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Dairy protein–ligand interactions upon thermal processing and targeted delivery for the design of functional foods

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Optimal protein performance in techno-functional and bio-functional foods is largely determined by thermal processing, leading to physical or chemical interactions with other constituents found in the commercial formulation. There is a need to understand at a fundamental level the kinetics of molecular transport of bioactive compounds, including vitamins, essential fatty acids, antioxidants and caffeine, from protein-based excipients in nutraceutical-type products. Physical interactions in these systems are further manipulated by crosslinking the protein network for controlled delivery in relation to the physicochemical environment of the release medium. Altering the processing conditions from ambient and pasteurisation temperatures to UHT treatment brings into play the denaturation of the milk protein, added to beverages that affects its association with phenolic compounds. These are found naturally in oat or wheat insoluble fibre, which is increasingly incorporated in formulations of added value foods, for example liquid breakfast. Potential formation of chemical interactions between hydroxycinnamic or hydroxybenzoic acids from insoluble dietary fibre and milk proteins following UHT processing and prolonged storage at ambient temperature may involve unexpected physiological and nutritional effects. We aim to review the significant results in this new and evolving field of dairy protein–ligand interactions in an effort to assist with planning further experiments for the design of convenient and nutritious foods.

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Introduction

Proteins are well known for their functional role as supporting materials in the physical structure of processed foods, assisting in the formation of a variety of gels, foams, and emulsions [1]. Additionally, they are utilised for their entrapping properties, bovine milk proteins, in particular, are well suited as protective excipients for bioactive materials, with wide applications in the delivery of natural bioactive compounds [2]. Part of this review aims to provide an overview of the current knowledge in the control and measurement of the kinetics involved in the controlled/targeted delivery of bioactives from milk-protein matrices.

In a similar vein, the fortification of convenience foods, including liquid breakfast, with additional dietary fibre from grains (mainly wholegrain oat and wheat) is also of growing industrial and consumer interest in improving nutrition and health [3]. This is not least due to the high content of phenolic compounds present, particularly phenolic acids associated with natural insoluble fibres, thought to be beneficial to well-being by assisting in the prevention of chronic disease including cardio vascular disease by lessening problems such as atherosclerosis, hypertension, and thrombosis [4]. In commercial formulations, phenolic acids are found in the vicinity of milk protein chains that leads inevitably to molecular interactions. These interactions have been investigated extensively and are fairly well understood at low processing temperatures. Thus, the molecular size of phenolic compounds, solution pH, temperature, and ingredient concentration are the main factors affecting the mostly reversible, that is, physical associations that take place during processing and subsequent storage [5].

Although challenging to reproduce at the laboratory scale, industrial processing of liquid food products commonly incorporates a UHT step at about 135°C to facilitate long shelf-life at ambient temperatures. The widely practised treatment should also result in molecular interactions between protein constituents and phenolics but it remains under researched. A recent investigation into such high temperature systems showed that the interactions might be chemical (covalent and irreversible) rather than physical (weaker and reversible) in nature [5]. Therefore, the second part of this is review aims to provide insights and possibilities for further research into the effect on structural and functional qualities that high temperature protein–phenolic processing may induce.

Overview of dairy protein as matrix for the controlled delivery of natural bioactive compounds

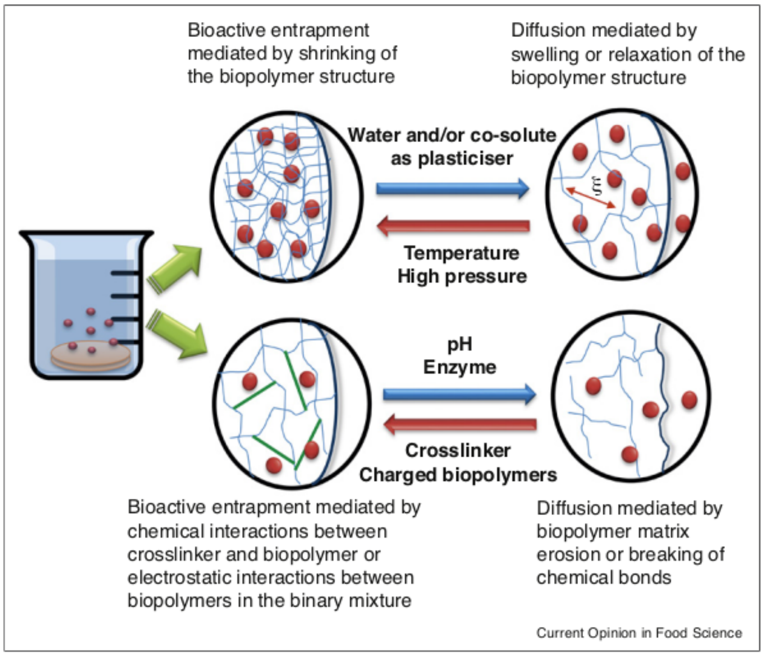
The formation of a delivery vehicle entails the creation of a barrier, protecting bioactive compounds against unstable environmental conditions during processing, subsequent storage and digestion [6]. As a protective excipient for bioactives, protein, specifically, has taken many forms that is films, nanocapsules and microcapsules, beads and electrospun fibres [7–10]. Theories surrounding the application of proteins for the delivery of drugs and food components have been reviewed by Chen *et al.* [11] and de Souza Simões *et al.* [12], with the diffusion of bioactives in model food systems being reviewed by Paramita and Kasapis [13].

Being a natural polymer, proteins exhibit certain physicochemical properties including vulnerability to enzymatic degradation, a balanced hydrophilic–hydrophobic nature, as well as sensitivity to ionic molecules/counter-ions and thermal treatment [14,15]. These properties can result in swelling, erosion or shrinking of the protein matrix during molecular transport phenomena. The former (swelling and erosion) creates enough space (hole free volume) between adjacent polymer chains to allow transport of bioactives [16]. The later reduces the mesh size of the polymeric network, impeding the diffusion of bioactives to the release medium [17]; these effects are illustrated in Figure 1.

The aforementioned properties can be manipulated by the modification of protein's surface area and three-dimensional structure through physical treatment (i.e. heating and/or pressurisation), addition of chemical agents (mainly charged materials), and permanent cross-linking with various compounds through chemical or enzymatic reactions [11,18]. These treatments can assist in altering ingredient functionality in formulations, for example, via protein aggregation leading to gelation, which makes proteins ideal systems in designing a controlled/desirable flux in bioactive release.

Protein gelation is associated with denaturation following two transformational stages, i.e. unfolding of the native protein conformation to expose reactive amino acid residues, and the intermolecular associations that reduce chain flexibility to strengthen the structural rigidity of an infinite molecular-weight network [19]. This creates a boundary condition surrounding the bioactive compound that can be adjusted to meet the requirements of the particular application for release within the human GI tract.

Figure 1



Effect of preparation treatment and experimental conditions on mesh size and network integrity governing bioactive compound release from biopolymer excipients.

Table 1

Release characteristics of bioactive compound from a biopolymer matrix

Biopolymer matrix	Bioactive compound	Geometry	Bioactive release profile	References
90% gelatin crosslinked with genipin	Vitamin B6	CH	Cumulative release (M_t/M_∞) was 18% when highly crosslinked (contained 3% genipin) compared to ~80% when not crosslinked	[17]
WPI	Lycopene	NP	At 4 hours, release of lycopene was ~25% at pH 1.2 and 60% at 7.4	[42][a]
9% WPA + κ-carrageenan gels	Curcumin	MG	33% for WPA alone and 9.6% and 3.5% for gels containing 0.1% and 0.55% κ-carrageenan	[24]
30% Whey protein	Bilberry anthocyanins	MC	Anthocyanin release at pH 1.2 was retarded by 90% in SGF, and by 80% in FeSSIF at pH 6.8	[22][a]
20% WPI	Ketoprofen	A	At pH 1.2, 40% ketoprofen was released, compared to 50% at pH 6.8	[43]
WPI microcapsules	Nicotinic acid (Vitamin B3)	MC	Diffusion coefficient, D: $8.5–18.4 \times 10^{-15}$ m^2/s at 30–60°C with 5°C intervals	[10]
15% WPI hydrogels, including XN, PC and GT	Black carrot extract	CG	Diffusion coefficient, D: 0.59×10^{-15} and 1.78×10^{-15} m^2/s for water bath and microwave-treated gels, respectively	[44]
79% WPI + WPI/GS at varying ratios	Linoleic acid (omega-6)	S	Diffusion coefficient, D: varied from $1.63–2.50 \times 10^{-10}$ m^2/s at −16ºC	[27]

WPI, whey protein isolate; NP, nanoparticle; WPA, whey protein aggregates; MG, microgel; CH, cylindrical hydrogel; MC, microcapsule; A, aerogel; CG, cylindrical gel; XN, xanthan; PC, pectin; GT, gum tragacanth; S, Slab; GS, glucose syrup.

[a] Chromatography was used (other references utilised spectrographic techniques).

There is ample evidence in the literature that phenolic compounds have a positive effect on human health, contributing in particular to the prevention of chronic disease by demonstrating an effective antioxidant capacity both *in-vitro* and *in-vivo* studies [49]. Phenolic compounds are formed as secondary metabolites in plants, with the term referring to a diverse range of structures classified into six main groups, the simplest of which are hydroxybenzoic acids and hydroxycinnamic acids with a single aromatic ring (phenolic acids). More complex multi-ringed structures exist being grouped as flavonoids, chalcones, stilbenes and lignans [50]. Pressurised hot water has been used in the extraction of bioactive compounds from industry by-products for many years, with the extraction of phenolic acids generally taking place between 80–150ºC [51]. There is strong evidence that this extraction of phenolic acids is not avoided in UHT treatments, and in fact Kaur *et al.* [5] note that the content of bound ferulic acid in oat-powder-enriched formulations decreases by around 20% upon UHT treatment and prolonged storage at ambient temperature. This is due to the deesterification of ferulic acid from oat arabinoxylan and dispersal into water bringing the phenolic acid into close proximity with the milk protein chain. The interactions between milk proteins and phenolic compounds form both reversible and irreversible complexes. The former is mainly through hydrogen bonds, hydrophobic interactions and Van Der Waals forces, while the latter is likely of covalent nature and occurs via the formation of the oxidation products of phenolic compounds, known as quinones [52–56].

Formation of quinones can be through a number of pathways, that is enzymatic, oxidative or thermal, allowing them to react with nucleophilic groups within the protein [57]. Covalent interactions between quinones and proteins are thought to preferentially occur with the side chains of lysine and cysteine (possessing amine and thiol groups, respectively), with Li *et al.* [53] arguing that thiol group reactions with 1,2-benzoquinones occur more rapidly without the need for oxygen involvement seen in amine reactions. However, other studies have shown that reactions between quinones and proteins or segments that do not contain cysteine or lysine do still occur [56]. For example, Prigent *et al.* [57] reported that oxidised caffeoylquinic acid reacted with tyrosine, lysine and to some extent with other residues.

Bovine milk or its fractions are highly nutritious, commonly used in the production of foods, hence being widely researched. They contain a number of unique proteinaceous fractions, with large variations in functional properties as well as structure being found amongst the caseins (making up 80% of the protein fraction of milk) and the whey proteins (making up the remaining 20%). Casein molecules tend towards more flexible orientations due to infrequent α-helical and β-sheet structures, frequent proline residues, and in recent years have been described by an unfolded model rather than as surfactants or relatively compact globular proteins [58].

As a result of this morphology, they show strong resistance to heat denaturation in milk and due to their hydrophobic domains tend to form micelles made up of their main protein fractions, i.e. $\alpha_{(s1)}$, $\alpha_{(s2)}$, β and κ-casein, a property that has been successfully exploited in the nanoencapsulation of bioactives [59,60]. While this structure remains remarkably resilient at lower (pasteurisation) temperatures, there is strong evidence that UHT treatment permanently destabilises this micelle structure, depletes irreversibly much of the κ-casein outer layer, as well as increases the amount of free casein in the aqueous solution [61,62].

Whey proteins are distinctly different to caseins in that they form more orderly, globular structures, and as a result they are susceptible to heat denaturation and aggregation [2]. They are made up of a number of variants, the most numerous being β-lactoglobulin (β-LG) (60%), of which eleven variants have been identified, α-lactalbumin (α-La) (20%), the most resistant of the whey proteins to heat induced unfolding, and bovine serum albumin (BSA) (3%), which is of particular interest for its affinity to lipids and free fatty acids as well as its similarity to human serum albumin (HSA) [63].

Numerous reviews for the evaluation of protein–phenolic interactions have been produced [64–66], yet all of which agree that currently a variety of molecular methods with their own strengths and pitfalls must be employed to gain a somewhat whole picture. *In silico* and spectroscopic methods including UV–vis, Fourier transform infrared (FTIR), fluorescence emission and circular dicroism (CD) spectroscopy are common, with more direct methods such as isothermal titration calorimetry (ITC), mass spectrometry and chromatographic techniques also being used. The newer technique of super resolution confocal microscopy may prove indispensable in the near future [67]. Table 2 describes some recent literature on low temperature interactions between casein or whey proteins with phenolic compounds [68–75].

UHT milk protein–phenolic interactions

Modern UHT techniques are able to rapidly heat and cool processed food products, holding them at the peak temperature (generally 135–145°C) for as little as a few seconds. This is a vast improvement on older, 'in container' methods, which took far longer allowing for extensive Maillard type interactions with potentially harmful, antinutritional or simply undesirable properties. It is important to note that while direct steam injection systems achieve this spending no longer than a few additional seconds above 100°C, more common indirect methods via tubular or plate heat exchanger take far longer, commonly more than 60 s [76]. Despite the relatively short processing time at high temperature, the interactions induced between macromolecular ingredients and bioactive compounds in the formulation are not negligible.

Research on the functionality of dairy ingredients and consistency of food products induced by UHT treatment indicates that permanent chemical interactions do occur in the case of both whey and casein proteins, creating unidentified protein fractions [77], affecting digestive

Table 2

Low temperature interactions between milk proteins and phenolics

Protein	Phenolic	Method	Main findings	References
BSA	Tannins (PGG)	RCL	PGG–BSA covalent complexes were formed more readily under oxidising conditions. Quantified using radio labelling of PGG	[68]
BSA and HSA	Grape seed polyphenols	FLQ	Larger phenolic compounds were found to quench more effectively, protein structure also played a role in the binding affinity	[69]
α-La	EGCC	DLS FTIR DSC	EGCC covalently linked to α-La at pH 8, as a result disordered secondary structure increased along with denaturation temperature	[70]
β-Lg	CA EGCC FA	UV–vis FLQ CD FTIR	EGCC had the strongest binding affinity, complexes were stabilised by physical interactions at neutral pH	[71]
α-La and β-Lg	FA Caf Cou	FLQ CD FTIR	A reduction in protein α-helical structure upon complexation, static FLQ indicated complex formation	[72]
WPI, CAS and β-Lg	Pelargonidin	CD FLQ	Changes in secondary structure not found, FLQ of WPI and CAS systems did not change with temperature (25–45°C)	[73]
β-Casein	BDMC	FLQ IS	β-Casein encapsulated BDMC, it was bound at the hydrophobic core, stabilised by physical interactions	[74]
Casein and MG	FA, PC, DHB, NH, SA and GA	TLC MS	TLC and MS techniques were combined to study protein–phenolic interactions, can be used as a way to identify binding sites	[75]

RCL, radiochemical labelling; PGG, 1,2,3,4,6-penta-*O*-galloyl-D-glucopyranose; HAS, Human salivary α-amylase; FLQ, Fluorescence quenching; EGCC, Epigallocatechin gallate; DLS, Dynamic light scattering; DSC, Differential scanning calorimetry; CA, Chlorogenic acid; FA, Ferulic acid; Caf, Caffeic acid; Cou, Coumalic acid; CAS, Caseinate; BDMC, Bismethocycurcumin; IS, *In silico*; MG, Myoglobin; PC, *para*-coumaric acid; DHB, 2,4-Dihydroxybenzoic acid; NH, Ninhydrin; SA, Sinapic acid; GA, Gallic acid; TLC, Thin layer chromatography.

properties [78] and sedimentation [76], among other things. Despite a wide body of literature on the topic of protein–ligand interactions and more specifically on protein–phenolic interactions at ambient or relatively low temperature, most work at UHT temperatures has been focused on interactions and resulting changes to functional properties of milk components exclusively. However, when it comes to incorporating other ingredients such as insoluble fibres from micromilled particles of whole grain oat and wheat flour that contain bound phenolic compounds, there is a large gap in knowledge in UHT treated materials.

The temperatures and pressures involved in UHT treatments change the physical properties of water molecules, for example, the viscosity as well as the dielectric constant of the aqueous phase decreases as temperature increases, reducing its polarity and allowing for greater solubility of less polar organic compounds [79]. In the presence of endogenous enzymes, this may aid in extracting previously bound phenolic compounds from insoluble fibres during processing, making them more available for protein interaction. Initial research assessing interactions between milk proteins and phenolic acids at high temperatures argues that they are likely covalent in nature [5,80], with molecular docking studies indicating that *para*-coumaric acid binds to the lysine47 residue of β-casein (Figure 2). As a result, the bio-functionality and techno-functionality of both protein and phenolic may be permanently affected following alterations in the UHT-treated solution. These findings contrast results generally observed at ambient or even below the boiling-point temperatures, in which covalent attachment is only noted at pH > 8 or under oxidising conditions in acidic environments.

Figure 2

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Image showing the best binding conformation between β-casein and *para*-coumaric acid, generated by using molecular docking techniques [77].

Analysis of high temperature protein–phenolic interactions should be conducted using highly pure single protein systems to understand the molecular basis of such interactions. This poses a problem, as the highly pure proteins required for accurate and molecular analysis are expensive and large volumes are needed by even the smallest scale UHT plants (typically 6 L for a mini-UHT); in practice, these large sample sizes are excessive/prohibitive considering the expense. To mitigate this problem, pressure resistant borosilicate glass tubes with a small (~10 mL) capacity can be utilised, being suspended in a glycerol bath for heating, with the small volume ensuring that heating is rapid. It is important that the tubes are a closed system and rated to withstand the vapour pressure created at the desired temperature, such that the bulk of the solution is kept in a liquid phase. Additionally, the hot pressurised glassware requires significant safety precautions and the temperature within the tube should be logged via a thermowell that allows the experimental setting to remain closed to the atmosphere. Once the samples have been processed, they can be analysed using the advanced methods already in place for molecular protein–phenolic interactions (see Section 'Milk protein–phenolic interactions'). It is noted that the system described is suitable for comparison to a UHT plant, but care should be taken to match the time/temperature curves by providing rapid enough heating and cooling to reproduce industrial conditions at a mini scale. Additionally, shear forces present within a UHT plant have been shown to have an impact on treated systems [81] and these would not be replicated due to a lack of flow within the tube.

A useful addition to analysing the resultant products of the interaction would be to monitor the reactions as they are taking place, that is obtaining spectral 'snapshots' for comparison as the temperature of the system moves in a controlled manner towards its maximum. An overlay of the resulting spectra may show a general trend and in the case of FTIR point to the increase or decrease of diatomic pairs. Such techniques are commonly used in the analysis of polymer formation, with Mikhaylova *et al.* [82] providing a good example. A similar approach could be invaluable in demonstrating both conformational changes in proteins as well as molecular protein–ligand interactions as a result of UHT processing. In the case of aqueous systems, most spectrographic devices do not come with the capability to analyse samples above temperatures of 100°C. However, FTIR analysis can be achieved under these conditions by using a 'flow through adaptor'. Brill and Savage [83] provide a good outline of the experimental protocol as well as descriptions of adaptor designs that have been employed in a variety of different studies.

Finally, pressure is also an important parameter in UHT treatment; the product passing through heat exchangers must remain in a liquid phase to maintain consistency and prevent biofouling of the process plant. To ensure this, back pressure greater than the vapour pressure of water at the maximum temperature achieved must be applied [76]. The application of pressure alone has been shown to alter the structural properties of milk protein preparations and when combined with temperature the effects seem to be enhanced [84]. Increased pressure favours hydrogen bonding while weakening hydrophobic and electrostatic interactions [85]. Examining the effect that pressure variation imparts on UHT treatments may provide interesting insights and another variable that favours or inhibits protein–phenolic interactions during food production.

Conclusions

Innovative food formulation engineering will be moving towards enhancing the bioavailability of selected components to satisfy the consumer's demand for wellbeing alongside improved sensory attributes. Understanding protein–ligand interactions is one of the key components to achieving these desirable food products. Controlled release and delivery of bioactive compounds following food manufacture and during prolonged shelf life can be achieved by modifying and utilising various physical treatments, primarily heating, crosslinking with other agents or changing the ionic environment surrounding the protein molecules. To predict their release, there are numerous methods and mathematical models available in literature. However, an outlook to the future direction is to elaborate on the coupling of stress relaxation and diffusion mechanisms to better predict the transport of bioactives in real food systems, taking into account the nature of interaction between bioactive agents with macronutrients.

UHT processing presents a further challenge, and protein–phenolic reaction pathways appear to become chemical rather than physical in nature, with unknown physiological and toxicological effects. Further experiments, using very small scale indirect UHT methods that emulate industrial production, should be planned do design high quality functional beverages incorporating dairy protein and insoluble dietary fibre in formulations. Future work should aim to characterise interactions that occur at different temperature levels, while traditional spectroscopic techniques should be adapted to directly analyse spectral changes at higher temperatures within a short timeframe. More direct techniques such as radio/fluorescent labelling and mass spectroscopy (MS) combined with thin layer chromatography (TLC) should also be considered for follow up analysis to profile interactions and attempt to identify binding sites of proteins derivatized with phenolic compounds.

Conflict of interest statement

Nothing declared.

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