. 2001). Besides, it is thought that Lf can further perform its functions intracellularly, but the mechanism for Lf entry into the cell is poorly understood (Haukland et al. 2001). It appears that the direct interaction between Lf and outer layer of bacteria disrupts membrane fluidity and destabilizes the packing of the phospholipids (Ellison 1994). Lf then enters the bacteria which is mainly driven by hydrophobic interaction, and the presence and number of Trp residue in N-terminal are essential to this process (Orsi 2004). Internalized Lf can then act on the cytoplasm and affect the related metabolic pathways (Vorland 1999). It has also been reported Lf receptors present on the surface of immune cells can mediate the entry of Lf. Internalized Lf can then move to the nucleus and act as a transcription factor to activate genes related to the host defense and immunology, and also balance the release of inflammatory factors (Legrand 2016). From this perspective, some high-density positively charged domains in Lf, including the N-terminal sequence GRRRR that is atypical to hLf, and other residues in the sequence 442–457 conserved in Lf from different species, are involved in localizing the internalized Lf to the nucleus to act as a transcription factor (Liu et al. 2019). In addition, Frontera et al. (2018) reported the internalized Lf halts the protozoan parasite *G. lamblia* to prevent infective cyst production. Recently, a timely and innovative study by Weber and Umansky (2019) provided important insights into a novel indirect action of Lf to the neonates and adults. They found that adoptive transfer of Lf-induced myeloid-derived suppressor cells (MDSCs) significantly decreased bacterial load in recipient mice, thus, prolonging their survival. Moreover, these Lf-MDSCs also showed potent anti-inflammatory properties in other experimental inflammation models, such as autoimmune lung inflammation, colitis, and hepatitis.

Another important structural feature of Lf are the glycans that are highly exposed on the surface of the glycoprotein, their location and number are a highly species-dependent property (Figure 1c). Previously, the structure and function of hLf and most commercially prepared bLf were thought to be little influenced by the presence or absence of these glycans (Ye, Nishimura, and Yoshida 1997). Recently, however, the relationship between N-glycans and Lf's physicochemical characteristics has attracted increasing attention. Some studies proposed that Lf's glycans are to help neutralizing the glycan-binding properties of certain viruses and protein toxins (Jose et al. 2018; Kieckens et al. 2017). Moreover, the purified *N*-Glycans present stronger anti-adhesion effect than the native Lf (Figueroa-Lozano et al. 2020). Due to the internalization effect of CD206 macrophages in mammals mediating by binding with the high-mannose N-glycans of Lf, glycosylation may also influence the half-life of Lf *in vivo* (Zlatina and Galuska 2021). Besides, Valk-Weeber, Eshuis-de Ruiter, et al. (2020) reported that the glycans of bLf changes over the course of lactation and understanding these changes might potentially assist in unraveling the functions of these Lf glycans.

There is a close relationship between the structure and function of Lf. The functions of iron binding and delivery are mainly dependent on the domains which distributed in both the C- and N-terminal of Lf and are composed of specific sequences of amino acids. In addition, some amino acid sequences and residues in the nonfunctional domains are essential for mediating Lf internalization, important for Lf to exert its intracellular activity. Based on these structure-function relationships, the structural integrity of Lf is pivotal to its multiple functions. It is noteworthy that the sequence on the N-terminus enriched positive charges in Lf, particularly that corresponding to Lfcin also retains the majority of the activities of Lf once separated from parent molecule. It is necessary to clarify whether Lfcin is produced during *in vivo* digestion

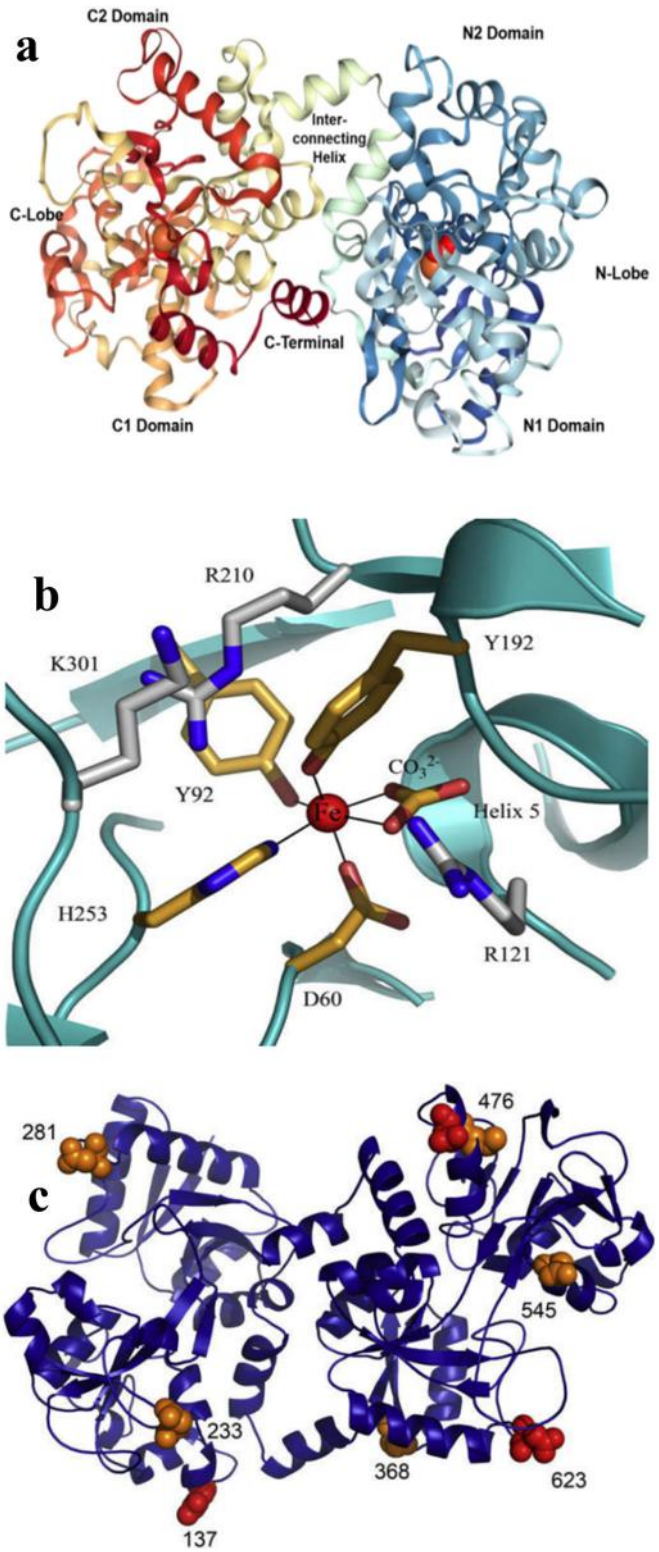

Figure 1. 3D crystal structure of bovine Lf (a): four domains are labeled N1, N2, C1 and C2; the iron and CO_3^{2-} binding sites are labeled as the red-orange spheres (Lu, Francis, et al. 2020); the iron binding site for N-lobe of human Lf (b) (Baker and Baker, 2005); The distribution of *N*-glycosylation sites in hLf (red spacing-filling mode) and bLf (orange spacing-filling mode) (Baker and Baker 2009).

process. Therefore, the GI degradation behavior of Lf is of great significance for the rational design of Lf-containing function foods.

Digestion behavior of Lf in GI tract

Extensive digestion experiments (i.e., *in vitro* and *in vivo*) in GI tract have shown that pH, an individual's age, enzyme source and the ratio of enzyme to substrate all significantly impact Lf digestion behavior (Wang, Timilsena, et al. 2019).

At present, *in vitro* simulation digestion experiments are most commonly used to investigate Lf degradation behavior in GI tract due to the easy accessibility of this method. The simplest method involves the preparation of simulated GI fluid with commercial pepsin and typsin and relies on a study of the retained intact Lf and produced fragments during digestion period by SDS-PAGE. Wang and coworkers (2012) studied the digestibility of recombinant hLf (rhLf) in simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.5). They found rhLf was digested within 2 min in SGF, but not after 60 min in SIF. Niu, Loveday, et al. (2019) compared the degradation of Lf and Lf-pectin complex in the presence or absence of an additional chitosan coating in SGF (pH 3.0, 60 min). They found that only 4% intact Lf was retained after gastric digestion, and the formation of Lf-polysaccharide complex significantly improved the stability of Lf in stomach. Notably, Eriksen et al. (2010) reported Lf-contained whey protein mixture was degraded more efficiently by purified porcine pepsin than by human gastric juice. Moreover, the bovine Lfcin, produced by *in vitro* enzymatic hydrolysis of nonhuman pepsin, was not identified from bLf-derived peptide after digestion in human GI juice (Furlund et al. 2013). Therefore, the use of human digestive juices might be the preferred method for studying the *in vitro* digestion behavior of Lf.

In addition, gastric pH significantly influences the pepsin activity and substrate conformation and, thus, the substrate digestion behavior. Lf is sensitive to the pepsin degradation in *ex vivo* human gastric juice at pH $<$ 4. It has been suggested that the release of iron at pH $<$ 4 can lead to looser conformation for Lf, making Lf easier to digest than at pH $>$ 4 (Mazurier and Spik 1980). Hence, Lf is only partly digested in neonates having a higher intragastric pH and emptying rate, and can be absorbed in its intact form from the gut of infants (Chatterton et al. 2004). In addition to individual variation, the gastric pH in the fed state, which is generally in the range from 2 to 4, depends greatly on the buffering capacity of ingested food. Eriksen et al. (2010) performed an *in vitro* two-step assay to simulate digestion of Lf in the stomach (pH 2, 4 or 6) and the duodenum (pH 8) with aspirated human gastroduodenal fluid, and found that gastric digestion at pH 2 followed by duodenal digestion resulted in the most efficient degradation for Lf. Furlund et al. (2013) further investigated the difference in Lf digestion by adjusting aspirated gastric juice pH either slowly or rapidly down to 2.5 or 4.0 to account for variation in the buffering capacity of different Lf-containing foods. The greatest *in vitro* degradation of Lf, leaving no intact protein, was observed when gastric pH was quickly adjusted to 2.5, while Lf was more resistant against gastric digestion when pH was slowly reduced to 2.5 or 4.0. An *in vivo* experiment involving two volunteers was performed to illustrate

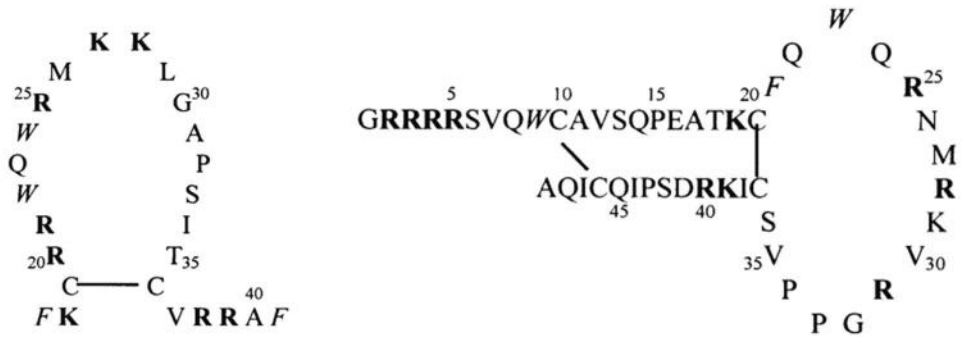

Figure 2. The primary structures of bovine Lfcin (left) and human Lfcin (right) (Bellamy et al. 1992; Hunter et al. 2005).

individual variations in degradation and compared to *in vitro* experiments results. Intragastric pH decreased to 3.4 after 10 min for volunteer 1 and to 3.8 for volunteer 2, which corresponded to the *in vitro* experiment for pH slowly adjusted to 4.0. But unlike *in vitro* digestion, no intact Lf was detected after 10 min gastric digestion despite variations in the peptide profiles for the two volunteers. The investigators speculated that the observed difference for intact Lf profile between *in vitro* and *in vivo* digestion may result from the lower enzyme concentration used in the *in vitro* digestion. Also, the activity of GI enzymes, especially pepsin, decreased rapidly in *in vitro* (Ulleberg et al. 2011).

In addition, it cannot be neglected that the reasonable detection method and *in vitro* model are also essential to pinpoint the digestion behavior of Lf. Grosvenor, Haigh, and Dyer (2014) developed a novel proteomic approach using isobaric labeling by isopropanol/iTRAQ reagent mixtures. In this study no prior artificial breakdown of protein structure took place to map and track Lf truncation and peptide release during simulated gastric digestion. Liquid chromatography and tandem mass spectrometry (LC-MS/MS) was used for separating and analyzing the labeled samples. In this new approach, the impact of prior pasteurization on peptide release was also assessed. They found that intact Lf of 80 kDa could not be detected even after digestion in SGF (pH 2.0 with commercial pepsin) for 30 s, the released peptides of Lf and their relative abundance had a significant change after pasteurization treatment. Lu, Ke, et al. (2020) designed the unmodified gold nanoparticles-based aptasensing assay for the positively charged protein detection (e.g., Lf). The pH of probe solution was slightly higher than pI of the targeted protein to prevent forming "protein corona" which interfering protein detection. The limit of detection for model proteins was comparable to that of other methods. Using the overall N-linked whey glycoprofile, Valk-Weeber, Eshuis-de Ruiter, et al. (2020) analyzed the difference in glycoprotein composition between concentrate, isolate and demineralized whey powders. IgG and Lf protein concentrations determined by N-glycoprofile analysis were confirmed being matched well with ELISA results. Lu, Zhang, et al. (2020) proposed a ratiometric electrochemiluminescence resonance energy transfer (ECL-RET) platform depended on novel dye BODIPY derivatives for rapid detection of Lf. Under optimal conditions, the constructed ECL-RET platform exhibited sensitive detection of Lf in the wide linear range of 10^{-4}- 850 ng/mL with a LOD of 42 fg/mL. Meanwhile, the proposed ECL-RET aptasensor demonstrated superior stability, specificity and reproducibility, displaying favorable application value in practical diagnosis of this method. In addition, the rapid and simple detection methods for Lf from milk samples were also reported by Ostertag et al. (2021) and Wang, Xu, et al. (2021), which included the sensitive immunoassays (i.e., ELISA method) based on specific monoclonal IgG and an RP-HPLC DAD method for simultaneous quantification of minor and major whey proteins (e.g., Lf). For the *in vitro* digestion assay, most of them have used batch reactors to quantify the fraction of the dose, which is liberated from the matrix in the absorption site. Generally, these digestions are performed with automated pH control and time-controlled conditions for the digestive juices. These systems are not autonomous and require a skilled technician to operate the system and collect samples. Miniaturization of such a system (leading to so called gut-on-a-chip devices) would allow for automation, as well as a reduced levels of expensive chemicals (such as enzymes) and solvents. Haan et al. (2019) developed the digestion-on-a-chip system for a continuous-flow modular microsystem recreating enzymatic digestion in the GI tract. Using Lf as the model bioactive protein, they demonstrated a complete digestion of this milk protein in a much shorter time than could be achieved by standardized *in vitro* digestion in a batch reactor. In addition to the *in vitro* model, the *in silico* modeling of protein hydrolysis by endoproteases is promising and could simplify research on the digestion behavior of bioactive proteins. Tonda et al. (2017) proposed a Monte-Carlo *in silico* simulation model for the investigation of bLf digestion behavior, simple enough to be performed without resorting to computational clusters or super-computers, and able to return useful results. But the quantitative prediction for a given peptide as a function of time still cannot match experimental observations.

At present, there are various findings for Lf digestion in animal experiments. Generally, the gastric enzyme (i.e., pepsin) has the greatest degradation capacity for Lf, but Lf and its fragments can retained for a prolonged time in the small intestine (Takeuchi, Kitagaw, and Harada 2004; Kuwata et al. 2001; Wang et al. 2012; Yao et al. 2013b). There are still limited *in vivo* human digestion studies. Troost et al. (2001) first investigated the bLf gastric digestion in 12 healthy volunteers ingesting a maltodextrin-containing beverage. They found the survival of Lf after gastric digestion

was above 60% for all forms bLf. However, despite a significant difference in the peptide spectrum produced, Furlund et al. (2013) found that bLf was completely digested in gastric environment for two volunteers within 10 min. The reasons for the differences within/between the performed *in vivo* experiments are complex. Furlund et al. (2013) found a large individual variation in gastric and duodenal secretions of two volunteers, which may be the main reason for the differences in degree of Lf hydrolysis and the peptide spectrum produced for two volunteers. Consistently, Ulleberg et al. (2011) reported the variability of GI environment. For majority volunteers, substantial volume of gastric juice leaked and mixed with duodenal juice. In contrast, Lf has been reported to be more resistant to protease hydrolysis by the formation of complex with carbohydrate (Niu, Loveday, et al. 2019). Hence, the interaction between bLf and maltodextrin may contribute to the high survival rate of bLf after gastric digestion for Troost et al. report (2001). It is also noteworthy that there is a significant difference in the amount of Lf used in the two *in vivo* experiments (Troost et al. 2001; Furlund et al. 2013), which could lead to a difference in the mass ratio of pepsin to Lf, thus, impacting the degradation of Lf in the stomach.

In summary, it is undeniable that Lf is degraded to a large extent in the upper GI tract especially in the stomach (Onishi 2011). It is noteworthy that bioactive peptide (i.e., Lfcin) is undetectable in the *in vivo* digesta (Furlund et al. 2013). Hence, improvement in the stability of orally administrated Lf is crucial to its multiple functions.

Intestinal absorption and transportation of Lf

Lf absorption mechanism

Generally, bioactives are first delivered to the intestinal site after oral administration and then absorbed into the blood circulation for systemic action on different tissues. Macromolecules can be transported across the enterocyte by two pathways, nonselective transcytosis and specific receptor-mediated transcytosis. In recent years, the Lf receptor (LfR) has been suggested to be present in different tissues and cells types. The characteristics of LfR vary among different types as previously described (Grey et al. 2004; Talukder and Harada 2006; Mu and Pang 2011). Suzuki, Shin, and Lönnerdal (2001) first reported the cloning and functional expression of human small intestinal LfR (i.e., intelectin-1) and evidence of its involvement in the iron metabolism. Subsequently, Talukder and Harada (2006) reported the distribution and characteristics of LfRs in the intestine of newborn calves. LfRs in brushy marginal membranous capsule (BBMV) were present in the duodenum, jejunum, ileum, colon, epithelium overlaying Peyer's patches (EOPP) in jejunum and ileum. They are saturable and specifically bind to Lf isolated from human and bovine sources. These studies suggest that Lf and Lf-bound Fe might bind to the LfR and then be absorbed through LfR-mediated pathways. To this end, researchers performed relative studies based on an *in vitro* intestinal model (i.e., Caco-2 cell monolayer, isolated BBMV of intestine). Lönnerdal and Jiang (2011) investigated the absorption of Lf and the Lf-bound Fe by an intestinal enterocyte model (Caco-2 cells) to verify the feasibility of adding CbLf to infant formula. They found the Lf-bound Fe was more efficiently absorbed than Lf protein, suggesting that the absorption mechanism might be different between Lf and the Lf-bound Fe. Jiang et al. (2011) reported that Lf can be absorbed into Caco-2 cells via intelectin-mediated endocytic process. Further, there was no intracellular degradation of Lf after being absorbed into the enterocyte, so that Lf and Lf bound Fe can be subsequently transported to tissues throughout the body to function (Kruzel et al. 2020). However, Akiyama et al. (2013) reported the Lf remained partially intact within the Caco-2 cells and was subsequently released into the medium and identified as degradation fragments of 30–50 kDa. Recently, an interesting discovery was reported by Matsuzaki et al. (2019). This group observed intact bLf was absorbed into 7 d and 21 d-cultured Caco-2 cells and successfully released back into the culture medium, even though the human intestinal Lf receptor, intelectin-1, was not immunochemically detectable. They speculated that a heparan sulfate-recognizing sequence on Lf played the role of endocytosis receptor for the protein internalization. It is noteworthy that current *in vitro* gut models generally lack physiological relevance. Various modifications of medium flow, three-dimensional scaffold, peristaltic motion and bacteria, and elements of the immune system have been used to promote current cell culture models. These experiments are promising when applied for more accurate characterization of the intestinal absorption behavior of bioactive proteins (Imura, Yoshimura, and Sato 2012; Ramadan et al. 2013; Shim et al. 2017; de Santiago et al. 2018). In addition, in view of the present contradictory results for Lf intracellular degradation in enterocytes, combining *in vivo* studies is important for further validating the absorption behavior of Lf. Meanwhile, due to the high pI and the positive charge of Lf, it is not possible to exclude the possibility that Lf can be internalized to enterocyte through nonspecific endocytosis.

Lf transportation mechanism

The absorbed nutrients leave the enterocyte and move to the blood capillary or lymphatic capillary in the lamina propria, where they enter the blood stream and are transported to various tissues in the body. There are three main pathways (portal vein, mesenteric lymphatics and enteroheptic cycle) for the intestinal transport of nutrients. The enteroheptic cycle prolongs the time for the bioactives entering the systemic circulation (Guan and Morris 2019). While portal vein transport generally results in the rapid elimination and, thus, a short half-life of bioactives (Feng et al. 2019). Comparatively, mesenteric lymphatic transport can increase the bioavailability of bioactives by reducing their first-pass metabolism, thus, it is advantageous for intestinal transport of bioactive protein in the presence of significant first-pass metabolism (Cai and Li 2013). Although it was previously reported there is intracellular degradation for Lf to some extent after absorbed by enterocyte (Akiyama et al. 2013), the *in vivo* pharmacokinetics assay have shown that

there are some antigenic macromolecules including Lf can be eventually transported to the blood circulation with the intact form especially in the early life. However, it is explicitly noteworthy that intestinal transportation of Lf differs among species. Harada et al. (1999) demonstrated that the Lf was transported into the liver and then excreted with bile to intestinal lumen, suggesting the possibility of enterohepatic circulation of Lf in neonatal pigs. Takeuchi, Kitagaw, and Harada (2004) investigated the characteristic transport system for Lf from intestinal lumen into the blood circulation. The results suggest that intraduodenally infused bLf is transported into the blood circulation purely through the lymphatic pathway in adult rats. But for the growing pigs, the absorbed bLf was reported being transported into systematic circulation via the portal vein and also the mesenteric lymphatics (Kitagawa et al. 2003). It is still not possible to draw definitive conclusions about Lf intestinal transport in the human body. It is also still not feasible to verify transport using human experiments. Recently, there has been progress in developing specific devices for determining the pharmacokinetics of medicinal drugs (Choe et al. 2017; Prot et al. 2014) and these should provide lessons in the case of nutraceuticals.

The assistive labeling techniques of Lf

The labeling techniques for visualization/traceability and the accompanying quantitative analytical analysis would be helpful in investigating the physicochemical properties and pharmacokinetics of bioactives (Gimona et al. 2017). The main labeling methods include fluorescent labeling and radiolabeling (Kim et al. 2019). The labeling principles and their applications in Lf tracing are described below.

Fluorescent labeling

Fluorescent molecules, possessing conjugated double bonds, can be activated to their excited state when exposed to the ultraviolet light or blue-violet light. Fluorescence is emitted when the excited state is restored to the ground state (Wang and Dong 2007). The attachment of fluorescent molecule to a target compound mainly requires a covalent connection. The positioning, tracing and content determination of the labeled molecules can be detected by optical measurements using fluorescent microscopy, laser confocal microscopy, and flow cytometry (Deng et al. 2020). Chaharband et al. (2018) prepared a curcumin-Lf conjugated nanostructures and incubated these with cancer cells (HCT116). By means of the fluorescent labeling technology (i.e., labeling Lf with fluorescein isothiocyanate), this nanostructured complex demonstrated a high efficiency of cellular uptake. It is also noteworthy that the attachment of a fluorescent molecule can drastically alter the pharmacology of a compound by changing its selectivity or resulting in a significant loss in affinity compared to parent compound (Soave et al. 2020). In the view of the close relationship between Lf structure and function, it is important to select suitable fluorescent molecule based on its binding site on Lf. For example, Mazurier et al. (1989) investigated the binding properties between membrane receptor on the activated peripheral-blood lymphocyte and the radio labeling ligand ^{125}I-labeled Lf. The membrane receptor, consisting of a single polypeptide chain, interacts with a well-defined domain in Lf (residue 4–52). But the fluorescein-isothiocyanate-derived Lf was unable to specifically bind to the lymphocyte receptor, which may be because the isothiocyanate, having a larger molecular weight, occupies/shelters the receptor binding site of Lf. The glycans present in Lf was another fluorescence-labeling site. Leveugle et al. (1993) used a fluorescent probe 5- ({[2-(carbhydrazino)methyl]- thio} acetyl)amino to label the aldehyde groups produced by mild periodic-acid oxidation of the glycan moieties on Lf. Such labeled Lf was used to purify the putative hLf receptor on platelets and its structural homology to the lymphocyte Lf receptor was investigated. A potential function of Lf binding on its platelet receptor was reported to result in the inhibition of platelet aggregation.

Radiolabeling

Even though fluorescence labeling is a convenient tool, radiolabeling enables easy quantitative detection of labeled molecules in deep organs (Kim et al. 2019). Radionuclide is used to replace a stable isotope or directly label the destination molecule. ^{125}I is the most commonly used radionuclide label for bioactives and for investigation of their *in vivo* fate. Chloramines t iodization is the original ^{125}I labeling method, but strong redox agents used in the labeling process and these can cause the deterioration of functionality of bioactives (Ogawa et al. 2018). Enzymatic iodization, especially lactoperoxidase-glucose oxidase method, allows bioactives to maintain relatively good biological or immunological activity because this oxidation process is controllable with few side reactions. Unfortunately, the enzymatic reaction mechanism is complex and the enzyme reagent is expensive. The Tyr is the binding residue for ^{125}I in both chloramines t iodization and enzymatic iodization methods. Some bioactives lack Tyr and bioactives with iodinated Tyr can lose their functionality. The Bolton-Hunter method appears an ideal alternative iodization strategy by forming the peptide bond between the terminal amino group and the iodized reagent. In recent years, the Iodogen method with the iodization occurring in the solid phase has been quickly developed. This method results in higher radioactivity than that of chloramines t iodization and is mild and simple and is quite suitable for protein labeling (Gong et al. 2020). Suzuki, Shin, and Lönnerdal (2001) expressed the recombinant LfR (rLfR) by the molecular cloning technology and found an apparent Kd of ~360 nM for ^{125}I-hLf binding to rLfR, similar to that for native LfR. Talukder and Harada (2006) carried out binding assays using ^{125}I-labeled bLf prepared with the Iodogen method and reported a specific and saturable Lf-R found in brushborder membrane vesicles in all the intestinal segments and in the choroid plexus. Lönnerdal, Jiang, and Du (2011) compared the digestion behavior, binding capacity to the intestinal enterocyte and bioactivities of commercially available bLf (CbLf) and HLf. Lf molecule labeled with ^{125}I by

the Iodogen method showed CbLF was biologically active and likely exerted several of hLf bioactivities when added to infant formula. Similarly, Jiang et al. (2017) labeled Lf with ^{125}I by the Iodogen method and investigated its role in the protection of skin. Lf could be internalized by human dermal fibroblasts and regulate gene expression to achieve its antioxidant, immunmodulatory activity and enhance wound healing. In addition, since Lf is capable of iron binding, the radioisotope ^{59}Fe can be used to label Lf-bound Fe to track iron absorption and transport processes. Lisiecki (2017) investigated the scavenging of iron by enterococci using ^{59}Fe-labeled Lf and revealed several ways to acquire iron from iron-binding protein. ^{125}I and ^{59}Fe are the most widely used labeling ligands for the investigation on intestinal absorption and transport of Lf and Lf-bound iron, respectively.

Other labeling methods

Huang, Lin, and Huang (2019) firstly labeled bLf with biotin and their investigation showed bLf promotes hair growth in mice and stimulates proliferation of dermal papilla cells through Erk/Akt and Wnt signaling pathways. Further, Almehdar et al. (2020) reported biotin can not only be used as a labeling ligand, but also cooperated with Lf to play an antibacterial role. In addition, AbdElhamid et al. (2018) developed highly fluorescent mercaptopropionic acid-capped cadmium telluride quantum dots, which were coupled to Lf and enabled tracing Lf's internalization. Xavier et al. (2010) reported the synthesis of highly luminescent, water soluble quantum clusters (QCs) of gold, which are stabilized by Lf. QCs/Lf clusters exhibited excellent stability in widely varying pH conditions, which made it feasible to be used for intracellular imaging.

Stabilization technologies

The fabrication of stabilization systems is important for improving the stabilization of bioactives during oral delivery and in achieving intestinal site-specific release or promoting intestinal absorption (Ibrahim et al. 2020). It is essential to design the stabilization systems based on the biological features of Lf. For the topical application (e.g., anticolon carcinoma), the concentration and residence time of Lf in the targeted sites are important for efficacy. For the systemic use, the efficient delivery of Lf to its intestinal receptors and its absorption into the circulation system are essential for Lf to effectively exhibit its biological functions, such as immunoregulation. The following sections provide an overview of the typical small intestine and colon-targeted stabilization systems for Lf.

Small intestinal-targeted stabilization system

Chemical modification

The introduction of conjugated ligands can change the immunogenicity of bioactives and elongate their biological half-life. Poly(ethylene glycol) (PEG) and its derivations are widely used conjugated ligands that have excellent biocompatibility. Nojima et al. (2008) developed the Lf conjugation (20 KDa-PEG-Lf) by modifying Lf with 20 KDa PEG-NHS (the *N*-hydroxysuccinimidil ester of PEG-COOH). The *in vitro* assay showed that the conjugated Lf possessed fully active iron binding and around 70% of the anti-inflammatory activity of Lf, and a pepsin hydrolysis half-life increased by 2-fold compared to unmodified Lf. In vivo pharmacokinetics analysis showed the absorption amount of 20 KDa-PEG-Lf from the intestinal tract increased by approximately 10-fold compared to unmodified Lf. Subsequently, Nojima et al. (2009) prepared a 40 KDa-PEG-Lf that retained 97% of the anti-inflammatory activity compared to parent Lf. It was found that this Lf derivative showed a 6-fold increase in pepsin hydrolysis half-life. Sugiyama, Sato, and Takeuchi (2009) further evaluated the *in vivo* hepatoprotective effect of PEGylated Lf using rats with CCl_4-induced liver injury. These investigators found that the protective effect was higher for PEGylated Lf than that of unmodified Lf, moreover, 40 KDa-PEG-Lf had the most protective effect. More recently, Wang, Xu, et al. (2021) used theaflavins to interact with bLf. And the effect of theaflavins on the structure and functionality of bLf was thoroughly observed. Comparing with the native protein, the addition of theaflavins significantly reduced the digestibility of Lf in the stomach and small intestine (11.2174% and 70.3030% vs. 26.7936% and 77.3946%). Although some progressive results have been achieved by chemical modification, the activity of Lf is affected to some extent because the conjugated site occupied or located near by the antigenic activity center of the protein.

Encapsulation methods

Development of encapsulation methods (Figure 3) is an important strategy for improving the stability of fragile molecules. The biocompatibility of carrier material is a prerequisite for its application in food field, while the encapsulation efficiency and the physicochemical stability are key evaluation indexes for carriers. In recent years, naturally abundant lipids and sterols have been used as materials to develop the encapsulation carriers (liposomes, solid lipid particles, etc.). Ishikado et al. (2005) prepared the Lf-loaded multi-lamellar liposome using the egg yolk phosphatidylcholine and phytosterol by two different methods (i.e., film dispersion and high pressure emulsion homogenization). In vitro digestive tests demonstrated liposomal incorporation improved the robustness of Lf toward the pepsin digestion. Oral pretreatment of liposomal Lf also showed greater suppressive effects than that of non-liposomal Lf on CCl_4-induced hepatic injury in rats. Using a film dispersion method, Liu et al. (2013) prepared positively charged Lf-loaded liposomes from milk fat globule membrane-derived phospholipids, which were isolated from dairy waste. Despite liposome aggregation, the Lf entrapped in these liposomes remained unchanged as a function of time and pepsin concentration when they were digested in SGF for 2 h. Additionally, liposomal Lf showed sustained release resulting from the hydrolysis of phospholipids in the small intestinal.

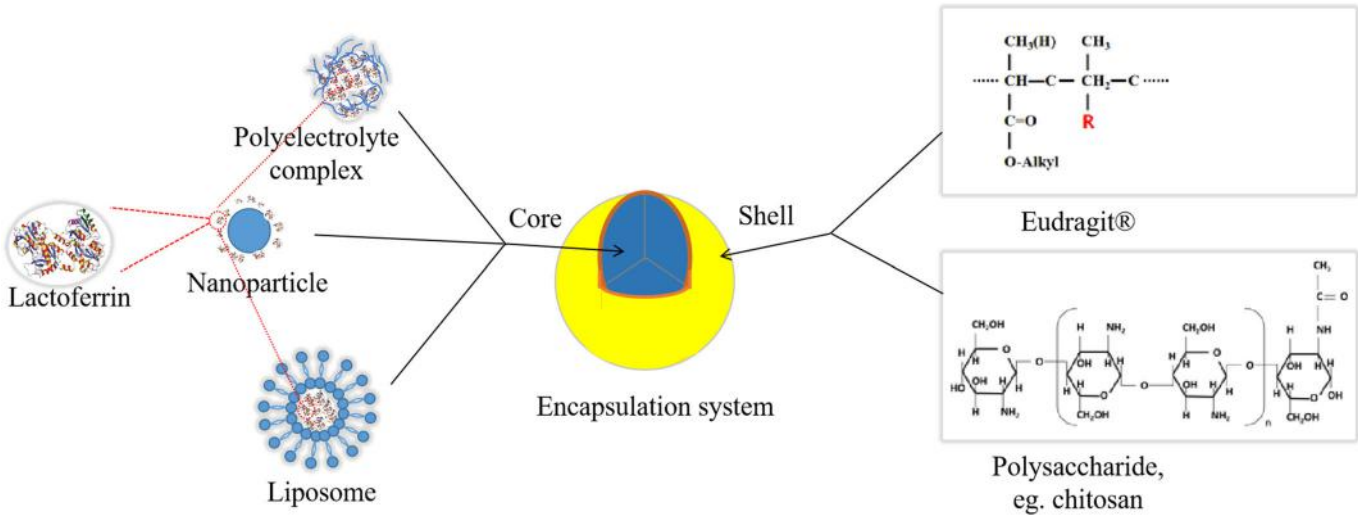

Figure 3. The encapsulation schematic: Core of Lf-loaded system such as liposome, nanoparticle and polyelectrolyte complex; Shell of single- or multi-layer assembly for targeted ligands.

Consistent with the digestion behavior of Lf, liposomal Lf shows less hydrolysis under infant model conditions (Liu, Lu, et al. 2018; Tian et al. 2019). Vergara et al. (2020) recently investigated the effectiveness of liposomes, prepared with rapeseed phospholipid extracted in residue of oil processing, for Lf encapsulation. The lamellar structure of these liposomes was organized in a liquid ordered phase with orthorhombic packing and 67%–80% of the initially encapsulated Lf remained intact after gastric digestion. Similar results were reported by Zhang et al. (2019), and they further systematically studied the effect of phospholipid structure (chain length, saturation) on the physicochemical and digestive properties of liposomes. This research may assist the development of more stable, highly dispersible liposomes in physiological *in vivo* environments. In addition, Yao et al. (2014) developed bioadhesive hydrophilic polysaccharides coated liposomes and solid lipid particles for Lf encapsulation. In vivo pharmacokinetics demonstrates the increased mean residence time and relative bioavailability of encapsulated Lf compared to free Lf.

In addition to acting as a coating material, a single polysaccharide component or polysaccharide complex has also used to develop a matrix (e.g., hydrogel particle) to directly encapsulate Lf. The polysaccharide-based matrix protects Lf against the pepsin hydrolysis and also possesses strong adhesion to the intestinal mucosa, improving the oral bioavailability of Lf. Raei et al. (2015) encapsulated isolated Lf from camel milk using alginate nanocapsules prepared by an emulsification-evaporation method. When evaluating the *in vitro* release of Lf from nanocapsules, it was revealed that there was no Lf release for the first 30 min at pH 2.0. Bokkhim et al. (2016) developed the alginate based micro-gel particles to encapsulate three forms of Lf (apo- native- and holo-Lf) using the novel impinging aerosol technique to prevent potential negative effects of emulsification process on Lf activity. Micro-gel particles retained significantly higher amount of Lf after digestion in SGF for 2 h as compared to pure Lf (76%–89% vs. 41%–58%). Niu, Thielen, et al. (2019) designed an electrostatic nanocomplex using naturally occurring Ɛ-poly-L-lysine and *β*-cyclodextrin sulfate to encapsulate four model proteins including Lf. Under the simulated gastric conditions with pepsin, these complexes protected Lf against proteolysis. Also, the complex had the potential to improve paracellular permeability of bioactive macromolecules due to a decreased trans-epithelial electrical resistance.

In addition to polysaccharides, protein-based carriers have been developed for Lf encapsulation. Kilic et al. (2017) first prepared negatively charged calcium carbonate particles for the adsorption of positively charged Lf. Lf-loaded particles were further coated with alternative layers of bovine serum albumin and tannic acid using a mild layer-by-layer process. The microcapsules obtained showed high stability in the simulated gastric conditions and effectively protected 76%–85% of the encapsulated Lf from gastric digestion. Moreover, *in vivo* studies showed the intestinal absorption ratio of encapsulated Lf increased by 2- to 4-times compared to free Lf. Considering that large amounts of Lf are released in the stomach, resulting from the hydrophilic swelling of polysaccharides and protein-based carriers, the introduction of enteric shell is expected to further decrease and even avoid the release of Lf in the stomach. Acrylic copolymer is the most commonly used enteric coating material and has been approved by FDA as GRAS (generally regarded as safe) (Thakral, Thakral, and Majumdar 2013). Onishi et al. (2015) coated the Lf-loaded chitosan particles using acrylic copolymer EL-100 by emulsification-evaporation method. The release amount of Lf after 1 h in the stomach significantly decreased (9%–15%) compared that (nearly 80%) of uncoated chitosan particles. Wei et al. (2020) constructed highly efficient intestinal-targeted microcapsule of Lf by introducing the enteric acrylic copolymer ES-100 coat using through a milder self-assembly process. In vitro release studies showed that the amount of Lf released in the stomach was less than 1%.

In view of the advantage of mesenteric lymphatic transport on the bioavailability of bioactive agents, investigations of strategies promoting lymphatic transport of bioactives is

of great significance. Especially, considering the active transport process of chylomicrons by intestinal lymphatics (Hokkanen, Tirronen, and Yl-Herttuala 2019), lipid based Lf-loaded nanoparticles may promote mesenteric lymphatic transport of Lf and, thus, improve the health benefit outcome.

Colon-targeted stabilization system

Colon-targeted delivery system has attracted much interest as it can result in more efficient treatment for colonic disease of bioactives and improve their intestinal absorption (Feng et al., 2021; Ohta et al., 1994). Colon-targeted stabilization systems mainly include pH-dependent, time-dependent, pressure-controlled, and bacteria/enzyme triggered delivery systems (Bourgeois, Richard, and Fattal 2005). It is noteworthy that the physiological characteristics (pH, food transit time, pressure etc.) of human GI tract are significantly correlated with individuals, including individual age and health status. However, intestinal flora mainly colonizes in colon (>99%), and can secrete various enzymes that catalyze colon-specific degradation (Mayur and Avani 2011). Hence, colonic bacteria and their secreted enzymes are the ideal triggers for the construction of colon-targeted stabilization systems. Azo compounds and non-digestible polysaccharides are commonly used polymer materials. In view of the toxicity of azo polymers, polysaccharides of natural origin seem to represent safer alternatives (Deb et al. 2018).

A successful colon-targeted stabilization system should use a high loading of bioactives and highly efficient targeted delivery. The evaluation of a colon-targeted stabilization system includes various *in vitro* (i.e., the dissolution tests in simulated GI fluid, cells studies) and *in vivo* methods (e.g., imaging observation). There are still few studies of the colon-targeted Lf delivery and the recent research has paid more attention to the inhibitory effect of stabilized Lf for colon diseases. Kanwar, Mahidhara and Kanwar (2012) developed a polysaccharides-based nanocarrier (NC) with Lf adsorbed to the negatively charged calcium phosphate nanoparticles as the nanocore and chitosan/alginate coating as a shell. This system was applied in the oral delivery of iron-saturated bovine Lf (Fe-bLf), an anti-colon carcinoma nutraceutical. SDS-PAGE and successive Western blots revealed these polymeric-ceramic NCs avoided Lf release in an acid environment (pH 1.2) and achieved cumulative release in intestinal alkaline environment (pH 7.4). The *in vitro* cell cytotoxicity and cell death assays were performed on the colon cancer cells (Caco-2) and the normal cells (FHs 74). Results showed that nano-encapsulted Lf had significant cytotocicity/apoptosis effects on colon cancer cells but not on normal cells. Analysis using flow cytometric suggested an increase in internalization of nano-encapsulted Lf. Moreover, *in vivo* tests revealed nano-encapsulted Lf were highly effective for the prevention and treatment of human xenograft colon cancer models. The chitosan-based stabilization system involves the use of organic acid that may exhibit cytotoxicity effects. Wu et al. (2013) developed a novel capsule system loaded with Lf using water soluble chitosan (chitosan hydrochloride), sodium cellulose sulfate (NaCS) and sodium polyphosphate. In vitro release studies showed that the capsules had a regular and sustainable release profiles in simulated colonic fluid (Wu, Li, and Yao 2014). While stabilized bioactives have a great potential for the treatment of colon diseases, a large amount of these (e.g., phycocyanin, theophylline, α-mangostin) are reportedly released in upper GI tract due to the hydrophilic swelling and dissolution of delivery systems (Manconi et al. 2010; Sullad, Manjeshwar, and Aminabhavi 2010; Samprasit et al. 2018). Comparatively, core-shell nanofiber system prepared by coaxial electrospinning was considered to be a more ideal targeted delivery system. The electric field force promotes the extension of polymer chains and the interaction between molecules, so the prepared materials showed strong water resistance (Mikaeili and Pelagia 2018). Using BSA as the protein model, Wen et al. (2017) prepared a polysaccharid-based nanofiber loading system. In vitro release demonstrated that about 75% of BSA achieved colon-specific release. While electrospinning technology has shown great potential for colon-targeted delivery of functional factors (Feng, Wei, et al. 2020; Wen et al. 2020; Feng et al. 2019), its use for oral delivery of Lf has not been reported. The hydrophilic swelling of polysaccharides-based nanofibers was still the main reason for protein release in the upper GIT. Hence, the future development of stabilization systems with high hydrophobicity in the upper GI tract and their degradable properties in the colon may be significant in Lf regulation of colonic diseases.

Concluding remarks

Lf, a bioactive protein with multiple biological functions, is promising for use in the nutritional and pharmaceutical areas. Oral delivery is the most accepted administration route, and the functional properties of orally administrated Lf intimately depend on its structural integrity, particularly its higher-order conformation. However, Lf is reportedly sensitive to the GI environment and is degraded in the stomach to a large extent. Correspondingly, the oral delivery systems have been developed, on the one hand, to improve the stability of Lf when going through the stomach. On the other hand, they are also designed for targeting Lf to the small intestine for its further absorption and transportation or to colon site for its local activity. A composite system with the enteric polymer as a shell and Lf-loaded nanocarrier as a core showed efficient small intestine targeting and controlled release properties for Lf. But currently available indigestible polysaccharide used in colon-targeted delivery system generally shows hydrophilic swelling resulting in the release of a large amount of encapsulated Lf in the upper GI tract and additional effort is needed to address this problem. The absorption of Lf by the small intestinal enterocyte followed by transport into the blood circulation has been investigated but no definite determination has been made on the transport pathway. The development of *in vitro* digestion, absorption, transport models and detection methods should promote a deeper understanding for the oral fate

of Lf. Considering the advantages of lymphatic transport for bioavailability of orally administrated bioactive agents, studies of strategies to promote the intestinal lymphatic transportation of Lf would be of great significance. In recent years, the colon has been regarded as a superior site for macromolecular protein absorption due to its unique physiological characteristics. Moreover, prebiotics can promote the absorption of trace elements through colon by regulating the colonic flora structure. Relevant research on Lf is still needed to lay the foundation to further improve the oral bioavailability of Lf.

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