β-lactoglobulin/linoleate complexes: binding properties and biological functions

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Poster

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LIST OF ABBREVIATIONS

αla α-lactalbumin
AFM Atomic force microscopy
ANOVA Analysis of variance
ANS Anilino naphthalene sulfonic acid
Asp Aspartic acid
ATR Attenuated total reflectance
AU Arbitrary units
βlg β-lactoglobulin
BAMLET Bovine Alpha-Lactalbumin Made LEthal to Tumor cells
BSA Bovine serum albumin
cAMP Cyclic adenosine 3',5'-monophosphate
CCK Cholecystokinin
CLA Conjugated linoleic acid
CLSM Confocal laser scanning microscopy
CMC Critical micelle concentration
CMP Caseinmacropeptide
Cys Cysteine
DHA Docosahexaenoic acid
DLS Dynamic light scattering
DMEM Dulbecco's modified Eagle medium
cDNA Complementary deoxyribonucleic acid
ε Extinction coefficient
EC_{50} Effective concentration 50
EDTA Ethylenediaminetetraacetic acid
EGCG Epigallocatechin gallate
ELISA Enzyme-linked immunosorbent assay
ESI-MS Electrospray ionization mass spectrometry
FA Fatty acid
FABPpm Plasma membrane-associated fatty acid-binding protein
FAME Fatty acid methyl ester
FAO Food and Agriculture Organization of the United Nations
FATP4 Fatty acid transport protein 4
FBS Foetal bovine serum
FTIR Fourier transform infrared spectroscopy
GC Gas chromatography
GLP1 Glucagon-like peptide-1
GP-HPLC Gel permeation high performance liquid chromatography
HAMLET Human Alpha-Lactalbumin Made LEthal to Tumor cells

IBMX 3-Isobutyl-1-methylxanthine
ISO International Organization for Standardization
ITC Isothermal titration calorimetry
K_a Association constant
LA Linoleic acid
LCFA Long chain fatty acid
LD_{50} Lethal dose 50
MCFA Medium chain fatty acid
MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
mRNA Messenger ribonucleic acid
M_w Molecular weight
n Reaction stoichiometry
NATA N-acetyl-tryptophanamide
NMR Nuclear magnetic resonance
PAGE Polyacrylamide gel electrophoresis
PBS Phosphate buffered saline
PEPT1 Peptide transporter 1
PYY Peptide YY
r_h Hydrodynamic radius
RP-HPLC Reversed-phase high performance liquid chromatography
RT-PCR Real time polymerase chain reaction
RTCA Real time cell analyzer
SANS Small-angle neutron scattering
SAXS Small-angle X-ray scattering
SCFA Short chain fatty acid
SD Standard deviation
SDS Sodium dodecyl sulphate
SEC Size exclusion chromatography
TEER Transepithelial electrical resistance
TEM Transmission electron microscopy
TFA Trifluoracetic acid
Trp Tryptophan
VLCFA Very long chain fatty acid
WHO World Health Organization

GENERAL INTRODUCTION

The nutritional quality of food is a major issue for the global food industry. A non-equilibrated diet has been associated with major health problems such as nutrient deficiencies, obesity, diabetes and other food related diseases. As such, an adequate and balanced intake of high quality nutrients is important for health. Food structure and digestion impact on the nutritive and bioactive properties of the individual components, which could have a high effect on health. For instance, the presence of grapefruit in the diet has been shown to impact on intestinal absorption of certain drugs such as cardiovascular medicines. This occurs as components present in the fruit, especially bergamottin, alter the metabolism of the drugs in the enterocyte cells changing their transport through the intestinal barrier. These phenomena can be dangerous as the control of the dose response of these medicinal products is essential, therefore the consumption of grapefruit by patients on certain medicinal products is contraindicated. Recent studies also show that the antioxidant properties of green tea may be reduced by the presence of milk. This is attributed to the interaction of the catechin epigallocatechin-3-gallate (EGCG) with milk proteins, which decreases its accessibility to intestinal cells. These examples highlight how the biological and nutritional properties of food components can be affected not only by the composition of food, but also by interactions between the ingested components.

Milk is extensively used in the food industry because of its palatibility, nutritional attributes and diversified functional properties such as gelling, emulsifying and foaming properties. Milk proteins caseins and whey proteins (β-lactoglobulin, βlg; α-lactalbumin, αla; and bovine serum albumin, BSA) are widely used ingredients. βlg, the major whey protein in bovine milk is an extensively studied protein, and is known to bind hydrophobic ligands such as fatty acids (FA) or vitamins. Nevertheless, its biological function beyond nutrition is unknown. The interaction of native βlg with some ligands such as retinol or palmitic acid are well described in the literature, but the impact of these interactions on the ligands biological function are weakly described. Additionally, βlg structure is highly sensitive to processing conditions used in food manufacture, especially heat treatments, which are routinely used to change food textures or reduce microbial load. Such treatments denature the protein, leading to the formation of non-native monomers, oligomers and higher aggregates of βlg in food products. Information regarding the interactions of hydrophobic ligands with these process-induced species of βlg are weakly described in the literature.

The interaction between milk protein and FA appears to increase FA digestion (Perez et al., 1992). Moreover, the binding of hydrophobic ligands to milk proteins can protect the ligands against oxidation (Futterman and Heller, 1972). It can also modify the kinetics of the enzymatic hydrolysis of milk proteins (Puyol et al., 1993; Mandalari et al., 2009). Complexes between human or bovine α-lactalbumin and oleic acid, called HAMLET/BAMLET (Human/Bovine Alpha-lactalbumin Made LEthal to Tumor cells), increase the FA cytotoxicity

to various cancerous cell line (Knyazeva et al., 2008), probably due to an increase in the FA bioaccessibility. In contrast palmitic acid bioaccessibility decreases when bound to βlg (Puyol et al., 1995; Riihimäki-Lampén, 2009).

In this thesis, **we investigated the interaction between βlg and the essential n-6 FA linoleic acid or its conjugated isomers, conjugated linoleic acids (CLA), and the effect of binding on the biological properties of the complexes**. This multidisciplinary project was performed between the Teagasc Food Research centre in Moorepark, Ireland, and INRA-Agrocampus Ouest STLO in Rennes, France. Teagasc has expertise in milk protein/FA interaction, impact of FA and peptides on gut signals, and the efficacy of dietary bioactives in food matrices. STLO has expertise in whey protein denaturation/aggregation, properties evaluation of the non-native protein structure and digestion. In this thesis, we combined the expertise of both laboratories to study the impact of βlg structure on its interaction properties with FA (linoleate and CLA) and the biological properties of the complex (bioaccessibility and satiety). Hence, the objectives of the project were to answer a number of key research questions, including:

- What are the binding properties of the βlg/FA complexes?
- Does the protein structure affect ligands interaction and biological properties?
- What is the impact of such complexes on the protein structure?
- Is the protein digested differently when βlg forms a complex with linoleate?
- How does the protein impact on linoleate bioaccessibility and transport in cell models?

Therefore, the thermodynamic properties of the complexes made with native βlg and linoleate were investigated. The impact of linoleate on the protein properties were studied, focusing on the structural changes of the protein. The biological functions of the FA, especially its bioaccessibility for intestinal absorption, in presence of βlg, were analysed. βlg aggregates of controlled size were used to elucidate the importance of the protein structure on linoleate binding parameters and the biological properties of the complexes. Finally, another complex made with βlg and CLA allowed us to understand the effect of the ligands properties on the complexes formation and properties (bioaccessibility and satiety).

The present PhD thesis is presented in four distinct parts:

- The literature review describes general aspects of milk and whey proteins, with a focus on pH and temperature structural changes of βlg. The thermodynamic properties of ligands/βlg interaction and the impact of the interaction on the ligands and protein properties are reported. Finally, biological properties of FA, which make them valuable nutritional components, are described. The FA/protein interaction section of

the literature review (Part 1, 3-Interaction between β-lactoglobulin and ligands) is currently being prepared for publication.

- Materials and methods used during the project are presented in the second part.
- Results and discussion are presented in three different chapters, each written in the form of publication complemented with supplementary data.

  The first chapter presents the binding properties of linoleate with native βlg and the impact of this interaction on the protein structure. It also describes the effect of the interaction on linoleate cytotoxicity and transport *in vitro*. The content of this chapter has been published in *Journal of Agricultural and Food Chemistry*, 2012, 60, 9476–9483.

  Chapter 2 focuses on the *in vitro* gastro-intestinal digestion of native βlg complexed with linoleate. The linoleate transport was determined using a Caco-2 cell monolayer, which mimics the intestinal barrier. The impact of the interaction on the ability of the FA to reach the cell membrane was also studied by detection of the hormone cholecystokinin (CCK). The result obtained in this chapter has been submitted to the *Journal of Nutritional Biochemistry*.

  In Chapter 3, the impact of βlg structure on the FA binding properties was evaluated. Different states of βlg aggregation were investigated, namely: native βlg, covalent dimers, and nanoparticles. The effect of such complexes on the FA cytotoxicity was assessed. This chapter is currently submitted for publication in *Food Chemistry*.
- Finally, a general conclusion summarises the main findings of this thesis and proposes future studies.

PART 1

LITERATURE REVIEW

Milk is a white liquid secreted by the mammary glands of all female mammals, which provides the nutrients for the growth and development of their infants. It is a complete food for the neonate supplying it with carbohydrates, proteins, fatty acids (FA), vitamins, inorganic elements, and water.[1] Milk composition varies amongst the species according to the physiological and nutritional needs of the neonate.[2] However, humans are the exception, consuming milk well past infancy. Global production of milk, primarily bovine, was 749 million tonnes in 2011.[3] Milk is consumed as liquid milk, butter, butter oil, cheese, whole milk powder, skim milk powder and as food ingredients.

Dairy proteins are used for their nutritional and techno-functional properties such as their ability to form stable foam or emulsion. Protein concentrations in milk vary with species from 1 % in human, to 23.7 % for white-tailed jack rabbit.[2] Bovine milk has an average protein concentration of 3.4 %. This concentration varies depending on cow breed, animal feeding strategies and lactation stage. Dairy proteins are divided into two categories based on their solubility at pH 4.6. The insoluble fraction is referred to as casein, whereas the soluble fraction is called whey or serum protein. Precipitation, centrifugation, coagulation or filtration can be used to separate casein proteins from whey proteins.[2]

Milk is a complex solution at pH 6.7 containing lactose, fat, caseins, whey proteins and ash (Table 1 and 2). Milk fat, mainly composed of triacylglycerols, exists as fat globules and is mainly composed of palmitic and oleic acids (22-35 % w/w and 20-30 % w/w in milk fat, respectively[4]). Different casein structures called αs1-, αs2-, β- and κ-casein differ in primary structure, type and degree of post-translational modification.[5] Associated caseins in complexes with calcium phosphate form a supramolecular organization called casein micelles. The exact structure of the casein micelles have been subject to controversy. Because of their large diameter (up to 400 nm[6]), casein micelles diffract light making the milk opaque and white.

Table 1: General composition of mature bovine milk. Modified fom Smithers (2008)[7].

Component	g/L
Total protein	
Casein	28
Whey protein	7
Lactose	49
Fat	37
Ash	7

Table 2: Protein composition of bovine milk and some of their properties. Modified from Farrell et al (2004)[8].

	Variant/class	Molecular weight	A % (1 cm at 280 nm)
Caseins			
αs1-casein	B	23,615	10.05
	C	23,542	10.03
αs2-casein	A	25,226	-
β-casein	A1	24,023	-
	A2	23,983	4.6, 4.7
	B	24,092	4.7
κ-casein	A	19,037	-
	B	19,006	10.5
Whey proteins			
β-lactoglobulin	A	18,363	9.6
	B	18,277	10, 9.6
α-lactalbumin	B	14,178	20.1 - 20.9
Serum albumin	A	66,399	6.3 - 6.9
Immunoglobulin	G1	161,000	13.6
	G2	150,000	13.6
	A	385,000-417,000	12.1
	M	1,000,000	12.1
Lactoferrin	-	76,110	9.91

1 WHEY PROTEINS

A significant portion of the milk produced globally (~35 %) is used for cheese production. Cheese manufacture results in the production of whey, as a by-product. This accounts for more than 80 % of the whey available in the world.[3] Additionally, whey production comes from the manufacture of casein ingredient.[3] In 2011, the United States and the European Union produced around 0.5 and 1.9 million tonnes of whey powder, respectively.[3] Whey proteins represent about 20 % (w/w) of the whole milk protein.[9] The major whey proteins are β-lactoglobulin (βlg), α-lactalbumin (αla) and bovine serum albumin (BSA). Minor whey proteins are immunoglobulins and lactoferrin.[6,8] If the whey comes from casein rennet coagulation, it may contain peptides coming from caseins called proteose peptone and caseinmacropeptide (CMP).

Historically, whey was considered a waste product of cheese manufacture and was used primarily for animal feeding. However, whey is now valued for its nutritional and functional properties and is added to infant milk formula, dietary supplements, sports foods, and animal food products.[7,10] Their amino-acid composition (high proportion of cysteins, tryptophan and branched amino acids), their ability to bind ligands and the presence of bioactive peptides within their sequence has resulted in the re-evaluation of whey as a functional food. Indeed, whey proteins have also important techno-functional properties in

food systems such as their ability to act as emulsifiers or to form gels.[7] Unlike caseins, which lack secondary structure, whey proteins have highly organized structures and most are globular proteins.

The α-lactalbumin (αla) is the second major protein of bovine whey proteins and accounts for about 20 % of the total whey proteins. It is the principle protein in human milk because of the absence of βlg. αla has 123 amino acids and a molecular weight of 14.2 kDa. It contains eight cysteine Cys residues, all engaged in disulfide bonds (Cys6-Cys120; Cys28-Cys111; Cys61-Cys77; Cys73-Cys91) that stabilize the tertiary structure of the protein. This protein has an important role in lactose synthesis, regulating the lactose synthase enzyme.[2,11] Moreover, αla can bind one calcium ion per protein.[12] In absence of calcium, αla can bind FA such as oleic acid, with these complexes being highly studied for their biological functions, especially their action against tumor cells.[13,14]

Bovine serum albumin (BSA), the third major protein of bovine whey proteins, constitutes about 10 % of total whey proteins. It consists of 582 amino acids with molecular weight of 66.4 kDa.[6] It is a monomeric protein containing one sulfhydryl group and 17 disulfide bonds. BSA binds large amounts of hydrophobic molecules. By its ability to bind free FA, BSA has an important role in milk fat digestion.

2 β-LACTOGLOBULIN, THE MAJOR WHEY PROTEIN IN BOVINE MILK

2.1 Structure of β-lactoglobulin

β-lactoglobulin (βlg) is the major whey protein in bovine milk. It is present in the milk of ruminants, but also in the milk of non-ruminants such as the pig, horse, dog, cat, dolphin, and marsupials.[15,16] Interestingly, it is absent from human, lagomorph and rodent milks.[16] With a concentration in bovine mature milk of 3.2 g/L, βlg accounts for about 10 % of the total milk protein and about 50 % of the total whey proteins. βlg was first isolated by Palmer in 1934[17]; since that time, several methods have been used for its purification.[18]

There are several genetic variants of βlg, A and B variants being the most common.[19] These two variants both contain 162 amino acids, but differ by two amino acids at positions 64 and 118. Variant A has an aspartic acid residue in position 64 and a valine residue in position 118, while variant B has glycine and alanine in these positions, respectively. The molecular weight of a monomeric βlg variant A is 18,362 Da and B is 18,277 Da (Table 3).[8] βlg contains five Cys, located at positions, 66, 106, 119, 121 and 160 (Figure 1). These cysteines form two disulphide bonds, between Cys66 and Cys160, and between Cys106 and

Cys119.[20,21] Cys121 is a free thiol, however, it is unavailable for reactions under physiological conditions as it lies buried in the centre of βlg structure.[22,23] Cys121 participates in protein stability and when exposed, it forms reactive monomers able to form aggregates with other proteins.[24-26]

Figure 1: Primary structure of βlg variant B. Disulphide bonds are shown with blue line. Swiss-Prot accession number P02754.

The secondary structure of the βlg shows that it is composed of 15 % α-helix, 50 % β-sheet, 15-20 % reverse turn.[16,27] The nine β-strands labelled from A to I, which form two β-sheets, and the three turns α-helix are arranged to form the βlg globular structure.

Tertiary structure of the βlg is shown in Figure 2A. In aqueous solution and at neutral pH, the β-sheets form a flattened and conical barrel, called a calyx.[20,21] This barrel is connected by strand A on one side, while a secondary connection is formed between strands D and E. The α-helix is stored between the strands A and H, and is followed by the ninth β-strand called I.[16] In the native protein, disulfide bonds link strands G to H, and strand D to the C-terminal. The free thiol group is located in the central cavity, inaccessible to solvent in the native protein structure.[22,23] This calyx is closed at one end by the N-terminal loop and at the other by the EF loop. βlg belongs to the lipocalin protein family, which typically contain a β-barrel, inside which they can bind small hydrophobic molecules.[28] Variant A and B of βlg have similar tertiary structure at neutral pH as the amino acids that are different on the two variants are in a mobile surface loop and in the hydrophobic core.[16,29] However, these

variants have different properties (ie. surface hydrophobicity and pI) making them dissociable using methods such as reverse phase-HPLC or ion exchange chromatography.

βlg has a quaternary structure which varies with pH, temperature, ionic strength or protein concentration.[30-32] At natural milk pH (pH 6.7) and at concentrations greater than 50 μM, βlg exists as dimers in equilibrium with monomers[33] (Figure 2B) with dissociation constants around 10^{-6} M as described in Table 3. Therefore, increasing βlg concentration increases the dimer content of the solution, the dimer interface being formed by the I β-sheet and the AB loop.[20-22,34] βlg variant A is also able to associate as octamers, at ambient temperature and at pH between pH 3.5 and 5.5, because the additional carboxyle Asp64 can be ionized.[31,32,35]

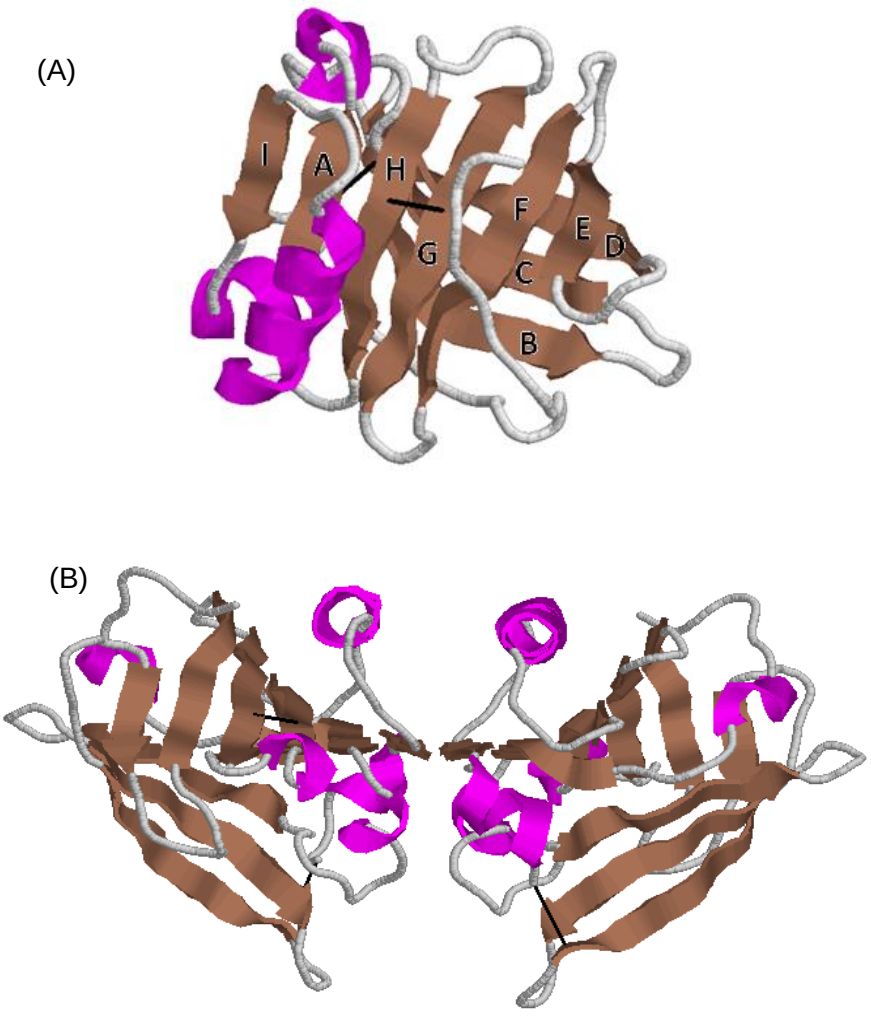

Figure 2: Schematic view of the main-chain fold of bovine βlg. (A) Native monomer with the strands names, (B) Native dimer. RCSB PDB code 1BEB.

Table 3: Some basic molecular properties of bovine βlg, modified from Sawyer and Kontopidis (2000)[16] and Saywer (2013)[35].

Number of amino acids	162
Monomeric M_w (A genetic variant)	18,362
Monomeric M_w (B genetic variant)	18,277
Isoelectric point	
B genetic variant, native	5.407
reduced and denaturing conditions	4.968
Extinction coefficient: 1 mg/ml at 280 nm	0.961
Monomer radius, Rg	1.75 nm
Axial ratio (dimer)	2:1
Dimer K_d (A genetic variant)	
pH 3.0	3.07×10^{-3} M
pH 6.5	4.93×10^{-6} M
pH 8.2	1.96×10^{-5} M
Dimer K_d (B genetic variant)	
pH 2.7	5.08×10^{-5} M
pH 6 7	7.04×10^{-6} M
pH 7.5	7.94×10^{-6} M
Octamer dissociation constant pH 4.7, 274 K	1.58×10^{-12} M^3

2.2 Influence of pH and temperatures on β-lactoglobulin structure

The structure of βlg changes with the pH. Structural changes of βlg can lead to the exposure of the free sulphydryl group Cys121 on the protein surface, thereby increasing its reactivity.[33] It is responsible for sulphydryl/disulphide exchange reactions and sulphydryl oxidation reactions leading to protein aggregation.[33,36]

At room temperature, pH plays an important role in the quaternary structure of βlg. Below pH 2, the protein is in a compact monomeric form. Between pH 2 and 3, βlg is dimeric, but becomes an octamer above pH 3. Around the natural pH of milk (between pH 5.1 and 7), the protein is in its dimeric form. Above pH 7 the protein undergoes the Tanford transition with βlg in the R-state. At pH above 9, the protein irreversibly unfolds and is able to form covalent aggregates.[6,16,18,32] The pH has an effect on the tertiary structure too. At low pH, the EF loop is in the closed position and ligand binding is inhibited, whereas at high pH, it is open allowing ligands to penetrate into the hydrophobic binding site.[37] Changes in the pH would allow the association or dissociation of the ligand in the calyx.[22]

Thermal denaturation of βlg at neutral pH is a multistep mechanism illustrated in Figure 3.[33,36,38-41] Native dimer dissociates to form native monomers at temperatures above 40°C. Between 40°C and 55°C, the globular monomer slightly unfolds yielding a looser conformation, this change being referred to as the Tanford transition. This conformation of

βlg (R-state) allows the formation of aggregates in small quantities. On heating to temperatures between 60°C and 70°C, βlg reversibly unfolds into a molten globule, exposing the sulphydryl group and making it available for the formation of irreversible aggregates. Irreversible unfolding of the monomers occurs above 70°C; these unfolded proteins are able to form aggregates. Different types of aggregates are formed depending on temperature.[33] Heating βlg at acidic pH predominantly leads to non-covalent aggregates as the thiol group is weakly reactive at these pH. The temperature of denaturation (irreversible formation of molten globule) varies with pH. Indeed, at pH close to the pI of βlg, the temperature of denaturation is highest. This is explained by a stabilisation of βlg structure through its oligomerisation under these conditions.

Controlling physico-chemical conditions, to which βlg is exposed, makes it possible to control the formation of specific types of βlg aggregates (ie. fibers, oligomers, nanoparticles, etc.).[33,42-44] For example, copper has a strong affinity toward sulfhydryl groups in a deprotonated state and promotes disulfide bond formation through oxidation reactions.[45] By heating a βlg solution at 80°C for 30 min in the presence of copper, βlg proteins unfold, exposing their free sulfhydryl group, which are oxidized by copper, resulting in stable covalent dimers.[43] Schmitt et al. (2009)[42] highlighted the importance of the balance between attractive and repulsive interactions occurring between unfolded βlg molecules. These interactions are strongly affected by environmental conditions such as pH and ionic strength. These authors[42] observed that by heating βlg at 85°C for 15 min at pH 5.8, βlg can form nanoparticles with a size of around 160 nm, stable to sedimentation and a ζ-potential more negative than native βlg.

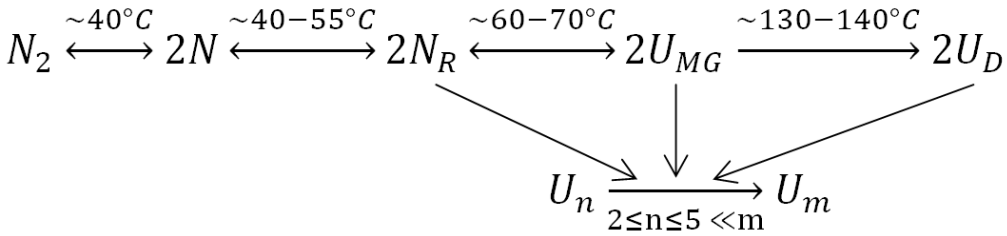

Figure 3: Mechanism of the thermal denaturation of βlg, modified from Tolkach and Kulozik (2007)[41].

2.3 Enzymatic digestion of β-lactoglobulin and its transport in the intestinal barrier

Following ingestion, proteins undergo digestion starting in the mouth and then continuing in the stomach, duodenum, and ileum (Figure 4). Digestion results in the release of small peptides and amino acids. It occurs through a combination of biological (enzymes), chemical (acidic pH) and mechanical (peristaltic) means. The main entero-enzymes responsible for protein hydrolysis are pepsin, trypsin, chymotrypsin, elastase, carboxypeptidase A and B, as well as peptidases from the enterocyte brush border of the intestine, which complete the digestion process.[46,47] Degradation products are absorbed by the intestinal barrier primarily in the jejunum, but also in the ileum. Such absorption is facilitated through the enterocytes using peptide transporters such as PEPT1 and HTP1.[48] However, peptides or even proteins can be absorbed using paracellular transport or by endocytosis. Indeed, Caillard and Tome (1995)[49] showed that 10 to 20 % of βlg can be absorbed intact by rabbit enterocytes.

Digestion and transport of proteins through the gut are influenced by digestive processes such as gastric emptying, enzyme secretion, bile salt, pH, and intestinal microflora. Additionally, digestion is also influenced by the protein structure and the food matrix. For example, Puyol et al. (1993)[50] demonstrated that the binding of palmitic acid to βlg has a protective effect on the protein against hydrolysis probably by increasing the amount of hydrophobic interactions. All these factors combine to influence the protein conformation, the activity of digestive enzymes and the binding of the enzyme to its catalytic site.[50-56]

βlg is more resistant to enzymatic hydrolysis in its native conformation than in an unfolded state.[52,57] In humans, Mahe et al. (1996)[58] have shown that 64 % of βlg is intact in the jejunum 30 min after ingestion in *in vivo* studies. *In vitro* studies have also indicated that βlg is highly resistance to pepsin hydrolysis, but as the conditions used differed substantially (ie. pH, concentrations, enzymes used), comparisons can be difficult. Moreover, the aim of several of these studies was not to mimic the intestinal conditions, but to show a decrease in the allergenicity of βlg and the production of bioactive peptides that could be achieved following hydrolysis. These studies did however all highlight the resistance of native βlg to pepsin hydrolysis possibly due in part to its compact globular form.[51] The protein is more extensively hydrolysed during intestinal digestion than gastric digestion, an observation that can be explained by the opening of the protein structure at higher pH.[22,57]

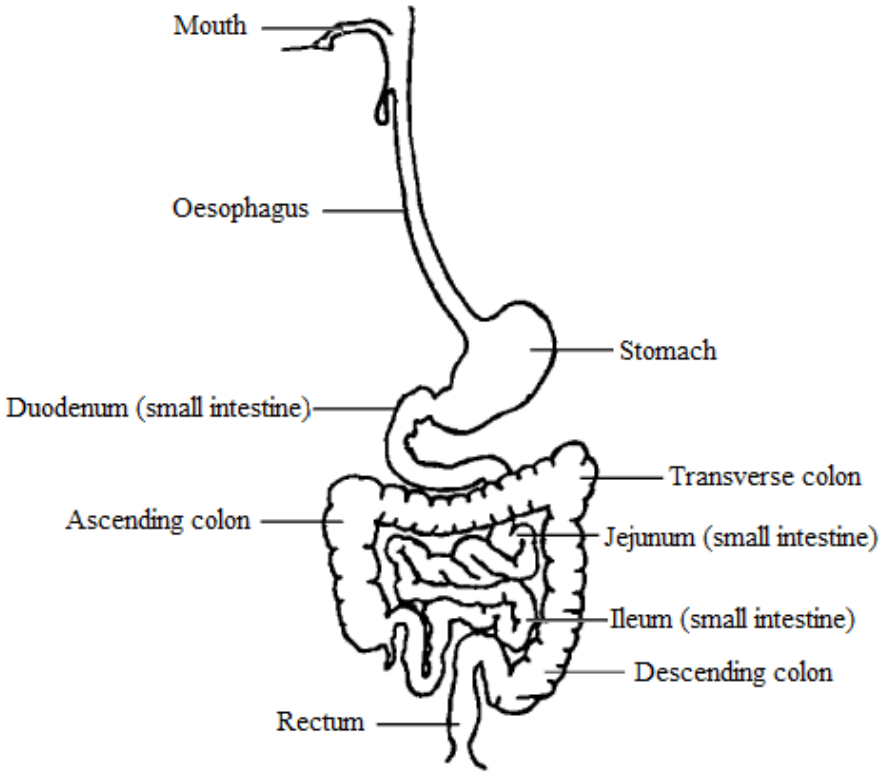

Figure 4: Schematic representation of the gastrointestinal tract. Modified from Liu et al. (2003)[59].

2.4 Biological functions of the β-lactoglobulin

βlg is the only major whey protein whose biological role, other than nutritional value, is still unknown. The most important biological properties attributed to βlg to date are related to its ability to (a) bind small hydrophobic ligands and (b) influence FA digestion. Indeed, Perez et al. (1992)[60] demonstrated that βlg participates in the digestion of milk lipids, during the neonatal period, by enhancing the activity of pregastric lipase. It does this by sequestering FA that inhibit this enzyme. *In vitro,* βlg also binds retinol.[61] It was thought for a time that βlg could have a role in the transport of retinol from the mother to the neonate because of the proteins homology to serum retinol binding protein. However, βlg was shown to bind mainly FA but not retinol in milk (retinol structure versus FA structure on Figure 7).[60,62] As the protein is not expressed in the milk of all species, it is unlikely that its ability to bind FA is its primary function. Indeed Perez and al. (1993)[63] described that pig and horse βlg could not bind FA. This failure to bind FA is attributed to structural modifications particularly at the C-terminus and the inability of porcine βlg to form native dimers.[64] βlg has also been demonstrated to bind other hydrophobic ligands such as vitamin D, cholesterol, curcumin, FA and their derivates.[64-70]

Digestion of βlg can form peptides with bioactivities such as antioxidant, antimicrobial activities, hypocholesterolemia benefits, antihypertensive and immunomodulating.[71-73] βlg or its peptides may also have satiating properties. Whey proteins are known to be more satiating that casein proteins or milk proteins.[74,75] In general, ingestion of protein induces secretion of satiety hormones by specialized enterendocrin cells that line the gut. Such satiety hormones include Cholecystokinine (CCK), glucagon-like peptide-1 (GLP1) and peptide YY (PYY). Hydrolyzed protein gives a higher CCK response compared to undigested protein.[76,77] However, other studies demonstrated a lower CCK response to hydrolysed protein or amino acids than undigested proteins, which could be due to the difference of the protein degree of hydrolysis.[78,79] Nevertheless, as βlg is resistant to hydrolysis in its native form, its value as a satiety enhancing protein is questionable.

3 INTERACTION BETWEEN β-LACTOGLOBULIN AND LIGANDS

In 1949, McMeekin et al.[80] described the binding of sodium dodecyl sulphate (SDS) to βlg. Since, βlg has been demonstrated to bind numerous hydrophobic ligands such as retinol, vitamin D, cholesterol, curcumin, FA and their derivates, polycyles (protoporphyrine IX), aromatic compounds, catechin and cations (Ca^{2+}).[66-68,81-90] Different methods have been used to study these interactions such as partition equilibrium, isothermal titration calorimetry (ITC), mass spectrometry, affinity chromatography, fluorescence, circular dichroism or nuclear magnetic resonance.

In this review, particular attention was given to binding of FA with bovine βlg. Molar ratio of free FA to βlg in milk is about 10 FA:βlg. After triglyceride hydrolysis, the molar ratio of linoleic acid (LA) to βlg in milk is about 10 LA:βlg.

3.1 Binding sites localisation

The main binding site of βlg for hydrophobic ligands is formed by the calyx of the protein (Figure 5).[37,91,92] Wu et al. (1999)[91] showed, by crystallography, the binding of palmitate in the calyx. Several studies have also indicated a secondary binding site on the protein monomer. Indeed, Dufour et al. (1990)[89] and Narayan & Berliner (1998)[93] observed that βlg can simultaneously bind two different ligands at two different sites, describing the binding of retinol and protoporphyrine IX and the binding of palmitic acid and retinoid, respectively. However, Puyol et al. (1991)[61] found that palmitic acid and retinol have the same binding site. To determine the competitivity between two ligands, these studies used

the difference of binding constants in presence of one or two ligands. They however did not use the same methods (ultrafiltration and fluorescence), which could explain the different results between these studies. The second binding site of βlg was hypothesized to be located in the hydrophobic pocket formed by the α-helix and the β-barrel, next to the dimer interface.[37,91,94] This was confirmed experimentally in 2008 when Yang et al. (2008)[94] studied the crystal structure of βlg with vitamin D_3. These authors described the second binding site between the α-helix and the I β-strand. Another possible binding site was suggested to be located at the dimer interface.[34] Using an ultrafiltration based methodology, Wang et al. (1998)[34] found that the binding of palmitate to βlg was affected by protein concentration. Indeed, an increase in protein concentration from 1 to 200 mM, which increased dimer content, resulted in a decrease of the association constant for a palmitic acid binding site. This binding site was described by the authors on the βlg dimer interface, binding 2 moles of palmitate per dimer of βlg. Another binding site with weaker affinity was described on the surface of the monomer, binding 24 moles of palmitate per mole of βlg. It is important to highlight that the same study found only one binding site by fluorescence at low protein concentration (20 µM). Similarly, Forrest et al. (2005)[65] studied βlg/vitamin D_3 binding by fluorescence at pH 6.6 and lower. This study showed that at low pH, when the EF loop is closed, binding can occur only at an external binding site. By varying pH and ionic strength, these authors showed that vitamin D_3 binds to the protein in the calyx and at the surface of the protein. This latter site exhibited a low affinity when the protein is monomeric, whereas affinity increased significantly when βlg is dimeric.

Location of binding sites and the binding properties remain controversial in the literature. This may simply be a methodological issue with methods such as fluorescence, partition equilibrium or ITC following different phenomena. For example, small association constants, similar association constants for different sets of binding sites, or other phenomena such as protein oligomerisation and change in ligand solubility may distort data and cause artefacts.[95]

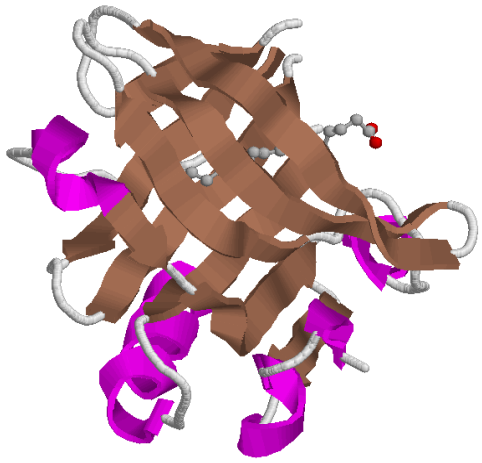

Figure 5: A schematic view of the main-chain fold of bovine βlg in interaction with linoleic acid in its central cavity. RCSB PDB code 4DQ4[96].

3.2 Binding properties: stoichiometry and association constant

An association between two molecular species, for instance a protein and its ligands, is characterized by its stoichiometry (molar ratio of ligand bound to the protein, n) and its association constant (K_a in M^{-1}). The constant K_a determines the ratio of protein/ligand (PL) to free protein (P) and free ligand (L) and is expressed by:

$$\mathrm{P} + \mathrm{nL} \overset{K_a}{\leftrightarrow} \mathrm{PL}_n$$

$$\mathrm{K}_a = \frac{[\mathrm{PL}_n]}{[P]\,[L]^n}$$

Perez et al. (1989)[15] demonstrated that bovine βlg binds FA in milk, at a molar ratio of one mole of FA per mole of βlg dimer. Palmitic acid and oleic acid, which are the major FA in milk were found to be the main lipids bound to βlg.

Table 4 is a non-exhaustive list of known FA that bind to native βlg but does not include binding data for βlg mutants or chemically unfolded βlg. Several studies showed that βlg does not bind hydrophobic ligands at low pH. Frapin et al. (1993)[64] demonstrated that the binding observed at pH 7 was not detectable at pH 3 for myristic, palmitic and oleic acids. Ragona et al. (2000)[92] demonstrated that the interaction between βlg and palmitic acid was reversible from pH 2.4 to 7.3. Similarly, Dufour et al. (1994)[97] highlighted that the interaction of βlg/retinol was pH-dependent in the range 3 to 8. Using fluorescence, Dufour et al. (1992)[88] found that the very long chain fatty acid (VLCFA) *cis*-parinaric acid binds βlg at pH 3 with a high K_a but low n (n of 2 and K_a of 4.7×10^7 M^{-1}). By increasing the pH to 7, these authors observed an increase in n values with no change in K_a. Interestingly, Collini et al. (2003)[98] showed an increase of K_a by increasing pH, from 6 to 8, for short, medium and long chain FA.

Collini et al. (2003)[98] and Loch et al. (2012)[99] demonstrated that the binding affinity of short chain fatty acids (SCFA) to βlg is low, probably due to the higher solubility of FA in aqueous solution and to the low number of hydrophobic interactions.[100,101] Association constant of FA to βlg increases with increasing chain length: between 10^2 M^{-1} to 10^4 M^{-1} for SCFA, between 10^3 M^{-1} to 10^6 M^{-1} for medium chain fatty acids (MCFA) and between 10^4 M^{-1} to 10^7 M^{-1} for LCFA and VLCFA. Frapin el al. (1993)[64] and Spector & Fletcher (1970)[102] studied binding of LCFA, showing the highest K_a for palmitic acid. This demonstrated that the βlg calyx is better adapted for a 16 length chain carbon. Frapin et al. (1993)[64] also observed that the structural constraints imposed by the number and position of double bonds within FA, only weakly affects the interaction of FA with βlg. Wang et al. (1998)[34] showed that the decrease of dimeric βlg, with decreasing protein concentration, increased the affinity constant.

Most authors agree that the number of ligands bound to the molecule (n) is close to one at the main binding site. Usually the n value was less than one but Loch et al. (2012)[99], who found a value of 0.7 by ITC, showed that the calyx contained one ligand per βlg monomer when examined by crystallography. A second binding site was observed in several studies resulting from the external binding site. This second binding site has a lower affinity (10^3 to 10^4 M^{-1}) but n can vary from 2 to 24. As Frapin et al. (1993)[64] clearly demonstrated, determination of both K_a and n are highly dependant on the fitting procedure.

Table 4: Parameters of the interactions between βlg and FA: Caprylic acid, Octanoic acid; Capric acid, Decanoic acid; Lauric acid, Dodecanoic acid; Myristic acid, Tetradecanoic acid; Myristoleic acid, *cis*-9-Tetradecenoic acid; Palmitic acid, Hexadecanoic acid; Palmitoleic acid, *cis*-9-Hexadecenoic acid; Stearic acid, Octadecanoic acid; Oleic acid, *cis*-9-Octadecenoic acid; Elaidic acid, *trans*-9-Octadecenoic acid; Rumenic acid (CLA), *cis*,*trans*-9,11-Octadecadienoic acid; Linoleic acid, *cis,cis*-9,12-Octadecadienoic acid; Linolelaidic acid, *trans,trans*-9,12-Octadecadienoic acid; γ-Linolenic acid, *cis,cis,cis*-6,9,12-Octadecatrienoic acid; Linolenic acid, *cis*,*cis*,*cis*-9,12,15-Octadecatrienoic acid; *Cis*-Parinaric Acid, *cis*,*trans*,*trans*,*cis*-9,11,13,15-Octadecatetraenoic acid; Arachidic acid, Eicosanoic acid; Arachidonic acid, *trans,trans,trans,trans*-5,8,11,14-Icosatetraenoic acid; Docosahexaenoic acid, all *cis*-4,7,10,13,16,19-Docosahexaenoic acid. Different methods were used to study the interactions: F, fluorescence; ITC, isothermal titration calorimetry; EP, equilibrium partition; ESI-MS, Electrospray ionization mass spectrometry; NMR, nuclear magnetic resonance; UF, ultrafiltration; FTIR, fourier transform infrared spectroscopy. n_1 K_{a1} and n_2 K_{a2} being the binding constant for the first and second binding sites, respectively.

Common name		pH	[βlg] (μM)	method	n_1	K_{a1}	M^{-1}	n_2	K_{a2}	M^{-1}	Reference
Caprylic acid	C8:0	6	5 to 10	F (ANS)		<3	10^2				Collini et al., 2003
Caprylic acid	C8:0	7	5 to 10	F (ANS)		<7	10^2				Collini et al., 2003
Caprylic acid	C8:0	8	5 to 10	F (ANS)		2.6	10^4				Collini et al., 2003

Common name		pH	[βlg] (μM)	method	n_1	K_{a1}	M^{-1}	n_2	K_{a2}	M^{-1}	Reference
Caprylic acid	C8:0	7.5	20	F	0.7	1.1	10^4				Loch et al., 2010
Capric acid	C10:0	7.5	20	F	0.6	6	10^3				Loch et al., 2010
Lauric acid	C12:0	6	5 to 10	F (ANS)		2.4	10^4				Collini et al., 2003
Lauric acid	C12:0	7	5 to 10	F (ANS)		1.6	10^5				Collini et al., 2003
Lauric acid	C12:0	8	5 to 10	F (ANS)		1.4	10^5				Collini et al., 2003
Lauric acid	C12:0	7	10 to 60	F	0.9	1.4	10^6				Frapin et al., 1993
Lauric acid	C12:0	7.5	50 to 100	ITC	0.7	1.7	10^5				Loch et al., 2012
Lauric acid	C12:0	7.4	200	EP	1	5.2	10^4	2	1.1	10^3	Spector and Fletcher, 1970
Lauric acid	C12:0	7.4	200	EP	1	4	10^4	24	0.1	10^3	Spector and Fletcher, 1970
Myristic acid	C14:0	3	10 to 60	F	0						Frapin et al., 1993
Myristic acid	C14:0	7	10 to 60	F	0.3	3	10^6				Frapin et al., 1993
Myristic acid	C14:0	8.5	12	ESI-MS		1.9	10^5				Liu et al., 2011
Myristic acid	C14:0	7.5	50 to 100	ITC	0.6	7.8	10^5				Loch et al., 2012
Myristoleic acid	C14:1	7	10 to 60	F	0.8	6.3	10^6				Frapin et al., 1993
Palmitic acid	C16:0	6	5 to 10	F (ANS)		6.6	10^4				Collini et al., 2003
Palmitic acid	C16:0	7	5 to 10	F (ANS)		4.7	10^5				Collini et al., 2003
Palmitic acid	C16:0	8	5 to 10	F (ANS)		5.1	10^5				Collini et al., 2003
Palmitic acid	C16:0	3	10 to 60	F	0						Frapin et al., 1993
Palmitic acid	C16:0	7	10 to 60	F	0.9	1	10^7				Frapin et al., 1993
Palmitic acid	C16:0	8.5	12	ESI-MS		3.8	10^5				Liu et al., 2011
Palmitic acid	C16:0	7.5	40 to 60	ITC	1.1	20	10^5				Loch et al., 2012
Palmitic acid	C16:0	6.5	10 to 150	F	1	2	10^6				Narayan and Berliner, 1998
Palmitic acid	C16:0	7	10 to 150	F	1	1.7	10^6				Narayan and Berliner, 1998
Palmitic acid	C16:0	8.5	10 to 150	F	1	2.5	10^6				Narayan and Berliner, 1998
Palmitic acid	C16:0	7.2	41.5	EP	1	4.2	10^6				Perez et al., 1992
Palmitic acid	C16:0	8.4 to 2.1	1500	NMR	1						Ragona et al., 2000
Palmitic acid	C16:0	7.4	200	EP	1	6.6	10^5	6	7.1	10^3	Spector and Fletcher, 1970
Palmitic acid	C16:0	7.4	200	EP	1	7	10^5	24	1.6	10^3	Spector and Fletcher, 1970
Palmitic acid	C16:0	7	1 to 200	UF	1	2.3	10^5	23	0.4	10^4	Wang et al., 1998
Palmitic acid	C16:0	7	20	F	1.2	2	10^6				Wang et al., 1998
Palmitic acid	C16:0	8	5	F	0.8	2.3	10^7				Yang et al., 2009
Palmitoleic acid	C16:1	7	10 to 60	F	0.8	3.9	10^6				Frapin et al., 1993
Stearic acid	C18:0	7	10 to 60	F	0.9	8.3	10^6				Frapin et al., 1993

Common name		pH	[βlg] (μM)	method	n_1	K_{a1}	M^{-1}	n_2	K_{a2}	M^{-1}	Reference
Stearic acid	C18:0	8.5	12	ESI-MS		1.6	10^6				Liu et al., 2011
Stearic acid	C18:0	7.4	200	EP	1	1.6	10^5	2	4.2	10^3	Spector and Fletcher, 1970
Stearic acid	C18:0	7.4	200	EP	1	1.8	10^5	4	17	10^3	Spector and Fletcher, 1970
Stearic acid	C18:0	7.4	200	EP	1	1.6	10^5	6	12	10^3	Spector and Fletcher, 1970
Stearic acid	C18:0	7.4	200	EP	1	1.5	10^5	12	5.9	10^3	Spector and Fletcher, 1970
Stearic acid	C18:0	7.4	200	EP	1	1.9	10^5	24	2.5	10^3	Spector and Fletcher, 1970
Oleic acid	C18:1	3	10 to 60	F	0						Frapin et al., 1993
Oleic acid	C18:1	7	10 to 60	F	0.8	7.7	10^6				Frapin et al., 1993
Oleic acid	C18:1	7.4	163 and 1630	FTIR	~8						Lišková et al., 2011
Oleic acid	C18:1	7.2	41.5	EP	1	2.3	10^6				Perez et al., 1992
Oleic acid	C18:1	7.4	200	EP	1	0.4	10^5	24	1.3	10^3	Spector and Fletcher, 1970
Elaidic acid	C18:1	7	10 to 60	F	0.8	6.7	10^6				Frapin et al., 1993
Rumenic acid (CLA)	C18:2	7	1	F	2.5	3.7	10^6				Jiang and Lui, 2010
Linoleic acid	C18:2	7	10 to 60	F	0.8	5.3	10^6				Frapin et al., 1993
Linoleic acid	C18:2	7.4	163	F	0.6	2.7	10^5	5.8	5.9	10^3	Le Maux et al., 2012
Linolelaidic acid	C18:2	7	10 to 60	F	0.9	3.3	10^6				Frapin et al., 1993
γ-Linolenic acid	C18:3	7	10 to 60	F	0.9	7.7	10^6				Frapin et al., 1993
Linolenic	C18:3	7	10 to 60	F	0.9	5.9	10^6				Frapin et al., 1993
Cis-Parinaric Acid	C18:4	3	1 to 20	F	0.2	4.8	10^7				Dufour et al., 1992
Cis-Parinaric Acid	C18:4	7	1 to 20	F	0.8	3.6	10^7				Dufour et al., 1992
Arachidic acid	C20:0	7	10 to 60	F	0.9	2.5	10^6				Frapin et al., 1993
Arachidonic acid	C20:4	7	10 to 60	F	0.8	3	10^6				Frapin et al., 1993
Docosahexaenoic acid	C22:6	7	1	F	2.7	6.8	10^5				Zimet and Livney, 2009

3.3 Impact of the fatty acid/protein binding on the biological function of the fatty acids

Binding FA to proteins such as βlg, can modify the bioaccessibility of FA for cells. This can be measured by looking at the FA transport into cells or by measuring FA cytotoxic effect when bound to a protein compared to that of free. LCFA are known to be cytotoxic, but need to be in the cells to have this effect.[103,104] The cytotoxic effect of different FA increase or

decrease when bound to βlg compared to unbound. Jiang et al. (2010)[69] demonstrated that when conjugated linoleic acid (CLA) is part of a CLA/βlg complex, it is 30 % more cytotoxic at a concentration of 100 µM after 48 h on Caco-2 cells. Similarly, the toxic effect of oleate/oleic acid was increased when it was bound to βlg or αla.[13,68] Oleic acid/αla complex also exhibited a ~40 % increase in cytotoxic effect on human larynx carcinoma cells compared to free oleic acid.[13] However, we have shown that at a concentration of 58 µM linoleate, free linoleate decreases Caco-2 viability by 50 %, whereas linoleate bound to βlg had no effect on the cell viability (see Part 3, Chapter 1[81]).

Jiang et al. (2010)[69] showed an increase of the CLA uptake from 45.8 µM to 85.9 µM when bound to βlg compared to free after 4 h of Caco-2 cells exposure to 100 µM of FA, in agreement with their viability tests on Caco-2 cells described above. However, using a Caco-2 monolayer that mimics the intestinal barrier in combination with confocal imaging, we have shown that linoleate transport into Caco-2 cells decreases in the presence of βlg, in agreement with the cytotoxic study (see Part 3, Chapter 2). Riihimäki-Lampén (2009)[105] and Puyol et al. (1995)[106] showed that palmitic acid was transported more efficiently across the monolayer in its free form than in complex with βlg. Riihimäki-Lampén (2009)[105] showed similar results for retinol, although no change was observed for cholesterol transport by Puyol et al. (1995)[106]. Interestingly, Levin et al. (1992)[107] observed that oleic acid in lipidic micelles with taurocholate was absorbed more efficiently than oleic acid bound to BSA. In this instance, the emulsifying properties of taurocholate may function to increase the solubility of the free FA, which explains the difference with the cytotoxicity assays previously presented.

Recent studies based on direct measurement of oleic acid in solution would argue that oleic acid alone or involved in a complex has comparable cytotoxic effects towards various cells, with the protein alone having no effect.[68,108] The measured cytotoxic effect observed with oleic acid/βlg or oleic acid/αla complexes maybe related to the solubility of the FA. Oleic acid has a poor solubility in aqueous solution, its critical micelle concentration (CMC) being at 20 and 69 µM at 17 and 45°C, respectively, for a pH of 8.3, 1 mM EDTA and no salts.[13] Therefore in the absence of protein, the amount of oleic acid available (soluble) for the cells would be low. The binding of oleic acid to βlg or other proteins increased the solubility of oleic acid on its own and possibly its bioaccessibility. Solubility of FA increases with the number of C=C double bond along the FA aliphatic chain.[101] Therefore, the solubility of LA (C18:2) is higher than that of oleic acid (C18:1). Under the experimental conditions of Collin et al. (2010) study[109], linoleate has a CMC of 2 mM. The impact of the FA solubility was highlighted by Norman et al. (1988)[110] who found a higher cytotoxic effect of linoleate, the soluble salt form of linoleic acid, on the epithelial mouse cells, Ehrlich Ascites Tumor, compared to linoleic acid. Study from Spector and Fletcher (1970)[102] supported the solubility

hypothesis demonstrating that palmitate bound to βlg was taken up twenty times faster by Ehrlich Ascites Tumor cells at a faster rate than palmitate bound to BSA for a palmitate/protein molar ratio of 0.8. This is due to the stronger binding of palmitate to albumin compared to βlg; therefore, the FA was less bioaccessible to the cells when bound to albumin. Hence, the FA bioaccessibility is altered by the presence of protein, which modifies its solubility in function of the binding properties of the FA/protein complex.

We have shown that even if the linoleate uptake by cells was decreased by its binding with βlg, the protein itself could help a small amount of FA to be transported into a Caco-2 cell monolayer.[81] This kinetic of transport would be much lower than the kinetic of transport of free FA.

Zimet and Livney (2009)[111] demonstrated that compared to docosahexaenoic acid (DHA) alone, the combination of DHA with βlg, protects DHA against oxidation at pH 7 and at 40°C. Similarly, Futterman and Heller (1972)[85] demonstrated that retinol was protected against enzymatic oxidation by BSA and βlg. It has also been shown that Retinol and β-carotene were protected against degradation by heating, oxidation and irradiation.[112]

3.4 Impact of the fatty acid/protein binding on the structural function of the protein

The βlg calyx closes at below pH 6.2 (Tanford transition). At this acidic pH, the EF loop region moves to a closed position, preventing the binding of a ligand.[22] Therefore, interaction of FA to βlg can only impact on the protein structure at non-acidic pH. Interestingly, Puyol et al. (1993)[50] demonstrated that by increasing the protein stability with the increase of hydrophobic interactions, the binding of palmitic acid to βlg has a protective effect on the protein against hydrolysis, but that the binding of retinol did not. Mandalari et al. (2009)[113] also observed that the binding of retinol to βlg did not have a protective effect toward hydrolysis. However, these authors demonstrated that the protective effect of the phosphatidylcholine binding to βlg could be due to the binding of this ligand next to the proteases cleavage sites.[113] Furthermore, Mandalari et al. (2009)[113] demonstrated that this protective effect was not effective if the protein was not in its native form.

Barbioli et al. (2011)[24] showed that the binding of palmitate to βlg protects the protein against temperature and chaotrope denaturation. It does this by stabilizing the calyx at the hydrophobic interface between the barrel itself and the long helix where the thiol group of Cys121 is buried. Other studies showed that binding of ligands such as myristic acid, CLA, SDS, anilino naphthalene sulfonic acid (ANS) or retinol to βlg, stabilised the protein against denaturation by heating or high pressure treatment.[114,115]

Binding of linoleate or phosphatidylcholine to βlg does not modify the protein secondary structure.[81,116] However, Tavel et al. (2008)[117] and Kanakis et al. (2011)[87] observed changes in the βlg amide I band with the presence of aromatic compounds and tea polyphenols, respectively. Kanakis et al. (2011)[87] showed that this was due to big size of ligands. However, protein aggregation has been reported for βlg and other protein systems in the presence of lipids.[118] CLA/βlg binding formed aggregates complexes with a size around 170 nm at pH 7.[69] Liskova et al. (2011)[68] and Le Maux et al. (2012[81], see Part 3 Chapter 1) described the formation of covalent dimers and trimers when the interaction between βlg and linoleate or oleate was formed under heating conditions at 60°C.

3.5 Impact of the non-native protein structure on binding properties of the fatty acid/protein complex

Modification of the Cys121 in the βlg protein weakens or totally eliminates the FA binding capacity of the βlg due to steric hindrance, showing the importance of the protein structural integrity to bind ligands.[93] βlg structure is influenced by the protein environment such as pH, temperature or pressure. Dufour et al. (1992)[88] studied the impact of pressure and demonstrated an irreversible dissociation of βlg/*cis*-parinaric acid complexes at 350 MPa. βlg denatured by urea, acetylation or unfolded by temperature, have a weaker affinity for palmitate.[102] Dufour et al. (1992)[88] showed that chemically modified βlg has a higher K_a for benzo(α)pyrene and ellipticine, whereas a higher n was observed for the binding of *cis*-parinaric acid to this denatured βlg. O'Neil and Kinsella (1998)[84] showed that binding affinity of the 2-nonanone for βlg was reduced but n was increased when the protein was heated at 75°C for 10 and 20 min. In this thesis, Part 3 Chapter 3 shows similar association bindings but an increase of linoleate bound to βlg when the protein size increased. Because of this binding difference, we found that linoleate uptake by Caco-2 cells was faster for FA bound to non-native βlg compared to the native protein (Part 3, Chapter 3). Similarly, Yang et al. (2009)[119] showed that heated βlg decreased the binding constant to vitamin D_3 and therefore decreased the uptake of FA in mouse. By heating βlg at 100°C for 16 min, Yang et al. (2008)[94] demonstrated that binding of vitamin D_3 to the calyx was ineffective but that the external binding site remained thermally stable.

With the exception of raw milk, βlg is often found as aggregates in food products. However, binding of FA to βlg aggregates has not been extensively reported yet. We have shown that βlg aggregates (covalent dimers and nanoparticles) bind a larger number of linoleate than native βlg (Part 3, Chapter 3). CLA binding to native βlg, covalent dimers or nanoparticles was also shown to differ (Part 3, Chapter 3). Shpigelman et al. (2010)[120]

demonstrated that binding of epigallocatechin gallate (EGCG) to βlg formed nanoparticles of ~50 nm when the protein was heat denatured. Binding of EGCG to denatured βlg was stronger compared to native, possibly due to the high ligand size.

Hetero-aggregates of βlg with chitosan or pectin were reported to form hydrogel particles ranging from nano to micro sizes. They were proposed to be potential vehicles for hydrophobic ligands.[121-123] Ron et al. (2010)[124] demonstrated that vitamin D2 was better protected by βlg-pectin aggregates (50 to 70 nm) than βlg alone, while Zimet and Livney (2009)[111] demonstrated that nanocomplexes of βlg and pectin stabilized DHA.

4 LONG CHAIN FATTY ACIDS PROPERTIES, EXAMPLES OF LINOLEIC ACID AND CONJUGATED LINOLEIC ACID

FA are carboxylic acids with hydrocarbon chains varying from 4 to 30 carbon units. In general, FA are poorly soluble in water but more soluble in organic solvant such as ethanol, chloroform or n-hexane. FA with a chain length ≥ 18 carbons can be synthetised from the saturated FA stearic acid. Synthesis of the major polyunsaturated FA oleic acid, mead acid, linoleic acid (LA), arachidonic acid, α-linolenic acid, eicosapentaenoic acid, docosapentaenoic acid and docosahexaenoic acid are outlined in Figure 6. However, mammalian cells cannot synthesise LA or α-linolenic acid and therefore these are regarded as essential FA which must be supplied in the diet.

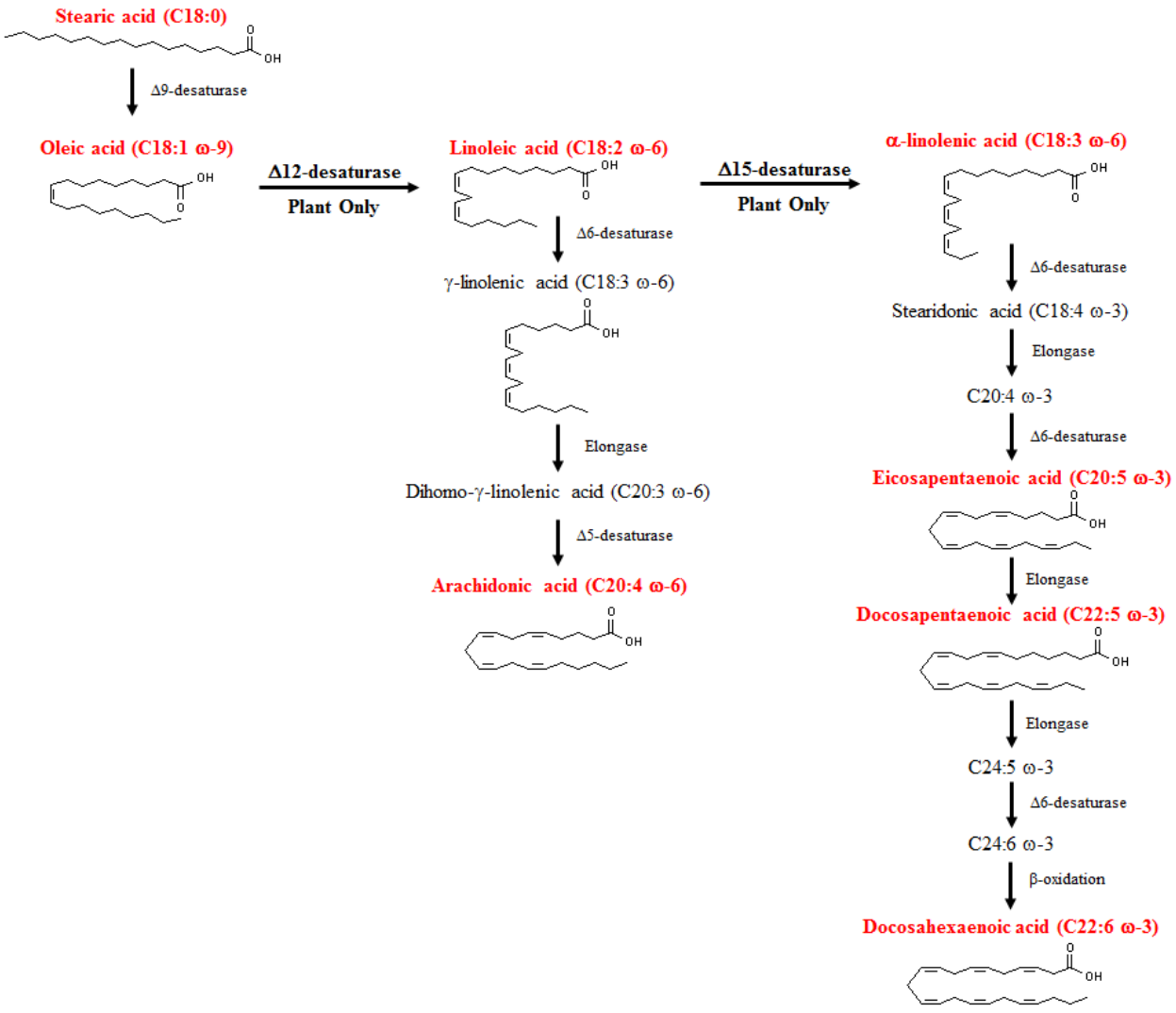

Figure 6: Synthesis of essential FA. Modified from Calder and Burdge (2004)[125].

4.1 Fatty acids properties

However, in aqueous environments their hydrophilic head allows the FA to be stabilized by organization into micelles and bilayers. Above the CMC and below the critical micelle temperature, FA exist in equilibrium between micellar and free FA states. FA solubility decreases with an increase in chain length. This is evident in the solubility of caprylic acid (C12:0) and stearic acid (C18:0) at 20°C, which are 0.08g/100g water and 0.0003g/100g water, respectively.[100] Indeed, the chain length increases the van der Waals interactions between the adjacent FA bringing the FA closer.[126] Moreover, the solubility of FA increases with the number of C=C double bonds in the aliphatic chain because the curvature of the FA increases the distance between FA.[101]

4.2 Linoleic acid, an essential long chain fatty acids

Essential FA are important constituents of cell membranes.[127] Two types of essential FA naturally occur in the body: n-3 essential FA are derived from α-linolenic acid, while n-6 essential FA are derived from linoleic acid (LA).[128] These essential FA are long chain unsaturated FA which by enzymatic action form several metabolites (Figure 6). The major polyunsaturated FA in most diets is the LA, *cis,cis*-9,12-octadecadienoic acid. The main dietary sources of LA are cereals, eggs, poultry, most vegetable oils, whole-grain breads, baked goods, and margarine. Sunflower, saffola, and corn oils are also rich in LA.[128] LA represents 1-3 % (wt) of the FA in bovine milk fat.[4]

World Health Organization (WHO) and Food and Agriculture Organization of the United Nations (FAO) recommend an adequate intake of LA of 2 % of the total energy.[129] The average LA intake in the United States and Western Europe has risen from < 3 % of energy in the 1950s to 6-7 % at present, with a commensurate decrease in saturated fat.[130] A few populations (Northern Belgium and Israel) habitually consume 8-12 % of their total energy intake as LA. Some experts have recommended that LA consumption be raised to 10 % of energy intake.[130]

4.3 Conjugated linoleic acid, isomer of linoleic acid

Conjugated linoleic acid (CLA) is the term used to describe the positional and geometric isomers of LA with either one or both of the double bonds in the *cis* (*c*) or *trans* (*t*) conformation and separated by simple carbon-carbon linkage. The major CLA isomers are *cis*9-*trans*11, called rumenic acid as well, and *trans*10-*cis*12 (Figure 7).

The presence of CLA in ruminant fat is a result of the microbial biohydrogenation of dietary LA and linolenic acids to stearic acid in the rumen via the action of the enzyme linoleic acid isomerase[131] or through the endogenous conversion of vaccenic acid to CLA.[132,133] Therefore, CLA isomers are naturally found in bovine milk fat at concentrations of 2-52 mg/g fat depending on cow's diet.[4,134] However, the CLA content of milk and dairy products is typically comprised of 85-90 % rumenic acid.[134]

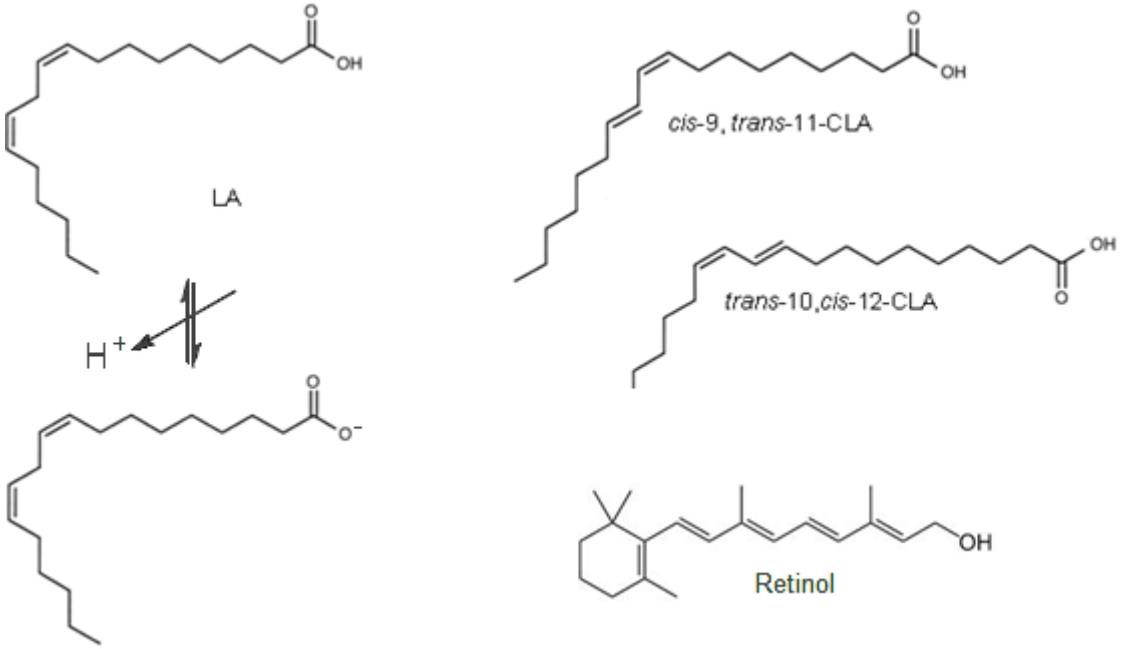

Figure 7: The structure of *cis*-9,*trans*-11-CLA, *trans*-10,*cis*-12-CLA, LA and retinol. Modified from Roche et al. (2001)[135].

4.4 Impact on heath

Polyunsaturated FA are necessary for the structure and function of cellular membranes and are precursors of lipid mediators. LA is a precursor to long chain metabolites such as γ-linolenic acid and arachidonic acid.[136,137] Polyunsaturated FA and their metabolites are a central subject for cancer and obesity.[128,138,139] Replacement of saturated fat with LA is advised to improve serum lipoprotein profiles and reduce the risk of developing cardiovascular coronary artery disease.[130] Some of these polyunsaturated FA such as LA or γ-linolenic acid have been shown to have anti-inflammatory properties.[140] LA is also cytotoxic at high concentrations to cancerous cells *in vitro*.[81,104,141] To date, it is not well understood how intestinal cells metabolize LA. A previous study showed that cytotoxic effect of LA was initiated by mitochondrial apoptotic pathway with cytochrome C release, indicating that uptake of LA is essential for its cytotoxic effect.[141] LA cytotoxicity may also occur by an adverse alteration of the n-6 to n-3 polyunsaturated FA ratio in the cells resulting in alterations to membrane permeability and fluidity.[104]

LCFA have been shown to have a positive health impact on the digestive tract. The satiety hormone CCK is secreted by I enteroendocrine cells in the proximal small intestinal mucosa in response to intraluminal nutrients including medium to long chain FA.[142] CCK is reported to have a satiety effect as it influences the digestive processes in the gut as it has

been associated with delay of gastric emptying, stimulation of gallbladder contraction, increases in pancreatic enzyme secretion, and reduced food intake.[143,144] Relatively little is known concerning the mechanisms whereby nutrients influence CCK synthesis and secretion. Several studies indicate that FA and proteins use a membrane receptor pathway, triggering GPR93, GPR120-coupled Ca^{2+} and TRPM5 membrane proteins, which in turn transduces a signal resulting in production and/or secretion of CCK [145-147] Secretion of CCK is dependent on FA structure, increasing with chain length, degree of unsaturation and when the FA is free compared to in triglycerides.[148-153]

In particular, CLA isomers have been reported to have multiple potential health benefits related to cancer, cardiovascular disease, diabetes, obesity, inflammation, bone density loss and immune dysfunction, as well as antioxidative and growth promoting properties.[134,135,154-157] However, the health benefit of CLA remains controversial due to the often opposing properties of the various isomers and the wide ranges of concentrations used *in vitro* and *in vivo* making it difficult for accurate comparative analysis across studies. It is estimated that our current CLA dietary intake is much lower than that needed for CLA to exert its health promoting activities. As a result dietary strategies have been designed in an attempt to increase CLA uptake.

4.5 Lipids digestion

Lipid absorption mainly takes place in the small intestine. Triacylglycerols, which are mainly composed of LCFA, represent 95 % of lipids in the human diet.[158] Triacylglycerol cannot cross the intestinal barrier, therefore digestion must occur pre-adsorption. Digestion consists of the emulsification of fat and its hydrolysis into free FA using salivary and pancreatic lipases. As LCFA are poorly soluble in aqueous phase, they are bound to proteins or exist in micelles with the aid of bile salts. Immediately prior to entry into the enterocyte, LCFA are found in their free form due to the acidic microclimate at the intestinal barrier. As this local pH is below their pKa, protonation of LCFA occurs.[158]

Uptake of LCFA remains complex because of their hydrophobicity (Figure 8). It was thought for a long time that their uptake by enterocytes occurred by diffusion. However, studies suggest the involvement of protein-transfer mechanisms with transport of LCFA reaching saturation at high concentrations in Caco-2 cells.[159-161] It is likely that an efficient LCFA uptake by cells requires both passive and facilitated transfer, with low LCFA concentrations favouring active transport.[159] Protein transporters used for facilitated transport included the plasma membrane-associated fatty acid-binding protein (FABPpm), the FA transport protein 4 (FATP4) and the FA transporter CD36.[158] The requirement of cyclic AMP

in LCFA uptake or metabolism was indicated by several authors, but it is controversial and appears to depend on FA, its concentration and cell type.[159,162-165]

After uptake by enterocyte cells, FA are bound to proteins for their intracellular trafficking and thio-esterification.[158] They are rapidly esterified into triacylglycerol in the endoplasmic reticulums, subsequently forming chylomicrons in the golgi before being released into the cytoplasm and finally into circulation for uptake by other cells.[158]

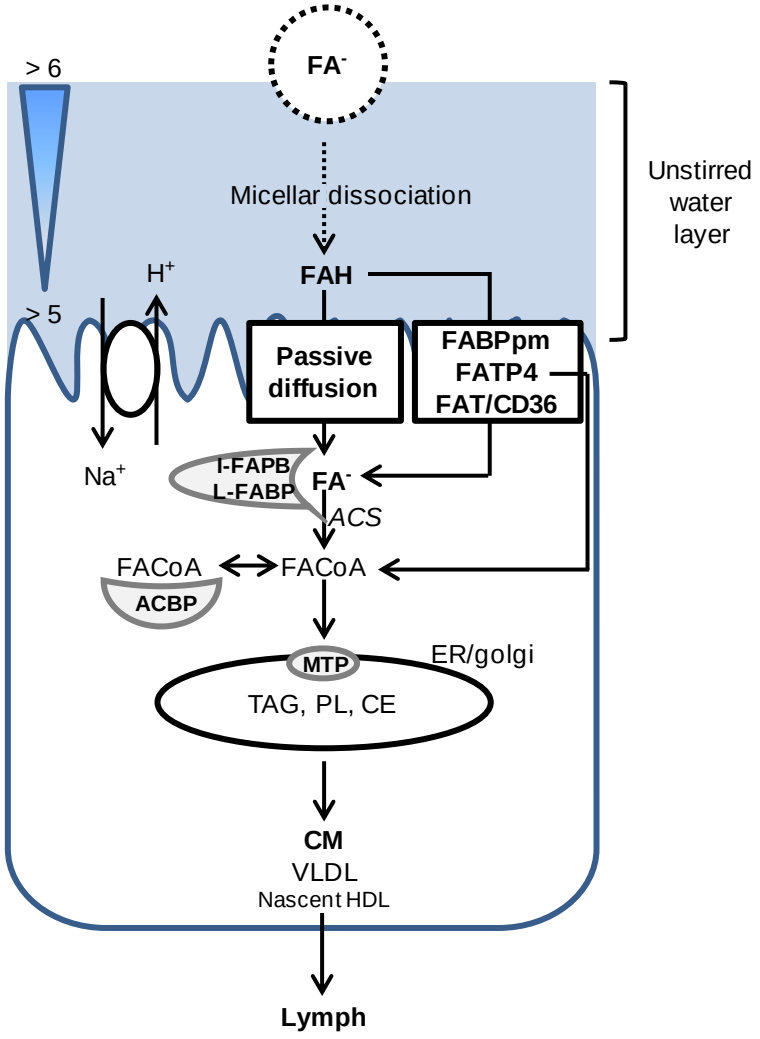

Figure 8: Schema of the transport of LCFA through enterocytes. Modified from Niot et al. (2009)[158]. Because of the acidic microclimate in the unstirred water layer of the enterocyte brush border membrane, LCFA protonation occurs leading to micellar dissociation. After their cellular uptake, LCFA are transported and converted into triacylgycerides, and then lipoproteins before their exocytosis into the lymph system. ACBP, acyl-CoA-binding protein; ACS, acyl-CoA synthetases; ER, endoplasmic reticulum; FA-, ionized long-chain fatty acids; FABPpm, plasma membrane fatty acidbinding protein; FAH, protonated long-chain fatty acids; FATP4, fatty acid transport protein 4; HDL, high density lipoprotein; I-FABP, intestinal fatty acid-binding protein; L-FABP, liver fatty acid-binding protein; MTP, microsomal triacylglycerol transfer protein; TAG, triacylglycerol; PL, phospholipids; CE, cholesterol esters; VLDL, very low density lipoproteins.

5 CONCLUSION

The molecular structure of bovine βlg has been extensively studied. Its globular structure allows it to naturally bind hydrophobic ligands such as FA. Its biological function other than nutrition is unknown but it may serve to deliver FA to the enterocyte. However, the ability to bind hydrophobic components is species dependent with some βlg species variants unable to bind FA.

The main site binding sites for hydrophobic ligands is the βlg calyx. A secondary binding site is located at the surface of the protein next to the dimer interface. This explains why βlg in its monomeric or dimeric native form does not have the same stoichiometry. Stoichiometry is difficult to measure as mainly indirect methods to analyze βlg complexes are used. Even if affinity constants are of the same order of magnitude between studies, ratios of FA bound to βlg are different.

The interaction of βlg with ligands such as FA modifies the bioaccessibility of the ligand. It would appear that when the FA solubility is low, βlg increases its bioaccessibility, whereas this interaction decreases the FA bioaccessibility when the ligand has a high solubility.

Whereas βlg is often found in non-native forms, the impact of the βlg aggregation on FA interaction and the ligand bioaccessibility are rarely reported in the literature.

LCFA are essential for a good health, being a source of energy, possessing a number of bioactive properties, and being important metabolites for other essential compounds in the body. During digestion, they are taken up by intestinal epithelial cells, mainly in the duodenum and jejunum. Because of their poor solubility, their digestion remains complex. Indeed, they are poorly bioaccessible for cells, but uptake of FA may be modified by the food matrix.[166,167] Therefore, FA bioaccessibility in presence of protein such as βlg is extensively studied in an attempt to modulate the FA properties.

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PART 2

MATERIALS AND METHODS

Sample concentrations used were generally specified in this section, unless otherwise detailed in each experimental section.

1 MATERIALS

β-lactoglobulin (βlg, 96% purity) was obtained from Davisco Foods International, Inc. (Eden Prairie, Minnesota). Non-native forms of βlg in the commercial sample were removed by precipitation at pH 4.6. Briefly, the pH of βlg solution was dropped to 4.6 using HCl 1 M. Following centrifugation in order to concentrate the denatured/aggregated proteins in the pellet, the supernatant was transferred to a clean vessel and its pH increased to 7. Dialysis of the supernatant was performed for 72 h against distilled water, which was changed three times. The βlg solution was freeze dried and stored at -20°C prior to use. After dialysis, the protein powder was composed of 85 % monomers, 10 % of oligomers and 5 % of α-lactalbumin (αla), determined by gel permeation-HPLC (GP-HPLC) as described below.

Sodium linoleate (purity ≥98%) and conjugated linoleic acid (CLA, a mixture of *cis*9-*trans*11 and *trans*10-*cis*12, 99 % purity) were purchased from Sigma-Aldrich (St. Louis, MO) and were used without further treatment.

Phosphate buffered saline (PBS), was a 0.01 M phosphate buffer at pH 7.4 containing 2.7 mM KCl, 137 mM NaCl. PBS was filtered through a 0.20 µm filter (VWR, Radnor, PA).

All other chemicals and solutions were purchased from Sigma-Aldrich, unless stated otherwise.

2 PROTEIN SAMPLE PREPARATION

2.1 Covalent dimers of β-lactoglobulin

Covalent dimers of βlg were formed by oxidation using the protocol described by Gulzar et al. (2009)[1]. Briefly, βlg was dissolved in a 5 mM Bis-Tris buffer (pH 6.7) to a final protein concentration of 5 g/L. Copper chloride ($CuCl_2$) was added to the protein solution at a Cu^{2+}/βlg molar ratio of 0.6. The solution was heated at 80°C for 30 min to form covalent dimers, then cooled on ice. Covalent dimers were first dialyzed against 10 mM NaCl (dialysis bath was changed every hour for 4 h) and then against distilled water for 48 h (water bath was changed twice). Samples were then freeze-dried and stored at -20°C.

The protein powder was composed of 74 % dimers (determined by GP-HPLC as described below).

2.2 β-lactoglobulin nanoparticles

Nanoparticles of βlg were formed according to the method described by Schmitt et al. (2009).[2] Briefly, βlg was dissolved in Milli-Q water (Millipore, Carrigtwohill, Ireland), to a final protein concentration of 10 g/L. The pH of the protein solution was adjusted to 5.9 using 1 M HCl. It was then heated to 85°C for 15 min, and rapidly cooled on ice. Samples were dialysed for 48 h against an excess of distilled water. Samples were freeze-dried and stored at -20°C.

2.3 Protein sample reconstitution

βlg solutions (native βlg, covalent dimers and nanoparticles) were obtained by solubilisation of an appropriate amount of βlg powder in PBS. The mixture was stirred at room temperature for 2 h or in the fridge overnight.

The concentration of native βlg and covalent dimers were determined by optical density using the extinction coefficient of βlg at 278 nm (ε_{278} = 0.96 L/g/cm) using the Beer-Lambert law:

$$A_{278}=\varepsilon_{278}\times l\times C$$

with A_{278} being the absorbance at 278 nm, l the path length in cm and C the protein concentration in g/L. The extinction coefficient of βlg is not modified when βlg is in a covalent dimer form.

To determine the concentration of βlg in nanoparticle sample, nanoparticle powder was dispersed at around 1 g/L in PBS. Sodium dodecyl sulphate (SDS, 5 µL of 10 %) and 25 µL β-mercaptoethanol were added to 470 µL of nanoparticle sample. The mixture was heated at 95°C for 5 min in order to achieve protein disulfide bond reduction. The protein concentration was quantified on reduced sample by the Bradford test following the manufacturer's instructions (Sigma-Aldrich).

3 FORMATION OF THE PROTEIN/LIGAND COMPLEXES

βlg/linoleate complexes were prepared by mixing a solution of βlg (native βlg, covalent dimers or nanoparticles) and sodium linoleate according to Lišková et al. (2011)[3]. Briefly, 0.163 mM βlg in PBS (expessed in monomer) and sodium linoleate (stock solution of

5 mM in PBS) were mixed together to reach a final linoleate/βlg molar ratio of 5, 7.5 or 10. Solutions containing native βlg were heated at 60°C for 30 min to facilitate βlg/linoleate interaction, then rapidly cooled on ice, whilst solutions containing covalent dimers or nanoparticles were placed overnight at room temperature. Samples were dialysed with a 3500 Da cut off, against distilled water for 72 h in order to remove free linoleate prior to freeze-drying. Freeze dried powders were stored at -20°C prior to further experimental use.

For CLA experiments, 0.163 mM βlg (expessed in monomer) dispersed in PBS and CLA (dissolved in ethanol) were mixed together to reach a final CLA/βlg molar ratio 2 and an ethanol concentration of 1 %. Because preliminary experiments showed that CLA was not removed by dialysis, solutions were placed overnight at room temperature the day prior to experimental use.

4 *IN VITRO* DIGESTION

Impact of FA binding on βlg digestibility was analysed using an *in vitro* adult digestion model adapted from Dupont et al. (2009)[4]. βlg samples (20 mL) were dissolved in simulated gastric fluid (0.15 M NaCl, pH 2.5) and the pH adjusted to 2.5 with 0.5 M HCl solution. Porcine gastric mucosa pepsin (Sigma-Aldrich P7000, activity: 837 U/mg of protein calculated using haemoglobin as a substrate) was added to give 182 U of pepsin/mg of βlg. The final concentration of βlg was 0.05 mM in 20 ml solution. During this gastric digestion, aliquots (1 mL) were removed at regular intervals over a 60 min period. Pepsinolysis was stopped by raising the pH to 7.0 using 0.5 M NaOH. For duodenal proteolysis, the pH was adjusted to 6.5. Duodenal digestion components were dissolved in simulated duodenal fluid (0.15 M NaCl, pH 6.5). They were added to give final concentrations as follows: 4 mM sodium taurocholate, 4 mM sodium glycodeoxycholate, 26.1 mM Bis-Tris buffer pH 6.5, 0.4 U/mg of βlg for α-chymotrypsin (Sigma-Aldrich C4129, activity 59 U/mg of protein using benzoyltyrosine ethyl ester as substrate) and 34.5 U/mg of βlg for trypsin (Sigma-Aldrich T0303, activity 14476 U/mg of protein using benzoylarginine ethyl ester as substrate). Aliquots of 1 mL were removed over a 30 min period of duodenal digestion. Proteolysis was stopped by addition of an excess of soybean Bowmann-Birk trypsin/α-chymotrypsin inhibitor (100 µL of 5 g/L in simulated duodenal fluid). Hydrolysed products were freeze-dried and stored at -20°C prior to analysis by GP-HPLC as described below.

5 BIOLOGICAL PROPERTIES ANALYSIS

Cell cultures were routinely cultured in a humidified 37°C incubator with 5 % CO_2 except where otherwise stated.

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5.1 Caco-2 cell line

The Caco-2 cell line was purchased from the European Collection of Cell Cultures (collection reference: ECACC 86010202) and was derived from human colonic adenocarcinoma cells.

Cells were routinely grown in 75 cm^2 plastic flasks in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/L glucose and 0.584 g/L L-glutamine. Media for subculture was supplemented with 10 % (v/v) foetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin. At 80 % confluency, cells were trypsinised with 0.25 % trypsin/EDTA, diluted 1:6 in media and reseeded. Media was changed three times a week. All cells used in these studies were between passage number 32 and 42.

5.2 STC-1 cell line

The STC-1 cell line was purchased from American Type Culture Collection (ATCC, Gaithersburg, MD). This enteroendocrine cell line originated from a double transgenic mouse tumour.[5] Because of the presence of I-cells, the murine enterocyte STC-1 cell line was used to study CCK gene expression and secretion.[6,7]

Cells were routinely grown in 75 cm^2 plastic flasks in DMEM containing 4.5 g/L glucose and 0.584 g/L L-glutamine. Media for subculture was supplemented with 20 % (v/v) FBS, 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were passaged at 80 % confluence. Cell passage numbers 15 to 20 were used in this project.

5.3 Cytotoxicity assay

Linoleic acid and CLA are toxic to cancerous cells.[8,9] To understand the impact of the interaction between FA and βlg on the FA cytotoxicity, cytotoxicity of test samples on Caco-2 and STC-1 cell proliferation was determined by MTS assay, a colorimetric assay using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Corporation Madison, Wisconsin, USA) according to the manufacturer's instructions. Viability was defined as the

ratio of absorbance of treated cells to untreated cells (cells incubated in media only) at 490 nm. Each cell exposure was repeated at least in triplicate.

For Caco-2 experiments, cells were seeded in 96-well plates, at a cell density of 2×10^4 cells/well, using serum-free media (DMEM only supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin) 24 h prior to experiment. Caco-2 cells were exposed for 24 h to tested samples.

Cytotoxicity assay was also performed on Caco-2 monolayers. Caco-2 cells were seeded in 96-well plates, at a cell density of 6.5×10^4 cells/well, using complete media. Initially, media was changed after 6 h, then every two days up to 21 days. Complete media was changed to serum-free media 24 h prior to experiment. Cells were exposed to 50 µM linoleate for 24 h.

For STC-1 experiments, STC-1 cells were seeded in 96-well plates, at a cell density of 2×10^5 cells/well, using complete media 24 h prior to experiment. STC-1 cells were treated with different concentrations of linoleate and βlg in serum-free media for 24 h.

The lethal dose 50 (LD_{50}) were determined by plotting the % viability versus the compound concentration on Graph-Pad Prism software 3.03 (GraphPad Software Inc., La Jolla CA, USA). The sigmoidal dose-response with variable slope was used to fit the measured curves and calculate LD_{50}.

5.4 Real-Time Cell Analyzer

Cell growth was monitored in real time using the Real Time Cell Analyzer (RTCA) SP instrument (xCELLigence, Roche Diagnostic Limited, West Sussex, UK). The RTCA system measures the impedance of the bottom of the well which is a function of cell number and cell morphology. As the cell number increases, the impedance increases. In addition, if the cell morphology changes (cells swell or shrink), the impedance will also be affected. The RTCA software generates a cell index value based on the level of impedance. Caco-2 cells in serum-free media were seeded in 16 E-Plates (Roche Diagnostic Limited) at a cell density of 1×10^4 cells/well. After 24 h in a humidified 37°C incubator with a 5 % CO_2 in air atmosphere, cells were treated for 48 h with different concentrations of linoleate, βlg or linoleate/βlg complexes in serum-free media. Data were analysed using the RTCA software 1.2 (Roche Diagnostic). Cell Index was plotted versus time and Effective Concentration 50 (EC_{50}) was determined by regression analysis of the cell index data versus the concentration of the compound after 48 h. Using the software, the time dependent EC_{50} curves were determined by calculating the EC_{50} values at 20 time points within 48 h and these EC_{50} values were plotted versus time.

5.5 Caco-2 transepithelial transport

5.5.1 Principle

The Caco-2 cell line is derived from human colonic carcinoma and has similar characteristics to the epithelial cells of the small intestine.[10] Cultured as a monolayer, these cells can differentiate into intestinal-like absorptive cells with tight junctions and a well differentiated brush border, which express nutrient transporters.[11] *In vivo* the apical side of these enterocyte-like cells, which contains the brush border, would be in direct contact with the lumen of the gut. The basal side of these cells would be in contact with the blood and lymphatic systems. As such, Caco-2 monolayers *in vitro* are a well-established intestinal barrier model to determine the bioavailability of substances and their transport into and out of the intestinal epithelium.[10-12] To study transport through the monolayer, Caco-2 are routinely differentiated on an insert between an upper chamber and lower chamber (Figure 9). Test samples are usually applied on the apical chamber and concentration of test sample measured in the basal chamber gives information on quantity transported across the Caco-2 monolayer.

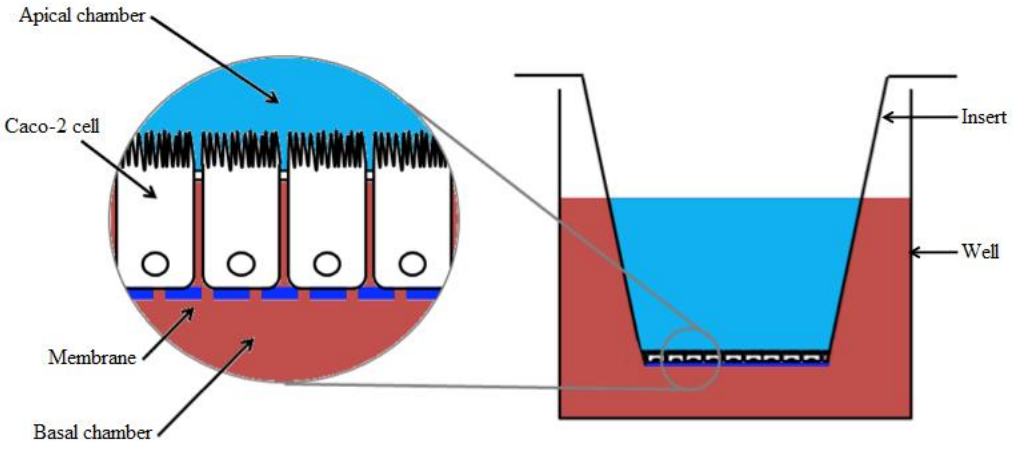

Figure 9: Schema of the monolayer experiment. Caco-2 cells are differentiated on the membrane of an insert, in a well-plate. The blue media represent the upper or apical chamber. The red media represent the lower or basal chamber.

5.5.2 Protocol

5.5.2.1 Cell culture

For transepithelial transport experiments, Caco-2 cells were seeded at a density of 3 × 10^5 cells/well into permeable Transwell filter inserts (24 mm diameter, 0.4 µm pore size; Costar, Cambridge, MA). Cell culture media was changed every two days for 21 days, upon which time the cells are fully differentiated. Serum free media was changed 24 h prior to experimentation in order to perform the experiment in media free of FA.

5.5.2.2 Transepithelial electrical resistance

Cell monolayer integrity was confirmed by measuring the transepithelial electrical resistance (TEER) at 37°C using a Millicell-ERS meter (Millipore Corporation, Bedford, MA, USA) according to the manufacturer's instructions. Inserts with a TEER value ≤ 1300 Ω.cm^2 were discarded.

5.5.2.3 Exposures of Caco-2 monolayers to test samples

Transepithelial transport studies were adapted from Hubatsch et al. (2007)[13]. Briefly, inserts were washed three times with serum-free media. Serum-free media (2.5 mL) was added to the basal side, 1.5 mL of tested compound (samples with a linoleate concentration of 50 µM) in serum-free media containing 5.3 mM fluorescein sodium salt was added to the apical side. Fluorescein acts as an indicator of paracellular transport across the Caco-2 monolayer.[14] Plates were then incubated in a humidified 37°C incubator on an orbital shaker at 60 rpm to minimise the impact of any unstirred layer. After 4 h, apical and basal media were removed for analysis. The linoleate concentration was chosen to avoid a cytotoxic effect of the FA after 4 h. Six experimental repeats were performed for each test sample.

5.5.2.4 Determination of paracellular transport in monolayer experiments

To determine paracellular transport, the concentration of fluorescein in the basal chamber was quantified by measuring fluorescence in a 50 µL sample taken from the basal chamber. Fluorescence was measured using excitation and emission wavelengths of 485 nm and 535 nm respectively, using a Synergy Biotek plate reader instrument (BioTek Instruments Inc., Winooski, VT). Fluorescein concentration was determined by interpolating fluorescence readings from a fluorescein standard curve. The concentration of fluorescein in the basal chamber was expressed as a percentage of the total fluorescein added to the apical side of the inserts. Paracellular transport was less than 0.1 % in all monolayer experiments performed.

5.5.2.5 Determination of the fatty acid uptake and transport out of the monolayer

To determine the transport of FA from the apical side to the basal side, FA were extracted and quantified by gas chromatography from both apical and basal chambers. In each individual experiment, the test sample data was corrected by subtracting the level of paracelullar transport measured and the level of FA in the control (cells with media only). The corrected linoleate data was then expressed as a percentage of the initial linoleate concentration applied to the apical side cells (50 µM).

5.6 Cyclic AMP assay

Cyclic AMP is a ubiquitous intercellular/intracellular messenger which may be involved in active FA uptake by cells.[15] A cAMP assay based on homogeneous time-resolved fluorescence, the cAMP HiRange kit (Cisbio Bioassays, Codolet, France), was performed according to manufacturer's instructions. Caco-2 cells were seeded into 96-well-half-area plates (Cruinn Diagnostics, Ireland) at a density of 1×10^5 cells/well in serum-free media. After an overnight incubation in a humidified 37°C incubator with 5 % CO_2 in air atmosphere, media was aspirated and 25 µL serum-free media/IBMX (3-Isobutyl-1-methylxanthine) was then added to all wells and the plate was pre-incubated for 30 min at 37°C, 5 % CO_2. Cells were incubated with 25 µL of linoleate (0 to 50 µM), βlg (0 to 50 µM) or a 3 linoleate/βlg complex (containing 0 to 50 µM linoleate) at room temperature for 30 min with gentle shaking. Forskolin (25 µL of 1 µM) was used as a positive control. Accumulation of the intracellular cAMP was measured using the cAMP HiRange kit following the manufacturer's instruction. Fluorescence was read using a FLUOstar Omega multi-mode microplate reader and analysed with the FLUOstar Omega software (BMG LABTECH GmbH, Ortenberg, Germany). Intracellular levels of cAMP (in the nM range) were determined by interpolating fluorescence readings from a cAMP standard curve generated in the same assay.

5.7 Gene expression by messenger RNA levels

Gene expression experiments were used to understand the impact of FA/βlg complexes on their transport and biological properties. Protein transporters used for facilitated transport included the plasma membrane-associated fatty acid-binding protein (FABPpm), the FA transport protein 4 (FATP4), the FA transporter CD36 and the intestinal fatty acid-binding protein (FABP2).[16] The peptide transporter PEPT1 was choosen to observe

the protein transport, and the cholecystokinin (CCK) was used as a biomarker as this peptide hormone secretion can be regulated by proteins and FA.

For quantifying gene mRNA levels, real time PCR (RT-PCR) was performed in a LightCycler 480 instrument (Roche Diagnostic Limited) based on the principles of absolute and/or relative quantification, depending on the target of interest

All primers were designed across intron/exon boundaries to prevent amplification from genomic DNA. Primers were designed using DNAstar Lasergene 8 software (DNAstar, Madison WI, USA) and based on the GenBank sequence as described in Table 5. Primers were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

Table 5: Primers sequences selected for gene expression.

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Species
FABPpm	CCGGAACAGTGGAAGGAAATAGC	TTGAGGGGAGGGTTGGAATACAT	Human
FATP4	CAGGGCGCCAACAACAAGAAGATT	GCAAAGCGCTCCAGGTCACAGT	Human
CCK	ATGTCTGTGCGTGGTGAT	AAATCCATCCAGCCCATGTA	Murine
E2D2	CATAAAGAGTAGCTGACCGAACCT	GCTGGCCTGGCTTACATTTAG	Murine
PEPT1	CACTGGCCTTTGGGGTTCC	AGCCGCTCATCGTATTTCTCTTTA	Human
FABP2	TGAAGCTGACAATTACACAAG	GTCTGGACTAGTTCATCACCT	Human
FABP2	ATGACAAGTTCATGGAAAAA	CATATACATAAGTCTGGA	Human
CD36	ACAGGCACAGAAGTTTACAGACAG	TGCCACAGCCAGATTGAGA	Human
CD36	AGGACGCTGAGGACAACACA	GTACGGAACCAAACTCAAAAATG	Human
Nested CD36	CTGGCTGTGTTTGGAGGTATTC	TCCATCTGCAGTATTGTTGTAAGG	Human

5.7.1 Messenger RNA extraction and complementary DNA synthesis

Cells were lysed using QIAzol Lysis Reagent (QIAGEN Ltd., West Sussex, UK) and RNA was isolated from cell suspensions using the QIAGEN miRNeasy Mini kit (QIAGEN Ltd.) according to the manufacturers' instructions. RNA was quantified spectrophotometrically using the Nanodrop 1000 (Thermo Fisher Scientific, USA) and the integrity assessed by electrophoresis in a 1.5 % glyoxyl gel with 1 X glyoxyl buffer (Ambion, Applied Biosystems, Foster City, USA). First strand cDNA synthesis was prepared from 1 μg of RNA using the QIAGEN QuantiTect reverse transcription kit (QIAGEN Ltd.) or the Bioline cDNA synthesis kit (Bioline, London, UK).

5.7.2 Absolute quantification real time-PCR

Levels of mRNA of *FABPpm* and *FATP4* transcripts in Caco-2 cells exposed to test samples were measured by absolute quantifcation. Complementary DNA standards of the

amplified gene were prepared. Plasmid standards were created by cloning an amplified PCR product into the pCR4-TOPO vector using the TOPO-TA cloning system (Invitrogen, Life Technologies, Carlsbad CA) according to the manufacturers' instructions. The cloned amplicon was (a) identified by PCR amplification, using gene specific primer pairs, and/or digestion of plasmid DNA using the *Eco*RI restriction enzyme and (b) confirmed by sequencing (Beckman Coulter Genomics, Essex, UK). For RT-PCR standards, plasmid DNA was linearized and quantified using the Nanodrop 1000. Standard curve preparation involved creating a series of dilutions from 10^9 to 10^2 copies/µL.

Caco-2 cells in serum-free media were seeded in 6-well plates at a density of 5.7×10^5 cells/well overnight in a humidified 37°C incubator with 5 % CO_2 in air atmosphere. Cells were then exposed to linoleate or linoleate/βlg complexes at different concentrations for 4 h in serum-free media with appropriate βlg controls. After removal of the supernatant, cells were washed with PBS and RNA was extracted as described below. Experiments were performed in triplicate.

5.7.3 Relative real time-PCR

Levels of *cholecystokinin* (*CCK*) mRNA in STC-1 cells exposed to test samples was measured by the protocol adapted from Hand et al. (2010)[6]. The target (*CCK*) to reference (*E2D2*) ratio expression was calculated for each test sample and normalised compared to untreated (media alone) controls.

Briefly, STC-1 cells were seeded into 12-well plates at a cell density of 2×10^6 cells/well, in complete media and incubated overnight. On the day of the experiment, the growth media was removed and the cells were washed three times with serum-free media and then incubated for 30 min with serum-free media at 37°C. The media was then removed and 400 µL of serum-free media containing the tested compound was added at different concentrations. After removal of the supernatant, cells were washed with PBS and RNA was extracted as described below. Experiments were performed in triplicate.

5.7.4 Messenger RNA quantification using the Lightcycler

Messenger RNA quantification was measured using cDNA and the LightCycler 480 (Roche Diagnostic Limited). For each 10 µl Lightcycler reaction, 1 µL of test cDNA or serially-diluted standard was used. The LightCycler 480 SYBR Green I Master kit (Roche Diagnostics Limited) was used for quantification according to the manufacturer's instructions using 0.5 µM of both the forward and reverse primers, as previously described in Table 5. All cDNA samples were tested in duplicates (technical repeat). Data were analysed using the LightCycler 480 Software (Roche Diagnostic Limited).

5.8 Determination of cholecystokinin secretion using ELISA

5.8.1 Principle

Enzyme-linked Immunosorbent Assay (ELISA) is a method used to determine antigen or antibody concentrations using antibodies or antigens coupled to an easily-assayed enzyme. A general ELISA uses a plate coated with antigen, primary antibodies then secondary antibodies are added to the wells. This secondary antibody is generally conjugated to an enzyme which catalyses the conversion of a substrate to produce a coloured product, thus indicating a positive and quantifiable results.

5.8.2 Protocol

CCK ELISA assay was adapted from Hand et al. (2010)[6]. STC-1 cells were seeded into 12-well plates at a cell density of 2×10^6 cells/well, in complete media and incubated overnight. On the day of the experiment, media was removed and cells were washed three times with serum-free media and then incubated for 30 min with serum-free media at 37°C. Media was removed and 400 µL of serum-free media containing 5 µM of free linoleate or linoleate/βlg complexes (molar ratio 1 to 3, undigested or digested complexes). After 4 hours incubation, 360 µL of the supernatant was removed, 40 µL of 10 % bovine serum albumin (prepared in PBS) was added and the solution centrifuged at 900 g for 5 min at 4°C to remove any cellular debris. The supernatant was collected and stored at -80°C prior to analysis by ELISA.

A CCK (26-33, non-sulphated) fluorescent ELISA immunoassay kit (Phoenix Pharmaceutical Inc., Burlingame, CA, USA) was used according to manufacturer's instructions. CCK content was determined using 25 µL of supernatant. Fluorescence (excitation 337 nm, emission 460 nm) was read using a FLUOstar Omega multi-mode microplate reader and analysed with the FLUOstar Omega software (BMG LABTECH GmbH, Ortenberg, Germany). The result as determined from standard curve, were expressed as a percentage of the control value (cells with media only). As STC-1 cells do not produce detectable levels of gastrin, the detected signal was attributed to CCK.[17] All CCK experiments were performed in triplicate.

6 METHODS FOR STUDYING PROTEIN/LIGAND INTERACTIONS

6.1 Isothermal titration calorimetry

6.1.1 Principle

Isothermal titration calorimetry (ITC) is a method which directly measures heat exchanges during the titration of a liquid solution into another in a range of temperature varying from 2 to 80°C. ITC allows to study molecular interactions that have an enthalpy contribution and having association constant between 10^3 to 10^9 M^{-1}. The ITC instrument is composed of an adiabatic envelope containing a reference cell and a sample cell, and a stirring syringe able to titrate a solution into the sample cell (Figure 10). Usually, the protein solution is in the sample cell, the ligand solution is in the syringe and the reference cell contains the buffer used during the experiment. The syringe mixes the cell during the experiment and injects required volumes of ligand solution in the protein solution at regular time intervals. Enthalpies of interactions are registered (peaks represented on the Figure 10) and are analysed for determination of the thermodynamic parameters of the interactions. The peaks of the heat exchange are integrated as a function of time. The energy exchanged per mole of injected ligand is then plotted as a function of the molar ratio of ligand/protein. The fitting of the binding isotherm gives the constant association (K_a) and the stoichiometry (n).

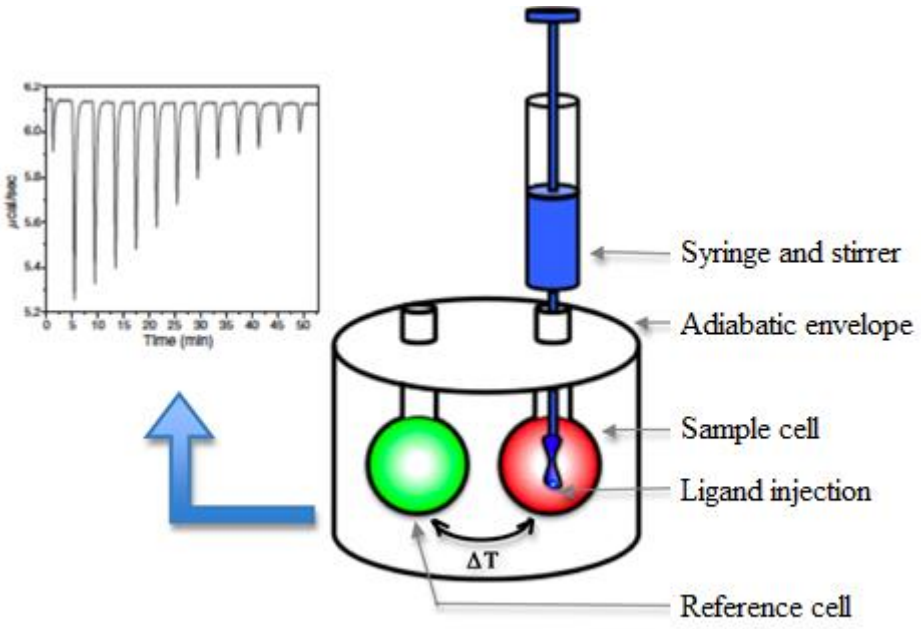

Figure 10: Schematic representation of ITC. During titration, difference of temperature is measured between the two cells, heat exchange is indicated in function of time.

6.1.2 Protocols

ITC was used to determine the interaction parameters between βlg and two FA, linoleate and CLA. ITC experiments were performed on a VP-ITC microcalorimeter (Microcal, Northampton MA). For linoleate/βlg interaction studies, solutions of βlg and linoleate in PBS were degassed under vacuum before the titration. Measurements were performed at a fixed temperature (25 or 60°C). For CLA experiments, solutions of βlg (97.4 µM) and CLA (964 µM) were dissolved in PBS containing 1 % ethanol (v/v) and were degassed under vacuum before titration. The reference cell was filled with PBS, and the sample cell (1.425 mL) with βlg solution. βlg was titrated with 29 successive 10 µL injections of FA. The injection time was 20 s, and the time between injections was 600 s to allow thermodynamic equilibrium. During titrations, the solution in the sample cell was stirred at 310 rpm to ensure complete mixing of the solution. The control measurement was obtained by titrating the FA into the buffer using the same injection procedure and was subtracted from the βlg titration with linoleate signal. To insure the amount of ligand injected in the cell, the first injection peak was ignored for the analysis. Data were analysed using MicroCal ORIGIN version 7.0 provided by the manufacturer with the integrated area of each peak plotted versus the linoleate/βlg molar ratio, providing binding constants. The “two sets of binding sites” model provided the best fitting for all the linoleate experiments, providing the binding parameters K_{a1}, K_{a2} and n_1, n_2; whilst K_a and n are the association constant and the stoichiometry, respectively. Each measurement was performed in triplicate and the results presented as mean ± SD.

6.2 Intrinsic fluorescence

6.2.1 Principle

Protein intrinsic fluorescence can be used to evaluate the structural state of a protein based on the amino acid tryptophan, which emits fluorescence between 300 and 450 nm in response to excitation at 278 nm. Other amino acids (tyrosine and phenylalanine) also fluoresce, but their intensity is negligible compared to that of tryptophan, even if the amount of tryptophan, tyrosine and phenylalanine per βlg is 2, 4 and 4, respectively. A change in the maximum emission wavelength of a protein indicates a change of hydrophobicity in the vicinity of tryptophan residues; a change in the peak intensity indicates a change of quenching of the tryptophan fluorescence.

6.2.2 Inner filter

The inner filter is the decrease in fluorescence signal caused by an excessive absorption of the excitation and/or emission wavelengths, by the ligand or a part of the

protein, which then does not reach the detector. When molecules in the surrounding of the protein absorb at the excitation wavelength, only a small proportion of the excitation light can reach the fluorophores. Therefore, the inner filter effect can be observed when the relation between absorbed light and fluorescence of the solution is no more linear. In addition, the absorbance spectrum of molecules in the solution can overlap with the wavelength at which fluorescence is emitted, contributing to the inner filter effect.

6.2.3 Protocol

Intrinsic fluorescence spectra were recorded using an excitation wavelength of 278 nm. For each titration a fluorescence spectra was recorded from 300-450 nm in order to check deviation in the fluorescent properties of the protein. Maximum emission wavelength was fixed at 345 nm. Experiments were performed at 25°C on a SPEX 112 spectrofluorometer (Jobin-Yvon, Longjumeau, France), using a 10 × 10 mm quartz cuvette. The slits were both set to 5 nm. βlg solutions in PBS (3 mL at 10 µM) were titrated with successive injections of 3 µL with linoleate at 5 mM up to a linoleate/βlg molar ratio of 10. For CLA experiments, CLA (4 mM) was dissolved in ethanol, the final ethanol solution was inferior to 1 % (v/v). βlg solutions did not have an absorbance at 278 nm superior to 0.1 in order to respect the linear range of fluorescence quantification. Solutions were agitated by pipetting up and down several times, and 5 min equilibrium time was respected prior to each measurement. An N-acetyl-tryptophanamide (NATA) blank was prepared and analysed following the same procedure in order to subtract the inner filter effect caused by the presence of FA in the titration sample. NATA fluoresces similarly to tryptophan but does not bind FA.[18] The concentration of NATA was chosen to have the same initial fluorescence (without FA) as the βlg solution. The fluorescence of NATA was subtracted from fluorescence intensity measurements of the ligand/protein complexes for all the FA/βlg molar ratios. Each measurement was performed in triplicate and the results presented as the mean ± SD.

6.2.4 Data fitting

Fluorescence data were fitted using two different methods. If L_{free}, L_{total} and L_{bound} represent the concentration of ligand free, total, and bound to βlg, respectively, n the molar ratio of ligand bound to protein at saturation, v is the fraction of ligands bound per mole of protein (v varies from 0 to n), and f_i the fraction of one site of the protein to be occupied by a ligand (f_i varies from 0 to 1), then:

$$L_{total} = L_{free} + L_{bound} \quad (1)$$

$$\nu = \frac{L_{bound}}{P_{total}} = nf_i \quad (2)$$

Combining equations (1) and (2) we deduce that:

$$L_{total} = L_{free} + nP_{total}f_i \quad (3)$$

The value of f_i is calculated as described in equation (4) using F_0, F_{max} and F_i which are the initial fluorescence, the maximum fluorescence at saturation, and the fluorescence at the ratio ligand/protein i, respectively. When F_{max} was not reached experimentally, it was determinated by fitting using an exponential phase decay model on Graph-Pad Prism software (GraphPad Software Inc., La Jolla CA).

$$f_i = \frac{F_i - F_0}{F_{max} - F_0} \quad (4)$$

By plotting L_{total} as a function of $P_{total}f_i$, n can be determined by fitting the data using a sequential linear regression in Graph-Pad Prism software 3.03.

The Scatchard plot is described below, with K_a the association constant:

$$\frac{\nu}{L_{free}} = nK_a - \nu K_a \quad (5)$$

Equations (3) and (5) can be rearranged as: $P_{total}(1-f_i) = \frac{L_{total}}{n}\left(\frac{1}{f_i} - 1\right) - \frac{1}{nK_a}$

By fitting this equation using Graph-Pad Prism software, n and K_a were determined.

7 BIOCHEMICAL AND PHYSICOCHEMICAL ANALYSIS

7.1 Gas chromatography

The FA content of the complexes was determined by gas chromatography (GC) following a protocol adapted from Palmquist and Jenkins (2003)[19]. Briefly, the internal standard tridecanoic acid (C13:0) was added to ~4 mg of complexes. FA were converted to fatty acid methyl esters (FAME) using an acid catalysed methylation by the addition of 1.5 mL of 10 % (v/v) methanolic HCl and 1 mL hexane. The samples were sealed under nitrogen, vortexed and heated to 90 °C for 2 h. After cooling on ice, 1 mL hexane and 3 mL 10 % (w/v) K_2CO_3 were added and samples were vortexed. After phase separation, an aliquot of the hexane phase (upper phase) containing the FAME was removed and analysed as previously described by Coakley et al. (2003)[20], using a CP-SELECT CB column for

FAME (100 m, 0.25 mm, 0.25 μm film thickness, Varian BV, Middelburg, The Netherlands), a Varian 3400 GLC (Varian, Walnut Creek, CA) and a flame ionization detector.

FA concentration in the apical and basal chambers of Caco-2 monolayer was determined by GC. Total FA were extracted according to the method of the International Organization for Standardization (ISO) standards 14156:2001 (ISO, 2001).[21] C13:0 (0.75 mg) was employed as an internal standard. Following removal of the diethyl ether (Fischer Scientific, Pittsburgh, PA) by heating at 45°C under nitrogen, extracted FA were converted to FAME by base catalysed methylation with 2 mL 0.5 N sodium methoxide in methanol at ambient temperature for 10 min. This was followed by acid catalysed methylation as described above. Following phase separation the upper layer (hexane), containing the FAME, was placed in a clean tube and the FAME were concentrated by heating under nitrogen as previously described. The concentrated FAME were transferred to clean glass GC vials and quantified as described above.

7.2 Gel Permeation-HPLC

Gel permeation-high pressure liquid chromatography (GP-HPLC) is a size exclusion chromatography (SEC), which separates mixtures of protein based on their size. The concentration of the different entities in βlg sample were determined by GP-HPLC using a TSK G SW guard column (7.5 × 7.5 mm, Tosoh Bioscience GmbH, Stuttgart, Germany) and a TSK G2000 SW column (7.5 × 600 mm, Tosoh Bioscience GmbH) connected to an HPLC system, consisting of a Waters 2695 Separations Module, a Waters 2487 Dual λ Absorbance Detector. Empower Pro software (Waters, Milford, MA) was used to acquire and analyse data. Sample (0.05 mg of protein injected previously dissolved in PBS) was injected and the protein entities were eluted using 30 % acetonitrile (LabScan Analytical Sciences, Dublin, Ireland) (v/v) and 0.1 % (w/v) trifluoracetic acid (TFA) in Milli-Q water (Millipore, Carrigtwohill, Ireland), at a flow rate of 0.5 mL/min. The use of acetonitrile ensured that native βlg was eluted in monomeric form. The method was calibrated using a set of protein molecular-weight standards (Sigma-Aldrich). The proportions of βlg monomers (including native and unfolded), dimers and oligomers of larger size, were deduced from GP-HPLC data by integration of the peaks area.

7.3 Fourier transform infrared spectroscopy

7.3.1 Principle

Fourier transform infrared spectroscopy (FTIR) measures the molecular absorption in the infrared spectrum, which corresponds to the frequencies of vibrations of the molecules. Each molecular chemical link has its own vibration frequencies expressed in wavenumber (v' in cm^{-1}). There are nine absorption wavenumbers characterizing amides group of proteins (amide A, B and I to VII). For conformational studies, amide bands I and II, between 1700-1600 and 1600-1500 cm^{-1}, respectively, are the most frequently used. Amide I band is sensitive to the secondary structures as it contains mainly the stretching vibrations of the C=O bond (70-85 %), and a contribution of the C-N bond (10-20 %) of the peptic link. Amide II band is mainly due to out of phase combination of the C-N-H in plane bend and C-N stretching vibrations.

7.3.2 Protocol

FTIR can acquire data on powders (transmission mode), and on liquid (reflection mode). For the reflection mode or Attenuated Total Reflection (ATR), the incident signal provides information on the secondary structure.

FTIR measurements were carried out using a Bruker Tensor 27 instrument (Bruker Optik GmBH, Ettlingen, Germany), equipped with a thermally controlled BioATR CellTM II, that was designed for the analysis of proteins in aqueous solutions. Protein samples were rehydrated in Milli-Q water at a concentration of 10 mg/mL and were filtered using 0.22 µm syringe filters before the measurement of FTIR spectra. Spectra were obtained at 20°C using an average of 180 scans at a resolution of 4 cm^{-1}. Data were processed to observe any difference in the spectra obtained compared to the control βlg (without FA), using the software OPUS 5.5 supplied with the instrument.

7.4 Polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins based on their ability to migrate in an electric field, which is function of their size and their charge.

Samples were analysed using SDS-PAGE. Mini-PROTEAN TGX precast Gels (4-20 % resolving gel, Bio-Rad Laboratories Inc., Hercules, CA) were used on a Mini Protean II system (Bio-Rad) according to the manufacturer's instructions. Samples diluted (10 µg of protein) in the denaturation solution were analyzed under reducing (in the presence of β-

mercaptoethanol in the denaturation solution) and non-reducing (in the absence of β-mercaptoethanol in the denaturation solution) conditions. Protein was visualized by staining with Coomassie blue (Bio-Safe Coomassie Stain G-250, Bio-Rad). An Amersham Low Molecular Weight Calibration kit (14.4 to 97 kg/mol, GE Healthcare UK Limited, UK) was used as molecular weight standards.

7.5 Dynamic light scattering

Dynamic light scattering (DLS) allows the measurement of the hydrodynamic radius (r_h) by measuring the time-dependent fluctuations in the scattering intensity caused by particles undergoing random Brownian motion.

To check the homogeneity of the nanoparticles preparation, the mean hydrodynamic diameter of the particles was measured by a dynamic light scattering technique using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, Worcestershire, UK) equipped with a 4 mW helium/neon laser at a wavelength output of 633 nm. Particles sizing was performed at 25°C at 10 s intervals in a particle-sizing cell using backscattering technology at a detection angle of 173°. Results were the mean of 13 runs. The intensity of light scattered from the particles was used to calculate the mean hydrodynamic diameter (z-average mean), based on the Stokes-Einstein equation, assuming the particles to be spherical.

7.6 Determination of linoleate pKa

The pKa of linoleate was determined by dissolving 1 mM linoleate in Milli-Q water and increasing the pH to 11 with 1 M NaOH solution. Linoleate was titrated with 0.1 M HCl (1.5 µmol/min) using an automatic 842 Titrando at 25°C. The pKa was determined being the inflection point of the plateau appearing during the decrease of pH from basic to acidic pH.

7.7 Determination of the critical micelle concentration of linoleate

The critical micelle concentration (CMC) of linoleate was measured by absorbance. Linoleate 10 mM was dissolved in PBS (pH 7.4) and titrated in PBS. The spectra were recorded at 400 nm on a UV-Vis Cary 1 (Varian Medical Systems Inc., Palo Alto, California) using 10 mm path length quartz cuvettes. The absorbance, which represents the turbidity of the solution, was plotted against the linoleate concentration in logarithm, each slopes intersection determined gives an indication on the FA organization into the solution.

8 MICROSCOPIC ANALYSIS

8.1 Confocal laser scanning microscope

8.1.1 Principle

Confocal imaging is a photonic microscopy method allowing focusing on the fluorescence emitted by one focal plan. Specific dyes are used depending on the aim of the study (detection of proteins, lipids, nucleus…) and the wavelengths of lasers employed. Confocal laser scanning microscope (CLSM) works by scanning a point of laser excitation using a pinhole. This eliminates out-of-focus light in samples that are thicker than the focal plan.

8.1.2 Protocol

Intracellular lipid accumulation was imaged using confocal microscopy. Caco-2 cells were seeded into 8-well chambered glass coverslips (Labtek, Nunc) at a density of 5×10^4 cells/well with serum-free media 24 h prior to experimentation. Cells were then treated with 50 µM linoleate or 50 µM linoleate in complex at a linoleate/βlg molar ratio of 3. Following 4 h exposure, cells were gently washed with PBS and stained with 4 µM Nile Red dye (prepared in PBS) to highlight intracellular lipid droplets.[22]

Cells were examined using a Nikon C1Si Laser Scanning Confocal Imaging System on inverted microscope TE2000-E (Nikon, Champigny-sur-Marne, France) equipped with a helium/neon laser emitting at 543 nm. Fluorescence emission was acquired with a 590/50 nm filter. Average fluorescence intensity was calculated from nine images.

8.2 Atomic Force Microscopy

The nanoparticles were imaged by atomic force microscopy (AFM), using an Asylum Research MFP-3DAFM (Asylum Research UK Ltd., Oxford, UK) in AC-mode. Samples were deposited on a freshly cleaved mica surface and then dried in a desiccator. An Aluminium reflex coated cantilever with a tetrahedral tip (AC 240 TS), spring constant of 2 N/m (Olympus Optical Co. Ltd, Tokyo, Japan), working frequency of 50-90 kHz, and scan rate of 0.4 Hz was used. The radius of curvature of the tetrahedral tip was 10 nm. Images were processed using AFM imaging software Igor 6.12A and Argyle Light for 3D images

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PART 3

RESULTS AND DISCUSSION

Chapter 1

β-lactoglobulin as a molecular carrier of linoleate: characterisation and effects on intestinal epithelial cells *in vitro*

1 INTRODUCTION

The major bovine whey protein, β-lactoglobulin (βlg), is able to form complexes with hydrophobic ligands such as fatty acids (FA). The aim of this chapter was to define the interaction between βlg and a FA. Linoleic acid was selected as the model FA. Linoleic acid is an essential FA found in milk. To date, the interaction between βlg and linoleic acid has been poorly studied. Different stoichiometries and association constants for native βlg and FA interactions have been previously reported. These discrepancies appear to be occurring because of the different experimental methodologies employed. In addition, how this interaction results in modifications to the ligand biological properties is not well established. This could come from a lack of characterisation of the ligand/protein complex before biological trials. In order to avoid the same lacks, we decided to combine a peer investigation of the interaction between βlg and linoleate with the study impact of the interaction on the biological properties of the linoleate.

Therefore the objectives of this chapter are:

- To characterize the binding properties of linoleate/native βlg complex.
- To define the protein structural changes due to the ligand binding.
- To determine the changes of FA bioaccessibility.

The water soluble form of linoleic acid, linoleate, was used to ensure direct contact between native βlg and linoleate allowing complex formation, whilst avoiding the use of ethanolic solutions to solubilise the FA. Isothermal titration calorimetry was used to determine the binding constants (stoichiometry and association constant) of a linoleate/βlg interaction. To evaluate the complexes properties only, samples with known linoleate/βlg molar ratio were dialysed to remove free FA. Molar ratios of bound linoleate/βlg were determined by gas chromatography and GP-HPLC. Protein structural changes resulting from the complexes formation were followed by FTIR, GP-HPLC and SDS-PAGE. The bioaccessibility of linoleate bound to βlg compare to free was observed by following the transport of FA into the cells. This was achieved by quantification of the messenger RNA levels of membrane transporters proteins (*FATP4* and *FABPpm*) and measuring the levels of cyclic AMP, an indicator of intercellular energy transfer. In addition, since linoleate must enter the cell to be cytotoxic, cytotoxicity of linoleate (free and bound to βlg) was measured.

Main results:

- Linoleate bound to βlg in two sets of binding sites.
- The binding of linoleate to βlg increased the protein oligomerisation without detectable modification of protein secondary structure.
- Linoleate cytotoxicity was delayed by its binding to βlg.
- Linoleate did not modify *FATP4* and *FABPpm* gene expressions nor cAMP level.

2 PAPER 1: β-LACTOGLOBULIN AS A MOLECULAR CARRIER OF LINOLEATE: CHARACTERISATION AND EFFECTS ON INTESTINAL EPITHELIAL CELLS *IN VITRO*

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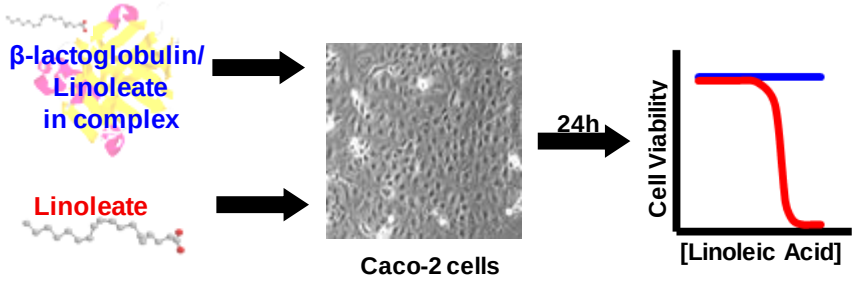

Table of content: βlg as a molecular carrier of linoleate: characterisation and effects on intestinal epithelial cells *in vitro*.

2.1 Abstract

The dairy protein β-lactoglobulin (βlg) is known to bind hydrophobic ligands such as fatty acids. In the present work, we investigated the biological activity *in vitro* of linoleate once complexed to bovine βlg. Binding of linoleate (C18:2) to bovine βlg was achieved by heating at 60°C for 30 min at pH 7.4, resulting in a linoleate/βlg molar binding stoichiometry of 1.1, 2.1 and 3.4. Two types of binding sites were determined by ITC titrations. Binding of linoleate induced the formation of covalent dimers and trimers of βlg. The LD_{50} on Caco-2 cells after 24 hours was 58 µM linoleate. However cell viability was unaffected when 200 µM linoleate was presented to the Caco-2 cells as part of the βlg complex. The Caco-2 cells did not increase mRNA transcript levels of long chain fatty acid transport genes, *FATP4* and

FABPpm, or increase levels of the cAMP signal, in response to the presence of 50 µM linoleate alone or as part of the βlg complex. Therefore, it is proposed that βlg can act as a molecular carrier and alter the bioaccessibility of linoleate/linoleic acid.

Key words: β-lactoglobulin; Sodium Linoleate/Linoleic Acid; Stoichiometry; Caco-2; Cytotoxicity

2.2 Introduction

Linoleic acid (LA, *cis,cis*-9,12-octadecadienoic acid, n-6, 18:2) is an essential long-chain fatty acid (LCFA). World Health Organization (WHO) and Food and Agriculture Organization of the United Nations (FAO) recommend an adequate intake of LA of 2 % of total energy.[1] LA is a precursor to long chain metabolites such as γ-linolenic acid, arachidonic acid and eicosapentaenoic acid.[2,3] Some of these polyunsaturated fatty acids (FA) such as LA or γ-linolenic acid have been shown to have anti-inflammatory properties.[4] Replacement of saturated fat with LA is advised to improve serum lipoprotein profiles and reduce the risk of developing cardiovascular coronary artery disease.[5] LA is also cytotoxic at high concentrations to cancerous cells *in vitro*.[6,7] LCFA are taken up by intestinal epithelial cells by both active transport via specific FA transporters and passive diffusion.[8] However, uptake and bioavailability of FA may be altered depending on the food matrix.[9,10]

Bovine β-lactoglobulin (βlg) is the major whey protein in bovine milk but absent in human milk. It is a globular protein with a monomeric molecular weight of 18.4 kDa, consisting of 162 amino acids.[11] Despite intensive studies on biological, chemical and physical properties of this protein, its biological function still remains unknown.[11-14] Structurally, βlg belongs to the lipocalin family[15], of which most are able to bind small hydrophobic molecules, such as FA, hydrophobic vitamins or curcumin.[11-19] βlg may be involved in transport, through the gastric tract, of hydrophobic substances naturally present in bovine milk, though clear evidence for this is lacking.[13,14] All members of the lipocalin family contain a β-barrel, shaped into a flattened calyx, composed of eight antiparallel β-strands. It has been suggested that βlg binds hydrophobic ligands in its internal calyx.[20,21] The existence of binding sites in a crevice near the α-helix on the external surface of the β-barrel has also been reported.[11,22-26] However, the binding sites and stoichiometry of several ligands have been controversial.[12,21,25]

βlg and LA are derived from food sources, βlg from milk and LA from many edible oils and fats, such as safflower oil, grape seed oil or corn oil and indeed milk fat.[27-29] On a daily basis, people in the Western world consume significant quantities of both βlg and LA. Little

information is known on the interaction between the water-soluble form of LA, linoleate, and βlg. Indeed how this interaction impacts on protein structure and on LA biological properties. This study investigated the βlg-linoleate complex formation in aqueous solution, the binding properties and the effect on protein structure. To investigate the bioavailability of the FA in the formed complexes, compared to FA alone, cytotoxicity was measured on intestinal epithelial cells *in vitro*. The active transport of FA in the cells was studied by two different methods. Intracellular cyclic adenosine 3',5'-monophosphate (cAMP) levels in viable Caco-2 cells were measured in the presence of linoleate alone or within βlg/linoleate complex, as an indication of active FA transport using cAMP signal transduction. Messenger RNA transcript levels of the LCFA transporter genes, Fatty Acid Binding Protein (*FABPpm*) and Fatty Acid Transport Protein 4 (*FATP4*), in the presence of linoleate alone or within βlg/linoleate complex were also measured.

2.3 Materials and methods

2.3.1 Materials

βlg (96 % purity) was obtained from Davisco Foods International, Inc. (Eden Prairie, Minnesota) and sodium linoleate (purity ≥ 98 %) from Sigma-Aldrich (St. Louis, MO). All other chemicals and solutions were purchased from Sigma-Aldrich unless stated otherwise.

2.3.2 Isothermal titration calorimetry

Isothermal Titration Calorimetry (ITC) was used to determine the interaction parameters between βlg and linoleate. ITC experiments were performed on a VP-ITC microcalorimeter (Microcal, Northampton MA). Solutions of βlg (0.163 mM) and linoleate (9.64 mM) in phosphate buffered saline (PBS; 0.01 M phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4) were degassed under vacuum before titration experiments. Measurements were performed at 60°C. The reference cell was filled with PBS, and the sample cell (1.425 mL) was filled with βlg solution. βlg was titrated at 60°C with 29 successive 10 µL injections of linoleate. The injection time was 20 s, and the time between injections was fixed at 600 s to allow thermodynamic equilibrium. During titrations, the solution in the sample cell was stirred at 310 rpm to ensure complete mixing of the solution. The control measurement was obtained by titrating sodium linoleate into the buffer. The first injection peak was ignored for the analysis. Data were analysed using MicroCal ORIGIN version 7.0 provided by the manufacturer: the integrated area of each peak was plotted versus the linoleate/βlg molar ratio, providing binding constants.

2.3.3 Preparation of linoleate/β-lactoglobulin complexes

βlg-linoleate complex were prepared by heating a solution of βlg and sodium linoleate according to Lišková et al. (2011)[30] with the following modifications. Briefly, 0.163 mM βlg was dissolved in Phosphate Buffer Saline (pH 7.4) and sodium linoleate was added to reach final linoleate/βlg molar ratios of 5, 7.5 and 10. Solutions were heated for 30 min at 60°C, then immediately cooled on ice. Samples were extensively dialysed against distilled water prior to freeze-drying. A control of the highest linoleate concentration (1.63 mM) was dialysed using the same conditions (Figure 11). No FA was detectable by gas chromatography in the control.

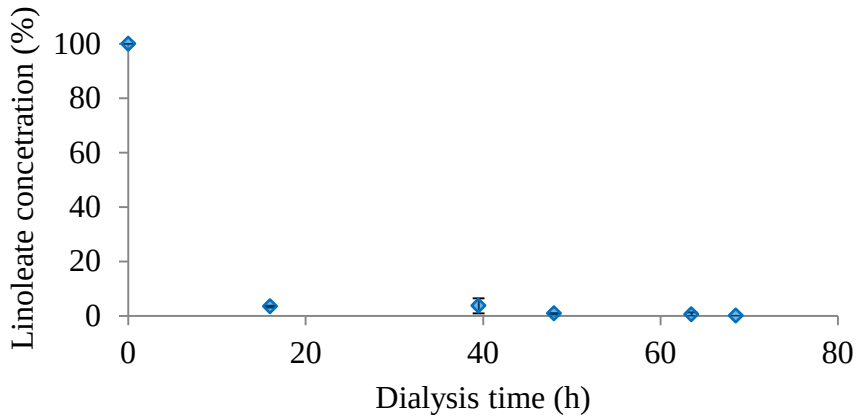

Figure 11: Dialysis of a linoleate solution at 1.63 mM versus time. Results are expressed relative to the initial concentration of linoleate. Results represent mean ± SD (n=2).

2.3.4 Determination of the fatty acid content by gas chromatography

The FA content of the complexes was determined by gas chromatography (GC) following a protocol adapted from Palmquist and Jenkins (2003)[31]. Briefly, the internal standard tridecanoic acid (C13:0) was added to ~4 mg of complexes. FA were converted to fatty acid methyl esters (FAME) by the addition of 1.5 mL 10 % methanolic HCl and 1 mL hexane. The samples were vortexed and heated to 90°C for 2 h. After cooling on ice, 1 mL hexane and 3 mL 10 % K_2CO_3 were added and samples were vortexed. After phase separation, the heptane phase (upper phase) containing the FAME were analysed as previously described by Coakley et al. (2003)[32], using a CP-SELECT CB column for FAME (100 m, 0.25 mm, 0,25 µm film thickness, Varian BV, Middelburg, the Netherlands), a Varian 3400 GLC (Varian, Walnut Creek, CA) and a flame ionization detector.

2.3.5 Gel permeation-HPLC

The concentration of monomers and aggregates were determined by gel permeation-HPLC (GP-HPLC) using a TSK G SW guard column (7.5 × 7.5 mm, Tosoh Bioscience GmbH, Stuttgart, Germany) and a TSK G2000 SW column (7.5 × 600 mm, Tosoh Bioscience GmbH) connected to an HPLC system, consisting of a Waters 2695 Separations Module, a Waters 2487 Dual λ Absorbance Detector and an Empower Pro software (Waters, Milford, MA) to acquire and analyse data. 0.05 mg of protein was injected using a solution of 30 % acetonitrile (LabScan Analytical Sciences, Dublin, Ireland) (v/v) and 0.1 % (w/v) trifluoracetic acid in Milli-Q water (Millipore, Carrigtwohill, Ireland) as an eluent, at a flow rate of 0.5 mL/min. The use of acetonitrile ensured that native βlg was eluted in monomeric form. The method was calibrated using a set of protein molecular-weight standards (Sigma-Aldrich).

The proportions of monomers (including native and unfolded) of βlg were deduced from GP-HPLC data by integration of the peaks area. The proportion of βlg oligomers in samples was calculated by subtraction of the concentration of monomer from the initial protein concentration, determined by GP-HPLC.

2.3.6 Polyacrylamide gel electrophoresis

Samples were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in order to determine the nature of the oligomers interaction. Mini-PROTEAN TGX precast Gels (4-20 % resolving gel, Bio-Rad Laboratories Inc., Hercules, CA) were used on a Mini Protean II system (Bio-Rad) according to the manufacturer's instructions. Samples were prepared under reducing (with β-mercaptoethanol) and non-reducing conditions. Protein was visualized by staining with Coomassie blue (Bio-Safe Coomassie Stain G-250, Bio-Rad). An Amersham Low Molecular Weight Calibration kit (14.4 to 97 kg/mol, GE Healthcare UK Limited, UK) was used as molecular weight standards.

2.3.7 Cell culture

The Caco-2 cell line was purchased from the European Collection of Cell Cultures (collection reference: ECACC 86010202) and was derived from human colonic adenocarcinoma cells. When fully differentiated, Caco-2 cells can mimic the enterocytes of the intestine.

Cells cultures were maintained in a humidified 37°C incubator with a 5 % CO_2 in air atmosphere. Cells were routinely grown in 75 cm^2 plastic flasks in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/L glucose and 0.584 g/L L-glutamine. Media for subculture was supplemented with 10 % (v/v) foetal bovine serum (FBS), 100 U/mL penicillin and 100

mg/mL streptomycin. At 80 % confluency, cells were trypsinated with 0.25 % trypsin/EDTA, diluted 1:6 in media and reseeded. Media was changed three times a week. All cells used in these studies were between passage number 25 and 40.

2.3.8 Cytotoxicity assay

Cytotoxicity of test samples on Caco-2 cell proliferation was determined by MTS assay, using CellTiter 96 Aqueous One Solution Cell Proliferation Assay according to the manufacturer's instructions (Promega Corporation, Madison, Wisconsin). Briefly, Caco-2 cells were seeded in 96-well plates, at a cell density of 2×10^4 cells/well, using serum-free media (DMEM only supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin). After 24 h, cells were treated with different concentrations of linoleate (0 to 150 μM), βlg (0 to 150 μM) or linoleate/βlg complexes (complex with a molar ratio of 1, 2 and 3, which contained 0 to 150 μM of linoleate) in serum-free media for 24 h. The One Solution Cell Proliferation reagent (20 μL) was then added to each well for a further 3 h. Viability was defined as the ratio of absorbance of treated cells to untreated cells (cells exposed to serum-free Media only) at 490 nm. Each cell exposure was repeated by six and intra-plate variation was accounted for by repeating the exposures on 3 different days (n=18). The Lethal Dose 50 (LD_{50}) values were determined using Graph-Pad Prism software 3.03 (GraphPad Software Inc., La Jolla CA). The sigmoidal dose-response with variable slope was used to fit the measured curves and calculate LD_{50}.

2.3.9 Real-time cell analyzer

Cell growth was monitored in real time using the Real Time Cell Analyzer (RTCA) SP Instrument, the xCELLigence system (Roche Diagnostic Limited, West Sussex, UK). The RTCA system measures the impedance of the bottom of the well which is a function of cell number and cell morphology. As the cell numbers increase the impedance increases. Correspondingly if the cell morphology changes (cells swell or shrink), the impedance will also be affected. The RTCA software generates a cell index value based on the level of impedance. Caco-2 cells in serum-free media were seeded in 16 E-Plates (Roche Diagnostic Limited) at a cell density of 1×10^4 cells/well. After 24 h in a humidified 37°C incubator with a 5 % CO_2 in air atmosphere, cells were treated for 48 h with different concentration of linoleate (0 to 100 μM), βlg (0 to 100 μM) or linoleate/βlg complexes (molar ratio of 3 linoleate/βlg containing 0 to 100 μM linoleate) in serum-free media. Data were analysed using the RTCA software 1.2 (Roche Diagnostic): Cell Index was plotted versus time and Effective Concentration 50 (EC_{50}) was determined by regression analysis of the cell index data versus the concentration of the compound after 48 h. Using the software, the time

dependent EC_{50} curves were determined by calculating the EC_{50} values at 20 time points within 48 h and these EC_{50} values were plotted versus time.

2.3.10 Cyclic AMP Assay

A cAMP assay based on homogeneous time-resolved fluorescence, the cAMP HiRange kit (Cisbio Bioassays, Codolet, France), was performed according to manufacturer's instructions. Caco-2 cells were seeded into 96-well-half-area plates (Cruinn Diagnostics, Ireland) at a density of 1×10^5 cells/well in serum-free media. After an overnight incubation in a humidified 37°C incubator with 5 % CO_2 in air atmosphere, media was aspirated and 25 µL serum-free media/IBMX (3-Isobutyl-1-methylxanthine) was then added to all wells and the plate was pre-incubated for 30 min at 37°C, 5 % CO_2. Cells were incubated with 25 µL of linoleate (0 to 50 µM), βlg (0 to 50 µM) or linoleate-βlg complexes (molar ratio of 3 linoleate/βlg containing 0 to 50 µM linoleate) at room temperature for 30 min with gentle shaking. Forskolin (25 µL of 1 µM) was used as a positive control. Accumulation of the intracellular cAMP was measured using the cAMP HiRange kit following the manufacturer's instruction. Fluorescence was read using a FLUOstar Omega multi-mode microplate reader and analysed with the FLUOstar Omega software (BMG LABTECH GmbH, Ortenberg, Germany). Intracellular levels of cAMP (in the nM range) were determined by interpolating fluorescence readings from a cAMP standard curve generated in the same assay.

2.3.11 Messenger RNA levels of FABPpm and FATP4

For quantifying *FABPpm* and *FATP4* mRNA levels, real time-PCR (RT-PCR) was performed in a LightCycler 480 instrument (Roche Diagnostic Limited) based on the principles of absolute quantification.

Caco-2 cells in serum-free media were seeded in 6-well plates at a density of 5.7×10^5 cells/well overnight in a humidified 37°C incubator with 5 % CO_2 in air atmosphere. Cells were then exposed to linoleate (0 to 50 µM) or linoleate-βlg complexes (molar ratio of 3 linoleate/βlg containing 0 to 50 µM linoleate) for 4 h in serum-free media. A control was done with βlg (0 to 50 µM). Supernatant was removed and total RNA was extracted from the cells using the QIAGEN miRNeasy Mini kit (QIAGEN Limited, West Sussex, UK). Quality and quantity of total RNA was measured by glyoxyl gel electrophoresis and spectrophotometrically using the NanoDrop 1000 (Thermo Fisher Scientific, Wilmington NC). First strand cDNA was generated from 1 µg total RNA using the Bioline cDNA synthesis kit (Bioline, London, UK).

All primers were designed across intron/exon boundaries and synthesised by Eurofins MWG Operon (Ebersberg, Germany). Primers for human *FABPpm* were designed

using DNAstar Lasergene 8 software (DNAstar, Madison WI, USA) and based on the GenBank sequence (accession number NM002080). *FABPpm* forward primer sequence was 5′-CCGGAACAGTGGAAGGAAATAGC-3′ and the reverse primer sequence was 5′-TTGAGGGGAGGGTTGGAATACAT-3′. The annealing temperature for amplification was 57°C. The forward primer sequence used for human *FATP4* was 5′-CAGGGCGCCAACAACAAGAAGATT-3′ and the reverse primer sequence was 5′-GCAAAGCGCTCCAGGTCACAGT-3′, both designed from the accession number NM002080. The annealing temperature for amplification was 58°C.

Plasmid standards for *FABPpm* and *FATP4* were created by cloning an amplified PCR product into the pCR4-TOPO vector using the TOPO-TA cloning system (Invitrogen, Life Technologies, Carlsbad CA) according to the manufacturers' instructions. The cloned amplicon was (a) identified by PCR amplification, using gene specific primer pairs, and/or digestion of plasmid DNA using the *Eco*RI restriction enzyme and (b) confirmed by sequencing (Beckman Coulter Genomics, Essex, UK). For RT-PCR standards, plasmid DNA was linearized and quantified using the Nanodrop 1000. Standard curve preparation involved creating a series of dilutions from 10^9 to 10^2 copies/µL.

For each 10 µl Lightcycler reaction, 1 µL of test cDNA or serially-diluted standard was used. The LightCycler 480 SYBR Green I Master kit (Roche Diagnostics Limited) was used for quantification according to the manufacturer's instructions using 0.5 µM of both the forward and reverse primers. All cDNA samples were tested in duplicates. Data were analysed using the LightCycler 480 Software (Roche Diagnostic Limited).

2.3.12 Statistical analysis

Results were compared using Minitab 15 statistical Software (Minitab Limited, Coventry, UK) and the ANOVA system with a Fisher's least significant difference comparison. Experiments were performed at least in triplicate.

2.4 Results

Linoleate/βlg complexes were produced at 60°C. The heating condition was below the denaturation temperature of βlg (~70°C at neutral pH), in a temperature range where βlg is in the R-state.[33,34] βlg R-state is characterized by small changes in βlg tertiary structure, a slight expansion in its volume and an increase in accessible surface area compared to native βlg.[35] The salt of LA was used because it is fully soluble in water up to its critical micelle concentration (CMC: 2 mM[36]). Using the water-soluble form of LA permits direct contact

between βlg and linoleate allowing complex formation whilst avoiding the use of ethanolic solutions to solubilise the FA.[37]

2.4.1 Interaction between linoleate and β-lactoglobulin

The thermodynamic parameters of linoleate binding to βlg were investigated by ITC. The changes in the enthalpy during the binding of βlg titrated with a solution of sodium linoleate was investigated at pH 7.4 and at 60°C. The heat exchange from the interaction of βlg with linoleate is shown in Figure 12. Each peak represents the heat exchange within the system after an injection and indicates that linoleate-binding to βlg is an exothermic process. Figure 12B depicts peak integration corrected by control (titration of sodium linoleate in buffer). The energy released during the titration decreased as the molar ratio increased. The curve levelled off at a linoleate/βlg ratio of ~3. The saturation point of βlg by linoleate was above a molar ratio of 13. For linoleate/βlg molar ratios of 3 to 13, the released heat did not plateau. Instead the heat decreased by 1.5 kcal/mol of linoleate. The binding titration curve was best fitted according to a “two set of binding sites model”, which yielded the thermodynamic constants for the two sites: K_{a1}= (2.70 ± 2.03) × 10^5 M^{-1}, n_1 = 0.62 ± 0.004; K_{a2} = (5.91 ± 3.85) × 10^3 M^{-1}, n_2 = 5.75 ± 0.51, where K_a is the association constant and n the stoichiometry. The return to thermodynamic equilibrium was very slow (> 1000 s). A longer equilibrium time did not affect the results (Figure 12C and 12D). The slow return to thermodynamic equilibrium could be due to the occurrence of other structural events, i.e. formation of oligomers (see below). No heat change was observed during direct injection of sodium linoleate in buffer solution (control sample).

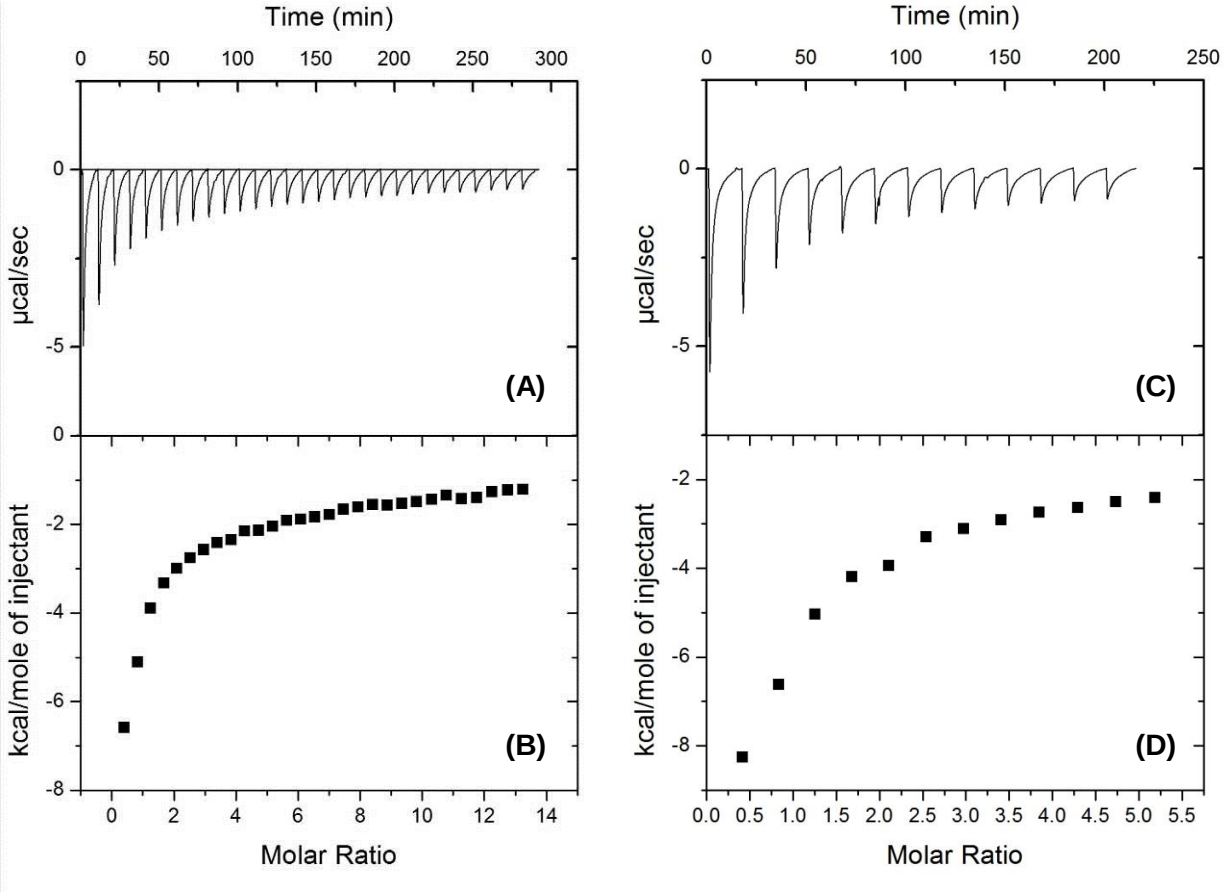

Figure 12: Microcalorimetric titration of βlg with linoleate in PBS buffer (pH 7.4) at 60°C. (A) and (C) represents the raw heat signal for the titration of βlg (0.163 mM) with 10 µL increments of 9.64 mM linoleate. (B) and (D) represent the area under each peak integrated and plotted against the linoleate/βlg molar ratio. (A) and (B) showed titration done with an intervalle of 600 s between each injection whereas (C) and (D) had an intervalle of 100 s.

2.4.2 Linoleate/β-lactoglobulin complex formation: stoichiometry and oligomerisation of the protein

βlg/linoleate complexes were prepared by heating 0.163 mM βlg at 60°C for 30 min at pH 7.4 in the presence of 5, 7.5 and 10 molar equivalent of linoleate. To remove excess of unbound FA, samples were extensively dialysed prior to freeze-drying.

Final stoichiometry of linoleate/βlg was determined from freeze-dried complexes using GC. A comparison of molar ratios of linoleate/βlg before the reaction, after dialysis and freeze-drying is shown in Figure 13A. The amount of linoleate bound to βlg increased by increasing the initial ratio of linoleate/βlg. For an initial linoleate/βlg molar ratio of 5, 7.5 and 10, the amount of linoleate bound to one protein after dialysis was 1.05 ± 0.10, 2.14 ± 0.06 and 3.35 ± 0.47 moles, respectively. Consequently, complexes with a linoleate/βlg molar ratio of 1, 2 and 3 were formed.

As shown by GP-HPLC analysis (Figure 13B and 13C), the presence of linoleate induced oligomerisation of the protein. The amount of oligomers increased significantly with the molar ratios of linoleate/βlg. In the absence of linoleate (control), the amount of oligomers in βlg samples was 12 %. In the presence of linoleate (3 linoleate/βlg), the amount of oligomers reached up to 45 % of total protein concentration. The oligomers were mainly dimers and trimers of βlg as shown in Figure 13C and confirmed by SDS-PAGE experiment (see below). However, no significant change in the protein secondary structures was associated with oligomer formation, as indicated by FTIR (see additionnal data below, Part 3, Chapter 1, 3.5-FTIR).

These results were confirmed by SDS-PAGE. Under non-reducing conditions, the SDS-PAGE analysis of native βlg and heated βlg (Figure 13C) showed a major band corresponding to the βlg monomer with small amount of dimers and trimers. In the presence of linoleate, the intensity of the bands corresponding to βlg dimers and trimers intensified. Under reducing conditions, no difference was observed between native βlg, heated βlg and linoleate/βlg complexes with a molar ratio of 1, 2 and 3 linoleate/βlg (Figure 13C). This indicated that dimers and trimers were covalently bound by disulfide links.

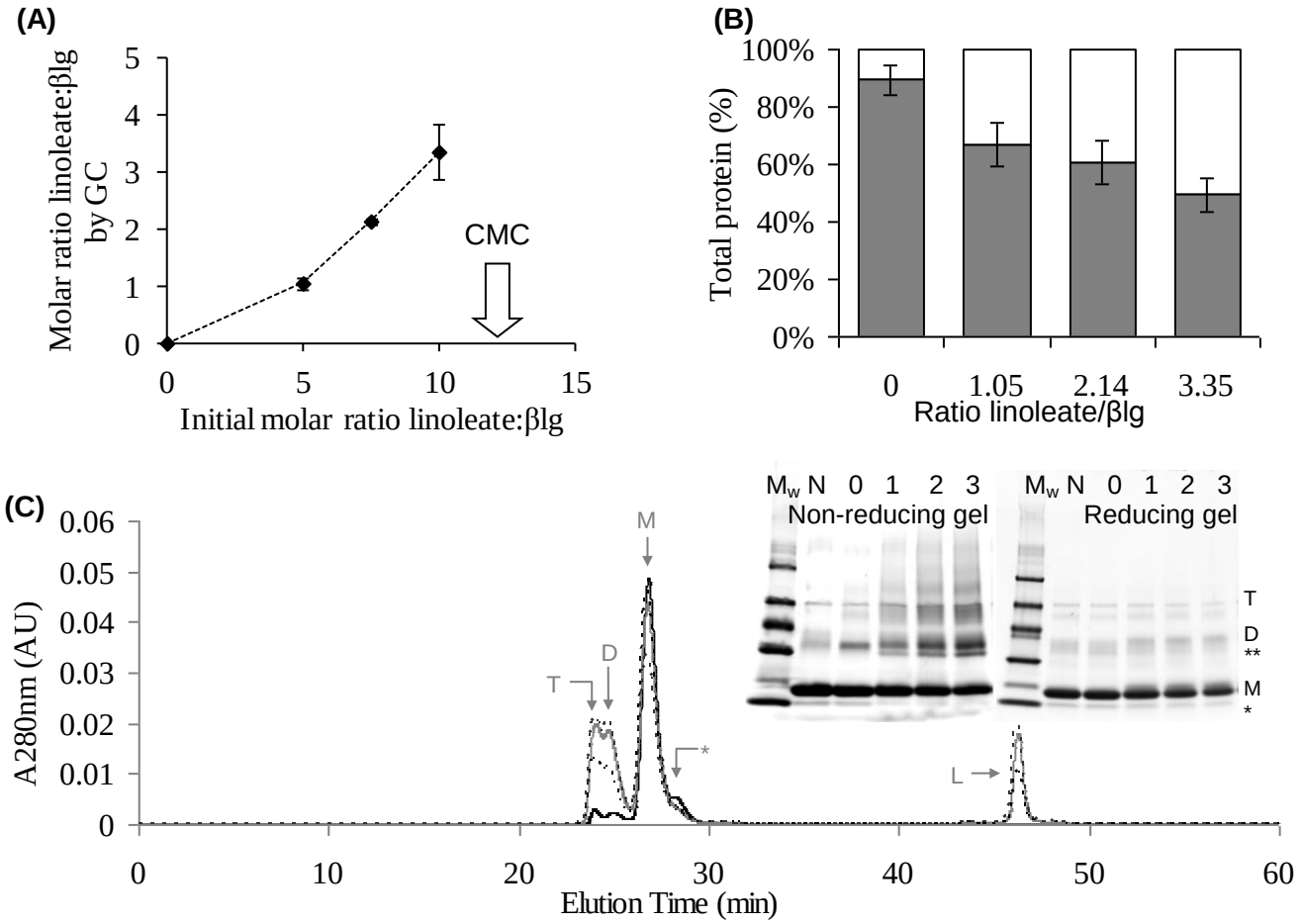

Figure 13: Linoleate/βlg complexes formations: 0.163 mM βlg in the absence or presence of linoleate were heat treated at 60°C for 30 min, extensively dialysed and freeze dryed. (A) Correlation of the molar ratios of linoleate/βlg added to the starting solutions with the molar ratios of linoleate/βlg that were detected by GC analysis in the βlg/linoleate samples after extensive dialysis and freeze-drying. The arrow represents the CMC of the sodium linoleate.[36] Complexes with a linoleate/βlg molar ratio of 1.05 ± 0.10, 2.14 ± 0.06 and 3.35 ± 0.47 moles were formed, referred as complexes with a linoleate/βlg molar ratio of 1, 2 and 3. (B) Protein composition observed by GP-HPLC (shown in C). The molar ratio of linoleate/βlg in dialysed solutions is indicated on the x-axis, the total protein content on the y-axis as follows: grey area, monomers; white area, oligomers. (C) Composition of solutions of βlg in presence or absence of linoleate observed by GP-HPLC chromatograms (30 % acetonitrile and 0.1 % TFA). Black line, βlg heated without sLA; dotted black line, complex of 1 linoleate/βlg molar ratio; Light grey line, complex of 2 linoleate/βlg molar ratio; dotted grey line, complex of 3 linoleate/βlg molar ratio. The inserts show SDS-PAGE profile of linoleate/βlg complexes under non-reducing (left gel) and reducing (right gel) conditions. M_w, molecular weight markers (14.4, 20.1, 30, 45, 66, 97 kDa); N, native βlg; 0, βlg control treated to complex formation conditions; lane 1, 2 and 3, complex with a final molar ratio of 1, 2 and 3 linoleate/βlg, respectively. T, βlg trimer; D, βlg dimer; M, βlg monomer; *, αla monomer; L, linoleate. A band with slightly lower molecular weight than βlg dimers was observed in the presence of linoleate under non reducing conditions (**). This maybe due to the formation of heterodimers of βlg and αla, as the electrophoretic band corresponding to αla monomers decreased in presence of linoleate.

2.4.3 Cytotoxicity on Caco-2 cells

To elucidate the effect of βlg/linoleate on human epithelial cell viability, the human colonic adenocarcinoma cells, Caco-2, were exposed to βlg, linoleate or complexes for 24 h (Figure 14A). LD_{50}, the concentration required to decrease the cell viability by 50 %, was then calculated. βlg was not toxic to Caco-2 cells at the concentrations tested (0 to 150 µM), as measured by MTS assay. In contrast, the LD_{50} of linoleate was 58.04 ± 4.21 µM. Linoleate/βlg complexes, where corresponding linoleate concentration varied from 0 to 150 µM, had no cytotoxic effect on Caco-2 cells after 24 h incubation.

Toxic effects of βlg, linoleate or complexes in real time over 48 h were studied using RTCA (Figure 14B). The cell index, which is function of the impedance at the bottom of the well, was measured. Cell index relates to cell viability and/or cell morphology. Results showed a decrease of cell index by 1.95 when the concentration of βlg increased from 0 to 100 µM, compared to the control cells without compound. However, the parallel MTS assay showed no change in cell viability (Figure 14A). Taken together, this allowed the authors to conclude that βlg alters cell morphology rather than cell viability. Interestingly, at the low concentrations of 5, 10 and 25 µM linoleate, an increase of cell index was observed for linoleate alone compared to control (Figure 14B-linoleate). At concentrations of 50, 75 and 100 µM, linoleate cell index decreased by 2.54 (for 100 µM linoleate) compared to control. However, the response of Caco-2 cells to βlg-linoleate complexes differed to linoleate alone at equivalent molar concentrations. A decrease in cell index was observed after 3 h of exposure to 100 µM of linoleate whereas the 3 linoleate/βlg complex (100 µM linoleate) required 12.5 h exposure to decreased the cell index (cf. arrows on Figures 14B-linoleate and 14B-cplx). βlg-linoleate complexes containing 5, 10, 25 and 50 µM linoleate increased Caco-2 cell index after 48 h. βlg-linoleate complexes containing 100 µM of linoleate in the complex decreased the cell index by 0.49 after 48 h. EC_{50}, the concentration required to obtain 50 % of the maximum effect, was calculated at different time points. After 48 h, EC_{50} was 35 µM for linoleate alone and 98 µM when linoleate was part of a 3 linoleate/βlg complex. A time dependent EC_{50} was calculated to indicate EC_{50} changes as a function of time (Figure 14C). At 13 h, the EC_{50} was reached with 74 µM linoleate alone. In contrast, it took 30 h for the EC_{50} to reach 78 µM linoleate when 3 linoleate was complexed to βlg.

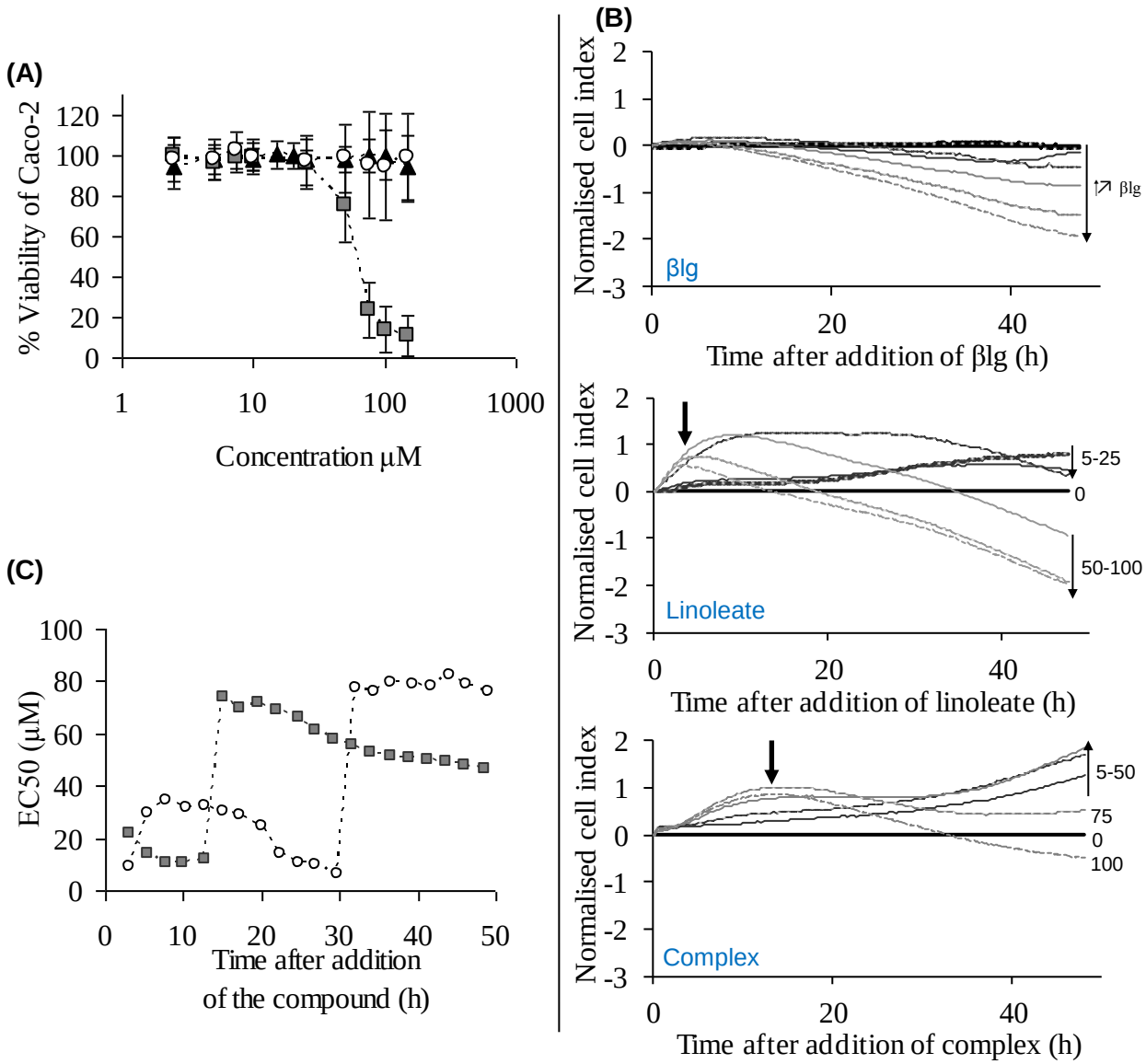

Figure 14: Cytotoxicity of βlg, linoleate and βlg-linoleate complexes to Caco-2 cells. Cytotoxicity was assessed using (A) MTS assay and (B, C) RTCA system. (A) % viability after 24 h on 2 × 10^4 Caco-2 cells compared to control cells. βlg concentration, filled triangle with dashed lines; linoleate concentration, gray box with dashed lines; and linoleate concentration in the linoleate/βlg complexes, diamond with dashed lines are given on the *x*-axis (0 to 150 µM). (B) Normalized cell index (difference between the cell index and the cell index without compound) over time in hours. 1 × 10^4 Caco-2 cells were exposed to 0 µM, thick solid line; 5 µM, wavy line; 10 µM, solid line; 25 µM, dashed line; 50 µM, thin solid line; 75 µM, dashed line; 100 µM, long dashed line of βlg (B-βlg), linoleate (B-linoleate), and linoleate in the 3 linoleate/βlg complex (B-complex). The large arrows on B-linoleate and B-complex indicate the start of the normalized cell index decrease for the highest concentration. (C) Time dependence EC_{50} is based on panel B; EC_{50} is calculated over a 48 h exposure to linoleate (gray box with dashed line) or the 3 linoleate/βlg complex (diamond with dashed line).

2.4.4 Cellular Response to β-lactoglobulin, linoleate and β-lactoglobulin/linoleate complexes

Cyclic AMP is a ubiquitous intercellular/intracellular messenger which may be involved in active FA uptake by cells.[38] Cyclic AMP levels were measured in Caco-2 cells exposed to linoleate, βlg, or complexes at non-toxic concentrations (50 µM linoleate). No changes were detected in cAMP levels by incubation with linoleate (0 to 50 µM), βlg (0 to 50 µM) or linoleate-βlg complexes (0 to 50 µM linoleate) as measured by a FRET-based time-resolved fluorescence assay (Figure 15). Messenger RNA transcript levels of the FA transporter genes, *FATP4* and *FABPpm*, in Caco-2 cells were investigated as an indication of active transport of linoleate across the cell membrane. Messenger RNA levels of *FATP4* and *FABPpm* were not significantly increased in Caco-2 cells after 4 h incubation with linoleate (0 to 50 µM) or linoleate/βlg complexes with a concentration of 0 to 50 µM linoleate (Figure 16).

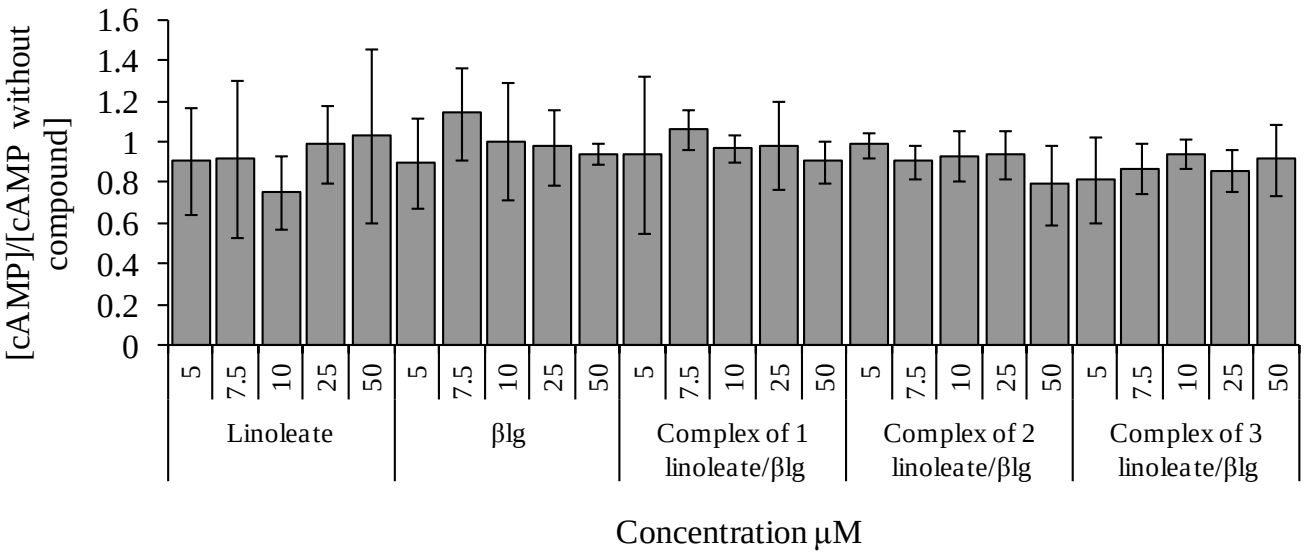

Figure 15: Relative intracellular cAMP levels in 1 × 10^5 Caco-2 cells treated with different concentrations of linoleate, βlg and linoleate/βlg complexes. cAMP without compound is defined as cAMP levels in Caco-2 cultured in medium without compound. No significant difference between samples and concentrations were found.

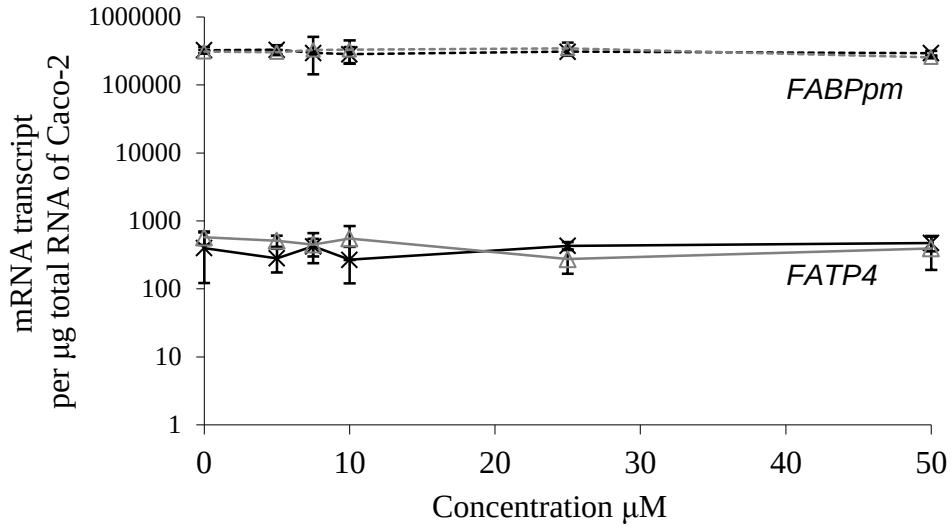

Figure 16: Levels of FABPpm and FATP4 mRNA transcripts in 5.7 × 10^5 Caco-2 cells after 4 h exposure to linoleate (cross with dashed or solid lines, 0 to 50 µM) or 3 linoleate/βlg complex (triangle with dashed or solid lines, 0 to 50µM linoleate). *FABPpm* mRNA transcripts are represented in dashed lines and *FATP4* mRNA transcripts in solid lines.

2.5 Discussion

βlg and linoleate formed complexes that protected Caco-2 cells from the cytotoxic effects of linoleate (Figure 14). Intracellular cAMP levels (Figure 15), mRNA *FATP4* and *FABPpm* levels were unaffected by the presence of linoleate either alone or in a protein complex (Figure 16). SDS-PAGE and HPLC analysis of the complexes revealed the formation of intermolecular disulfide bonds between protein molecules, which increased with higher molar ratios of linoleate/βlg (Figures 13B and 13C). These observations, combined with those from ITC (Figure 12), allowed us to suggest a binding mechanism between βlg and linoleate which modifies the cytotoxic effect of the FA.

Linoleate interacted with βlg via two different binding sites with respective affinity constants of 2.7 × 10^5 and 5.9 × 10^3 M^{-1}. These association constants are similar to those reported for other hydrophobic ligands.[39,40] Spector and Fletcher (1970)[40] reported two binding sites with association constant in the order of 10^5 M^{-1} and 10^3 M^{-1} for the binding of βlg to palmitate, oleate, stearate and laurate. Concomitantly to linoleate binding, we also observed the formation of covalent protein oligomers, i.e. dimers and trimers, that could explain the stoichiometry value (n=0.62) determined from ITC experiments. This value could result from a mixture of complexes such as $(βlg)_2$-$(linoleate)_1$ (n=0.5) and $(βlg)_3$-$(linoleate)_2$ (n=0.67). This hypothesis is consistent with the simple shape of the ITC peaks, with slow return to equilibrium attributed to the induced oligomerisation step. Recently, a

crystallographic structure of native βlg/linoleate complex, showing the FA located at the protein calyx (stoichiometry=1) was published.[41] Our results suggest that heating of βlg and linoleate mixture may lead to the formation of other types of complexes.

βlg has a weak aptitude to aggregation below the temperature of denaturation.[42] At 60°C, the presence of linoleate increased the formation of disulfide-linked dimers and trimers without formation of larger aggregates. This work confirms previous work in our laboratory[30] where binding of sodium oleate to βlg at 60°C decreased monomeric βlg and increased the formation of dimers and trimers. Protein aggregation in the presence of lipids has also been reported for other protein systems.[43] βlg oligomerisation into covalent dimers and trimers may be triggered by slight structural changes induced by linoleate binding to βlg monomers, as suggested by previous studies.[44,45]

From the presented results, we propose a hypothetic binding mechanism where the interaction of the FA with the protein and the oligomerisation of βlg take place in a single step:

- At 60°C, native βlg monomers reversibly unfold to form non-native R-state monomers.[33]
- Negatively charged linoleate molecules interact with positively charged regions at the surface of βlg monomers, as suggested from the exothermic signal of ITC experiments. Consequently, the formation of a linoleate/βlg complex would favour additional hydrophobic interactions between proteins.
- Two or three βlg molecules were then non covalently “cross-linked” by one or two linoleate molecules bound to a high affinity binding site this favours the formation of βlg oligomers by intermolecular disulfide bonds, making the unfolding irreversible after cooling.

This assumption is in agreement with the single peak structure showing a slow return to thermodynamic equilibrium of ITC results. It probably indicates that several physico-chemical phenomena can contribute simultaneously to the measured signal including: (i) binding of linoleate to βlg, (ii) conformational changes of βlg following binding of linoleate molecules, (iii) oligomerisation of the protein βlg and (iv) counterion release.

The protection provided by βlg/linoleate complexes to Caco-2 cells from linoleate differs to βlg:Conjugated Linoleic Acid (CLA) complex. Although the different CLA isomers display varying effects on biological functions,[46,47] a 2.46 *cis*9,*trans*11-CLA/βlg molar ratio complex resulted in a 30 % increase in cytotoxicity after 48 h of exposure to *cis*9,*trans*11-CLA at a concentration of 100 µM, compared to *cis*9,*trans*11-CLA alone.[17] HAMLET/BAMLET (Human/Bovine Alpha-lactalbumin Made LEthal to Tumor cells), a complex formed of oleic acid and α-lactalbumin (αla), is more cytotoxic than oleic acid on its own.[48] Oleic acid and LA by themselves exhibit cytotoxic effects on various cell lines.[48,49] However, binding these FA to proteins such as βlg, modify their cytotoxic effect compared to

the FA on its own. Indeed oleic acid/αla complex is ~40 % more cytotoxic to human larynx carcinoma cells compared to free oleic acid.[48] However, recent studies based on direct measurement of oleic acid content in the incubation mixture would argue that oleic acid alone or involved in a complex have comparable cytotoxicity effects on various cells, with the protein alone having no effect.[30,50] Frapin et al. (1993)[39] showed that the structural constraints imposed by the double bonds of FA only weakly affects the interaction of FA with βlg. The reduced cytotoxic effect observed with linoleate/βlg complexes may relate to the solubility of the FA. oleic acid has a poor solubility in aqueous solution, its CMC is between 20 and 69 µM at pH 8.3 at the temperature and salt concentrations tested by Knyazeva et al. (2008).[48] Therefore in the absence of protein, the amount of oleic acid available to the cells would be low. The binding of oleic acid to βlg or other proteins such as αla (HAMLET, BAMLET) increased the solubility of oleic acid (Joseph J. Kehoe, personal communication) and possibly its bioavailability. The solubility of FA increases with the number of C=C double bonds in the aliphatic chain.[51] Consequently, the solubility of LA (C18:2) is higher than that of oleic acid (C18:1). Under the experimental conditions used by Collin et al. (2010)[36], sodium linoleate has a CMC of 2 mM. Hence, the binding of linoleate to βlg is unlikely to alter solubility, but potentially alters linoleate uptake by altering levels of free FA.

The cytotoxicity of linoleate was concentration dependent, in agreement with that observed with LA.[6] Norman et al. (1988)[52] showed that sodium linoleate was more cytotoxic to the epithelial mouse cells, Ehrlich Ascites Tumor, than emulisified LA. This effect may be explained by the higher solubility of sodium linoleate in aqueous bioassays with greater access to the Caco-2 cells. Prior to uptake, LCFA enters a low pH microclimate at the enterocyte surface. As this local pH is below their pKa, protonation of LCFA will occur with LCFA entering in the FA form rather than the salt form.[53] To date, it is not well understood how intestinal cells metabolize LA. A previous study showed that FA cytotoxic effect was initiated by mitochondrial apoptotic pathway with cytochrome C release, indicating that uptake of LA is essential for its cytotoxic effect.[7] FA cytotoxicity may also occur by an alteration of the cellular n-6 to n-3 polyunsaturated FA ratio adversely affecting membrane permeability and fluidity.[54]

LCFA are hydrophobic and so uptake by enterocytes was thought to occur by diffusion. However, recent studies suggest the involvement of a protein-transfer mechanisms, with transport of LCFA reaching saturation at high concentrations in Caco-2 cells.[8,38,54] It is likely that an efficient LCFA uptake by cells requires both passive and facilitated transfer, possibly using a cAMP pathway.[38] However, in our study, no change in intracellullar cAMP levels was observed by viable intestinal cells exposed to different concentrations of linoleate and βlg-linoleate complexes (0 to 50 µM linoleate). Nevertheless, the requirement of cAMP in a facilitated LCFA uptake or metabolism is controversial and

appears to depend on the FA and cell type used. Bovine oocytes treated with 100 μM LA for 6 or 24 h decreased intracellular cAMP levels.[55] A perfusion of 1 mmol/L plasma of eicosapentaenoic acid (EPA; C20:5 n–3) during 150 min decreased cAMP level by 0.27 nmol/g tumor on MCF-7 human breast cancer xenografts perfused in situ in nude rats.[56] In contrast, C6 glioma cells incubated with 100 μM EPA for 48h, increased cAMP levels by ~250 %.[57] The FA transporters, FABPpm and FAPT4 have been involved in the uptake of LCFA by intestinal cells.[54,58] Messenger RNA transcript levels of *FATP4* and *FABPpm* were not increased upon exposure to linoleate which suggests that either (a) there is sufficient quantities of FATP4 and FABPpm transporter proteins to transport linoleate or (b) these transporters are not involved in linoleate transport, at the concentrations tested.[39]

This study has demonstrated that βlg can bind at least three linoleate per βlg monomer at two different sets of binding sites. According to cell proliferation assays, linoleate can inhibit the viability of Caco-2 cells, but linoleate/βlg complexes appear to protect cells from the cytotoxicity effect of linoleate. This effect could be due to the relatively high solubility of linoleate. Caco-2 exposure to linoleate or linoleate/βlg complexes did not modify intracellular cAMP levels or mRNA transcript levels of the LCFA transporter genes, *FABPpm* and *FAPT4*, suggesting another mechanism for FA to enter into the cells.

2.6 Abbreviations used

αla, α-lactalbumin; βlg, β-lactoglobulin; cAMP, cyclic adenosine 3',5'-monophosphate; CLA, conjugated linoleic acid; CMC, critical micelle concentration; DMEM, Dulbecco's modified Eagle medium; EC_{50}, effective concentration 50; FA, fatty acids; FABPpm, fatty acid binding protein; FAME, fatty acid methyl ester; FATP4, fatty acid transport protein 4; FBS, foetal bovine serum; GC, gas chromatography; GP-HPLC, gel permeation high performance liquid chromatography; HAMLET/BAMLET, human/bovine α-lactalbumin made lethal to tumor cells; ITC, isothermal titration calorimetry; K_a, association constant; LA, linoleic acid; LCFA, long chain fatty acid; LD_{50}, lethal dose 50; n, reaction stoichiometry; RTCA, real time cell analyzer; RT-PCR, real time polymerase chain reaction.

2.7 Acknowledgements

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3 ADDTITIONAL DATA

3.1 Linoleate properties

Extinction coefficient of linoleate was determined by measuring its absorption at 232 nm as a function of its concentration. Using the Beer-Lambert law, the linoleate extinction coefficient value of 6050 L/mol/cm was determined.

Linoleate (5 mM at pH 11.5) was titrated with HCl to observe linoleate conformation as a function of pH (Figure 17). We observed a similar titration curve than the one shown in studies for FA, phospholipids, and bilirubin.[59-61] Altought curves are similar, the pKa determination is different in function of the studies. Each change of slope, on Figure 17, represents a physical and/or chemical modification of the FA. We calculated the pKa of linoleate at the neutralization endpoint (Figure 17C), as Boiadjiev et al. (2004), because the pKa is equivalent to the pH when there is an equilibrium between the basic and acidic form of the molecule, creating a buffer effect. We calculated a pKa of 7.2 ± 0.1. This value is similar to that observed by Bild et al. (1977)[62] who found a pKa of 7.9. However, Kanicky et al. (2002)[59] reported a pKa of 9.24 calculated at half neutralisation endpoint for LA (Figure 17B). The half endpoint of our study is in the same order than Kanicky et al. (2002)[59]. Even if the determination of the pKa was different, the same FA conformation can be described in their study and ours.

Kanicky et al. (2003)[63] demonstrated that the pKa of LCFA was concentration dependant decreasing to a value of around 4.8 at low FA concentration, the same pKa as short chain FA. The increase in apparent pKa value is due to submicellar FA association, which can occur at concentrations lower than the CMC. Therefore both pH and FA concentration have an important role on the FA conformation.

The linoleate critical micellar concentration (CMC) of 2 mM was determined by absorbance at 400 nm (Figure 18), as obtained by Collin et al. (2010)[36]. Moreover, the existence of an aggregation of linoleate was determined from 80 µM. Indeed, it is generally assumed that by increasing the concentration, FA progress from monomeric to dimeric forms, polymers and then micelles.[64]

Therefore, at pH 7.4 and below 2 mM, linoleate is mainly soluble and only few of them are under micelle/aggregates form. These results explain the rapid removal of free linoleate by dialysis as observed in Figure 11.

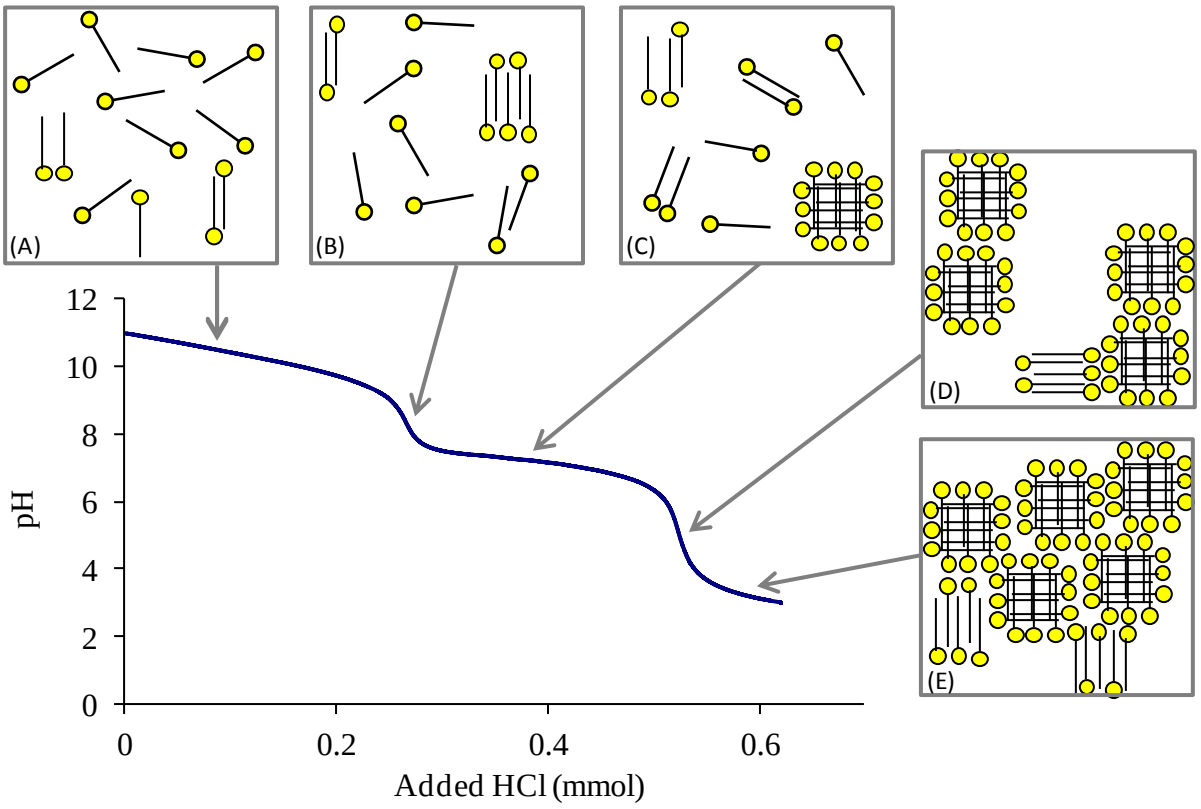

Figure 17: Titration of 1 mM linoleate with HCl at 25°C to determine the linoleate conformation. (A) Soluble FA. (B) Crystals begin to appear in solution. (C) Bigger crystals keep forming in solution. (D) and (E) FA become insoluble. Illustrations modified from Kanicky and al. (2002)[59].

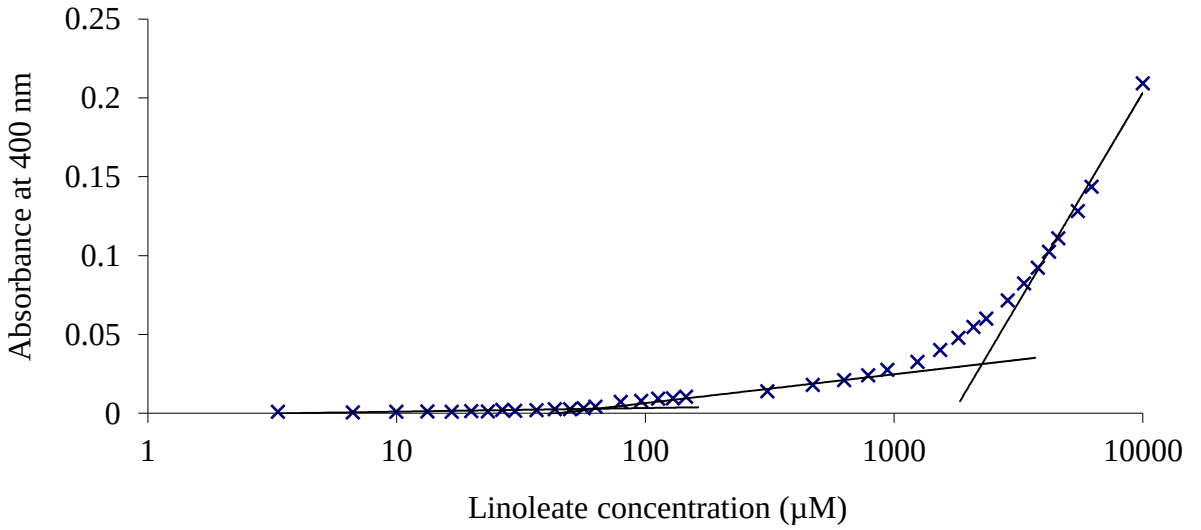

Figure 18: Experimental measure of linoleate CMC determined by absorbance at 400 nm for different concentrations.

3.2 Linoleate/β-lactoglobulin molar ratio determined by gel permeation-HPLC

The amount of linoleate bound to βlg after dialysis was determined by GP-HPLC (Figure 19). Linoleate/βlg molar ratios detected were 1.06 ± 0.11, 1.54 ± 0.18 and 2.41 ± 0.27, for initial molar ratios of 5, 7.5 and 10, respectively. Thes values were slightly lower than those determined by GC (Figure 13A). Only the sample with 10 linoleate/βlg initial molar ratio exibits a significant difference for bound linoleate detected by GP-HPLC and GC methods ($p < 0.05$). It may be due to the poor solubility of linoleate at the low pH of the eluent (30 % acetonitrile, 0.1 % TFA).

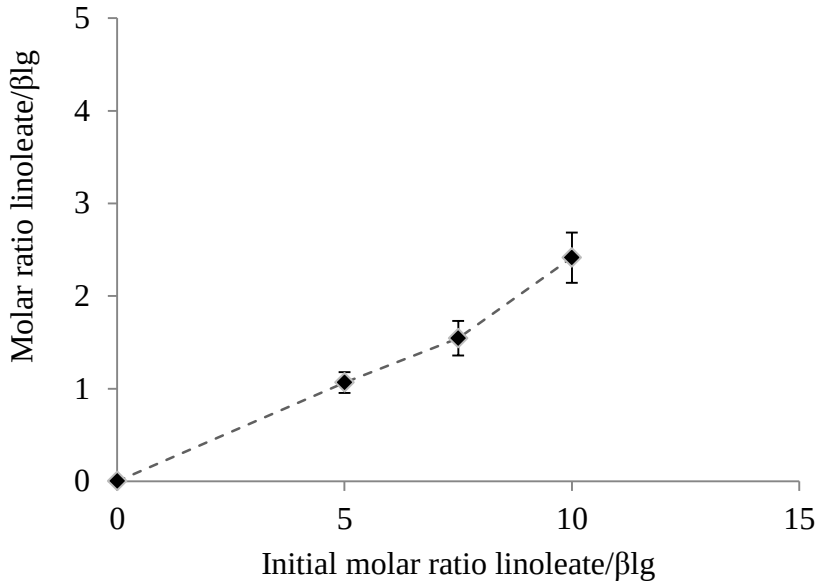

Figure 19: Linoleate/βlg complexes formations. Correlation of the molar ratios of linoleate/βlg added to the starting solutions with the molar ratios of linoleate/βlg that were detected by GP-HPLC analysis in the βlg/linoleate samples after extensive dialysis and freeze-drying. βlg (0.163 mM) in the absence or presence of linoleate was heated at 60 °C for 30 min, extensively dialysed and freeze dryed.

3.3 Cytotoxicity of linoleate at different time points

To interprete RTCA data shown in Figure 14B, cytotoxicity was measured by MTS assay. RTCA data showed an increase in the cell index after 2 h of exposure to high linoleate concentration (≥ 50 µM) compared to untreated cells. This increase could be linked to the increase in cell number or changes in conformation before cell death. To discriminate between these two cell death pathways, cytotoxicity MTS tests were performed at 2, 4, 6 and

24 h exposure to linoleate concentration of 5, 50, 100 and 200 µM (Figure 20). Decreases in viability were already observed after 2 h with increasing linoleate concentration. This shows that the increase in cell index observed by RTCA was not due to cell proliferation but due to the cell swelling before cell death. There are two main programmed cell death pathways namely, apoptosis and oncosis, both leading to necrosis (Figure 21).[65,66] Apoptosis is a non-inflammatory cell death where the cell shrinks and the chromatin condenses, whereas oncosis is an inflammatory pathway where the cell swells and becomes leaky. FA n-6 and n-3 are known to be cytotoxic by both apoptotic and oncotic pathways. In general, as n-6 FA form precursors of several pro-inflammatory molecules, they cause cell via oncosis death, whereas n-3 FA, which are less inflammatory, cause cell death via apoptosis.[7,67]

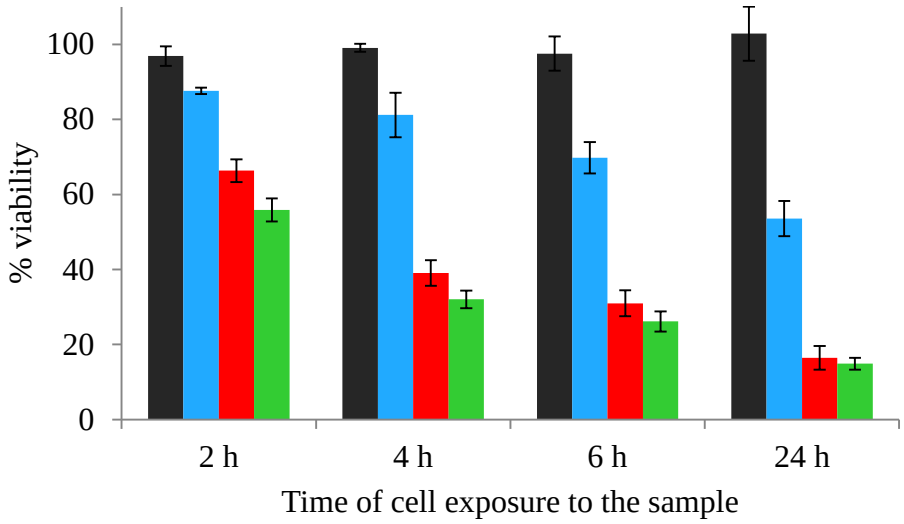

Figure 20: Cytotoxicity of varying concentrations of linoleate at different times of exposure to Caco-2 cells, determined by MTS assay. % viability after 2, 4, 6 and 24 h on 2 × 10^4 Caco-2 cells compared to control cells. Black bars, 5 µM; blue bars, 50 µM; red bars, 100 µM; green bars, 200 µM. Results represent mean ± SD (n=2).

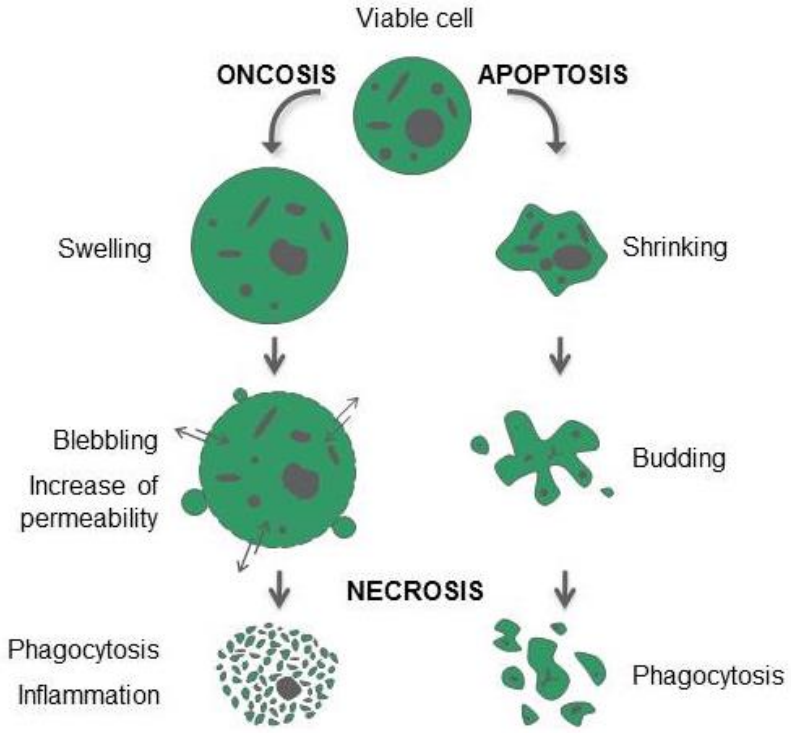

Figure 21: Schema of the two main pathways of cell death, apoptosis and oncosis, both leading to necrosis. Modified from Majno and Joris (1995)[65].

3.4 Formation of complexes, protein aggregation at the different stages

Because of the presence of oligomers after the formation of complexes, we quantified the proportion of monomers and oligomers after each step involved in the formation of 10 linoleate/βlg complex by GP-HPLC. Monomers and oligomers proportions as determined in the native βlg, a mixed but non-heated solution, a heated solution, a heated and refrigerated for 72 h solution and a heated and dialysed for 72 h solution of 10 linoleate/βlg are represented in Figure 22. Oligomerisation was already increased by just mixing the linoleate to native βlg, this was again increased by heating at 60°C for 30 min and increased further by a holding time of 72 h with or without dialysis, as both refrigerated and dialysed have similar proportions of oligomers. These results suggest that heating is not necessary for the interaction of the FA to βlg as suggested by previous studies[17,68], and confirmed by ITC measurements performed (Part 3, Chapter 3). Heating fasters the complex formation and concomitantly increase the proportion of oligomers. Finally, this highlighted the slow reaction of oligomerisation as observed by the shape of the ITC peak and the long time necessary to return to thermodynamic equilibrium during the ITC experiments (Figure 12A).

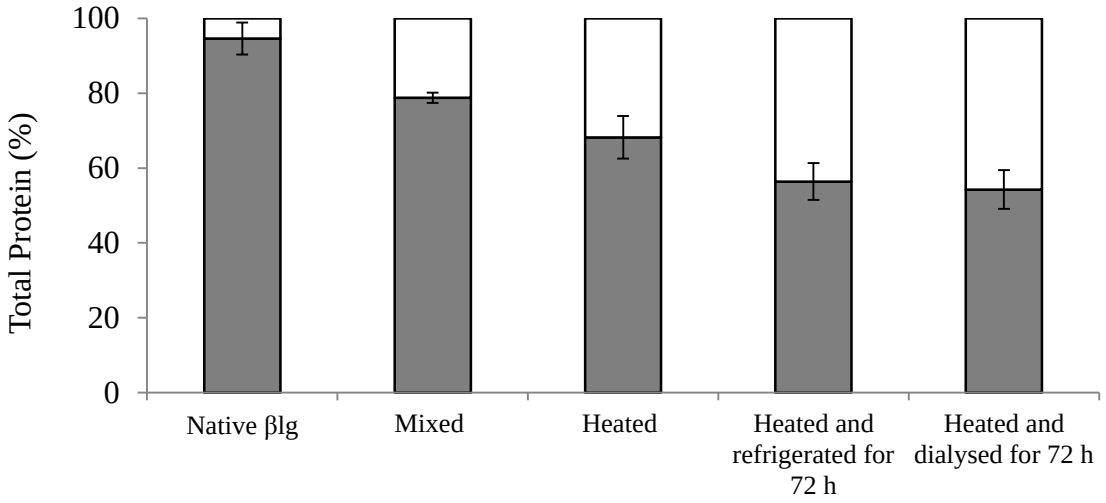

Figure 22: Protein composition of βlg during the formation of a linoleate/βlg complex observed by GP-HPLC. βlg (0.163 mM) was mixed with linoleate (molar ratio of 10 linoleate/βlg), the solution was then heat treated at 60°C for 30 min, then the solution was refrigerated or dialysed for 72 h. The solutions (native βlg and linoleate and βlg mixed, heated, heated and refrigerated for 72 h, and heated and dialysed for 72 h) are indicated on the x-axis. The total protein content is represented on the y-axis as follows: grey area, monomers; white area, oligomers.

3.5 FTIR

The secondary structure of βlg complexed with linoleate (molar ratio of 1, 2 and 3 linoleate/βlg) were examined using infrared spectra. Even if an aggregation was observed in the presence of linoleate, no difference was observed in the secondary structure of βlg in complexes compared to native βlg (Figure 23). The secondary structure was similar for βlg alone and in presence of phosphatidylcholine.[69] However, Tavel et al. (2008)[70] observed changes in the βlg amide I band with the presence of aromatic compounds. Secondary structure changes were also observed when βlg was in interaction with tea polyphenols.[71] These authors attributed the modifications of the protein secondary structure to size of ligands.

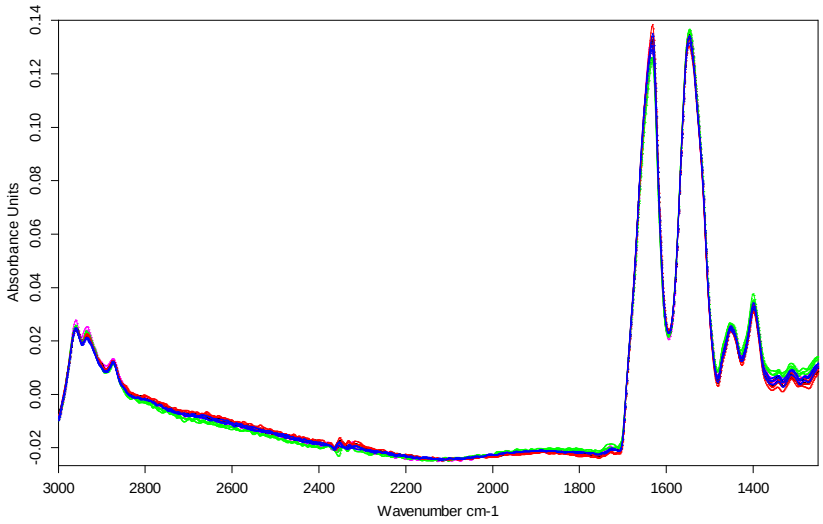

Figure 23: FTIR spectra of βlg and linoleate/βlg complexes. βlg control (green lines), 1 linoleate/βlg (purple lines), 2 linoleate/βlg (blue lines) and 3 linoleate/βlg (red lines) using native βlg.

3.6 FA transporters PCR

FA transporters CD36 and FABP2 were also chosen to elucidate the impact of βlg on FA transport via quantification of gene expression. The two sets of primers for each target gene are described in Table 5. However, the use of these primers did not allow the amplification of the target genes, even under various gradients of melting temperature, magnesium concentration, and primer concentration. This lack of amplification of the respective target genes may have occurred as the targeted genetic sequence was not accessible to the primers. Therefore, a nested PCR method was used on the CD36 gene target in order to amplify a genetic sequence larger that containing the target gene. Regretfully, the use of the primers described in Table 5 did not allow the amplification of the CD36 gene either. Gradients of melting temperature, magnesium concentration and primers concentration were also used for this experiment without success.

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Chapter 2

β-lactoglobulin/linoleate complexes: *in vitro* digestion and role of the protein in fatty acids uptake

1 INTRODUCTION

In the previous chapter, interactions between linoleate and native β-lactoglobulin (βlg) were shown to induce protein aggregation and to delay fatty acid (FA) cytotoxicity though reduced FA bioaccessibility. Being part of food products, these complexes are destined to be ingested and consequently exposed to the gastro-intestinal tract environment. Hence, in this chapter, we followed the digestion kinetics of linoleate/βlg complex under gastro-duodenal conditions. The ability of the digested complexes to induce the satiety hormone cholecystokinin (CCK) was measured. The bioaccessibility of the FA, the protein and the peptic digests were also investigated.

Therefore the objectives of this chapter are:

- To characterize the gastro-duodenal *in vitro* digestion of the linoleate/βlg complex.
- To measure the ability of linoleate, βlg and a linoleate/βlg complex to modify CCK response from enteroendocrine cells.
- To analyse the FA and βlg uptake.
- To determine the FA cytotoxicity after digestion of the complex.

A gastro-duodenal *in vitro* digestion was followed by GP-HPLC. The cytotoxicity of linoleate in a digested complex was observed by MTS assay. The bioaccessibility of linoleate free or bound was studied by mimicking the intestinal barrier using a Caco-2 monolayer. Using confocal imaging, we observed the uptake of FA by the cells. The effect of FA/βlg complexation was investigated by measuring CCK gene expression and secretion in enteroendocrine cells.

Main results:

- Linoleate increased βlg hydrolysis during gastric digestion probably by increasing its denaturation/oligomerisation.
- Linoleate uptake in Caco-2 cells was faster when free compared to bound.
- βlg aided to the uptake of linoleate, it occurred at a slower kinetic than free linoleate.
- CCK gene expression and secretion was similar for free and bound linoleate.
- The digestion of the complex increased linoleate cytotoxicity.

2 PAPER 2: β-LACTOGLOBULIN/LINOLEATE COMPLEXES: *IN VITRO* DIGESTION AND ROLE OF THE PROTEIN IN FATTY ACIDS UPTAKE

The content of this chapter has been submitted to *Journal of Nutritional Biochemistry*.

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2.1 Abstract

The dairy protein β-lactoglobulin (βlg) is known to bind fatty acids such as the salt of the essential long-chain fatty acid linoleic acid (LA, *cis,cis*-9,12-octadecadienoic acid, n-6, 18:2). The aim of current study was to investigate how bovine βlg/linoleate complexes of various stoichiometry affect the enzymatic digestion of βlg and the intracellular transport of linoleate into enterocyte-like monolayers. Duodenal and gastric digestions of the complexes indicated that βlg was hydrolysed more rapidly when complexed with linoleate. Digested, as well as undigested, βlg/linoleate complexes reduced intracellular linoleate transport as compared to free linoleate. To investigate whether enteroendocrine cells perceive linoleate differently when part of a complex, the ability of linoleate to increase production or secretion of the enteroendocrine satiety hormone cholecystokinin (CCK) was measured. *CCK* mRNA levels and CCK secretion were not altered by whether linoleate was presented to the cells alone or as part of a protein complex.

Key words: β-lactoglobulin; Linoleate; Digestion; Caco-2 Monolayer; Cholecystokinin.

2.2 Introduction

Bovine β-lactoglobulin (βlg) is the major whey protein in bovine milk. It is a globular protein with a monomeric molecular weight of 18.4 kDa, consisting of 162 amino acids.[1] βlg is a member of the lipocalin family, which are able to bind small hydrophobic molecules, including fatty acids (FA) and hydrophobic vitamins.[1-8] All members of the lipocalin family contain a β-barrel, shaped into a calyx, composed of eight antiparallel β-strands.[5] It has been suggested that βlg binds hydrophobic ligands in its internal calyx, in a crevice near the α-helix on the external surface of the β-barrel and near the interface of βlg dimers.[1,9-15] However, the binding sites and stoichiometries of ligands have been controversial.[2,10,14] βlg may modify the transport, through the gastric tract of hydrophobic substances naturally

present in bovine milk.[3,4] βlg was reported to enhance pregastric lipase activity by binding FA that inhibit the enzyme.[16] The binding of hydrophobic ligands has been shown to modify the proteins digestibility, as such interactions alter the accessibility of the protein to digestive enzymes.[17,18]

Previously, the salt of the essential long-chain fatty acid (LCFA) linoleic acid (LA, *cis,cis*-9,12-octadecadienoic acid, n-6, 18:2) was shown to bind βlg.[19] LA represents 1-3 % (w/w) of the FA in milk fat.[20] The mechanisms by which LCFA are absorbed by cells remains controversial. Some studies suggest that LCFA are taken up by intestinal epithelial cells by both active transport via specific FA transporters, and by passive diffusion.[21-23] However, uptake and bioavailability of FA may be altered depending on the food matrix.[24-26] We observed that linoleate, the salt form of LA, bound to βlg was less bioaccessible compared to free linoleate.[19] After uptake in enterocyte cells, FA are bound to proteins for their intracellular trafficking and thio-esterification.[27] They are rapidly esterified into triacylglycerols in the endoplasmic reticulum. In the golgi, these triacylglcerols form chylomicrons (lipid droplets) before being released into the cytoplasm and finally into circulation for uptake by other cells.[27] FA also trigger internal signals in the intestinal epithelium to allow the body to control food intake for example, secretion of satiety hormones by specialised entroendocrine cells.

Cholecystokinin (CCK) is a peptide hormone reported to have satiety effect as it influences the digestive processes in the gut.[28] CCK has been associated with delay of gastric emptying, stimulation of gallbladder contraction, increases in pancreatic enzyme secretion, and reduced food intake.[29] CCK is secreted by I enteroendocrine cells located predominantly in the proximal small intestinal mucosa in response to intraluminal nutrients such as FA and proteins.[30] Relatively little is known concerning the mechanisms whereby nutrients influence CCK synthesis and secretion. Several studies have shown that peptides and FA can use an external receptor to induce CCK secretions.[31-33] *In vivo* and *in vitro* studies indicate that proteins increase CCK secretion.[34-36] This effect is further improved when proteins are hydrolysed, however individual amino acids do not contribute to CCK release.[37] Secretion of CCK by FA is dependent on FA structure, chain length, degree of unsaturation and whether it is in the free or bound form.[38-42]

This study investigated whether βlg/linoleate complexes alter βlg digestion, linoleate bioaccessibility and intracellular transport of linoleate into intestinal epithelial cells Caco-2. To investigate whether enteroendocrine cells perceive linoleate differently when free or part of a complex, the production and secretion of the satiety hormone secretion CCK was measured in STC-1 cells.

2.3 Materials and methods

2.3.1 Materials

βlg (96 % purity) was obtained from Davisco Foods International, Inc. (Eden Prairie, Minnesota) and sodium linoleate (purity ≥ 98 %) from Sigma-Aldrich (St. Louis, MO). All other chemicals and solutions were purchased from Sigma-Aldrich unless stated otherwise.

2.3.2 Complex preparation

Linoleate/βlg complexes were prepared by mixing a solution of βlg and sodium linoleate adapted from Lišková et al. (2011)[43] and described by Le Maux et al. (2012)[19]. Under these conditions complexes with 1, 2 and 3 linoleate bound to βlg are formed. βlg without FA was used as a βlg control.

2.3.3 *In vitro* digestion

In vitro adult digestion model was adapted from Dupont et al. (2009)[44]. Briefly, βlg samples were dissolved in simulated gastric fluid (0.15 M NaCl, pH 2.5) and the pH was adjusted to 2.5 with 0.5 M HCl solution. Porcine gastric mucosa pepsin (Sigma-Aldrich P7000, activity: 837 U/mg of protein calculated using haemoglobin as a substrate) was added to give 182 U of pepsin/mg of βlg. The final concentration of βlg was 0.05 mM in 20 ml solution. During this gastric digestion, aliquots (1 mL) were removed at regular intervals over a 60 min period. Pepsinolysis was stopped by raising the pH to 7.0 using 0.5 M NaOH. For duodenal proteolysis, the pH was adjusted to 6.5. Duodenal digestion components were dissolved in simulated duodenal fluid (0.15 M NaCl, pH 6.5). They were added to give final concentrations as follows: 4 mM sodium taurocholate, 4mM sodium glycodeoxycholate, 26.1 mM Bis-Tris buffer pH 6.5, 0.4 U/mg of βlg for α-chymotrypsin (Sigma-Aldrich C4129, activity 59 U/mg of protein using benzoyltyrosine ethyl ester as substrate) and 34.5 U/mg of βlg for trypsin (Sigma-Aldrich T0303, activity 14476 U/mg of protein using benzoylarginine ethyl ester as substrate). Aliquots of 1 mL were removed over a 30 min period of duodenal digestion. Proteolysis was stopped by addition of an excess of soybean Bowmann-Birk trypsin/α-chymotrypsin inhibitor (100 µL of 5 g/L in simulated duodenal fluid), which ensured inhibitor excess. The final solution was freeze-dried.

2.3.4 Gel permeation-HPLC

The relative proportion of protein monomers, aggregates and peptides in the digested samples were determined by gel permeation-HPLC (GP-HPLC) using in tandem a TSK G SW guard column (7.5 × 7.5 mm, Tosoh Bioscience GmbH, Stuttgart, Germany) and a TSK

G2000 SW column 130 (7.5 × 600 mm, Tosoh Bioscience GmbH) connected to an HPLC system (Waters 2695 Separations Module, Waters 2487 Dual λ Absorbance Detector, at 214 and 280 nm) with Empower Pro software (Waters, Milford, MA). A quantity of 0.05 mg protein was injected using a solution of 30 % (v/v) acetonitrile (LabScan Analytical Sciences, Dublin, Ireland) and 0.1 % (w/v) trifluoracetic acid in Milli-Q water (Millipore, Carrigtwohill, Ireland) as an eluent, at a flow rate of 0.5 mL/min. The method was calibrated using a set of protein molecular-weight standards (Sigma-Aldrich). Digestion solutions without βlg or FA served as control chromatograms and were subtracted from chromatograms generated from test samples to reduce background noise. Chromatograms were integrated and peaks expressed by size (> 30 kDa, 30 to 10 kDa, 10 to 1 kDa and < 1 kDa) as a percentage of protein content.

2.3.5 Extraction and determination of the fatty acid content in digested samples by gas chromatography

FA concentration in samples was determined by gas chromatography (GC). To determine FA concentration in digested complexes, total FA were extracted and methylated according to the protocol of Palmquist and Jenkins (2003)[45] with some modifications described by Le Maux et al. (2012)[19].

Fatty Acid Methyl Esters (FAME) was quantified using a CP-SELECT CB column (100 m, 0.25 mm, 0,25 µm film thickness, Varian BV, Middelburgh, the Netherlands) on a Varian 3400 GLC (Varian, Walnut Creek, CA, USA), which was fitted with a flame ionization detector as previously described by Coakley et al. (2003)[46].

2.3.6 Cell culture

Cell lines were cultured in a humidified 37°C incubator with a 5 % (v/v) CO_2 in air atmosphere.

2.3.6.1 Caco-2 cell line

The Caco-2 cell line was purchased from the European Collection of Cell Cultures (collection reference: ECACC 86010202) and was derived from human colonic adenocarcinoma cells.

Cells were routinely grown in 75 cm^2 plastic flasks in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/L glucose and 0.584 g/L L-glutamine. Media for subculture was supplemented with 10 % (v/v) foetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin. At 80 % confluency, cells were trypsinised with 0.25 % trypsin/EDTA,

diluted 1:6 in media and reseeded. Media was changed three times a week. All cells used in these studies were between passage number 32 and 42.

2.3.6.2 STC-1 cell line

The STC-1 cell line was purchased from American Type Culture Collection (ATCC, Gaithersburg, MD). This enteroendocrine cell line originated from a double transgenic mouse tumour.[47]

Cells were routinely grown in 75 cm^2 plastic flasks in DMEM containing 4.5 g/L glucose and 0.584 g/L L-glutamine. Media for subculture was supplemented with 20 % (v/v) FBS, 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were passaged at 80 % confluence. Cells at passage numbers of between 15 to 20 were used in this study.

2.3.7 Viability assay

Cytotoxicity of test samples on Caco-2 and STC-1 cell proliferation was determined by MTS assay, using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Corporation Madison, Wisconsin, USA) according to the manufacturer's instructions. Viability was defined as the ratio of absorbance of treated cells to untreated cells (cells incubated in media only) at 490 nm. Each cell exposure was repeated in triplicate, at a minimum.

For Caco-2 experiments, cells were seeded in 96-well plates, at a cell density of 2×10^4 cells/well, using serum-free media (DMEM only supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin) 24 h prior to experiment. The Lethal Dose 50 (LD_{50}), concentration required to decrease the cell viability by 50 %, of free linoleate is 58 µM linoleate.[19] As such, Caco-2 cells were exposed for 24 h to digested complexes (molar ratio of 1, 2 and 3 linoleate/βlg) containing 58 µM linoleate.

Cytotoxicity assays were also performed on Caco-2 monolayers. Caco-2 cells were seeded in 96-well plates, at a cell density of 6.5×10^4 cells/well, using complete media. Initially, media was changed after 6 h, then every two days up to 21 days. Complete media was change to serum-free media 24 h prior to experiment. Cells were exposed to 50 µM linoleate for 24 h.

For STC-1 experiments, STC-1 cells were seeded in 96-well plates, at a cell density of 2×10^5 cells/well, using complete media 24 h prior to experiment. STC-1 were treated with different concentrations of linoleate (0 to 200 µM) and βlg (0 to 200 µM) in serum-free media for 24 h. LD_{50} were determined using Graph-Pad Prism software 3.03 (GraphPad Software Inc., La Jolla CA, USA). The sigmoidal dose-response with variable slope was used to fit the measured curves and calculate LD_{50}.

2.3.8 Caco-2 transepithelial transport

The Caco-2 cell line is derived from human colonic carcinoma and has similar characteristic to small intestine epithelial cells.[48] Cultured as a monolayer, these cells can differentiate into intestinal-like absorptive cells with tight junctions and a well-differentiated brush border, which express nutrient transporters.[49] The apical side of these enterocyte-like cells, which contains the brush border, would be in contact with the lumen of the gut. The basal side of the cells would be in contact with the blood and lymphatic systems. As such, Caco-2 monolayers are a well-established intestinal barrier model to determine the bioavailability of substances and their transport into and out of the intestinal epithelium *in vitro.*[48,49]

For transepithelial transport experiments, Caco-2 cells were seeded at a density of 3 × 10^5 cells/well into permeable Transwell filter inserts (24 mm diameter, 0.4 µm pore size; Costar, Cambridge, MA). Cell culture media was changed every two days for 21 days, upon which time the cells are fully differentiated. Serum free media was changed 24 h prior to experimentation. In these conditions, Caco-2 cells were exposed to media devoid of FA in order to minimise the impact of exogenous FA, and maximise the impact of added linoleate.

Cell monolayer integrity was confirmed by measuring transepithelial electrical resistance (TEER) at 37°C using a Millicell-ERS meter (Millipore Corporation, Bedford, MA, USA) according to the manufacturer's instructions. Inserts with a TEER value ≤ 1300 Ω.cm^2 were discarded.

Transepithelial transport studies were adapted from Hubatsch et al. (2007)[50]. Briefly, inserts were washed three times with serum-free media. Serum-free media (2.5 mL) was added to the basal side, 1.5 mL of tested compound (1, 2 and 3 linoleate/βlg complexes digested and undigested with a linoleate concentration of 50 µM) in serum-free media containing 5.3 mM fluorescein sodium salt was added to the apical side. Fluorescein acts as an indicator of paracellular transport across the Caco-2 monolayer.[51] Plates were then incubated in a humidified 37°C incubator on an orbital shaker at 60 rpm to minimise the impact of unstirred layer. After 4 h, apical and basal media were removed for analysis. Six replicates of each experiment were performed.

2.3.9 Determination of paracellular transport in monolayer experiments

Fluorescein concentration in the basal chamber was determined by detecting fluorescence in a 50 µL aliquot. Fluorescence was detected with a Synergy Biotek plate reader (BioTek Instruments Inc., Winooski, VT) at excitation and emission wavelengths of 485 nm and 535 nm respectively. Fluorescein concentration was determined by interpolating fluorescence readings from a fluorescein standard curve. The concentration of fluorescein in

the basal chamber was expressed as a percentage of the total fluorescein added to the apical side of the inserts. Paracellular transport was always inferior to 0.1 %.

2.3.10 Extraction and determination of the fatty acid content in monolayer experiments by gas chromatography

FA concentration in the apical and basal chambers was determined by GC. Total FA were extracted according to the method of the International Organization for Standardization (ISO) standards 14156:2001 (ISO, 2001)[52]. C13:0 (0.75 mg) was employed as an internal standard. Following removal of the diethyl ether (Fischer Scientific, Pittsburgh, PA) by heating at 45°C under nitrogen, extracted FA were converted to FAME by base catalysed methylation with 2 mL of 0.5 N sodium methoxide at ambient temperature for 10 min. This was followed by acid catalysed methylation using an adaptation of the method of previously described by Palmquist and Jenkins (2003)[45]. The concentrated FAME was transferred to clean glass gas chromatography vials and quantified as described above.

The amount of linoleate remaining in the apical chamber and its transport in the basal chamber were corrected by the paracellular transport and the control (cells with media only). The corrected linoleate data was then expressed as a percentage of the initial linoleate applied to the apical side cells (50 µM).

2.3.11 Confocal fluorescence microscopy

Intracellular lipid accumulation was imaged using confocal microscopy. Caco-2 cells were seeded into 8-well chambered glass coverslips (Labtek, Nunc) at a density of 5×10^4 cells/well with serum-free media 24 h prior to experimentation. Cells were then treated with 50 µM linoleate or 50 µM linoleate in a complex with a molar ratio of 3 linoleate/βlg. Following 4 h exposure, cells were gently washed with phosphate buffered saline (PBS) and stained with 4 µM Nile Red (prepared in PBS) to highlight intracellular lipid droplets.[53] Cells were examined using a Nikon C1Si Laser Scanning Confocal Imaging System on inverted microscope TE2000-E (Nikon, Champigny-sur-Marne, France) equipped with a helium/neon laser emitting at 543 nm. Fluorescence emission was acquired with a 590/50 nm filter. Average fluorescence intensity was calculated from 9 images.

2.3.12 Cholecystokinin experiments on STC-1 cells

In order to assess the impact of samples on CCK secretion *in vitro*, STC-1 cell line was employed.[40,54]

The CCK assay was adapted from Hand et al. (2010)[54]. Briefly, STC-1 cells were seeded into 12-well plates at a cell density of 2×10^6 cells/well, in complete media and

incubated overnight. On the day of the experiment, media was removed and cells were washed three times with serum-free media and then incubated for 30 min with serum-free media at 37°C. Media was removed and 400 µL of serum-free media containing 5 µM of linoleate, free or complexed to βlg (molar ratio 1 to 3), either digested or undigested were added. All CCK experiments were performed in triplicate.

After a 4 hour incubation, 360 µL of the supernatant was removed, 40 µL of 10 % bovine serum albumin (prepared in PBS) was added and the solution centrifuged at 900 g for 5 min at 4°C to remove cellular debris. The supernatant was collected and stored at -80°C prior to analysis by ELISA.

For determination of *CCK* mRNA levels, cells were washed with PBS. Cells were lysed using QIAzol Lysis Reagent (QIAGEN Ltd., West Sussex, UK) and RNA was isolated from cell suspensions using the QIAGEN miRNeasy Mini kit (QIAGEN Ltd., West Sussex, UK) according to the manufacturers' instructions. RNA was quantified spectrophotometrically using the Nanodrop 1000 (Thermo Fisher Scientific, USA) and the integrity assessed by electrophoresis in a 1.5 % glyoxyl gel with 1X glyoxyl buffer (Ambion, Applied Biosystems, Foster City, USA). Complementary DNA synthesis was prepared from 1 µg of RNA using the QIAGEN QuantiTect reverse transcription kit (QIAGEN Ltd., West Sussex, UK).

2.3.13 Determination of cholecystokinin secretion using ELISA

A CCK (26-33, non sulphated) fluorescent ELISA immunoassay kit (Phoenix Pharmaceutical Inc., Burlingame, CA, USA) was used according to manufacturer's instructions. CCK content was determined using 25 µL of supernatant. Fluorescence (excitation 337 nm, emission 460 nm) was read using a FLUOstar Omega multi-mode microplate reader and analysed with the FLUOstar Omega software (BMG LABTECH GmbH, Ortenberg, Germany). Concentration of CCK in samples was determined by extrapolation to a standard curve. Data were expressed as a percentage of the control value (cells with media only). STC-1 cells do not produce detectable level of gastrin, so the detected signal was attributed to CCK.[55] Experiments were performed in triplicate.

2.3.14 Determination of c*holecystokinin* mRNA using real time-PCR

CCK mRNA was quantified using Lightcycler and SyBr green technology (Roche Diagnostics Ltd, Mannheim, Germany) and by the principles of relative quantification using the housekeeping gene ubiquitin-conjugating enzyme (*E2D2*). Protocol and primers for murine *CCK* were previously described by Hand et al. (2010)[54].

The target (*CCK*) to reference (*E2D2*) ratio expression was calculated for each FA treatment and normalised compared to untreated (media alone) controls. Experiments were performed in triplicate.

2.3.15 Statistical analysis

Where appropriate, results were compared using R software package version 2.15.1 (R Foundation for Statistical Computing, Vienna, Austria) and the ANOVA system with a Tukey's least significant difference comparison. *P*-Values < 0.05 were deemed to be statistically significant.

2.4 Results

2.4.1 Protein digestion

Gastric and duodenal *in vitro* digestion was performed on native βlg, βlg control and linoleate/βlg complexes (linoleate/βlg molar ratio of 1, 2 and 3, Figure 24). As linoleate/βlg complexes had similar chromatograms, only the 3 linoleate/βlg molar ratio complex was compared to βlg control. The GP-HPLC chromatogram was divided into four fractions: protein oligomers (molecular weight, $M_w \geq 30$ kDa); protein monomers (30 kDa $\geq M_w \geq 10$ kDa); large peptides (10 kDa $\geq M_w \geq 1$ kDa); small peptides ($M_w \leq 1$ kDa).

The initial HPLC profiles (prior to *in vitro* digestion) of βlg control and complexes were different regarding oligomer and monomer categories. The proportion of oligomers in the initial samples was 15 % for βlg control and 31 % for complex with a molar ratio of 3 linoleate/βlg. These βlg oligomers were rapidly hydrolysed during the gastric digestion. Their proportion decreased from 31 % to 7 %, and from 15 % to 5 %, within the first minute of gastric digestion, for the βlg control and 3 linoleate/βlg complex, respectively. The proportion of monomers decreased from 85 % to 61 % for βlg control and from 65 % to 45 % for a complex with a molar ratio of 3 linoleate/βlg. Small and large peptides were produced during the gastric digestion for all the samples. They represent about 10 % for βlg control and 20 % for the 3 linoleate/βlg complex at the end of the gastric digestion process.

During the duodenal digestion, the proportion of βlg monomers decreased from 61 % to 53 % for the βlg control sample while it remained unchanged in samples with linoleate. A decrease in the proportion of small peptides (< 1 kDa) was observed for all the samples prior the addition of enzymes of the duodenal digestion. For example, a decrease from 20 % to 12 % of small peptides was observed for the 3 linoleate/βlg complex. Concomitantly, an increase of oligomers (M_w ~ 66 kDa) was detected. Since there was no change in monomer and large peptide populations, these oligomeric species were attributed to the aggregation of

small peptides. This result was confirmed by SDS-PAGE experiments, which showed the formation of aggregated molecules that disappeared after chemical reduction (Data not shown).

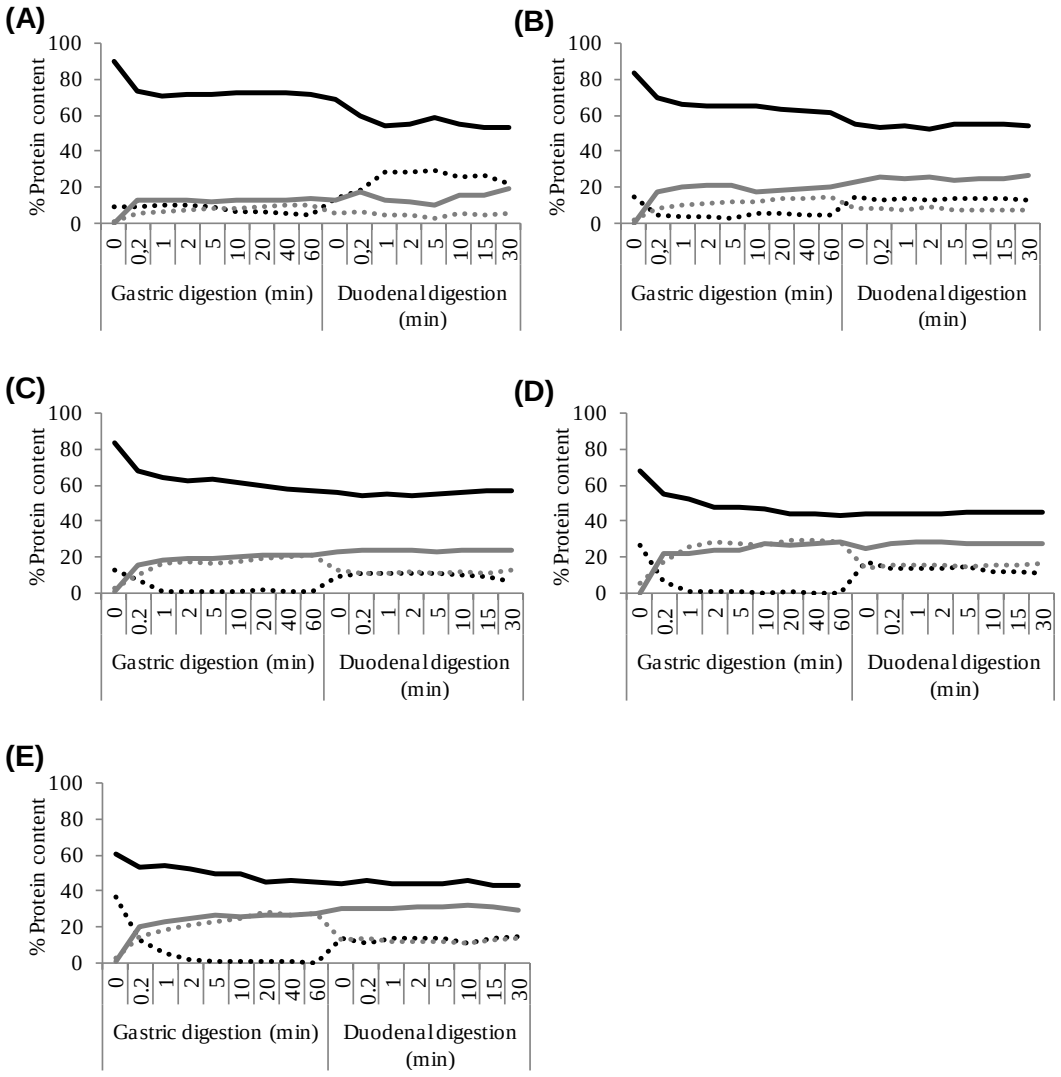

Figure 24: Characterisation of *in vitro* gastric and duodenal digestion of βlg alone or in complexes with linoleate determined by GP-HPLC. Proportion of βlg monomers, aggregates, peptides (1-10 kDa), and small peptides (< 1 kDa) are represented by the black line, the black dotted line, the grey line and the grey dotted line, respectively. (A) native βlg, (B) βlg control, (C) 1 linoleate/βlg, (D) 2 linoleate/βlg and (E) 3 linoleate/βlg.

2.4.2 Cytotoxicity of *in vitro* digested complexes on Caco-2 cells

Exposure of Caco-2 cells to the digested complexes, containing 58 µM linoleate, the LD_{50} of linoleate on Caco-2 cells in these conditions[19], resulted in a significant decrease in

Caco-2 viability after 24 h compared to digested βlg alone (Figure 25). Cells exposed to digested βlg control had a viability of 90.8 ± 1.8 % whereas cells exposed to digested complexes had a viability of 71.7 ± 2.6 %, 75.0 ± 1.7 % and 67.9 ± 3.2 % for 1, 2 and 3 linoleate/βlg, respectively. Digested complexes did provide a protective effect to the cells, compared to free linoleate. However undigested complexes, similarly to βlg alone had minimal effect on cell viability under the experimental conditions used (96.6 ± 5.6 %).[19] Consequently, the level of cell viability with digested complexes is intermediate between undigested complexes and free linoleate.

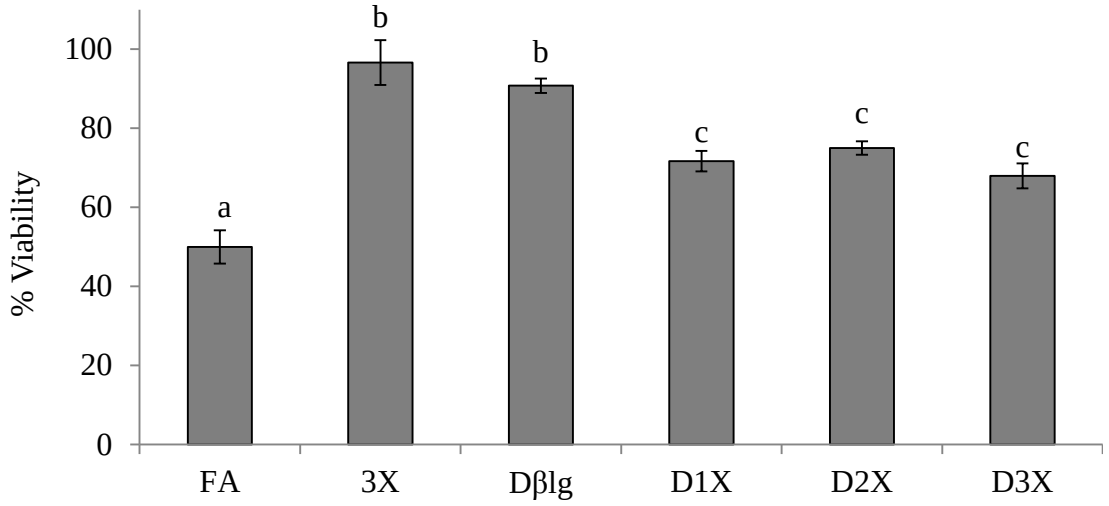

Figure 25: Viability of Caco-2 cells exposed to digested βlg /linoleate complexes for 24 hours. Cells ($2x10^4$ cells/well) were treated with digested complexes (molar ratio of 1, 2 and 3 linoleate/βlg each of which contained 58 μM linoleate). Cell viability is calculated as a percentage of viability of cells incubated in media alone. FA, linoleate; 3X, 3 βlg /linoleate complex; Dβlg, digested βlg control; D1X, D2X, and D3X, linoleate/βlg digested complexes with a molar ratio of 1, 2 and 3, respectively. Results represent mean ± SD (n=6). Multiple Comparisons of means was performed using Tukey contrasts (p<0.001).

2.4.3 Uptake of linoleate by Caco-2 cells

Uptake by Caco-2 cells of linoleate free or bound to βlg was followed both by the use of Caco-2 cell monolayers, which mimic the intestinal barrier, and by confocal imaging.

Levels of linoleate in the apical and basal chamber of the Caco-2 monolayer was quantified by GC analysis (Figure 26). Preliminary cytotoxic assays demonstrated that a 4 h exposure of differentiated Caco-2 cells to 50 μM linoleate free or in complex did not disturb the Caco-2 monolayer (data not shown). Therefore, this sub-lethal concentration was selected for the Caco-2 transepithelial transport experiments. It was observed that the presence of βlg did not affect the transport of linoleate through the basal chamber as the

basal level of linoleate was similar for all the samples (ie. 3.86 ± 0.7 %). However, the molar ratio of linoleate/βlg complexes affected the proportion of linoleate remaining in the apical chamber. As shown in Figure 26, 4.9 ± 0.9 % linoleate remained in the apical chamber when cells were exposed to 50 µM of free linoleate compared to 10.4 ± 0.4 %, 13.7 ± 0.3 % and 15.3 ± 2.5 % linoleate when cells were exposed to complexes of 1, 2 and 3 linoleate/βlg, respectively. Interestingly, the proportion of linoleate remaining in the apical chamber was similar for all digested samples (12.2 ± 0.6 % for 3 linoleate/βlg experiment). Hence, binding of βlg with linoleate significantly decreased the uptake of linoleate by the cells.

Lipid accumulation in Caco-2 cells was measured by Nile Red fluorescence via confocal imaging (Figure 27). Cells were incubated for 4 h with 50 µM linoleate free or in a 3 linoleate/βlg complex. A significant increase in the amount of lipid accumulated in the cells exposed to free linoleate compared to the cells exposed to linoleate complexed to βlg was observed. Fluorescence intensity was 333 ± 25 AU and 2203 ± 570 AU after 4 h of incubation with bound and free linoleate, respectively. For both treatments, the formation of lipid droplets was observed in the confocal images, with larger lipid droplets in cells exposed to free linoleate (Figure 27).

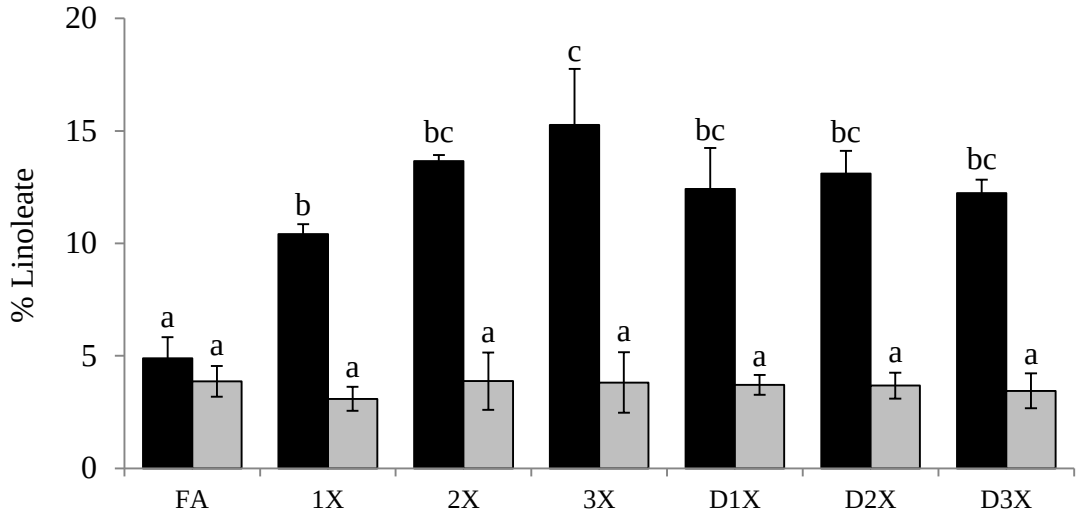

Figure 26: Transport of linoleate across the Caco-2 monolayer after 4 h of exposure to 50 µM linoleate free or bound to βlg (undigested and digested complexes with a molar ratio of 1, 2 or 3 linoleate/βlg). Proportion of linoleate in apical or basal chamber was expressed as the percentage of the initial linoleate content exposed to cells. Linoleate was measured by GC in the apical chamber (black bars) and in the basal chamber (grey bars). FA, linoleate; 1X, 2X and 3X, undigested linoleate/βlg complexes with a molar ratio of 1, 2 and 3, respectively; D1X, D2X, and D3X, digested linoleate/βlg complexes with a molar ratio of 1, 2 and 3, respectively. Results represent mean ± SD (n=6). Multiple Comparisons of means was performed using Tukey contrasts ($p < 0.05$).

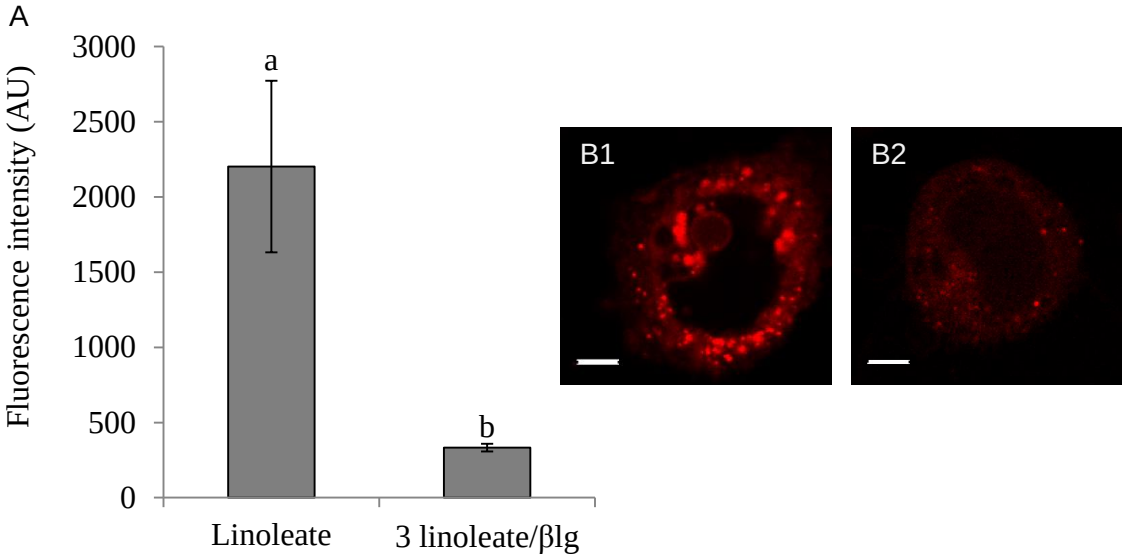

Figure 27: Confocal analysis of Caco-2 cells (5 x 10^4 cells/well) exposed to 50 µM linoleate free or bound in a complex with a 3 linoleate/βlg molar ratio for 4 h. Lipid droplets were stained with Nile Red dye. (A) Fluorescence intensity of Nile Red in Caco-2 cells. (B) confocal imaging depicting the difference in the level of fluorescence from cells exposed to free (B1) or bound (B2) linoleate (bar, 5 µm). Results represent mean ± SD (n=9 pictures). Multiple Comparisons of means was performed using Tukey contrasts (p <0.01).

2.4.4 Impact of linoleate/β-lactoglobulin on the regulation of the satiety hormone cholecystokinin, secretion and mRNA level

To determine if linoleate bound to βlg can alter downstream cellular response, the enteroendocrine cell line STC-1 was incubated with free linoleate or complexes of βlg-linoleate. Preliminary experiments showed that βlg was not cytotoxic to STC-1 cells at the concentrations tested (0 to 200 µM) after 24 h, as measured by MTS assay (data not shown). In contrast, STC-1 cells were sensitive to linoleate with a LD_{50} of 12.68 ± 2.56 µM after 24 h (Figure 28).

There was no significant difference in the levels of CCK peptide secreted from STC-1 cells exposed to untreated control, free linoleate, undigested and digested complexes (Figure 29) for 4 h. In contrast, all the samples tested increased *CCK* mRNA transcripts levels compared to untreated control after 4 h (Figure 30). No significant difference was observed between the normalised mRNA response of undigested 1, 2 and 3 linoleate/βlg molar ratios used. In the same manner, digested complexes did not show significant difference between the three linoleate/βlg molar ratios. Normalised mRNA levels increased was of 1.007 ± 0.004 for free linoleate and increased to 1.041 ± 0.013 and 1.072 ± 0.014 when cells were exposed 3 linoleate/βlg complex undigested and digested, respectively (P<0.05). However, the normalised *CCK* mRNA levels were 1.027 ± 0.016 and 1.059 ± 0.009 for undigested and digested βlg control, respectively. Therefore, undigested linoleate/βlg complex (3

linoleate/βlg molar ratio) increased normalised *CCK* mRNA levels by 0.014 compared to the undigested βlg control. Digested complexes with a 3 linoleate/βlg molar ratio, increased normalised *CCK* mRNA level by 0.013 above those measured for cells exposed to digested βlg control. Linoleate control (5 µM linoleate) increased *CCK* mRNA level from 0.007 compared to the untreated control. As such, no significant difference were observed between increases of mRNA level due to linoleate free or bound to βlg.

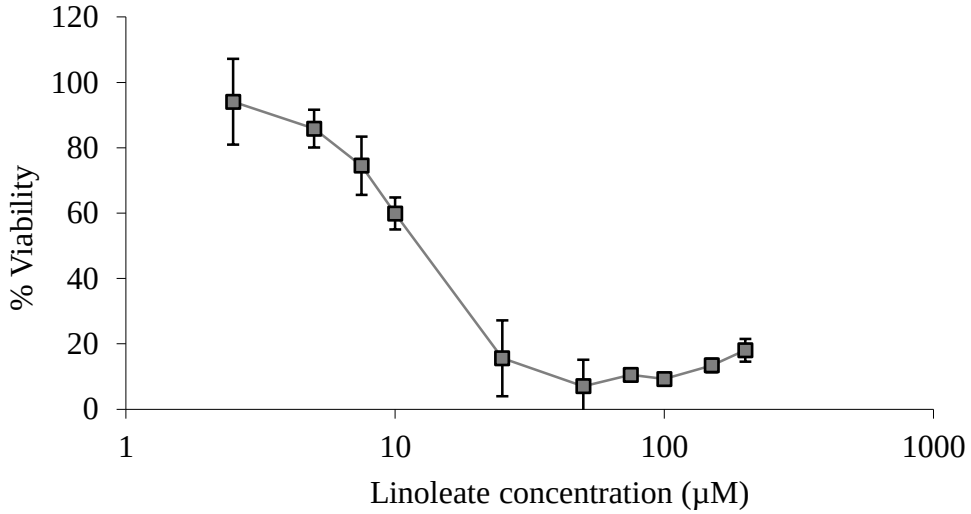

Figure 28: Cytotoxicity of linoleate to STC-1 cells. Cytotoxicity was assessed using an MTS assay. % viability after 24 h on 2 × 10^5 STC-1 cells was expressed relative to control cells.

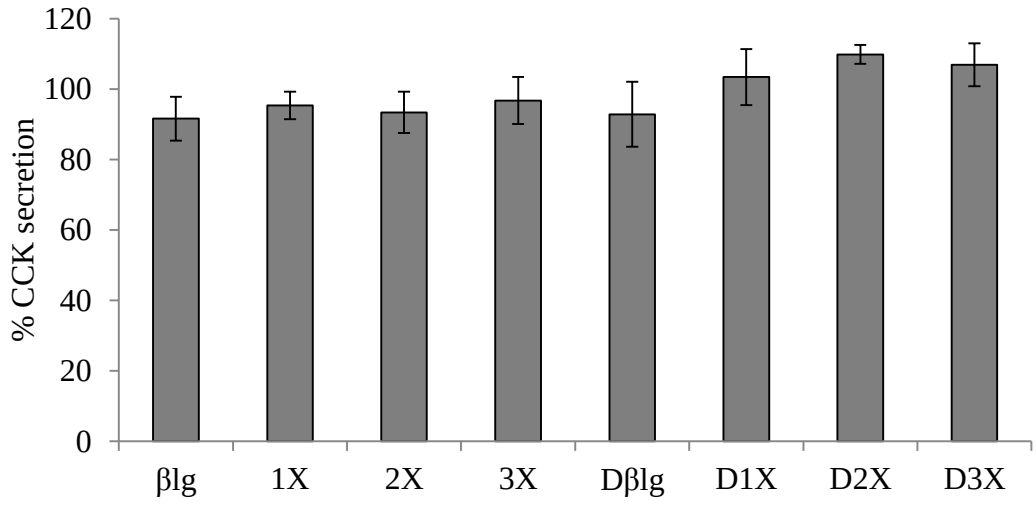

Figure 29: CCK secretion by 2x10^6 STC-1 cells/well exposed to linoleate/βlg complexes with a linoleate concentration of 5 µM for 4 h. CCK secretion was normalised by cells exposed to media only. βlg, βlg control; 1X, 2X and 3X, undigested linoleate/βlg complexes with a molar ratio of 1, 2 and 3, respectively; Dβlg, digested βlg control; D1X, D2X, and D3X, linoleate/βlg digested complexes with a molar ratio of 1, 2 and 3, respectively. Results represent mean ± SD (n=3).

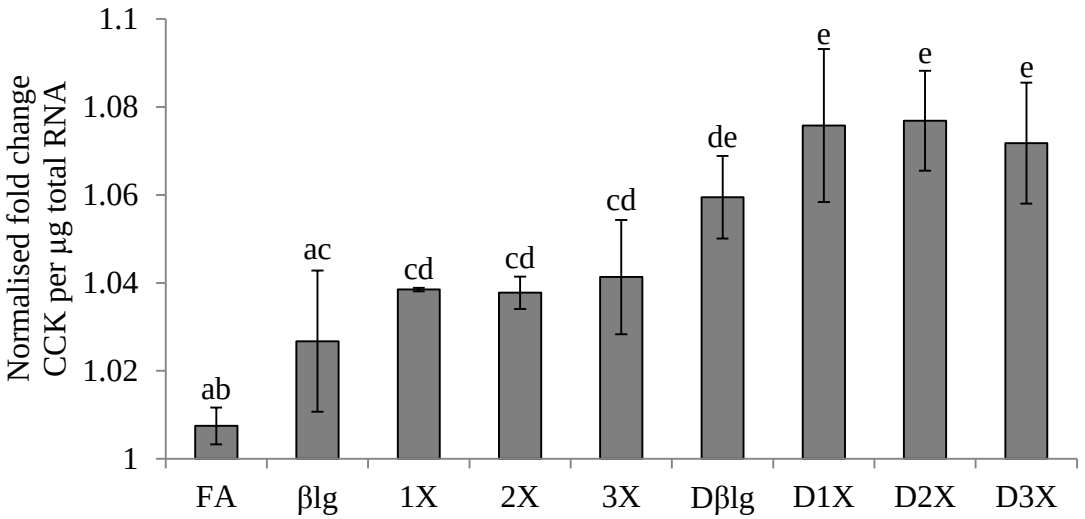

Figure 30: CCK mRNA levels in 2 x 10^6 STC-1 cells/well exposed to linoleate/βlg complexes with a linoleate concentration of 5 µM for 4 h. Normalised fold change in CCK mRNA levels in STC-1 cells exposed to test samples are normalised to levels of mRNA of the housekeeping gene, E2D2, and to CCK and E2D2 mRNA levels in untreated cells. FA, linoleate; βlg, βlg control; 1X, 2X and 3X, undigested linoleate/βlg complexes with a molar ratio of 1, 2 and 3, respectively; Dβlg, digested βlg control; D1X, D2X, and D3X, linoleate/βlg digested complexes with a molar ratio of 1, 2 and 3, respectively. Results represent mean ± SD (n=3). Multiple Comparisons of means was done using Tukey contrasts ($p<0.05$).

2.5 Discussion

Digested complexes of βlg/linoleate were found to be more cytotoxic to Caco-2 cells compared to undigested complexes but less cytotoxic than free linoleate.[19] As linoleate has to be transported into the cell to be cytotoxic[56], this would infer that a portion of the linoleate remains bound to the protein or peptides after *in vitro* gastro-duodenal digestion. Even if it is assumed that linoleate dissociates from the protein under gastric conditions (pH 2.5), complexes could reform under duodenal digestion conditions (pH 6.5).[4] The interaction between FA and βlg is pH-dependent: at low pH (below pH 6), FA are released from the βlg but the interaction is reversible when the pH is increased. As up to 50 % of βlg monomers remained after digestion, it is highly probable that reformed complexes consisted of linoleate and undigested monomers.

In its native state, βlg conformation is resistant to pepsin hydrolysis.[57] However, βlg was hydrolysed faster under gastric digestion conditions when FA/βlg molar ratio was increased. This is probably due to the increased proportion of oligomers in the sample.[19] During the duodenal digestion, no hydrolysis was observed for linoleate/βlg complexes. Only the βlg control undergoes peptic hydrolysis during duodenal digestion suggesting a protective effect of linoleate. Puyol et al. (1993)[17] demonstrated that the binding of palmitic

acid to βlg has a protective effect on the protein against hydrolysis, whereas the binding of retinol does not. Mandalari et al. (2009)[18] also demonstrated that phosphatidylcholine could protect βlg from hydrolysis possibly because the binding sites are located close to protease cleavage sites. Furthermore, Mandalari et al. (2009)[18] observed that this protective effect was only effective if the protein was in its native form.

During duodenal *in vitro* digestion, we observed the appearance of aggregates greater than 30 kDa and a concomitant decrease in the population of small peptides less than 1 kDa. The proportions of large peptides and monomers remained constant. These aggregates disappeared under reducing conditions, suggesting the aggregation of a fraction of the low M_w peptides via disulphide bonds occurs under duodenal conditions. Aggregates were observed for all complexes and βlg control samples, suggesting that the presence of linoleate did not influence peptide aggregation. Jiang and Lui (2010)[7] also observed the formation of aggregates of around 36 kDa during trypsin treatment of CLA/βlg while Bateman et al. (2011)[58] showed formation of βlg fibrils after pepsin hydrolysis and a subsequent pH increase to 6.9.

Caco-2 cells are an appropriate model to investigate FA transport across the intestinal gut.[59,60] Our results showed a decrease in the transport of the linoleate, when bound to βlg, into and out of the Caco-2 monolayer. Confocal microscopy confirmed that more linoleate was accumulated in the cells when free, compared to when linoleate was complexed to βlg. This is in agreement with the results of Riihimäki-Lampén (2009)[60] where the authors demonstrated that free retinol and free palmitic acid were transported more efficiency, from the apical to the basal side of Caco-2 monolayer, than when complexed to βlg. However, no such change was observed for cholesterol. Opposite results were found for conjugated linoleic acid (CLA).[7] These authors showed that the level of CLA in cells was significantly greater when CLA was provided to the cells as a βlg/CLA complex compared to free CLA. The low solubility of CLA and the concentration range used in the various studies could explain the discrepancy.

Our results suggest that a considerable quantity of FA was metabolised or stored by the cell. Riihimäki-Lampén (2009)[60] and Puyol et al. (1995)[59] showed that palmitic acid-bovine βlg complex was principally stored in the cell (more than 90 % after 24 h in Puyol et al., 1995[59]) and its transport in the basal chamber was low (ie. 5 % to 7 % in the presence of bovine βlg[59,60]). However, our confocal analysis showed a substantial difference in the lipid accumulation in the cells compared to the monolayer experiment which showed up to 10 % difference between free and bound linoleate in the apical chamber. This might be accounted for by our use of Nile Red dye, as this Nile Red dye fluoresces better in lipid droplets.[53] This

confocal result suggests that linoleate from the βlg/linoleate complexes is slower to form lipid droplets.

When in complexes, an increase in linoleate uptake was observed with increasing concentrations of βlg content using Caco-2 monolayer. As linoleate concentration was constant (50 µM linoleate), βlg concentration was three times smaller for a linoleate/βlg molar ratio of 3 compare to a molar ratio of 1. Native βlg has been shown to be transported into the cells.[61] This could explain why the kinetics of linoleate transport from the medium to the cells is affected by βlg concentration. However, some authors reports the existence of another mechanism involving a pH dependant dissociation of the ligand bound to βlg immediately prior to transport into the cell, as the pH of the environment close to the cells is more acidic (pH<5).[4,27]

Digestion of linoleate/βlg complexes seems to impact linoleate uptake by Caco-2 cells. Linoleate remaining in the apical chamber increased when the linoleate/βlg complex with a molar ratio of 1 was digested compared to undigested. This could be explained by the fact that after digestion, the βlg monomer concentration was reduced by a factor of 2. Under these conditions, the concentration of residual βlg monomers was sufficient to load linoleate (as indicated for complex with linoleate/βlg molar ratio of 2). However, transport of FA to the cells facilitated by βlg would be lower in the digested sample as the amount of βlg monomer was lower. Interestingly, the concentration of linoleate remaining in the apical chamber decreased when the complex with linoleate/βlg molar ratio of 3 was digested compared to undigested. Under this condition, it is possible that the concentration of residual βlg monomer after digestion was too low to bind all the linoleate molecules in the sample increasing the amount of free linoleate. As free linoleate is quickly transported to the cells an overall faster transport is observed for digested complex than undigested.

The ability of STC-1 cells to produce and secrete CCK is thought not to require internalisation of the FA as an external receptor seems to induce CCK secretions.[31-33] After 4 h exposure to the studied protein/FA complexes, no effect on CCK secretion was detected, whereas mRNA *CCK* levels differed between samples. This supports that CCK synthesis and secretion are distinct. This was highlighted by Hand et al. (2010)[54] who showed that after STC-1 cells were exposed to 100 µM linoleic acid, there were differences between the mRNA *CCK* levels and CCK content in the cell or secreted after either 30 min or 72 h. Another hypothesis is that CCK antibodies may be not specific to the CCK peptides. We found that digested samples induced higher *CCK* mRNA levels than undigested samples. This is probably due to the protein digestion, as it is already reported that protein hydrolysis leads to higher CCK response compared to undigested protein.[36] Digested or undigested linoleate/βlg complexes did not show modification of the linoleate impact on *CCK* mRNA levels, which may be caused by the high signal of the protein compare to the linoleate signal.

However, the *CCK* mRNA data seem contradictory with the cytotoxicity and the transport experiments. Indeed, these experiments demonstrated that less linoleate was free in presence of βlg. Thus, we would expect less linoleate to be in contact with the cell membrane and therefore a lower CCK response. However; the denaturation of the βlg is likely to have exposed unfolded proteins, which could increase the *CCK* mRNA response compared to the native protein.

This study has demonstrated that βlg modifies linoleate transport and FA metabolism in Caco-2 cells by altering FA bioaccessibility. Binding of linoleate to βlg induces protein oligomerisation and consequently influences protein susceptibility to digestion. Digested linoleate/βlg complexes delayed the cytotoxicity of the FA suggesting that part of FA is still in a complexed form. Binding of linoleate to βlg potentially alters linoleate uptake by altering levels of free FA. However, no effect on CCK secretion and *CCK* mRNA level was observed when linoleate was free or bound to βlg.

2.6 Abbreviations used

βlg, β-lactoglobulin; CCK, cholecystokinin; CLA, conjugated linoleic acid; DMEM, Dulbecco's modified Eagle medium; FA, fatty acids; FAME, fatty acid methyl ester; FBS, foetal bovine serum; GC, gas chromatography; GP-HPLC, gel permeation high performance liquid chromatography; LA, linoleic acid; LCFA, long chain fatty acid; LD_{50}, lethal dose 50; M_w, molecular weight; PBS, phosphate buffered saline; TEER, transepithelial electrical resistance.

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3 ADDITIONAL DATA

Peptide transporter *PEPT1* gene expression was studied to see if the presence of linoleate induced a change in the protein (digested and undigested) transport into Caco-2 cells (Table 6). Proteins have to be hydrolyzed into small peptides or amino acids in order to be taken up by the transporter PEPT1. Indeed, no protein transporter is known to exist because proteins are weakly transported intact, and when it does occur, it is performed by endocytosis or paracellular transport. As expected, non-digested samples of complexes and βlg (50 µM βlg) did not show any difference in the concentration of *PEPT1* mRNA. However, digested complexes showed an increase in the mRNA level compared to undigested samples, because of the formation of peptid which are known to be better transported in to enterocytes cells. Moreover, digested complexes increased the mRNA level in parallel to increasing linoleate/βlg molar ratio. Indeed, by increasing the linoleate/βlg molar ratio, we previously demonstrated an increase in oligomer generation. Therefore, it is likely that the increase in the amount of small peptides appearing after the *in vitro* digestions (Figure 24) are responsible for the increase of *PEPT1* mRNA level.

Table 6: PEPT1 mRNA transcript per µg total RNA from Caco-2 cells after 4 h exposure to undigested of digested linoleate/βlg complexes.

Sample	**10^3 mRNA transcript per µg total RNA of Caco-2**
βlg control	1992 ± 183
1X	2153 ± 607
2X	2071 ± 435
3X	1834 ± 308
Digested βlg control	4030 ± 371
Digested 1X	4246 ± 492
Digested 2X	4413 ± 406
Digested 3X	6680 ± 891
linoleate	328 ± 29

An increase of the mRNA level of *PEPT1,* leading to a higher peptide uptake, would highlight an increase in the FA uptake by βlg whether linoleate is able to bind to peptides. This could signify that a high range of FA uptake kinetics could be targeted, depending on the βlg hydrolysis.

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Chapter 3

Complexes between linoleate and native or aggregated β-lactoglobulin: Interaction parameters and *in vitro* cytotoxic effect

1 INTRODUCTION

Complexes formed between native β-lactoglobulin (βlg) and linoleate highlighted the impact of the molecular interaction on the structural and biological functions of the ligand and protein. However the different processes used in food industry, to reach specific microbial standards and/or food functionality, often result in βlg denaturation and aggregation. These non-native protein structures may have different affinities than native βlg for the ligand and consequently affect its biological properties. Hence in order to investigate interaction and biological properties of linoleate/βlg in food products, selected βlg aggregates were analysed. Additionally, results obtained in Part 3-Chapter 1 highlighted that the fatty acid (FA) solubility may be an issue to explain change in FA bioaccessibility when free or bound to proteins. To test this hypothesis, CLA was used in FA/βlg complexes, as an alternative FA to LA. CLA is an isomer of linoleic acid present in bovine milk. CLA has different physico-chemical properties, especially in term of solubility as it is about ten times less soluble than its isomer counterpart.

Therefore the objectives of this chapter are:

- To characterize the binding properties of linoleate to different βlg structures.
- To determine the changes of linoleate bioaccessibility when free or bound to different forms of βlg.
- To analyse the importance of the FA solubility on the complexes properties.

The interaction of hydrophobic ligands with native βlg, a βlg oligomer (covalent dimers of βlg) and βlg nanoparticles of controlled size were investigated. The different molecular structure of βlg provided insight into the importance of the protein structure on the ligand binding and the biological properties. Interaction constants were measured by intrinsic fluorescence and isothermal titration calorimetry. Linoleate bioaccessibiliy bound to the different βlg forms was measured using cytotoxicty assays. Finally, CLA/βlg complexes using native βlg, covalent dimers and nanoparticles were used to understand the impact of FA solubility on the complex binding properties and the FA cytotoxicity.

Main results:

- Linoleate binding capacity of βlg increase through its aggregation.
- Cytotoxicity of linoleate was modulated in function of the βlg form used as a carrier.
- Unlike linoleate, CLA was more cytotoxic when bound to βlg compared to the free form.

2 PAPER 3: COMPLEXES BETWEEN LINOLEATE AND NATIVE OR AGGREGATED β-LACTOGLOBULIN: INTERACTION PARAMETERS AND IN VITRO CYTOTOXIC EFFECT

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2.1 Abstract

The dairy protein β-lactoglobulin (βlg) is known to bind hydrophobic ligands such as fatty acids (FA). However, this protein is sensitive to environmental conditions commonly used during industrial processing. Therefore βlg is often in non-native form in food products. Structural changes of βlg can modify its binding affinity and capacity for ligands and thus impact on the biological properties of βlg/ligand complexes. The current study investigated the interaction of bovine βlg in different selected structural forms (native βlg, covalent dimer and nanoparticles) with sodium linoleate (C18:2). Using intrinsic fluorescence and isothermal titration calorimetry, linoleate was found to bind to βlg in two distinct sets of binding sites. Regardless of the structural state of βlg, the linoleate/βlg association constants remained in the same order of magnitude. However, the stoichiometry increased up to six fold for nanoparticles, compared to that of native βlg. The impact of these structural changes on FA bioaccessibility *in vitro* was measured by cytotoxic assays on Caco-2 cells. The order of cytotoxicity of linoleate was as follow: free linoleate > linoleate complexed to covalent dimers > linoleate complexed to βlg nanoparticles > linoleate complexed to native βlg. Therefore, it is proposed that the *in vitro* bioaccessibility of linoleate can be modulated by altering the state of βlg aggregation, which in turn affects its binding capacity to the FA.

Key words: β-lactoglobulin; Linoleate; Interaction; Aggregation; Cytotoxicity.

2.2 Introduction

β-lactoglobulin (βlg), the major whey protein in bovine milk, is present in a large number of food products. βlg is a member of lipocalin family, composed of 162 amino acids with a monomeric molecular weight of 18.4 kDa.[1] It contains nine β-strands labelled from A to I, and a three turns α-helices, that are arranged to form a globular protein structure.[2,3] Eight antiparallel β-strands are organised in a β-barrel, shaped into a hydrophobic calyx. Under physiological conditions, native βlg exists in a non-covalent dimer/monomer equilibrium. However, βlg structure is highly sensitive to processing conditions used in food industries, especially the heat treatments that are applied during food manufacture to reach specific food textures or to reduce microbial load.[4-7] Such treatments denature native βlg, leading to the formation of non-native monomers and aggregates of βlg in food products.[6]

βlg is able to bind small hydrophobic molecules such as fatty acids (FA),[3,8] and the formation of such complexes modifies FA digestion.[1,3,9] It has been suggested that native βlg binds hydrophobic ligands in its internal calyx and on surface binding sites. However, FA binding to the βlg is sensitive to the physicochemical conditions of the medium. Several studies related the decrease of association constants between βlg and binding FA with a decrease in pH. Below pH 6.2, the calyx binding site is closed by the EF loop region, decreasing interaction with hydrophobic components.[10-13] Additionally, Wang et al. (1998)[4] demonstrated that a decrease in the proportion of native βlg dimer increased βlg affinity constant for palmitate. A number of studies have assessed the interaction of ligands with heat treated βlg. However, these different studies have shown inconsistencies changes in the binding constants of such ligands with heat treated βlg compared to native form. This may be due to the nature of the ligand[12,14], or to differences in the applied heat treatments[14,15]. In fact, aggregates differ in the parts of protein exposed and therefore differ in how they react to heat.[6]

The essential long-chain fatty acid (LCFA) linoleic acid (LA, *cis,cis*-9,12-octadecadienoic acid, n-6, 18:2) constitutes 1-3 % (w/w) of the total FA found in bovine milk fat.[16] LA serves as an essential precursor to a number of long chain metabolites.[17,18] Its health benefits include anti-inflammatory effects, improvements in serum lipoprotein profiles and reduction in the risk of cardiovascular coronary artery disease.[19,20] Furthermore, LA, at high concentrations, is cytotoxic to cancerous cells *in vitro*.[21,22] However, bioaccessibility of FA is altered according to the structure of the food matrix.[23-25] We previously demonstrated an interaction between the water soluble form of LA, linoleate, and native βlg.[25] This binding alters the transport of linoleate into the cell by decreasing its bioaccessibility. However, as βlg is often in non-native forms in food products, we investigated whether βlg structure alters

the βlg/linoleate interaction and consequently the linoleate bioaccessibility. This could be of interest in relation to optimising the design of food products from a sanity, textural and health benefit perspective. Selected βlg aggregates of controlled size, covalent dimers and nanoparticles, were formed. Binding properties of native βlg, covalent dimers and nanoparticles with linoleate were measured by both isothermal titration calorimetry and intrinsic fluorescence. Bioaccessibility of Linoleate either free in solution or in complexes was measured by cytotoxicity assays.

2.3 Materials and methods

2.3.1 Materials

βlg (96 % purity) was obtained from Davisco Foods International, Inc. (Eden Prairie, Minnesota) and sodium linoleate (purity ≥98 %) from Sigma-Aldrich (St. Louis, MO). All other chemicals and solutions were purchased from Sigma-Aldrich unless stated otherwise.

2.3.2 Protein sample preparation and characterisation

2.3.2.1 Formation of β-lactoglobulin dimers and nanoparticles

Covalent dimers of βlg were formed using the protocol reported by Gulzar et al. (2009)[26]. Briefly, βlg was dissolved in a 5 mM Bis-Tris buffer (pH 6.7), the final protein concentration was 5 g/L. Copper chloride ($CuCl_2$) was added to the βlg solution at a Cu^{2+}/βlg molar ratio of 0.6. The solution was heated at 80°C for 30 min to form covalent dimers, then cooled on ice. Covalent dimers were first dialyzed against 10 mM NaCl (dialysis baths were changed every hour for 4 h) and then against distilled water for 48 h (water bath was changed twice). Samples were then freeze-dried and stored at -20°C prior to experiments.

Nanoparticles of βlg were formed according to the method of Schmitt et al. (2009).[27] Briefly, βlg was dissolved in Milli-Q water (Millipore, Carrigtwohill, Ireland), to a final protein concentration of 10 g/L. The pH of the protein solution was adjusted to 5.9 using 1 M HCl, before heating the solution at 85°C for 15 min, and then rapidly cooling on ice. Samples were dialysed for 48 h against an excess of distilled water, freeze-dried and stored at -20°C prior to experimental use.

2.3.2.2 Characterization of native β-lactoglobulin, covalent dimers and nanoparticles

2.3.2.2.1 Quantification of β-lactoglobulin concentration in reconstituted solutions

The concentration of native βlg and covalent dimers (expressed in monomer) were determined by optical density using the extinction coefficient of βlg at 278 nm (ε_{278} = 0.96 L/g/cm).

For nanoparticles, the concentration of βlg monomers was quantified on a reduced sample by the Bradford test following the manufacturer's instructions (Sigma-Aldrich). For reduction, 470 µL of nanoparticle sample (1 mg of powder/ml) was dissolved in phosphate buffered saline (PBS; 0.01 M phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4), 5 µL of 10 % SDS and 25 µL β-mercaptoethanol, and the mixture was heated at 95°C for 5 min.

2.3.2.2.2 Characterisation of β-lactoglobulin samples using gel permeation-HPLC

The proportion of monomers, dimers, oligomers and aggregates in βlg samples were determined by gel permeation-HPLC (GP-HPLC) using a TSK G SW guard column (7.5 × 7.5 mm, Tosoh Bioscience GmbH, Stuttgart, Germany) and a TSK G2000 SW column (7.5 × 600 mm, Tosoh Bioscience GmbH) connected to an HPLC system, consisting of a Waters 2695 Separations Module (Waters, Milford, MA) and a Waters 2487 Dual λ Absorbance Detector (Waters) working at 280 nm using Empower Pro software (Waters) to acquire and analyse data. Solvent with 30 % (v/v) acetonitrile (LabScan Analytical Sciences, Dublin, Ireland) and 0.1 % (w/v) trifluoracetic acid in Milli-Q water were used for protein elution at a flow rate of 0.5 mL/min. The molecular-weight of the different molecular entities in the samples was determined using a protein molecular-weight standard calibration set (Sigma-Aldrich).

The molecular entities present in each βlg sample were determined as follows: solutions of native βlg, covalent dimers and nanoparticles were prepared at 1 g/L in PBS. Nanoparticle solutions were centrifuged at 12000 g in order to separate nanoparticles (pellet) from smaller molecular entities (supernatant). Solutions of native βlg, covalent dimers and the supernatant of nanoparticle solutions were filtered (0.22 µm filter) prior to injection onto GP-HPLC. The proportions of monomers, dimers and higher size oligomers of βlg were determined from their relative GP-HPLC chromatographic peak area obtained using Apex Track integration, and the sample total chromatographic peak area. The proportion of monomers and aggregates in the nanoparticle samples were determined from their chromatographic peak area in the supernatant of the nanoparticle sample and the total chromatographic area of a solution of native βlg prepared at 1 g/L. The proportion of the different molecular entities for each of the βlg samples (native βlg, covalent dimers and nanoparticles) and of αla (impurity) are listed in Table 7. Native βlg sample contains 84.6 ± 1

% monomers, 5.4 ± 0.5 % dimers, 5.4 ± 0.4 % oligomers and 4.6 ± 0.4 % of α-lactalbumin (αla). Covalent dimers sample has 74.4 ± 3.1 % of dimers, 15.5 ± 1.4 % of residual monomers, 6.5 ± 1.6 % of oligomers. Nanoparticle sample has 77.6 ± 1.4 % of aggregates and 22.4 ± 1.4 % of monomers.

Table 7: Composition of the three structural forms of βlg used in this study determined by GP-HPLC. βlg M, βlg monomers; βlg D, βlg dimers; βlg Olig, βlg oligomers; NanoP, βlg nanopaticles; αla, α-lactalbumin (impurity). Results represent mean ± SD (n=3).

	Native βlg	**Covalent dimers**	**Nanoparticles**
αla	4.6 ± 0.4	3.6 ± 0.4	0.0 ± 0.0
βlg M	84.6 ± 1.0	15.5 ± 1.4	22.4 ± 1.4
βlg D	5.4 ± 0.5	74.4 ± 3.1	0.0 ± 0.0
βlg Olig	5.4 ± 0.4	6.5 ± 1.6	0.0 ± 0.0
NanoP	0.0 ± 0.0	0.0 ± 0.0	77.6 ± 1.4

2.3.2.2.3 Mean hydrodynamic diameter of nanoparticles

To check the homogeneity of the preparation, the mean hydrodynamic diameter of the aggregates in the nanoparticle sample was measured by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, Worcestershire, UK) equipped with a 4 mW helium/neon laser at a wavelength output of 633 nm. Particles sizing was performed at 25°C at 10 s intervals in a particle-sizing cell using backscattering technology at a detection angle of 173°. Results were the mean of 13 runs. The intensity of light scattered from the particles was used to calculate the mean hydrodynamic diameter (z-average mean), based on the Stokes-Einstein equation, assuming the particles to be spherical. The mean hydrodynamic diameter of aggregates (nanoparticles) was centered around 130 nm (Figure 31).

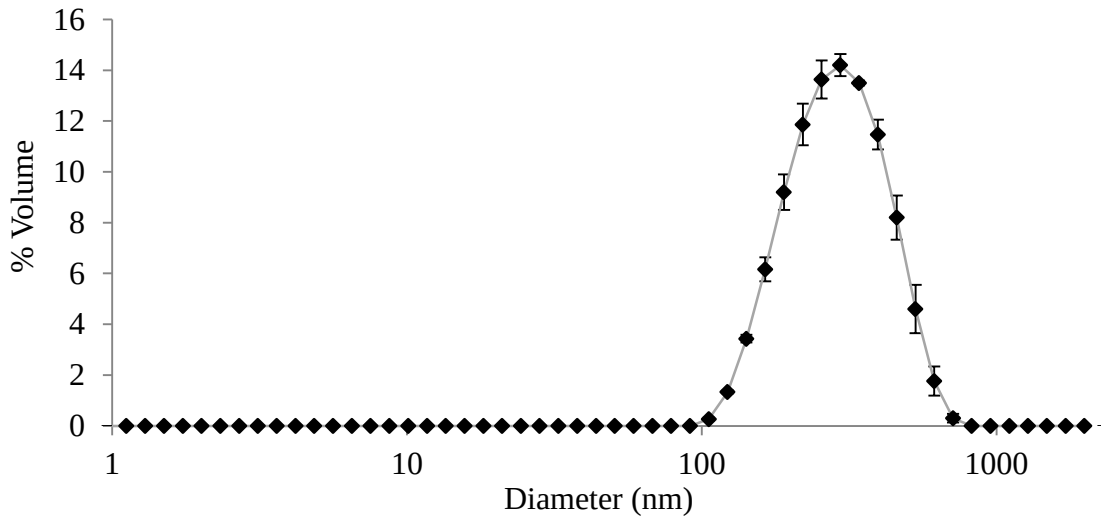

Figure 31: Size distribution of nanoparticles based on the volume of light scattered at 25°C. Nanoparticles were prepared by heating βlg (10 g/L, pH 5.9) at 85°C for 15 min. Results represent mean ± SD (n=3).

2.3.3 Linoleate/ β-lactoglobulin structure interaction

2.3.3.1 Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) was used to determine the interaction parameters between the different forms of βlg and linoleate. ITC experiments were performed on a VP-ITC microcalorimeter (Microcal, Northampton MA). Solutions of βlg (0.027 mM) and linoleate (1.65 mM) in PBS were degassed under vacuum before titration experiments. The reference cell was filled with PBS, and the sample cell (1.425 mL) was filled with βlg solution. βlg was titrated at 25°C with 29 successive linoleate injections of 10 µL. The injection time was 20 s, and the time between injections was fixed at 600 s to achieve thermodynamic equilibrium. During titrations, the solution in the sample cell was stirred at 310 rpm to ensure complete mixing. The control measurement was obtained by titrating sodium linoleate into PBS buffer using the same injection procedure. The control measurement was subtracted from the βlg titration with linoleate and the first injection peak was systematically ignored for the data analysis. Data were analysed using MicroCal ORIGIN version 7.0 (Microcal). The integrated area of each peak was plotted versus the linoleate/βlg monomer molar ratio. The “two sets of binding sites” model was the best fit for all experiments, providing the binding parameters K_{a1}, K_{a2} , n_1, and n_2 (K_a and n are the association constant and the stoichiometry, respectively). Each measurement was performed in triplicate.

2.3.3.2 Intrinsic fluorescence

Intrinsic fluorescence spectra were recorded at 345 nm using an excitation wavelength of 278 nm. For each titration, a fluorescence spectrum was recorded from 300-450 nm in order to observe deviation in fluorescence properties of the protein. Experiments were performed at 25°C on a SPEX 112 spectrofluorometer (Jobin-Yvon, Longjumeau, France), using 10 × 10 mm quartz cuvette. Excitation and emission slits were both set to 5 nm. βlg solutions in PBS (3 mL at 10 µM) were titrated with successive 3 µL injections of 5 mM linoleate, upto a linoleate/βlg molar ratio of 10. The solution was agitated by pipetting up and down several times and a 5 min equilibrium time was respected prior to each measurement. An N-acetyl-tryptophanamide (NATA) blank was titrated following the same procedure in order to subtract the inner filter effect caused by the FA. NATA fluoresces similarly to tryptophan but does not bind FA (Cogan et al., 1976)[28]. The concentration of NATA was chosen to have the same initial fluorescence (without FA) as the fluorescence of βlg solutions. Fluorescence of NATA was subtracted from fluorescence intensity measurements of the ligand/protein complexes for all the linoleate/βlg molar ratios tested. Each measurement was performed in triplicate. Fluorescence data were fitted using two different methods.

In method 1, L_{free}, L_{total} and L_{bound} represent the concentration of free, total and bound linoleate, respectively, P_{total} is the concentration of βlg, v is the fraction of linoleate molecules bound per mole of protein (v varies from 0 to n), n the number of linoleate bound to βlg at saturation (number of sites), and f_i the fraction of one site of the protein to be occupied by a ligand (f_i varies from 0 to 1). Then:

$$L_{total} = L_{free} + L_{bound} \quad (1)$$

$$v = \frac{L_{bound}}{P_{total}} = nf_i \quad (2)$$

Combining equations (1) and (2) we deduce that:

$$L_{total} = L_{free} + nP_{total}f_i \quad (3)$$

The value of fi is determined using the initial fluorescence intensity (F_0), the fluorescence intensity at saturation (F_{max}) and the fluorescence intensity at the ratio ligand/protein i (F_i) as indicated in equation (4):

$$f_i = \frac{F_i - F_0}{F_{max} - F_0} \quad (4)$$

When F_{max} was not reached experimentally, it was determinated by fitting using an exponential phase decay model on Graph-Pad Prism software. The value of n was determined by plotting L_{total} in function of $P_{total}f_i$. The data were fitted using a sequential linear regression in Graph-Pad Prism software 3.03 (GraphPad Software Inc., La Jolla CA).

Method 2 is an adaptation of the Scatchard plot. In the Scatchard plot described below, K_a is the association constant:

$$\frac{\nu}{L_{free}} = nK_a - \nu K_a \qquad (5)$$

Equations (3) and (5) can be rearranged as: $P_{total}(1-f_i) = \frac{L_{total}}{n}\left(\frac{1}{f_i} - 1\right) - \frac{1}{nK_a}$ (6)

By fitting this equation using Graph-Pad Prism software, n and K_a were determined.

2.3.4 Preparation of linoleate/β-lactoglobulin complexes for biological assay

2.3.4.1 Preparation of complexes

Linoleate/βlg complexes were prepared by mixing βlg samples with sodium linoleate according to Lišková et al. (2011)[29] with modifications as described in Le Maux et al. (2012)[25]. Briefly, 0.163 mM βlg, in its native form, covalent dimers or nanoparticles, were dissolved in PBS, and sodium linoleate was added to reach final linoleate/βlg molar ratios of 5, 7.5 or 10. Solutions containing native βlg were heated at 60°C for 30 min to facilitate βlg/linoleate interaction and rapidly cooled on ice. Solutions containing covalent dimers or nanoparticles were mixed overnight at room temperature. Samples were dialysed against distilled water for 72 h with dialysis bags of nominal cut-off of 3500 Da. Samples were freeze-dried and powders stored at -20°C prior to experiments.

2.3.4.2 Determination of fatty acid content by gas chromatography

The FA content of the complexes was determined by gas chromatography (GC) following a protocol adapted from Palmquist and Jenkins (2003)[30] and Coakley et al. (2003)[31] and described in detail previously[25]. Briefly, the internal standard tridecanoic acid (C13:0) was added to ~4 mg of complexes. FA were converted to fatty acid methyl esters (FAME) and were analysed using a CP-SELECT CB column for FAME (100 m, 0.25 mm, 0,25 µm film thickness, Varian BV, Middelburg, the Netherlands), adaptated on a Varian 3400 GLC (Varian, Walnut Creek, CA) connected to a flame ionization detector.

2.3.4.3 Complexes analysis by polyacrylamide gel electrophoresis

Samples were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Mini-PROTEAN TGX precast Gels (4-20 % resolving gel, Bio-Rad Laboratories Inc., Hercules, CA) were used on a Mini Protean II system (Bio-Rad) according to the manufacturer's instructions. Samples were prepared under non-reducing (in the absence of β-mercaptoethanol) and reducing (in the presence β-mercaptoethanol)

conditions. Protein was visualized by staining with Coomassie blue (Bio-Safe Coomassie Stain G-250, Bio-Rad). An Amersham Low Molecular Weight Calibration kit (14.4 to 97 kg/mol, GE Healthcare UK Limited, UK) was used as molecular weight standards.

2.3.5 Cell Culture and cytotoxicity assay

The Caco-2 cell line was purchased from the European Collection of Cell Cultures (collection reference: ECACC 86010202). It was derived from human colonic adenocarcinoma cells and can mimic the enterocytes of the intestine.

Cells cultures were maintained in a humidified 37°C incubator with a 5 % CO_2 in air atmosphere. Cells were routinely grown in 75 cm^2 plastic flasks in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/L glucose and 0.584 g/L L-glutamine. Media for subculture was supplemented with 10 % (v/v) foetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin. At 80 % confluency, cells were trypsinised with 0.25 % trypsin/EDTA, diluted 1:6 in media and reseeded. The growth medium was changed three times a week. All cells used in these studies were between passage number 20 and 31.

Cytotoxicity of test samples on Caco-2 cell proliferation was determined by MTS assay, using CellTiter 96 Aqueous One Solution Cell Proliferation Assay according to the manufacturer's instructions (Promega Corporation, Madison, Wisconsin) and previously described in Le Maux et al. (2012)[25]. Briefly, 96-well plates were seeded with 2×10^4 Caco-2 cells/well, using serum-free media. After 24 h, cells were treated with different concentrations of linoleate (0 to 200 µM) or linoleate/βlg complexes (higher linoleate/βlg complex which contained 0 to 200 µM linoleate as determined by GC) in serum-free media for 24 h. After the use of One Solution Cell Proliferation reagent, viability was defined as the ratio of absorbance of treated cells to untreated cells (cells exposed to serum-free Media only) at 490 nm. Cells exposed to the different controls of βlg were subtracted to the corresponding samples. Each cell exposure was performed in triplicate.

The Lethal Dose 50 (LD_{50}) values, the concentration required to decrease the cell viability by 50 %, were determined using Graph-Pad Prism software 3.03 (GraphPad). The sigmoidal dose-response with variable slope was used to fit the measured curves and calculate LD_{50}.

2.3.6 Statistical analysis

Where appropriate, results were statistically analysed using the R software package version 2.15.1 (R Foundation for Statistical Computing, Vienna, Austria) and the ANOVA system with a Tukey's least significant difference comparison. *P*-Values less than 0.05 were deemed to be statistically significant.

2.4 Results

2.4.1 Binding properties of the different β-lactoglobulin forms with linoleate

Binding parameters, determined at 25°C using ITC and intrinsic fluorescence spectroscopy, were expressed on the basis of βlg monomeric units.

ITC data revealed an exothermic signal for the interaction between linoleate and all the βlg forms tested. Increasing the amount of linoleate in the titration cell resulted in a progressive decrease of the exothermic signal due to the saturation of the binding sites (Figure 32). Regardless of the states of βlg aggregation, the data were best fitted with a two sets of binding sites model. The number of binding sites for each set of binding sites (n) and the corresponding association constant (K_a) could be determined from the fitted curves (Table 8). Similar association constants were observed for all βlg forms for each set of binding sites. The K_a values for the first and second sets of binding sites were close to 10^6 M^{-1} and 10^4 M^{-1}, respectively. Molar ratio of linoleate bound to βlg monomer (n) varied for the first set of binding sites between 0.53 ± 0.08 for covalent dimers and 0.92 ± 0.29 for nanoparticles. For the second set of binding sites, n varied somewhat more: native βlg (6.79 ± 0.05), covalent dimers (8.64 ± 0.54) and nanoparticles (10.25 ± 1.64).

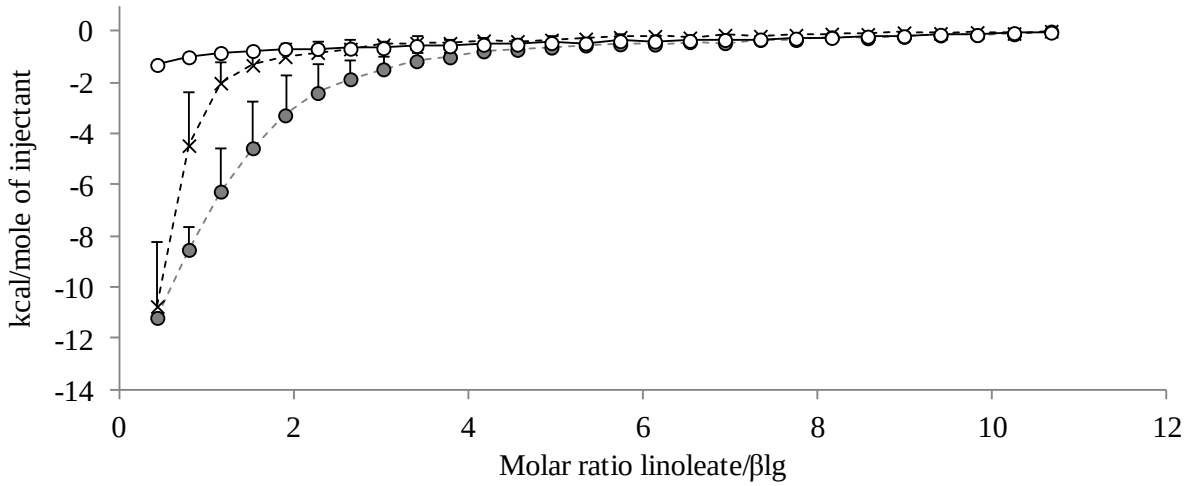

Figure 32: ITC titration of linoleate in different forms of βlg in PBS buffer (pH 7.4) at 25°C. βlg (0.027 µM) were titrated with increments of 10 µL linoleate (1.65 µM). The raw heat signals were integrated and plotted against the linoleate/βlg molar ratio. Linoleate/native βlg, white circle with black plain line; linoleate/covalent dimers βlg, black cross with dashed black line; linoleate/nanoparticles βlg, grey circle with dashed grey line.

Table 8: Stoichiometry of linoleate/βlg with different forms of βlg obtained by ITC. Linoleate (1.65 µM) was titrated in 0.027 µM βlg (native, covalent dimers and nanoparticles) at 25°C. Association constant K_a and molar ratio n of linoleate/βlg were determined per βlg monomer with a "two set of binding sites" model, n_1 K_{a1} and n_2 K_{a2} being the binding constant for the first and second binding sites, respectively. Results represent mean ± SD (n=3).

	Linoleate/native βlg	Linoleate/covalent dimers	Linoleate/nanoparticles
n_1	0.60 ± 0.01	0.53 ± 0.08	0.92 ± 0.29
$K_{a1}\times10^5$ M^{-1}	17.95 ± 6.29	15.13 ± 9.53	15.83 ± 3.35
n_2	6.79 ± 0.05	8.64 ± 0.54	10.25 ± 1.65
$K_{a2}\times10^5$ M^{-1}	0.41 ± 0.05	0.50 ± 0.42	0.57 ± 0.22

Intrinsic fluorescence titration is based on the change in the intensity of βlg tryptophan fluorescence. The maximum emission wavelength was 345 nm, 353 nm and 350 nm for native βlg, covalent dimers and nanoparticles, respectively; therefore aggregated βlg caused a red shift. However, the fluorescence spectra had a similar shape for all the βlg forms tested and the changes in fluorescence intensity consecutive to linoleate addition were correlated at the three wavelengths. Therefore the fluorescence changes were followed at 345 nm, which is the wavelength of maximal fluorescence intensity of native protein. In the titration range used in this study, the change in fluorescence intensity reached a maximum of 10.5 ± 1.3 %, 21.7 ± 1.6 % and 32.2 ± 2.1 % from the initial fluorescence intensity for native βlg, covalent dimers and nanoparticles, respectively (Figure 33). Increasing the linoleate concentration in native βlg samples induced an increase in fluorescence intensity at 345 nm. This increase levels off when the linoleate/βlg molar ratio reaches 3. In contrast, the fluorescence intensity of the covalent dimers and of the nanoparticles decreased continuously up to a linoleate/βlg molar ratio of 10. For each titration, fluorescence data were fitted with two different models.

In the first model the total concentration of linoleate is plotted as a function of total concentration of protein and variation in fluorescence intensity ($P_{total}.f_i$). It gave access to the number of binding sites (n), which are determined from the slope of the graphical representation. For the entire titration, the graphical representation can be fitted with two straight lines, indicating the presence of two sets of binding sites (Table 9). The number of binding sites varied according to the βlg forms. From linoleate/native βlg to linoleate/nanoparticles complexes, n_1 increased from 2.38 ± 0.12 to 15.74 ±0.55 and n_2 from 6.02 ± 0.29 to 40.73 ± 2.17.

The second model was an adaptation of the Scatchard plot, in which the maximum fluorescence (F_{max}) was required for the plot construction. However, F_{max} was not reached with a 10 linoleate/βlg molar ratio for the complexes made with the covalent dimers and the

nanoparticles. Therefore, the fit of the Scatchard plot was obtained using the experimental F_{max} only for the complex made of linoleate and native βlg. An extrapolated F_{max} was used for the Scatchard plot of the linoleate/covalent dimers complex (Table 9). Unfortunately, the fluorescence data for the linoleate/nanoparticles complexes could not be fitted correctly using extrapolated F_{max}. The thermodynamic constants (K_a) for the two sets of binding sites were 9.20 ± 2.65 × 10^5 M^{-1} and 0.62 ± 0.49 × 10^5 M^{-1} for the linoleate/native βlg complex and 14.67 ± 2.12 × 10^5 M^{-1} and 0.37 ± 0.13 × 10^5 M^{-1} for the linoleate/covalent dimers complex. These values of association constants were in the same range than those deduced from ITC data. The stoichiometry n_1 was 2.45 ± 0.07 and 10.31 ± 0.05 while n_2 was 5.27 ± 1.5 and 15.29 ± 0.71 for linoleate/native βlg and linoleate/covalent dimers complexes respectively.

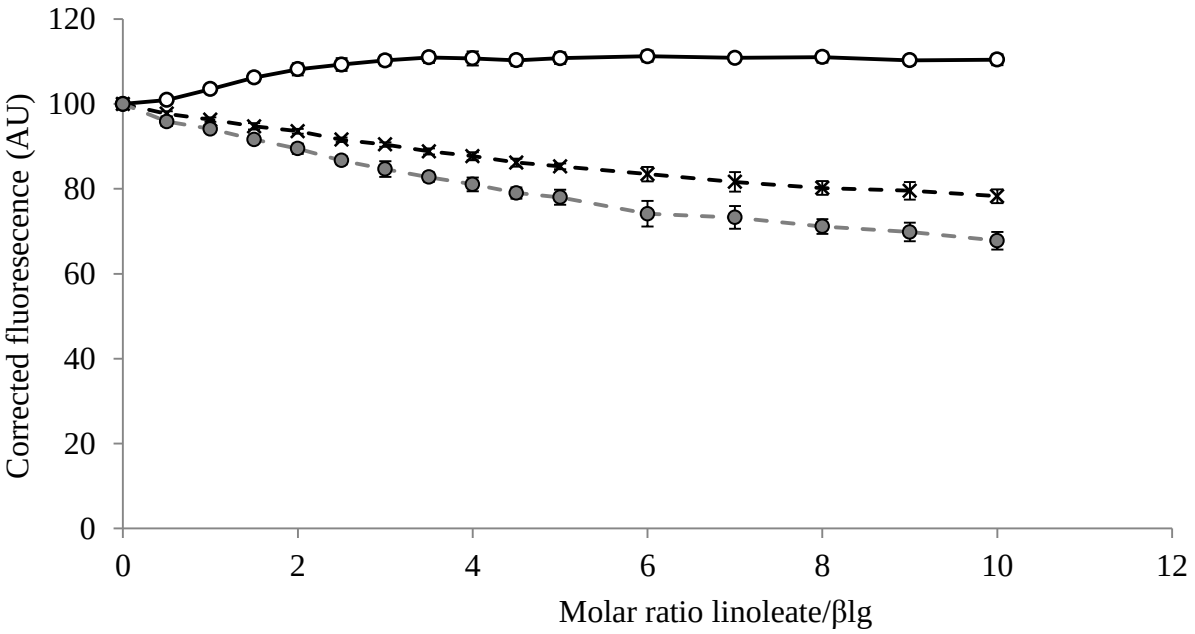

Figure 33: Intrinsic fluorescence of different forms of βlg titrated with linoleate. Native βlg, covalent dimers and nanoparticles (10 µM) were titrated with linoleate (5 mM) at 25°C. Linoleate /native βlg, white circle with black plain line; linoleate/covalent dimers βlg, black cross with dashed black line; linoleate /nanoparticles βlg, grey circle with dashed grey line. Results represent mean ± SD (n=3).

Table 9: Stoichiometry of linoleate/βlg with different forms of βlg obtained by fluorescence. Linoleate (5mM) was titrated in 10 µM βlg (native, covalent dimers and nanoparticles) at 25°C. Sequential linear regression (L_{total} = f(F × P_{total})) model was used to determine n. Association constants K_a and molar ratio n of linoleate/βlg were determined per βlg monomer using a modified Scatchard method. Experiments with linoleate binding to βlg nanoparticles could not be fitted using modified Scatchard method (non applicable, NA). n_1 K_{a1} and n_2 K_{a2} being the binding constant for the first and second binding sites, respectively. Results represent mean ± SD (n=3).

	L_{total} = f(F×P_{total})			**Modified Scatchad**		
	Linoleate/ native βlg	**Linoleate/ dimer**	**Linoleate/ nanoparticle**	**Linoleate/ native βlg**	**Linoleate/ dimer**	**Linoleate/ nanoparticle**
n_1	2.38 ± 0.12	9.8 ± 0.21	15.74 ± 0.55	2.45 ± 0.07	10.31 ± 0.05	NA
K_{a1}×10^5 M^{-1}				9.20 ± 2.65	14.67 ± 2.12	
n_2	6.02 ± 0.29	12.54 ± 0.76	40.73 ± 2.17	5.27 ± 1.50	15.29 ± 0.71	
K_{a2}×10^5 M^{-1}				0.62 ± 0.49	0.37 ± 0.13	

2.4.2 Changes in the structure of the linoleate/β-lactoglobulin complexes

Complexes of linoleate with native βlg, covalent dimers and nanoparticles were analysed by SDS-PAGE and GP-HPLC in order to identify changes in the aggregation state of βlg following linoleate interaction. Previously we demonstrated that native βlg aggregated into dimers and oligomers in the presence of linoleate (Le Maux et al., 2012[25]). Figure 34A confirms this observation with SDS-PAGE analysis of native βlg, under non-reducing conditions, showing a major band corresponding to the βlg monomer with small amount of dimers and trimers. The presence of linoleate increases the amount of βlg dimers and oligomers at the expense of βlg monomers. In contrast, the presence of linoleate had almost no effect on covalent dimers except a slight decrease in the intensity of the residual βlg monomer band (Figure 34B). A similar result is obtained for the SDS-PAGE of the complexes made with nanoparticles (Figure 34C). Under reducing conditions, SDS-PAGE for all the complexes and the βlg controls (without linoleate) were similar. Figure 34D is a representation of these results depicting nanoparticles and linoleate/nanoparticles complexes prepared at three different linoleate/βlg molar ratio (5, 7.5 or 10), under reducing conditions. Taking the non-reducing and reducing results together, linoleate induced aggregation of βlg stabilised by intermolecular disulphide bonds.

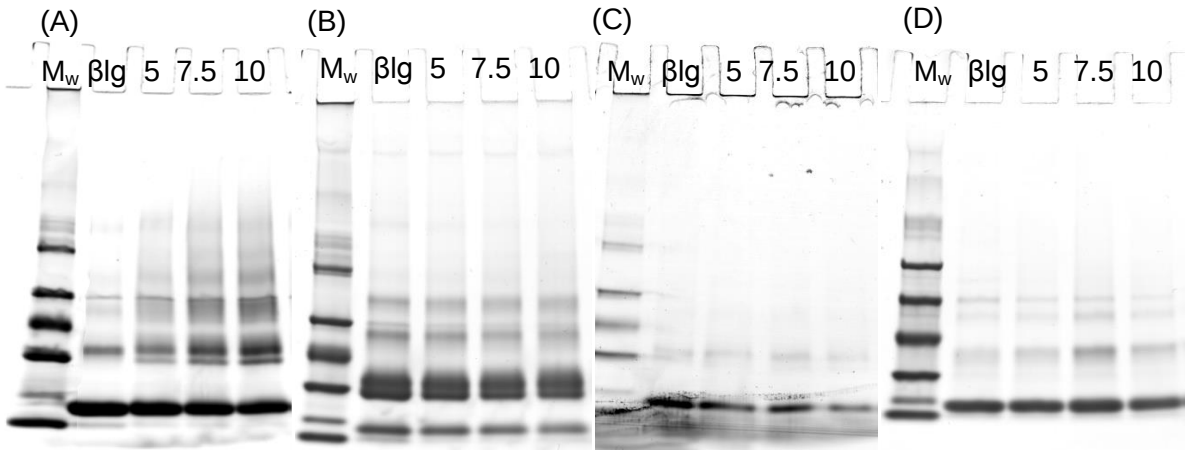

Figure 34: SDS-PAGE profile of linoleate/βlg complexes under non-reducing and reducing conditions. (A) Non-reducing gel of linoleate/native βlg complexes. (B) Non-reducing gel of linoleate/covalent dimers complexes. (C) Non-reducing gel of linoleate/nanoparticles complexes. (D) Reducing gel of linoleate/nanoparticles βlg complexes, reducing gels of other complexes are not presented due to their similitudes. M_w, molecular weight markers (14.4, 20.1, 30, 45, 66, 97 kDa); βlg, βlg control; lanes 5, 7.5 and 10, complexes with an initial molar ratio of 5, 7.5 and 10 linoleate/βlg, respectively.

GP-HPLC chromatograms of complexes formed with native βlg, covalent dimers and nanoparticles were integrated and the proportion of βlg monomers, dimers and oligomers (trimers and tetramers) as a function of the initial linoleate/βlg molar ratio are shown in Table 10. A decrease in the concentration of monomers in the presence of linoleate were observed for all the βlg forms, in agreements with the SDS-PAGE experiments. The monomeric proportion decreased from 88.5 ± 5.2 % to 51.1 ± 4.9 % using native βlg, from 16.3 ± 1.5 % to 13.4 ± 0.6 % for complexes using covalent dimers and from 22.4 ± 1.4 % to 10.6 ± 1.9 %, for complexes using nanoparticles with an initial molar ratio of linoleate/βlg varying from 0 to 10. Concomitantly, an increase of the protein aggregation was also observed. As predicted the difference in aggregation by increasing the linoleate/βlg molar ratio was more pronounced for native βlg than the other forms of βlg assayed.

Table 10: Protein proportion of linoleate/βlg complexes with different forms of βlg (native, covalent dimers and nanoparticles), obtained by GP-HPLC. 0, 5, 7.5 and 10 represents the initial molar ratios of linoleate/βlg. βlg M, βlg monomers; βlg D, βlg dimers; βlg O, βlg oligomers; NanoP, βlg nanopaticles. Results represent mean ± SD (n=3).

Initial linoleate/βlg		0	5	7.5	10
Linoleate/ native βlg	βlg M	88.45 ± 5.24	66.44 ± 8.24	60.43 ± 8.17	51.09 ± 4.95
	βlg D	6.64 ± 2.63	23.13 ± 6.94	28.22 ± 9.57	34.94 ± 6.78
	βlg O	4.92 ± 2.78	10.43 ± 4.51	11.35 ± 2.03	13.97 ± 3.97
Linoleate/ dimer	βlg M	16.29 ± 1.50	14.43 ± 0.50	14.05 ± 0.19	13.39 ± 0.61
	βlg D	78.27 ± 3.21	78.62 ± 4.23	79.55 ± 5.80	77.88 ± 6.48
	βlg O	5.43 ± 1.65	6.95 ± 2.70	6.41 ± 3.74	8.72 ± 4.22
Linoleate/ nanoparticle	βlg M	22.41 ± 1.36	15.82 ± 2.48	11.96 ± 1.03	10.65 ± 1.95
	NanoP	77.59 ± 1.36	84.18 ± 2.48	88.04 ± 1.03	89.35 ± 1.95

2.4.3 Cytotoxicity of linoleate bound to the different forms of β-lactoglobulin

The effect of linoleate (0 to 200 μM), bound to the different forms of βlg, on Caco-2 cell viability was measured. For quantifying the effect of the bound linoleate only, the complexes were dialysed to remove unbound linoleate. After dialysis, the exact stoichiometry of linoleate/βlg complexes was determined from freeze-dried complexes using GC (Figure 35). The amount of linoleate bound to βlg increased when the initial linoleate/βlg molar ratio was increased. This increase varied depending on the βlg form with more linoleate binding increasing in the order of nanoparticles > covalent dimers > native βlg. Only the complexes prepared with the higher linoleate/βlg molar ratio were used for cytotoxicity experiments (Figure 36). No cytotoxic effect was detected for any of the βlg forms used at the concentrations assayed when employed in the absence of linoleate (data not shown). Free linoleate has a LD_{50} of 58.0 ± 4.2 μM[25]. Comparatively, the LD_{50} of the complexes were all significantly different (p<0.001). The linoleate/native βlg complex was not cytotoxic to Caco2 cells at the concentrations tested (LD_{50} >> 200 μM complex). LD_{50} was 80.0 ± 3.1 μM for linoleate/covalent dimers complex, and 189.0 ± 4.1 μM for linoleate/nanoparticles complex.

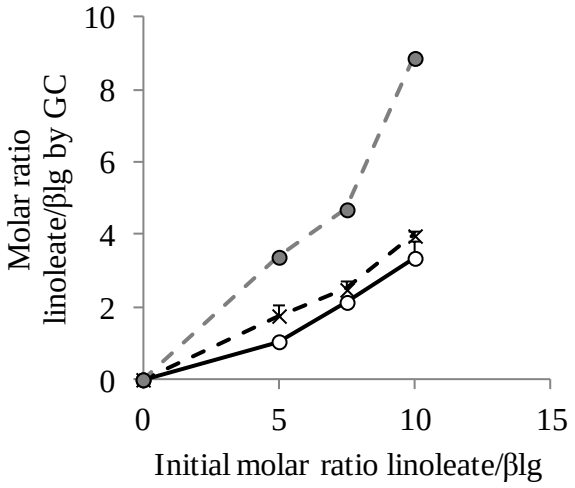

Figure 35: Stoichiometry of linoleate/βlg with different forms of βlg (native, covalent dimers and nanoparticles) as determined by GC after dialysis. Correlation of the molar ratios of linoleate/βlg added to the starting solutions with the molar ratios of linoleate/βlg that were detected by GC analysis in the linoleate/βlg samples after extensive dialysis and freeze-drying. Linoleate/native βlg, white circle with black plain line; linoleate/covalent dimers βlg, black cross with dashed black line; linoleate/aggregates βlg, grey diamond with dashed grey line.

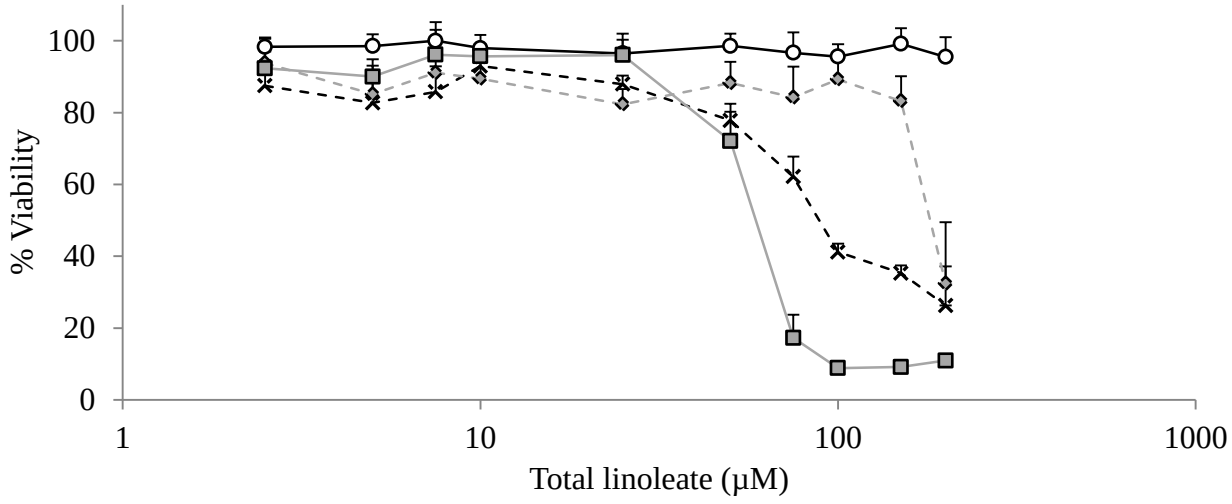

Figure 36: Cytotoxicity of linoleate, free or bound to different forms of βlg, using Caco-2 cells. Cell viability after 24 h on 2 × 10^4 Caco-2 cells compared to control cells was assessed using an MTS assay. Linoleate concentrations in the tested sample varied from 0 to 200 µM. Free linoleate, grey square with black plain line; linoleate/covalent dimers βlg, black cross with dashed black line; linoleate/aggregates βlg, grey diamond with dashed grey line; linoleate/native βlg, white circle with black plain line. Results represent mean ± SD (n=3).

2.5 Discussion

The structural state of βlg modified its binding properties to linoleate. This was demonstrated using βlg intrinsic fluorescence and ITC measurements albeit the determined stoichiometry of the two techniques differed slightly. The number of binding sites determined from ITC data for the interaction between linoleate and native βlg showed lower n_1 value, but a higher n_2 value compared to those deduced from intrinsic fluorescence data. However, the total number of binding sites (n_1 + n_2) for linoleate to native βlg was similar (around 7.5 to 8 linoleate bound to the βlg native protein) regardless of technique and method used for data fitting. The binding parameters from βlg intrinsic fluorescence titration gave a higher number of binding sites for linoleate to covalent dimers and to nanoparticles than the ITC data. This discrepancy may have resulted from (i) the intrinsic fluorescence data that cumulates inner filter and non-specific quenching of the fluorescence spectrum of the complex under study and/or (ii) the ITC signal complexity that includes all energetic changes occurring during the titration such as structural changes of protein, modifications to protein and/or ligand hydration.[32] Similarly, Loch et al. (2012)[33] found a stoichiometry lower than 1 mole for lauric and myristic acids per mole of βlg when the interaction was studied by ITC while one FA was found in the calyx of native βlg by Xray crystallography with resolution 1.9-2.1 Å. According to these authors, this may be related to the weak interaction between the FA and βlg. Spector and Fletcher (1970)[34] demonstrated that stearic acid exhibited a secondary set of binding sites to βlg with the number of sites varying from 2 to 24, using the same set of data analyzed with different fitting parameters.

Comparative analysis of the fluorescence data show differences in the fluorescence changes for the native βlg experiments relative to the aggregated βlg experiments. The intrinsic fluorescence of covalent dimers and nanoparticles decreased the titration of linoleate due to tryptophan quenching by the FA. Conversely, the intrinsic fluorescence of native βlg increased in the presence of linoleate. This can be explained by the compensation of the tryptophan quenching effect by the denaturation of the protein caused by the binding with linoleate, which reduced the tryptophan quantification by Cys-Cys disulphide bonds.[35-37]

The number of linoleate bound per βlg molecule increased with the degree of aggregation (native βlg < covalent dimers < nanoparticles) but the association constants for each sets of binding sites remained similar. Several studies have demonstrated the impact of βlg denaturation/aggregation for ligand binding, but were dependent on the type of ligands and/or the structure of the aggregates.[38,39] Hydrophobic ligands are able to bind native βlg on hydrophobic patches of the protein surface and in the internal calyx if specific structural

properties of the ligands are respected.[1] The changes in binding parameters are related to the structural changes of βlg, which occur during heat denaturation/aggregation.[6] Heat-induced protein unfolding exposes internal hydrophobic patches[6] that constitute additional potential binding sites for hydrophobic ligands. Even if they are usually of low specificity and low affinity, these hydrophobic patches could be responsible for the higher ratio of linoleate bound per βlg molecule in the covalent dimers and nanoparticles compared to the native form of βlg. The higher degree of aggregation in the nanoparticles, compared to covalent dimers, could also create hydrophobic pockets, trapping more ligands with weak affinity. Indeed, nanoparticles are microgels, which have more hydrophobic binding sites available compared to native βlg as shown by anilino naphthalene sulfonic acid (ANS) fluorescence.[27] In addition, the internal calyx of βlg is modified during the heat-denaturation and aggregation of βlg. Consequently the specific affinity to the ligand at this site could be affected. The formation of covalent dimers involves the displacement of the free Cys121 that potentially distorts the calyx, decreasing its affinity for linoleate. O'Neil and Kinsella (1988)[15] showed that heat-denaturation of βlg (75°C up to 20 min) increased the number of binding sites for 2-nonanone but decreased its association constant. Yang et al. (2009)[40] found a weaker binding, with a lower n, when vitamin D_3 was bound to heat denatured βlg (100°C for 16 min) compared to native βlg. Similar conclusions were reported by Spector and Fletcher (1970)[34] who found lower binding constants between palmitate and βlg when the protein was heat treated from 55 to 80°C. These different ligands were shown to specifically interact in the calyx of βlg that is affected by the βlg denaturation. Unlike these studies, conformational changes of βlg do not lead to a change in the affinity for linoleate at the first set of binding sites. This is rather surprising, since the central cavity contains the binding site with strongest affinity for linoleate, as shown by 2.1 Å resolution crystallography (PBD ID: 4DQ4[41]). However, it is possible that some specific protein structure are selected for crystal formation leading to different results when protein in solid or liquid states are compared.

Cytotoxic assays represent an excellent method for determining changes in the bioaccessibility of FA to Caco-2 cells since the linoleate must enter cells to be cytotoxic.[21,22,42] In the present study, exposure of the cells to linoleate/βlg complexes resulted in a decrease in cytotoxicity compared to free linoleate. Therefore, we can postulate that binding of linoleate to all the βlg forms decreased the bioaccessibility of the FA. After a 24 h exposure period, linoleate bound to βlg nanoparticles had a higher cytotoxic effect compared to linoleate bound to native βlg. This could be explained by the higher binding capacity of nanoparticles for the FA compared to native βlg: 8.9 linoleate per βlg nanoparticles versus 3.3 linoleate per native βlg. As only 0.6 or 0.9 linoleate is strongly bound per 1 βlg molecule

in the nanoparticular or native state, respectively (ITC data), the fraction of linoleate bound with a lower affinity is much higher for the nanoparticles. This may explain the higher bioaccessibility of linoleate when bound to the nanoparticles. Spector and Fletcher (1970)[34] demonstrated that palmitate bound to βlg was taken up faster by Ehrlich ascites tumor cells compared to palmitate bound to bovine albumin because palmitate binds to bovine albumin with a higher affinity than to βlg. Consequently, the FA was more bioaccessible to the cells when bound to βlg. Interestingly, linoleate/covalent dimer complexes were more cytotoxic than linoleate/nanoparticles complexes, even though the amount of linoleate bound with higher K_a was similar, as determined by ITC. As native βlg protects the cells against the linoleate cytotoxicity, this difference in cell viability may be the result of the different proportions of βlg monomers present in the test samples (22.4 % βlg monomers in the nanoparticle sample compared to 16.3 % βlg monomers in the covalent dimer sample, prior to the addition of linoleate). In addition, to obtain the same linoleate concentration, a higher quantity of complex was needed for the linoleate/covalent dimers complex. The molar ratios were 8.9 linoleate per βlg in the nanoparticles versus 4.0 linoleate per βlg in covalent dimers. However, we have previously demonstrated that increasing βlg concentration increased the linoleate uptake by Caco-2 cells even if the kinetic of transport is slower than free linoleate (Chapter 2). Other studies have reported the opposite effect, with the binding of a given ligand to βlg increasing the ligand bioaccessibility. Indeed, Yang et al. (2009)[40] observed that vitamin D_3, which is practically insoluble in water, was transported more effectively bound to βlg than free vitamin D_3 in a mouse model. Proteins may affect differently the bioaccessibility of the ligand in function of the ligand solubility. The potential contribution of residual copper used to prepare βlg covalent dimer in the cytotoxic effect of this oligomer cannot be excluded. Copper by itself at concentrations up to 5 mg/L was not cytotoxic (see additional data below, Chapter 3 3.3-Copper detection and impact on linoleate cytotoxicity). However, copper was reported to be a potent catalyst of FA oxidation.[43,44] Peroxidated FA are reported to be more cytotoxic than FA.[45] Hence, the occurrence of a peroxidated form of linoleate which would increase its cytotoxicity cannot be ruled out.

This study has demonstrated that linoleate can bind to different structural states of βlg (native, covalent dimers, nanoparticles). Binding capacity but not affinity was affected by the protein structure. Stoichiometries increased with the size of the protein aggregates. This is probably due to the exposure of hydrophobic sites during the protein denaturation and the formation of hydrophobic pockets at the surface or in the inner structure of the aggregates. Changes in the binding properties modified the cytotoxicity of the complexes. Consequently, it is proposed that the *in vitro* bioaccessibility of linoleate can be modulated by changing protein structures, which subsequently modifies the ligand binding parameters.

2.6 Abbreviations

αla, α-lactalbumin; βlg, β-lactoglobulin; CLA, conjugated linoleic acid; CMC, critical micelle concentration; DMEM, Dulbecco's modified Eagle medium; FA, fatty acid; FAME, fatty acid methyl ester; FBS, foetal bovine serum; GC, gas chromatography; GP-HPLC, gel permeation high performance liquid chromatography; ITC, isothermal titration calorimetry; K_a, association constant; LA, linoleic acid; LCFA, long chain fatty acid; n, reaction stoichiometry; NATA, N-acetyl-tryptophanamide, PBS, phosphate buffered saline;

2.7 Acknowledgements

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3 ADDITIONAL DATA

3.1 Atomic force microscopy

Atomic force microscopy (AFM) was used to visualise the homogeneity of structure and size of βlg nanoparticles fromed by heating at 80°C for 15 min at pH 5.9 (Figure 37). Length and height were of 400 and 100 nm, respectively, which is coherent with the dynamic light scattering analysis. Indeed, lengths are overestimated and heights are underestimated by AFM, especially if particles are soft and therefore slightly spread during deposition or AFM plate surface scanning. The surface of nanoparticles exhibits irregularities probably created by lumps sticking together in the nanoparticles. Between them appear clefts that could be responsible for the FA binding. Further investigations would be necessary to confirm this hypothesis, maybe by analysing the linoleate/nanoparticles binding by AFM.

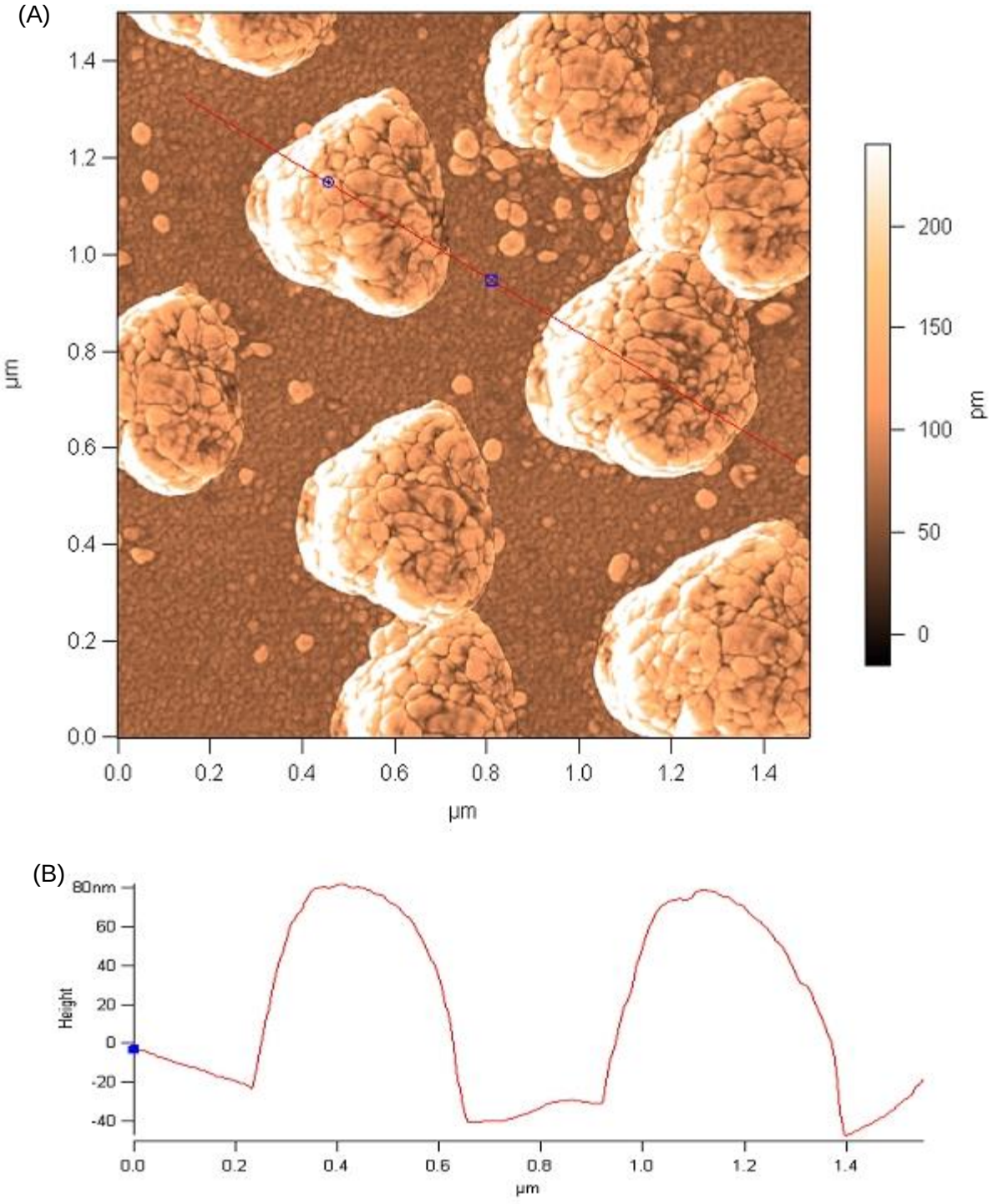

Figure 37: AFM images of nanoparticles (1.5 × 1.5 µm). (A) illustrates the surface topography and the size of βlg nanoparticles formed by heating at 80°C for 15 min at pH5.9. (B) shows the height line profile of the red bar represented on (A).

3.2 Conjugated linoleic acids interaction with β-lactoglobulin and bioaccessibility

Our investigations have shown that linoleate bound to βlg was less cytotoxic and had a lower uptake by Caco-2 cells compared to free linoleate. However, other studies reported that oleic and conjugated linoleic acids (CLA) had a higher cytotoxicity when bound to βlg compared to free.[42,46] Interestingly, Levin et al. (1992)[47] observed that the uptake of oleic acid emulsified with taurocholate was more efficient than oleic acid bound to bovine serum albumin. This study suggests that cytotoxicity is a solubility issue as oleic acid is more soluble and more cytotoxic in the presence of the emulsifier taurocholate. Difference in the bioaccessibility of ligands in these studies could be related to their solubility as linoleate has a higher solubility than vitamin D, CLA and oleic acid. Therefore, the addition of βlg did not increase its solubility, but functioned to reduce its availability to be taken up by the cells.

To support this hypothesis (see Figure 44), we investigated the binding properties of the fatty acid CLA, which has a critical micelle concentration (CMC) of 0.2 mM at pH 9.2[48], with βlg using ITC and intrinsic fluorescence. Its subsequent cytotoxicity was measured using MTS assay. CLA are isomers of LA with low solubility compared to linoleate. CLA and linoleate experiments were conducted under identical conditions.

Measurement of the interaction between CLA and βlg by ITC was challenging due to the poor solubility of this FA. Ethanol was initially used to solubilise CLA but this resulted in a high heat exchange peak during the titration of CLA in βlg by ITC. This peak was generated by the dilution of ethanol. Indeed, this concurs with peak generation in the control experiment where ethanol is titrated in PBS (Figure 38A and 38B) as previously reported.[49] A reverse titration (titration of the protein into the ligand) was then employed to avoid the ethanol dilution peaks.[50] However, the signal of interaction is masked by the heat exchange due to the βlg dimer dissociation[51] as observed by the titration of βlg (0.3 mM) into PBS (Figure 38C). Finally, PBS supplemented with 1 % ethanol was used to dissolve the protein and CLA. To avoid interference peaks due to dilution, low concentrations of both protein and CLA were used. Although a heat exchange peak from the interaction of the FA with βlg was observed and integrated (Figure 39), the fitting of the data were not possible due to the background noise. Hence, binding properties could not be properly calculated. However, the raw data indicated differences between the complexes formed with native, covalent dimers and nanoparticles.

Intrinsic fluorescence of βlg was measured and the normalized raw data are shown in Figure 40. Preliminary studies were performed to quantify the inner filter effect of CLA. As N-

acetyl-tryptophanamide (NATA) fluoresces similarly to tryptophan but does not bind FA[28], it was used as a blank in order to subtract the inner filter effect caused by the presence of CLA in the titrated sample. However, the titration did not show a linear relationship between NATA fluorescence and the CLA concentration. This is probably because of low solubility and micellisation of the FA. However, because the properties of the FA probably changes (difference of solubility limit and micellisation concentration) with the presence of βlg, data could not be accurately corrected and consequently binding constants could not be determined (Figure 40). Nevertheless, the data shows different interactions with the different βlg forms.

Consequently, selection of a method avoiding the drawbacks highlighted by ITC and fluorescence measurements is necessary for quantifying the interaction between CLA and βlg although Jiang et al. (2010)[42] determined the CLA/βlg stoichiometry using intrinsic fluorescence. Thermophoresis seems a more suitable method as it allows the quantification of the mobility of proteins in a temperature gradient. This mobility is dependent of the charge, size and hydration of the protein, such parameters being affected by the binding of a ligand onto the protein.

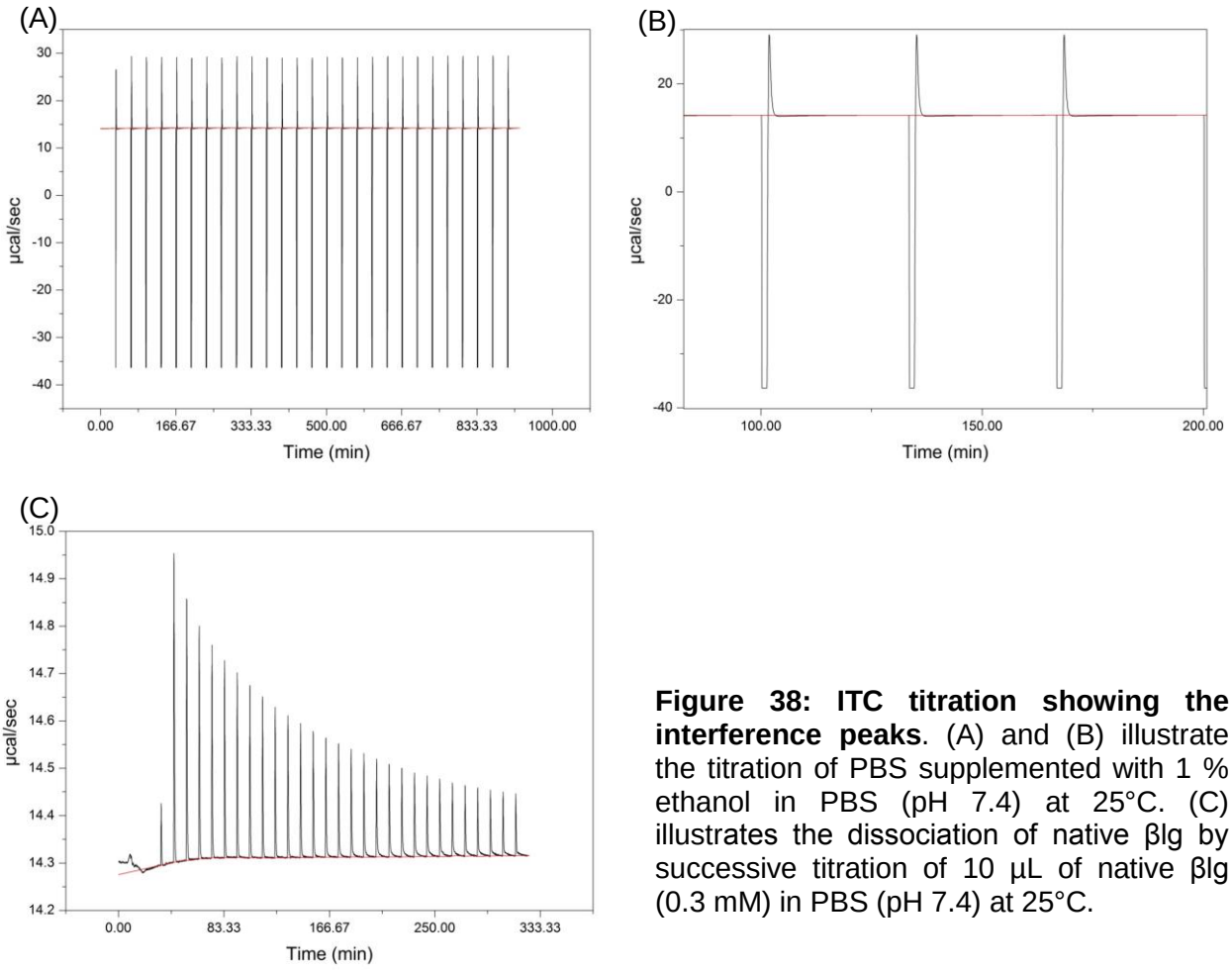

Figure 38: ITC titration showing the interference peaks. (A) and (B) illustrate the titration of PBS supplemented with 1 % ethanol in PBS (pH 7.4) at 25°C. (C) illustrates the dissociation of native βlg by successive titration of 10 µL of native βlg (0.3 mM) in PBS (pH 7.4) at 25°C.

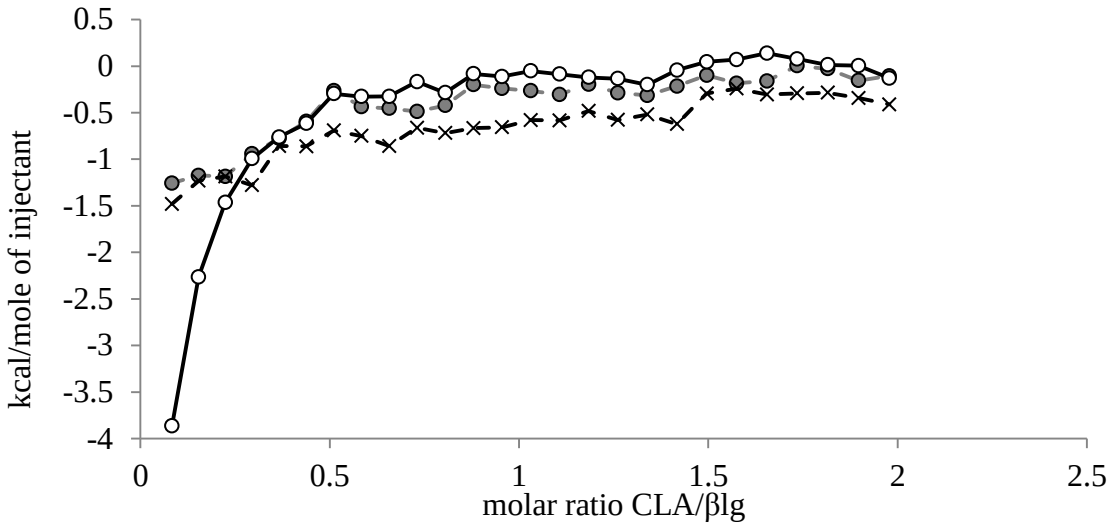

Figure 39: Microcalorimetric titration of CLA in different forms of βlg using PBS buffer (pH 7.4) supplemented with 1 % ethanol (v/v) at 25°C. βlg (97.4 µM) were titrated with increments of 10 µL CLA (964 µM). The raw heat signals were integrated and plotted against the linoleate/βlg molar ratio. CLA/covalent dimers βlg, black cross with dashed black line; CLA/nanoparticles βlg, grey circle with dashed grey line; CLA/native βlg, white circle with black plain line. Experiments were performed in duplicate.

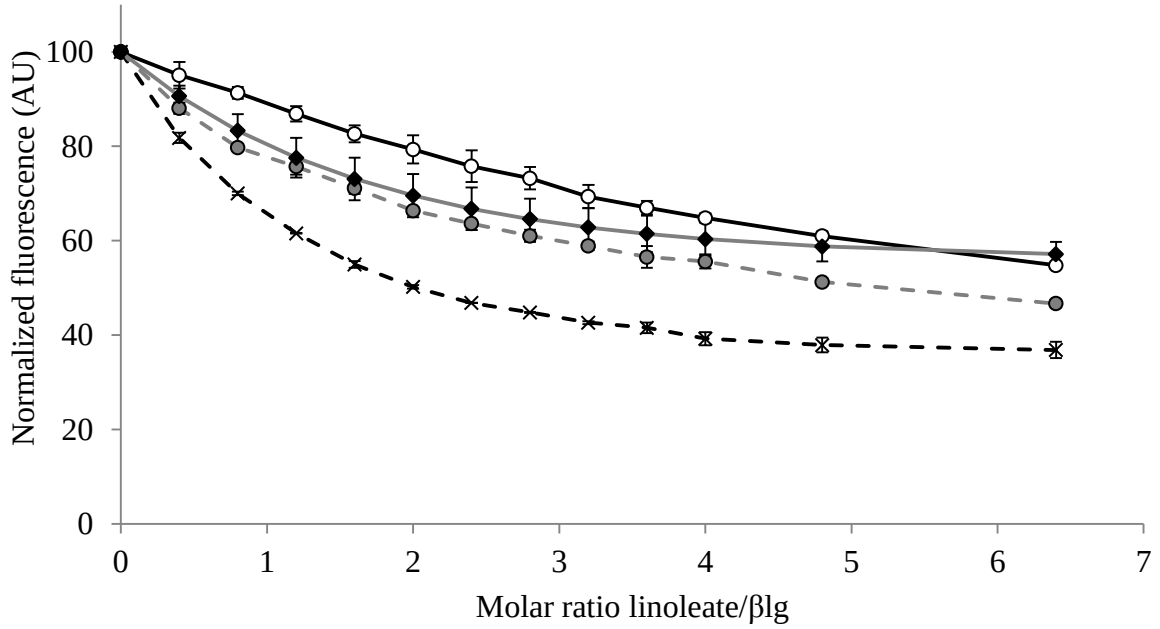

Figure 40: Intrinsic fluorescence of βlg titrated with CLA. Different forms of βlg (native, covalent dimers and nanoparticles, 10 µM) were titrated with 4 mM CLA at 25°C. CLA/covalent dimers βlg, black cross with dashed black line; CLA/nanoparticles βlg, grey circle with dashed grey line; CLA/native βlg, white circle with black plain line; CLA-NATA, black diamond with grey plain line. Results represent mean ± SD (n=3).

To investigate the bioaccessibility of the CLA free and bound to βlg, cytotoxic MTS assays were conducted.The viability of the colonic epithelial cell line, Caco-2, was measured in the presence of various CLA/βlg test samples with CLA concentrations of up to 200 µM. Preliminary studies showed no cytotoxic effect of ethanol at the concentration used. The experiment was confounded by the fact that free CLA could not be removed by dialysis due to its micellisation. Hence free and bound CLA were present in test samples. A 2 CLA/βlg molar ratio was used for all the complexes tested (Figure 41A). CLA in complexes had similar cytotoxicity regardless of βlg form. The lethal dose 50 (LD_{50}) for CLA/native βlg, CLA/covalent dimers and CLA/nanoparticles were 138.9 ± 3.1 µM, 123.1 ± 5.2 µM and 156.2 ± 3.6 µM, respectively. Free CLA was less cytotoxic (LD_{50} > 200 µM). For comparison purposes, cytotoxicity of linoleate on Caco-2 was also measured by MTS assay using a 2 linoleate/βlg molar ratio without dialysis to remove free linoleate. Free linoleate had an LD_{50} is of 59.7 ± 2.3 µM. In the presence of blg covalent dimers and nanoparticles, linoleate had a cytotoxicity of LD_{50} of 173.4 ± 2.1 µM and 171.0 ± 1.5 µM, respectively. Linoleate/native βlg had a LD_{50} > 200 µM (Figure 41B).

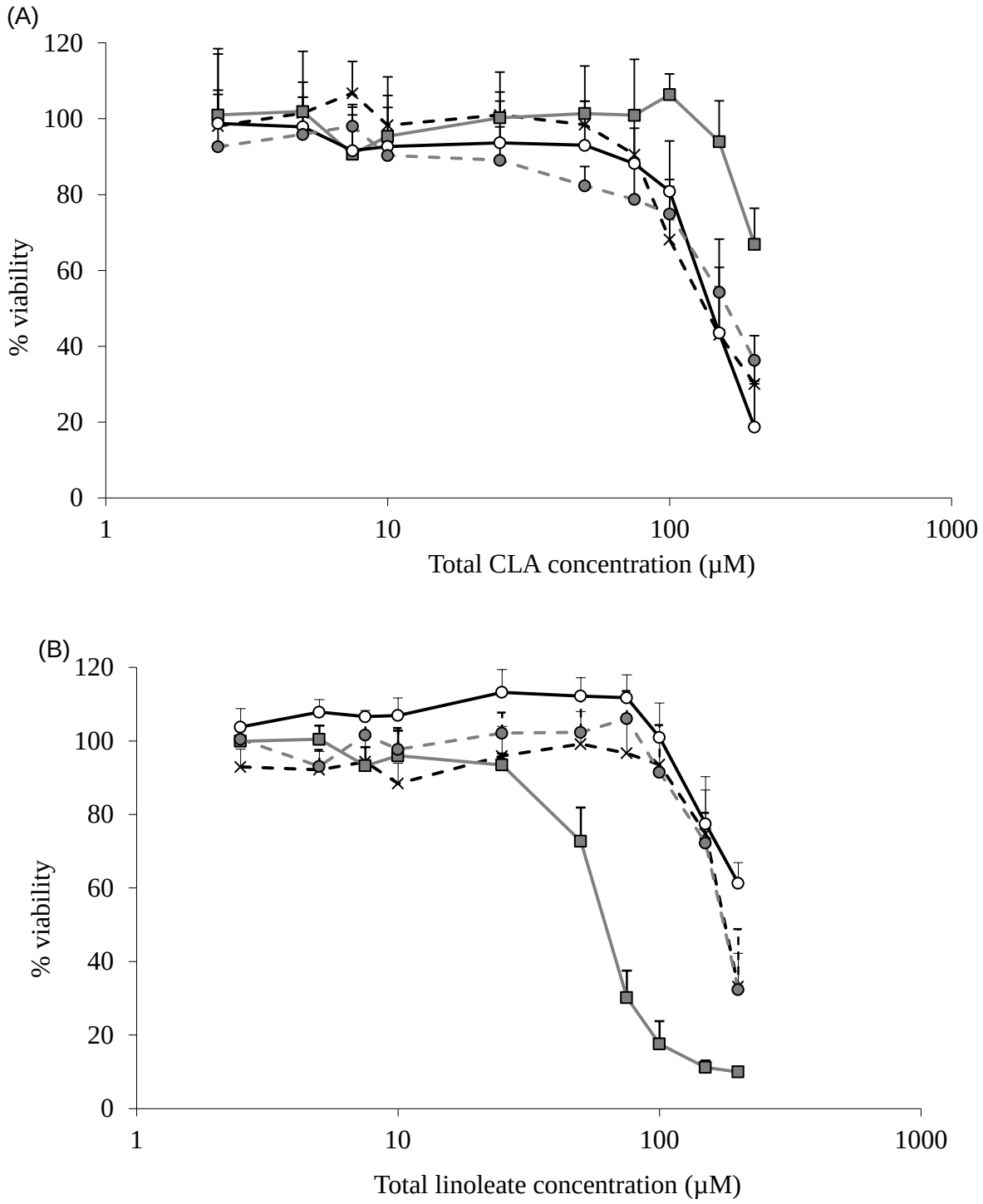

Figure 41: Cytotoxicity of CLA (A) and linoleate (B), free or in presence of different forms of βlg with a 2 FA/βlg molar ratio, on Caco-2 cells. Cell viability after 24 h on 2×10^4 Caco-2 cells compared to control cells was assessed using an MTS assay. The FA concentrations in the tested sample were of 0 to 200 µM. Free FA, grey square with black plain line; FA/covalent dimers βlg, black cross with dashed black line; FA/aggregates βlg, grey diamond with dashed grey line; FA/native βlg, white circle with black plain line. Results represent mean ± SD (n=6).

In conclusion, CLA was more cytotoxic, when presented to the cell bound to βlg compared to free CLA. In contrast linoleate was less cytotoxic when bound to βlg compared to free. For CLA, the amount of FA accessible to the cells is low in the absence of protein because of its poor solubility. The interaction of CLA with βlg adds an equilibrium between free and bound FA, increasing the CLA solubility, and thus its cytotoxicity. This is not the case for linoleate. Free Linoleate being water soluble, the addition of βlg creates an equilibrium between free linoleate and bound, decreasing the proportion of free linoleate. As such, it is less bioaccessible and therefore less cytotoxic.

The differences in LD_{50} values for dialysed and undialysed linoleate/βlg test samples were noteworthy except with the use of nanoparticles (Table 11). The curves of cytotoxicity of linoleate with nanoparticles before and after dialysis were similar because of the aptitude of the nanoparticles to bind almost all the added linoleate. Indeed, we observed by GC that the molar ratio of linoleate/nanoparticles after dialysis was of 8.9 for an initial molar ratio of 10 (before dialysis). The difference of LD_{50} observed in these cases (LD_{50} of 138.9 and 189.0 µM for undialysed and dialysed samples, respectively) could be due to the curve fitting as the sigmoids were not completed for the range of concentrations used. We observed an increase in cytotoxicity when linoleate was in presence of native βlg (not dialysed) compared to when it was bound to the βlg (dialysed complex). This could be explained by the presence of free linoleate in the solution which was not dialysed, as GC data highlighted the removal of some free FA by dialysis. Surprisingly, LD_{50} of undialysed linoleate/covalent dimer complex was lower (173 µM) than dialysed linoleate/covalent dimer complex (80 µM). One possible explanation could be due to the presence of trace of copper used to catalyse the formation of covalent dimers. Copper ions were reported to cause the peroxidation of FA and generating more cytotoxic peroxidated FA.[45] However, it seems necessary to do further experiments to understand the influence of peroxidation on cell death.

Table 11: LD_{50} of linoleate free or bound to βlg with and without dialysis reported in Figures 36 and 41B.

	LD_{50} after dialysis (µM)	**LD_{50} without dialysis (µM)**
Free linoleate	–	58.0
Linoleate/native βlg	>> 200	> 200
Linoleate/covalent dimers	80.0	138.9
Linoleate/nanoparticles	189.0	123.1

3.3 Copper detection and impact on linoleate cytotoxicity

The amount of copper remaining in the covalent dimer powder was determined using atomic absorption spectrometry (model AA 1275; Varian, F-91941 Les Ulis, France). Copper concentration in covalent dimer powder after dialysis was 0.96 g/kg. It could be responsible for linoleate peroxidation. Therefore 10 mL of linoleate (final concentration of 1.63 mM, equivalent to the amount of linoleate during the formation of 10 linoleate/βlg complex) with a copper concentration up to 5 mg/L were analysed by GC (Figure 42). Data showed a decrease in the linoleate peak and the appearance of mainly two new peaks which were not identified. These new peaks could correspond to the peroxided forms of FA. Indeed, copper is known to be a high oxidant of FA[52], and peroxided FA are highly cytotoxic.[45] A MTS assay with constant linoleate concentration, which is not cytotoxic on its own (10 µM), became cytotoxic by increasing the copper concentration even if copper is not cytotoxic on its own (Figure 43). This highlights the possible formation of linoleate peroxides, which are therefore more toxic to the cells. However, Figures 36 and 41B show differences in the cytotoxicity between linoleate/covalent dimers dialysed and non dialysed, the peroxidation phenomena in different conditions (ie. time, freeze-drying, concentrations) should therefore be identified in order to control it.

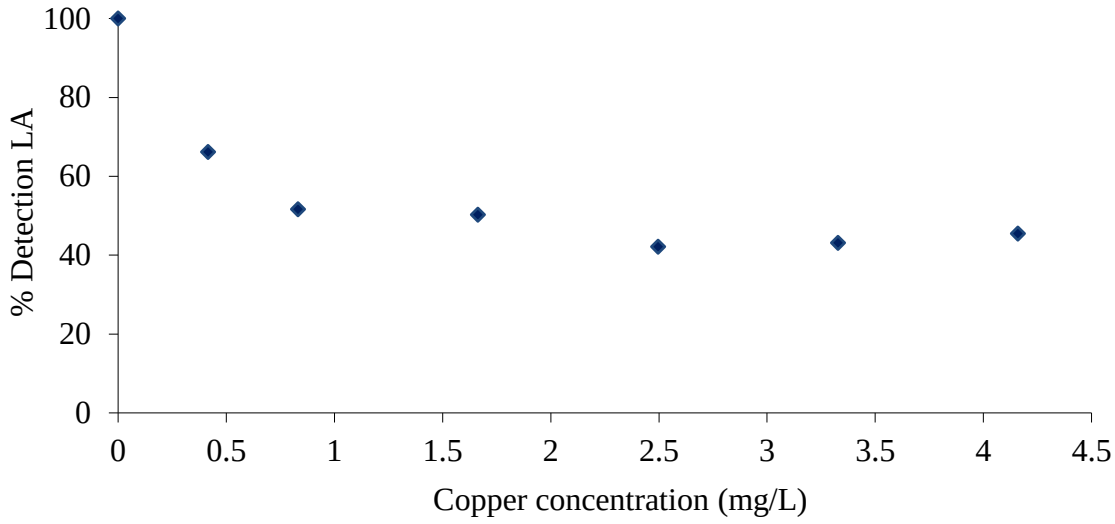

Figure 42: Detection of linoleate in the presence of copper by GC. The concentration of linoleate (1.63 mM) detected was decreased in the presence of copper, illustrating its peroxidation.

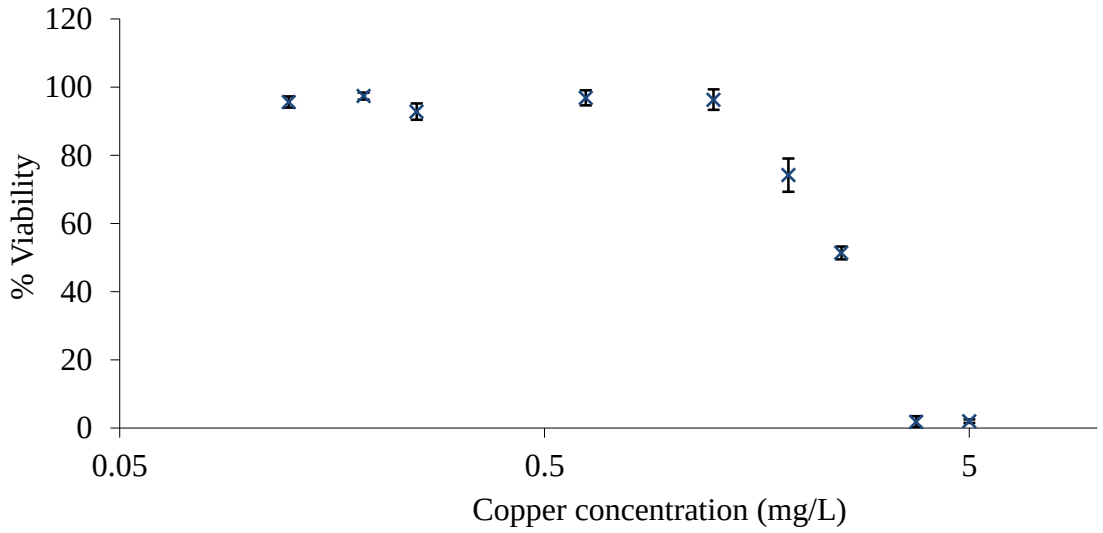

Figure 43: Cytotoxicity of linoleate free or in presence of different concentration of copper on 2 × 10^4 Caco-2 cells after 24 h. Cells viability compared to control cells was assessed using an MTS assay. The linoleate concentration in the samples assayed was of 10 µM. Results represent mean ± SD (n=3).

3.4 Differences in isothermal titration calorimetry data at 25°C and 60°C

ITC data of linoleate/native βlg binding in the experiment conducted at 25°C (Figure 32) exhibits slight differences relative to the data from our previous experiment conducted at 60°C (Figure 12). The first binding site has the same n but a higher Ka at 25°C than 60°C. Additionally, on the second binding site, one more linoleate molecule binds to the native βlg and K_a is ten times higher at 25°C compared to 60°C. This decrease in affinity constant, when the temperature is increased from 25°C to 60°C, could be due to different βlg structure (non covalent dimers versus monomeric R-states βlg) at 60°C. These changes in the calyx structure could lead to a decrease of affinity. These changes in binding properties are related to the structural changes of βlg which occur with increases of temperature and with aggregation.[6] During aggregation, hydrophobic exchange occurs resulting in an exposition of hydrophobic regions.[6] The hydrophobic surface is known to have a smaller affinity but a higher affinity stoichiometry when the βlg is in its native dimeric structure compared to the native monomer.[4,53] These changes in hydrophobicity, from the native βlg to aggregates, could form hydrophobic pockets and therefore increase the ratio of ligand bound to the protein.

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PART 4

GENERAL CONCLUSION AND PERSPECTIVES

The biological properties of food products are affected by their composition and structure. Additionally, interactions between food components within food products have been shown to modify their bioaccessibility (Riihimäki-Lampén, 2009). Therefore, the design of food products with targeted biological properties requires a complete understanding of component interactions and their impact on the molecule biological properties during the manufacture, storage, consumption and digestion. β-lactoglobulin (βlg), the main bovine whey protein, has been extensively studied because of its capability to bind hydrophobic ligands including fatty acids (FA). βlg is frequently added to foods for nutritional and functional properties. However, this protein is highly sensitive to processes used in food manufacture and is often found in an aggregated form. The FA binding properties of βlg aggregates have not been studied extensively, but suggest differences may exist between the binding of FA with native βlg or βlg in aggregated states.

Changes in binding parameters of molecules are supposed to affect the complexes overall biological properties. Hence, in order to progress our understanding of the impact of molecular interaction on molecules biological properties, this project examines the interactions between different forms of βlg and an essential FA, linoleate, or its conjugated isomers, conjugated linoleic acids (CLA). The objective of this project was to study FA/βlg complexes using native βlg and βlg aggregates. The βlg aggregates were selected to have different states of aggregation (covalent dimers and nanoparticles) in order to understand the impact of the aggregate structure on FA binding properties and the resulting biological properties of the complexes. The biological properties of the complexes (undigested and digested) were investigated *in vitro* through the measurement of FA bioaccessibility, its uptake into intestinal epithelial cells, and its effect on the gene expression expression of fatty acid transporter proteins and secretion of the satiety hormone cholecystokinin (CCK). Table 12 summarises the main results obtained through this PhD.

Table 12: Summary of the main results.

Experimental chapters	Main results
Chapter 1	• Linoleate bound to βlg in two sets of binding sites, a hypothetic mechanism of binding was described page 86. • The binding of linoleate to βlg increased the protein oligomerisation without detectable modification of protein secondary structure. • Linoleate cytotoxicity was delayed by its binding to βlg, which could be due to the FA solubility. • Linoleate did not modify FATP4 and FABPpm gene expressions nor cAMP level.
Chapter 2	• Linoleate increased βlg hydrolysis during gastric digestion probably by increasing its denaturation/oligomerisation. • Linoleate uptake in Caco-2 cells was faster when free compared to bound. • βlg aided to the uptake of linoleate, it occurred at a slower kinetic than free linoleate. • CCK gene expression and secretion was similar for free and bound linoleate. • The digestion of the complex increased linoleate cytotoxicity.
Chapter 3	• Linoleate binding capacity of βlg increase through its aggregation. • Cytotoxicity of linoleate was modulated in function of the βlg form used as a carrier. • The copper remaining in the covalent dimer solutions may modify the linoleate properties. • Unlike linoleate, CLA was more cytotoxic when bound to βlg compared to the free form, which could be due to the FA solubility.

Linoleate binds to all βlg forms tested (native βlg, covalent dimers, nanoparticles) through two distinct sets of binding sites. The first set of binding sites has a strong affinity for linoleate (approximately 10^6 M^{-1} at 25°C) and is likely to be located within the calyx of the protein, as suggested by 2.1 Å X-ray crystallography (Loch et al., 2012). The second set of binding sites has a lower affinity for linoleate (approximately 10^4 M^{-1} at 25°C) and is likely to be located on the hydrophobic surface of the protein. The association constants were similar regardless of βlg form. Overall, the molar ratio of linoleate bound to βlg was close but lower than one for the first set of binding sites. The molar ratio at the second set of binding sites increased depending on βlg form with βlg nanoparticles > covalent dimers > native βlg. This is probably due to the presence of an increasing number of hydrophobic patches on the surface of heat-aggregated βlg forming unspecific sites, of low affinity, for linoleate to bind to (Schmitt et al., 2009).

Native βlg undergoes oligomerisation in the presence of linoleate whereas aggregated βlg do not oligomerize. The oligomers formed in presence of linoleate are stabilized by intermolecular disulfide bonds, as indicated by SDS-PAGE. This suggests that native βlg has to modify its conformation leading to exposition of a free sulphydryl for linoleate to bind. However, the structure of βlg in linoleate/native βlg complex viewed by crystallography does not confirm this (Loch et al., 2012). We can therefore postulate that the binding of linoleate on the surface of native βlg increases βlg hydrophobicity. This allows the proteins to move closer together to establish intermolecular disulfide bonds. The formation of intermolecular disulfide bonds could be favoured at that temperature (60°C) and the high linoleate concentration used in our studies. However, the influence of temperature and linoleate concentration on βlg oligomerisation needs further investigation. Another plausible explanation suggests that the free thiol group of βlg may act as linoleate free radical scavenger. In fact, linoleate is very sensitive to oxidation and it is possible that free radicals of linoleate are formed during the preparation of our sample. In the presence of βlg, the free thiol group of βlg that has reducing activity may transfer a hydrogen atom to linoleate ensuring its reduction; simultaneously, the thiol radical is stabilized by oxydation reaction resulting in mainly covalent dimers or very small aggregates of βlg.

During *in vitro* digestion of linoleate/native βlg complexes, the presence of βlg oligomers was not detetected during the gastric phase while βlg monomers remained intact even after duodenal digestion. Gastric digestion occurred at pH 2.5, where FA dissociate from complexes. The oligomers of βlg formed in the presence of linoleate are rapidly digested by the gastric enzyme pepsin. The increase of pH to 6.5 under duodenal conditions allows the reformation of the linoleate/native (undigested) βlg that is more resistant to duodenal digestion than native βlg. Therefore, linoleate can protect the protein against further hydrolysis under duodenal conditions. Whether non-native forms of βlg are also protected by linoleate from the duodenal digestion remains to be determined. In agreement with other studies, βlg peptides generated during gastric digestion associate as oligomers under duodenal conditions (Jiang and Liu, 2010; Bateman et al., 2011). Microscopic (AFM, TEM) and diffusion techniques (SANS, SAXS) could be employed to investigate the kinetics of formation and the structural properties of these oligomers.

The impact of linoleate/βlg interactions on FA biological properties was studied using enterocytes, monolayers, enteroendocrine excretory signals, and cytotoxicity assays. Cytotoxicity assays evaluated the bioaccessibility of linoleate, as the FA has to enter the cell to be toxic to the cell (Lu et al., 2010a; Lu et al., 2010b). The study showed that free linoleate is more cytotoxic than linoleate bound to βlg. As free linoleate is soluble under our

experimental conditions, we assume that its binding to βlg delays its uptake into the cell by adding an equilibrium between free and bound FA. This theory is supported by the use of another FA, CLA, which is much less soluble in aqueous phase (CMC of 0.2 mM (Fan and Fang, 2011) versus 2 mM for linoleate). The presence of βlg increases the amount of CLA in the solution, increasing its entry into the cell resulting in increased cytotoxicity. As the linoleate affinity constant for βlg was similar for the different βlg forms tested, the complexes cytotoxic effect is similar as long as linoleate/βlg molar ratio is below the saturation of binding sites. In contrast, the cytotoxicity of the various complexes differ with the structure of βlg when the linoleate binding sites on βlg are saturated. This is attributed to the larger amount of linoleate weakly bound to nanoparticles than to native βlg, which is consequently more bioaccessible for cells. This suggests that the protein aggregation could be chosen in function of the desired bioaccessibility of hydrophobic ligands.

Linoleate/covalent dimers complex are more cytotoxic than linoleate/nanoparticles complex which appears to contradict the above hypothesis. It is possible that traces of pro-oxidant copper ions (used to form covalent dimers) in the samples of linoleate/covalent dimer complex increases the concentration of highly cytotoxic peroxidated linoleate. Further studies are needed to confirm the presence of peroxidated linoleate in the samples, such as determining the LD_{50} of this linoleate form, and examining the interaction linolate peroxides Alternatively preparing covalent dimers in the absence of copper or the use of an anti-oxidant such as copper chelator (i.e. citrate and EDTA), are required to definitely assign the contribution of covalent dimers on the FA bioaccessibility.

Uptake of linoleate into Caco-2 cells was visualised by confocal imaging. The transport of linoleate was decreased in the presence of native βlg, in agreement with cytotoxicity data. However, increasing the amount of βlg increases linoleate uptake but the kinetics of transport is slower compared to the uptake of free linoleate (Figure 44). Further investigations would involve tracking the proteins transport into the cell or the manner by which it presents the FA to the cell to support and understand how the FA uptake is increased by βlg. Such investigations would be interesting, in particular to identify which mechanism is responsible for the FA/βlg dissociation if any, before transfer to the cell.

Uptake of linoleate by Caco-2 cells decreased when linoleate/native βlg complex underwent proteolytic digestion compared to free linoleate. Under identical conditions and after 24 h of exposure, free linoleate decreased Caco-2 cell viability (LD_{50} of 58.0 µM), whereas the linoleate/native βlg complex had an LD_{50} superior to 200 µM. However, the same complex post gastro-duodenal *in vitro* digestion reduced cell viability with a linoleate cytotoxicity included between free linoleate and undigested complex. We can therefore infer that linoleate still binds to βlg post digestion albeit with a reduced cytotoxicity compared to free linoleate. Moreover, it would be interesting to know if linoleate binds to specific peptides

or only to remaining undigested proteins, and what the binding constants and bioaccessibility of FA under these conditions would be. The use of radiolabelled FA and protein/peptides during digestion of the complexes may offer a more complete picture of FA/protein binding and the FA bioaccessibility to the intestinal cell lining.

We tried to follow the FA uptake into Caco-2 cells by active transport using FATP4, FABPpm, cAMP, and the contact of linoleate with the cell membrane of STC-1 cells by studying the gene expression and secretion of the satiety hormone CCK. No difference could be observed in the conditions used between the controls, free linoleate and bound linoleate. It could be due to the low concentration in linoleate used not to be cytotoxic and/or to a lack of specificity of the CCK antibody. However, it would be interesting to understand how linoleate enters into cells (active, facilited and/or passive transport) to observe if its binding to a protein orientates its uptake to certain pathways.

This thesis focused on the impact of FA/βlg complexes on protein digestibility and on FA accessibility *in vitro*. Along with the questions answered by *in vitro* research, there are others that require *in vivo* studies. Food products contain FA primarily in the form of triglycerides. Further research should determine if complexes can be formed in the digestive tract, after triglycerides are hydrolysed by lipases. Additionally, the digestion protocol used in our project was a static *in vitro* digestion. A dynamic *in vitro* or an *in vivo* digestion would address the dilution impact on the protein hydrolysis and FA bioaccessibility. As the FA uptake by the intestinal barrier decreases in the presence of βlg, more FA would remain in the gut. Only an *in vivo* study could determine if this build-up altered gastric transit and/or food intake.

We chose to study a model interaction of βlg with two FA present in milk. However, it would be of interest to characterise such interactions and biological properties in a more complex matrix.

In a complex matrix, the presence of pro-oxidants could damage the FA. Further investigations could investigate the protective effect of the interaction of ligand with proteins against oxidation. Other hydrophobic nutrients present in food products, such as vitamins or other FA, could bind to βlg and could perturb the linoleate/βlg complex, and the amount of free linoleate in solution. Additionally, linoleate is able to bind other proteins such as bovine serum albumin (Fletcher et al., 1971). Therefore, linoleate could be in equilibrium between several proteins and its free form, having a different impact on the FA bioaccessibility. Moreover, the presence of ligand may modify the proteins functional properties, such as its ability to form stable emulsion and foam. Consequently, the interaction of linoleate with βlg in a food matrix should be studied.

Hydrophobic ligands/βlg interactions provide an opportunity for food manufacturers to deliver, in reduced fat foods, essential hydrophobic components or indeed hydrophobic bioactives, albeit at low molar concentrations. Indeed, low fat or fat free products can be deficient in necessary fat soluble molecules. Hence, this type of interaction between proteins and hydrophobic ligands can allow an equilibrated intake of hydrophobic molecules in low fat alimentation.

Food products also tend to reduce their animal protein content, one of the reasons being the excessive amount of ingested protein in Western countries. Therefore, in the perspective of a healthy diet, it is necessary to reduce the protein and fat contents of the food products. We show that it is possible to increase the ratio of ligands bound to protein by inceasing the protein aggregation in order to make bioaccessible a maximum of essential hydrophobic molecules. Consequently, it is important to understand the relationship between the protein structure and its ability to increase the binding of hydrophobic ligands.

To conclude, research into food structure will help formulate products with enhanced efficacy from a nutritive and bioactive perspective.

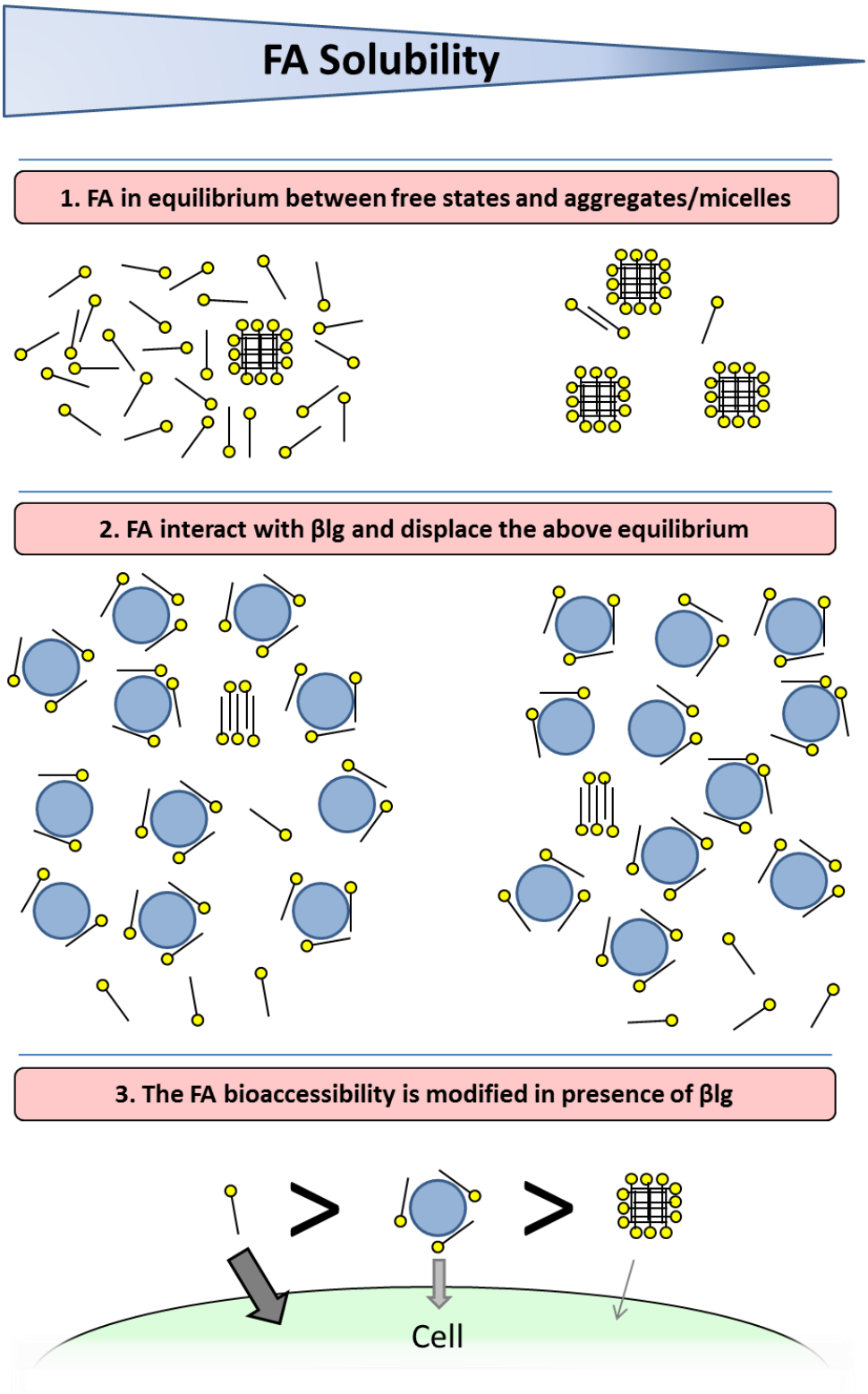

Figure 44: Graphical representation of the impact of βlg on FA bioaccessibility. 1) In solution, FA exist in equilibrium between free FA and micelle/aggregate states. Under high solubility conditions this equilibrium favours the free FA state, whereas under low solubility conditions it favours the micelle/aggregate states. 2) In the presence of βlg, FA interact with the protein. This binding of FA to βlg displaces the above equilibrium. 3) Comparatively, free FA are more bioaccessible than micellar/aggregated FA. The presence of βlg therefore decreases FA bioaccessibility in conditions where the FA solubility is high, whereas it increases the FA bioaccessibility in conditions where the FA solubility is low.

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ABSTRACT

Food structure can have a profound influence on delivering health benefits. Bioaccessibility of nutrients can be affected by their interaction with food components. The dairy protein β-lactoglobulin (βlg) is known to bind hydrophobic ligands such as fatty acids (FA). However, this protein is highly sensitive to the process conditions used in the dairy industry. Therefore βlg is often present in non-native or aggregated form in processed food. This structural change may modify the protein affinity for FA and the biological properties of the FA/protein complexes. The aim of this thesis was to investigate the interaction of bovine βlg in different structural forms (native, covalent dimer and nanoparticles) with linoleate (C18:2, *cis,cis*-9,12-octadecadienoic acid) and conjugated linoleic acids (CLA, C18:2), and the impact of those complexes on their biological activity *in vitro*. Two different sets of binding sites were determined for the interaction between linoleate and βlg, regardless of its state of aggregation, using intrinsic fluorescence spectroscopy and isothermal titration calorimetry. By increasing the level of βlg aggregation, the linoleate/βlg stoichiometry increased but the association constants remained similar for both sets of binding sites. In the presence of linoleate, the native protein was more sensitive to gastric *in vitro* digestion, due to the increased level of denaturation/aggregation of βlg. Transport of linoleate in Caco-2 cells was decreased in presence of the native βlg as observed by confocal microscopy and a monolayer that mimics the intestinal barrier. Cytotoxicity of linoleate on Caco-2 cells was reduced when the FA was bound to βlg compared to free FA. CLA, which is less water soluble than linoleate, is more cytotoxic when complexed by βlg than in its free form. Therefore, it is proposed that βlg can act as a molecular carrier and alter the bioaccessibility of FA depending on their solubility.

Key words: β-lactoglobulin; Fatty acids; Interaction; Binding constants; *In vitro* digestion; Bioaccessibility.