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REVIEW

Nanocarriers for β-Carotene Based on Milk Protein

Carini Aparecida Lelis[1,2,3,4] · Diego Galvan[1,3,5] · Carlos Adam Conte-Junior[1,2,3,4,5,6,7]

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Abstract
For many years, milk proteins have been included in human nutrition and as an ingredient in several products. In recent years, studies have sought to propose milk proteins as natural carriers for bioactive compounds and develop nanostructures containing these compounds. Although most bioactive compounds have beneficial effects on health, some limitations such as low solubility in aqueous media, low stability to pH and temperature variations, and low bioavailability have hampered their use. We carried out a systematic review analyzing interaction mechanism studies and nanoencapsulation studies to analyze the progress of using milk proteins as carriers of bioactive compounds. To better understand the subject, a brief review of the characteristics of milk proteins and the techniques used in interaction mechanism studies were also included in this review. We initially discuss interaction mechanism studies involving milk proteins and β-carotene (BC), a bioactive compound chosen to be explored. These studies are essential because they report the different mechanisms of milk proteins in BC complexation. Thus, it made it possible to understand that hydrophobic forces were the main ones involved in the interaction mechanism between milk proteins and BC. Changes in pH and/or temperature can cause changes in the structure of proteins, which influence the complexation process. Finally, we analyzed the studies that formed nanostructures containing BC from milk proteins. Emulsification was the outstanding methodology in nanoencapsulation of BC by milk proteins. The use of polysaccharides and polyphenols improved the milk proteins' performance in formingation and stabilizing nanostructures containing BC.

Keywords Binding · Bioactive compounds · Nanoemulsion · Interaction mechanisms · Nanoencapsulation

✉ Carlos Adam Conte-Junior
conte@iq.ufrj.br

[1] Center for Food Analysis (NAL), Technological Development Support Laboratory (LADETEC), Federal University of Rio de Janeiro (UFRJ), Cidade Universitária, Rio de Janeiro, RJ 21941-598, Brazil

[2] Nanotechnology Network, Carlos Chagas Filho Research Support Foundation of the State of Rio de Janeiro (FAPERJ), 20.020-000, Rio de Janeiro, RJ, Brazil

[3] Laboratory of Advanced Analysis in Biochemistry and Molecular Biology (LAABBM), Department of Biochemistry, Federal University of Rio de Janeiro (UFRJ), Cidade Universitária, Rio de Janeiro, RJ 21941-909, Brazil

[4] Graduate Program in Food Science (PPGCAL), Institute of Chemistry (IQ), Federal University of Rio de Janeiro (UFRJ), Cidade Universitária, Rio de Janeiro, RJ 21941-909, Brazil

[5] Graduate Program in Chemistry (PGQu), Institute of Chemistry (IQ), Federal University of Rio de Janeiro (UFRJ), Cidade Universitária, Rio de Janeiro, RJ 21941-909, Brazil

[6] Graduate Program in Veterinary Hygiene (PPGHV), Faculty of Veterinary Medicine, Fluminense Federal University (UFF), Vital Brazil Filho, Niterói, RJ 24230-340, Brazil

[7] Graduate Program in Sanitary Surveillance (PPGVS), National Institute of Health Quality Control (INCQS), Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, RJ 21040-900, Brazil

Introduction

Bioactive compounds have been the subject of much research in recent years, demonstrating potential health benefits. The general population is increasingly concerned about their food, seeking healthier, more natural foods that can offer health benefits. This has driven research on applying these bioactive compounds in foods (El-Saber Batiha et al., 2021; Volino-Souza et al., 2020). Studies propose to replace synthetic ingredients by natural or add functionality to the food and consequently benefit the consumer's health. In both cases, limitations related to their applications are extensively reported, such as insolubility in water, sensitivity to light, pH, oxygen, and heat (Anguelova & Warthesen, 2000; Krinsky & Johnson, 2005; Li et al., 2021a, b, c; Moran et al., 2018; Zhou et al., 2020a, b). Degradation of the bioactive compound can alter the possible beneficial health effects, as well as its functionality in food during processing and storage.

Given the limitations that bioactive compounds have shown for application in the food and pharmaceutical industry (Barba et al., 2017; Berni et al., 2015; Hempel et al., 2016; Song et al., 2018), research proposing nanocarriers (nanovehicles) has been the key to acting in the transport and protection of bioactive compounds. Various vehicles (zein, ovalbumin, bovine serum albumin, chitosan, whey protein, gelatin, Arabic gum, maltodextrin, rice starch, inulin, β-lactoglobulin, soybean polysaccharide, etc.) for various bioactive compounds (astaxanthin, *Tinospora cordifolia* extract, lycopene, brown bioactive compounds, extracts of *Passiflora edulis f. edulis*, camu-camu extracts, anthocyanins) have been studied in recent years (Bassijeh et al., 2020; Carvalho et al., 2021; Cui et al., 2021; de Abreu Figueiredo et al., 2020; El Ghazzaqui Barbosa et al., 2022; Anshika Jain et al., 2021; Jiang & Zhu, 2019; Kuhn et al., 2019; Li et al., 2020a; Mar et al., 2020; Mekhloufi et al., 2022; Nkurunziza et al., 2021; Qi et al., 2022; Ren et al., 2022; Wang et al., 2022).

Among the natural compounds that have gained prominence are carotenoids. In addition to acting such natural dyes, the carotenoids have attributes that aid health and function as antioxidants (Selig et al., 2018). Among carotenoids, β-carotene (BC) is the predominant form in most green leaves and has shown the solid antioxidant capacity and high provitamin A activity (Böhm et al., 2002; Santos et al., 2019). β-carotene deficiency is still a concern in developing countries, and hence, efficient fortification strategies and delivery systems are needed.

Of the proposed vehicles mentioned above, milk proteins have been studied for years and demonstrate the enormous potential to be used as ready-made vehicles and/or in constructing nanostructures containing nanoencapsulated bioactive compounds.

Milk proteins are naturally used as an ingredient in many foods due to their techno-functional properties, low cost, and sustainability (Zhou et al., 2020a, b). They act as natural carriers for some compounds in milk, such as calcium and phosphorus, and when isolated, they can form a complex with several compounds carrying them. These complexes can be developed through different interactions. The interactions and their magnitude may vary on the type of milk protein used. The proposal of a natural carrier is interesting because it makes the process easier and faster. Understanding the mechanisms related to the interaction between milk proteins and BC helps to better choose the nanocarrier material depending on the conditions of the application medium. Incorporating bioactive compounds in these nanostructures can reduce and even eliminate these limitations (Al-Hanish et al., 2016; Esmaili et al., 2011; Lang et al., 2021; Liu et al., 2018; Syama et al., 2019; Taghavi Kevij et al., 2019).

In this context, this systematic review aimed to group studies on milk proteins as natural nanocarriers for BC, a carotenoid with enormous potential for application in food and the pharmaceutical industry. After understanding the mechanisms involved in forming the milk protein and BC complexes that make milk proteins promising as a natural carrier, we also brought forward the potential to be used as a wall material in the BC nanoencapsulation process. As far as we know, there is no scientific document in the literature that gathers data regarding the use of milk proteins as natural nanocarriers and as a wall material for nanostructures aimed at BC transport.

Methodology

Based on the PICO (population, intervention, comparison, and outcome) method, we developed our question: Which milk proteins are used to form a complex with BC and as a wall material in the nanoencapsulation process? What were the thermodynamic parameters of complex formation? What are the main forces involved in forming the BC-milk protein complex? What techniques are used to determine the thermodynamic parameters of interaction? What methodology is used for nanoencapsulation of BC? What is the diameter of the formed nanostructures? What effects does nanoencapsulation have on BC?

The results are reported in agreement with the Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) statement flow diagram (Moher et al., 2015). The protocols' guidelines PICO and PRISMA have been carefully followed to help identify and prevent possible bias. Among, bias risks may also be associated with a lack of data regarding the diameter of the structures formed, eligibility criteria, database, and article type.

Screening: Title, Keywords, and Abstract

Inclusion: Studies published in English, at least one encapsulation strategy, and at least one natural antimicrobial were encapsulated and applied in a food matrix.

Exclusion: Studies that are not research articles, no encapsulation methodology has been studied, no natural antimicrobials have been learned, they have not been applied to food, no antimicrobial activity has been inspected, application to packaging, films, sachets, coating materials, and for cleaning.

Eligibility: Full-Text Reading

Exclusion: nanostructures containing BC with a diameter > 200 nm.

The systematic review was done in Science Direct, PubMed, Scopus, and Web of Science to find published articles between 2015 and March 3rd, 2021. The following search terms were used as groups of Keywords and Boolean operators ("AND," "OR" and "NOT"):

Search component 1: interaction OR complex OR nanocomplex OR binding OR encapsulation OR nanoencapsulation OR microencapsulation OR carrier OR microcarrier OR nanocarrier OR delivery OR capsules OR microcapsules OR nanocapsules OR structures OR microstructure OR nanostructure;
Search component 2: carotene OR carotenoids OR β-carotene;
Search component 3: "whey protein" OR "milk protein" OR albumin OR casein OR caseinate OR lactoferrin OR lactoglobulin OR lactalbumin.

A manual search of the articles and their reference sections was also conducted to avoid missing essential studies. In the initial screening, based on the reading of the title, abstract, and keywords, articles that did not investigate carotenoids and milk proteins were eliminated, as well as letters, reviews, editorials, commentaries, monographs, and Ph.D. thesis. Based on the entire reading of the paper, we excluded articles that did not approach BC (carotenoid selected for this review). Taking into account that the vast majority of the BC encapsulation process involves the formation of emulsions and considering nanoemulsion structures with a diameter < 200 nm (López-Monterrubio et al., 2021; Zhou et al., 2018), articles that did not present the diameter of the structure obtained or that the diameter of the structures was greater than > 200 nm were rejected. In the other articles, the complete reading was carried out. Any questions regarding the selection of articles were resolved through discussion among the authors.

Results and Discussion

Initially, 1,489 articles were identified, of which 200 were duplicated/tripled. After reading the title, keyword, and abstract, 1,154 were excluded, and 135 were selected to read the full text. After careful reading, 81 articles were excluded for not meeting the eligibility criteria, and 54 were selected (Fig. 1). Other papers were added during the review discussion to explain the subject further.

Characteristics of Milk Proteins that make them the Potential for the Transport of Compounds

In milk are found two main categories of proteins, the casein micelles (~ 80%) and the whey proteins (~ 20%) (Huppertz et al., 2018). The range of functional and structural properties of milk proteins can facilitate nanostructure development to carry bioactive compounds. According to Tang (2021), the proteins have shown the most promising and effective nanovehicles. Besides being suitable for food are sustainable, low cost, eco-friendly, easy to prepare, and natural (chemical-free).

Complex heterogeneous associations of α_{S1}-, α_{S2}-, β-, k-casein, and calcium phosphate, held through non-covalent intermolecular binding, form the casein micelles. The four fractions of α_{s1}-, α_{s2}-, β-, and k-casein is a molar ratio of 4:1:4:1.3. About 95% of the caseins are naturally self-assembled, forming colloidal micelles, spherical colloidal particles, 50–500 nm (average 150 nm) in diameter, and molecular mass between 10^6 and 3×10^9 Da, present in significant amounts in milk, 10^{14}–10^{16} micelles/mL milk. The caseins are kept in solution due to electrostatic and steric stabilization. Casein micelles' interior is composed of water-filled cavities (Allahdad et al., 2020; De Kruif et al., 2012; Horne, 2020; Fox & McSweeney, 2006). The casein proteins showed the amphiphilic nature that self-associate. The α_{s1}-casein can join, forming dimers, tetramers, and larger polymers, depending on the pH and ionic strength. The α_{s2}-casein is also self-associated with ionic strength and decreases again at higher ionic strength (> 0.3 M). Unlike whey proteins, the casein micelles are formed for rheomorphic proteins and do not have a well-defined tertiary structure. However, the structure of casein micelles is highly pH-dependent, being used as a strategy for nanovehicles for delivery of hydrophobic compounds, where pHs above the isoelectric point, e.g., 4.6, the structure becomes more compact as the pH decreases and more open as the pH increases (Livney, 2010; Nicolai & Chassenieux, 2021; Tang, 2021). Casein's self-assembly behavior or individual components can be utilized to fabricate tunable micelles or micelle-like nano-architectures as potential nanovehicles for nutraceuticals. The micellar casein porous

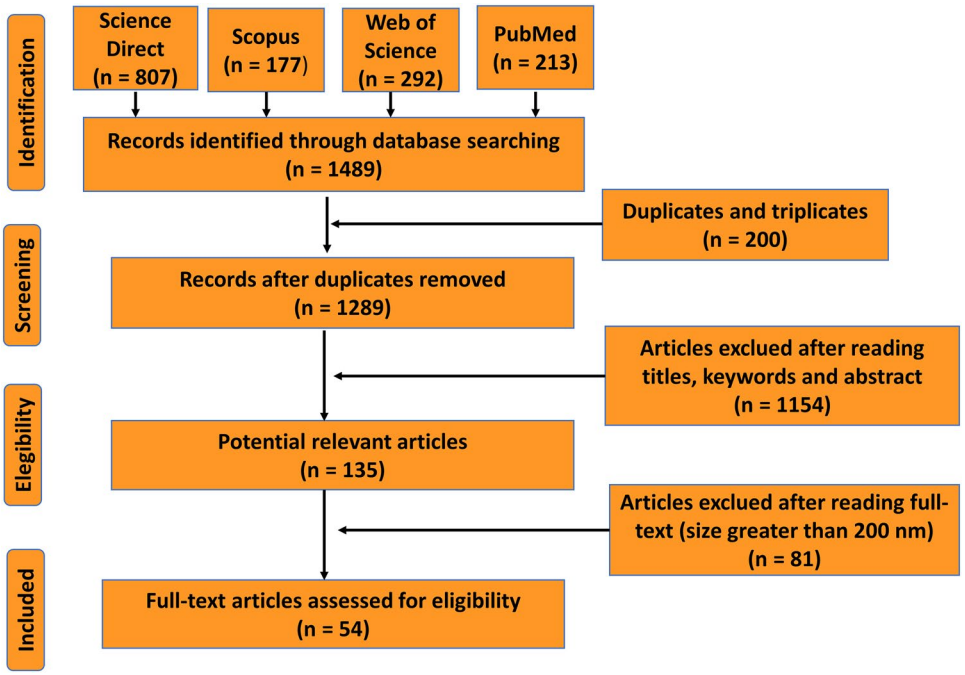

Fig. 1 PRISMA flow diagram displaying the results of the systematic search

structure and the amphiphilic nature may offer advantages for the nanoencapsulation of bioactive compounds (Yang et al., 2020).

The reassembly process can be performed by forming caseinates, similar to casein micelles. Salts of caseinates are commercially available and are practical for application in food formulations. Redispersion of caseins in acid (pH 5.3) with the aid of NaOH or $Ca(OH)_2$ allows the formation of sodium or calcium caseinates (Tang, 2021).

Recently studies have shown the potential that casein micelle and caseinate with nanovehicles and wall material for compounds diversity with lutein, β-carotene, vitamins, curcumin, anthocyanins, resveratrol, eugenol, quercetin, lysozyme, propolis extract, tannic acid (Antonov et al., 2017; Casanova et al., 2021; Ma et al., 2021; Mantovani et al., 2020; Nagaraju et al., 2021; Zhan et al., 2018; Zhou et al., 2021), aflatoxin M1 (Indyk et al., 2021), and microorganism with *Lactobacillus paracasei*, *Lactobacillus reuteri*, and *Lactobacillus zeae* (Li et al., 2021a, b, c; Liu et al., 2016a, b; Zhao et al., 2020).

After casein precipitation at pH 4.6, the whey proteins (WPs) are obtained from milk. WPs include α-lactoalbumin (α-la), β-lactoglobulin (β-lg), bovine serum albumin (BSA), lactoferrin, and lactoperoxidase (Anema, 2020). Unlike casein micelle, the WPs are heat-labile due to their globular nature. β-lactoglobulin, the main protein present in the whey protein isolate (WPI), is a smaller globular protein, a member of the lipocalin family, featuring a large hydrophobic cavity in the barrel calyx-shaped that serves as a suitable carrier, especially for poor water-soluble molecules (Allahdad et al., 2019; Livney, 2010). The pH influences this globular protein's reversible association/disassociation behavior; its pI (isoelectric point) is ~ 5.2. At pH < 2 and pH > 8, and the secondary and tertiary structures of β-lg are largely preserved. Between pH 2 and 8, with moderate ionic strength (e.g., 100 mM NaCl) and temperature above 20 °C, the β-lg is in the form of dimeric. In pH < 3 and ionic strength < 10 mM, β-lg is predominantly monomeric, and at pH between 3.5 and 5.5, it tends to exist as an octamer (Allahdad et al., 2019; Labra-Núñez et al., 2021; Livney, 2010; Tang, 2021; Taulier & Chalikian, 2001). A conformational rearrangement occurs between pH 6.5 and 7.8, the Tanford transition. In this rearrangement, the main conformational change is restructuring the E–F loop, a great place to acess the binding site. Free access to the binding site occurs at pH > 7 due to open conformations in the E–F loop. Already in pH < 7, occluding of the hydrophobic cavity occurs (Labra-Núñez et al., 2021). The second major protein component in serum is α-la, a small globular metalloprotein with a molecular mass of 1.42×10^4 Da. The α-la has Ca^{2+} binding in its structure to assume its functional fold (Allahdad et al., 2019; Livney, 2010). Its pI is between 4.3 and 4.7. The calcium ions are released at pH below 5 due to the protonation of the aspartate residues (Tang, 2021). BSA is found both in blood serum and in milk (Livney, 2010; Tang, 2021). BSA is a natural carrier of small molecules in the blood and contains three domains (I-III, each one divided into two subdomains (A and B)) specified for binding like the hydrophobic cavities of Sudlow's site I (on subdomain IIA) and Sudlow's site II (on subdomain IIIA) (Javaheri-Ghezeldizaj et al.,

2020; Poór et al., 2014; Precupas et al., 2021; Sergio et al., 2021). BSA has a 75.6% sequence homology with HSA, which makes it a model protein in interaction studies with bioactive compounds (Majorek et al., 2012). Lactoferrin is a basic glycoprotein belonging to the family of iron-binding proteins (with pI of 8.5). However, it can also bind other metal ions like copper, chromium, manganese, and aluminum in vivo. At neutral pH show a highly positive charge, and it exhibits a high tendency to associate with oppositely charged proteins, offering a promising strategy for forming nanocarriers for hydrophobic molecules (Livney, 2010; Fox & McSweeney, 2006). β-lg and α-la were able to preserve the two antibacterial regions within the apolactoferrin structure (lactoferrin and lactoferampin), and free energy calculations found electrostatic attractions be the primary mode of interaction (Darmawan et al., 2020). β-lg monomer interacted with vanillic acid (Abdollahi et al., 2021). The internal cavity of the β-barrel of β-lg was the local target for astaxanthin binding (Liu et al., 2021). β-lg has also been shown to be promising for the transport of phenolic acids, anthocyanins, flavonoids, allyl-isothiocyanate, xylitol, lycopene, and anthraquinones (Gheona (Dima) et al., 2018; Ersöz & Dudak, 2020; Geng et al., 2020; Kong et al., 2020; Li et al., 2020b; Salah & Xu, 2021; Wang et al., 2021; Xu et al., 2019). The chlorogenic acid showed binding in surface, cleft, and subdomain I for β-lg, α-la, and BSA, respectively (Zhang et al., 2021a, b). Site I in BSA is also the binding site for farrerol, epigallocatechin gallate, camptothecin, 10-hydroxycamptothecin, and butylated hydroxyanisole (Gu et al., 2021; Tang et al., 2020; Yang et al., 2021). Already the lutein showed binding in site III of BSA (Paiva et al., 2020). Both β-lg, α-la, and BSA demonstrated potential as nanovehicles for fucoxanthin (Zhu et al., 2017). Lactoferrin also showed the potential for natural nanovehicles of naringenin, curcumin, dihydromyricetin, and myricetin (Huang et al., 2020; Lelis et al., 2020; Nunes et al., 2020). WPI composed of the proteins aforementioned showed to be natural nanovehicles for norbixin, flavonoids (naringin, rutin, quercetin, puerarin), sucralose, and hyaluronic acid (Li et al., 2021a, b, c; Møller et al., 2020; Yin et al., 2020; Zhang et al., 2017a; Zhong et al., 2021).

Due to the intrinsic amphiphilic nature, self-assembly, and co-assembly molecular capacity, many milk proteins spontaneously bind to hydrophobic compounds or can serve as molecules to form nanostructures able to encapsulate and transport molecules such as eugenol, vitamin D_3, curcumin, *trans*-cinnamaldehyde, thyme oil, krill oil, anthocyanins, lycopene, clove oil, and caffeine (Abbasi et al., 2014; El-Messery et al., 2020; Fuciños et al., 2017; Jain et al., 2018; Liao et al., 2021; Nagaraju et al., 2021; Salah et al., 2020; Sari et al., 2015; Sharma et al., 2017; Zhang & Zhong, 2020).

β-Carotene

BC comprises 40 carbon atoms in a core structure of conjugated double bonds substituted with two β-ionone rings. The orange to red color is due to 9 fully conjugated double bonds. There is a predominance of all-*trans* β-carotene (E-isomer), but *cis*-isomers are also found in living organisms and food (Bogacz-Radomska & Harasym, 2018; Grune et al., 2010). It works by protecting food and biological tissues from oxidation due to the long chain of unsaturated conjugated hydrocarbons that effectively quench singlet oxygen and free radicals. Despite the strikingly intense colorations of BC, a large number of double bonds makes the BC compounds quite susceptible to oxidation, causing loss of biological activity and change in color. It is widely used in medicine due to its high bioactivity. The primary nutritional function of the BC is a vitamin A precursor. Vitamin A is essential for the vision process also systemic functions in the growth and reproductive efficiency. The antioxidant property is associated with its binding capacity with singlet oxygen by conjugated double bond systems (Bogacz-Radomska & Harasym, 2018; Ribeiro et al., 2011; Shahidi & Zhong, 2010). Industrially BC is used as a food coloring contributing to the yellow to the orange color of foods. Food matrices are varied, and BC application occurs in hydrophilic matrices such as juices and beverages and lipophilic matrices such as butter, margarine, and cheese. It is also used in the cosmetic industry to protect against skin lesions, oxidation, and ultraviolet radiation and in the pharmaceutical industry as a colorant. Natural BC can be produced by biotechnological processes using filamentous fungi, yeasts, bacteria, or microalgae or through extraction from vegetable sources (Bogacz-Radomska & Harasym, 2018; Grune et al., 2010; Ribeiro et al., 2011).

Despite having gained prominence in recent years in the supplementation of food products (Niu et al., 2020), its poor stability against heat, light, and oxygen and extremely low solubility in the aqueous phase make applications difficult (Fan et al., 2017; Yi et al., 2016). For example, they are not stable under processing and storage conditions such as light, heat, and oxygen or gastrointestinal conditions (Du et al., 2019) and low in vivo bioavailability (Aprodu et al., 2017).

One of the reasons for the drop-in functionality of BC in aqueous media is the formation of aggregates. Like other hydrophobic molecules, BC in water forms aggregates through hydrophobic effects and the interaction of van der Waals and hydrogen bonds. Two aggregates can be formed, J (weakly coupled) and H (strongly associated), and they are distinguished by absorption measures (Allahdad et al., 2020; Hempel et al., 2016). The H aggregate has a highly blue-shifted absorption spectrum relative to the BC monomer, and the J aggregate has a red-shifted absorption spectrum (Allahdad et al., 2020; Wang et al., 2012).

The oxidation process stands out when it comes to the low stability of BC under different environmental conditions. In addition to the loss of bioactivity, the BC oxidation process can cause a loss of food quality as color and flavor. When the oxidative process starts, BC can react with itself or other chemical species present in food, forming many products (Boon et al., 2010; Kanasawud & Crouzet, 1990; Mortensen, 2002).

Given the wide range of limitations related to the application of BC in food matrices, carrier molecules, or those forming delivery systems, it has highlighted the scientific community's interest. Complexes/structures containing BC can be inserted in different types of food. This process facilitates dispersion, action and protections, helping to reduce degradation and remain stable. Depending on its purpose in the food, it is crucial to consider the controlled release of BC complexed or coated by milk proteins during the digestive process.

Interaction Between Milk Proteins and β-Carotene

Studies involving the comprehension of the interaction between milk proteins and BC are highly relevant to their subsequent application in food. Basic science makes it possible to understand all thermodynamic parameters and the forces involved in forming milk protein-BC complexes. The comprehension of the kinetics involved in the formation of complexes has also grown in recent years, bringing greater scientific clarity to the dynamics of complex formation between milk proteins and bioactive molecules. The study of the effect of pH, temperature, salt concentration, and light also helps develop efficient nanocarrier systems for BC and potential applications in food.

Table 1 shows the pH used in the interaction study, the binding constant, and the main forces present in forming the milk protein-BC complex.

β-Lactoglobulin

β-lg has been shown to interact with BC with a binding constant of the order of 10^7 for both pHs studied, 4.25 and 7.0 (Hosseini et al., 2015). A binding constant on the order of 10^7 is considered high affinity (Gu et al., 2021; Rodrigues et al., 2020). At pH 7.0, two β-lg binding sites per mole of BC are needed. Already at pH 4.25, almost twice as many protein sites are required. Although at pH 4.25, the EF loop is in a closed conformation, and the binding constant was higher than pH 7.0, indicating that hydrophobic interactions are increased. This larger binding constant also assumes that a BC molecule is tightly bound at the interfaces of monomers found within octamers. Based on the binding constant obtained at different pHs, we suggest that the hydrophobic interactions have stood out in the formation of the complex (Hosseini et al., 2015). Allahdad et al. (2019) showed values 3.5 times lower for the interaction between β-lg and BC. When analyzing the studies, we suggest some factors that may have influenced the data found: (i) in the study by Hosseini et al. (2015), the authors filtered the β-lg to get aggregate free β-lg dispersion, which was not described by Allahdad et al. (2019), (ii) excitation wavelengths in the fluorescence analysis were different, and (iii) amount (%) of ethanol added to the protein solution (from BC solution) were different (about 1.8 times). A binding constant of 9.0×10^7 M^{-1} was obtained at pH 7.7. As mentioned in item 3.1, at pH > 7.0, there is free access to the binding site due to open conformations of the E–F loop (β-lg), which may justify the higher value of the binding constant (Aprodu et al., 2017).

In general, in addition to pH, other factors may have influenced the different values found, such as (i) the type of buffer (tris–HCl, acetate, phosphate); (ii) the solvent used to solubilize BC such as ethanol and hexane. The solubility of BC in the two solvents is different, which can provide a different amount of BC in free or aggregate form; and (iii) the amount of organic solvent in the protein solution after the addition of BC. These data are of great relevance since β-lg can be used to carrier bioactive compounds such as BC both in food and in the pharmaceutical industry.

Bovine Serum Albumin

Binding constants of 2.63×10^5 M^{-1}, 4.67×10^5 M^{-1}, and 1.19×10^5 M^{-1} have been obtained at pH 7.4 (Chang et al., 2016; Li et al., 2015; Silva et al., 2018). The pH 7.4 represents the blood where BSA is considered a natural vehicle, so the primary justification for its application is as a BC transport protein in the human body. The highest value found of 4.67×10^5 M^{-1} was determined by photooxidation (UV–vis) and the others by fluorescence. Only interactions that occur with or near tryptophan and tyrosine are determined by fluorescence. Therefore, it is suggested that BC binds in regions beyond those involving tryptophan and tyrosine. The binding constants in these regions contribute to a higher complex formation constant, leading to higher values obtained by photooxidation. By fluorescence spectroscopy, Silva et al. (2018) and Allahdad et al. (2019) found *n* close to 1, suggesting that 1 BC molecule binds to each BSA binding site. Already Reszczynska et al. (2015) by absorption (UV–vis) found a stoichiometry of 3, suggesting that BC molecules bind at other binding sites on BSA different from tryptophan surroundings.

To assess the effect of protein structure as a carrier for BC, Silva et al. (2018) performed the study with denatured BSA (heating 80 °C/10 min). After denaturation, they found an approximately 900-fold reduction in the binding constant and a threefold reduced *n*, suggesting that about

Table 1 Interaction study between milk proteins and β-carotene

Protein	pH	Binding constant M^{-1} (25 °C)	*n* (25 °C)	Thermodynamic parameters	Types of interactions	Technique	References
β-Lactoglobulin	7.00	4.70×10^{7}	0.48	-	-	Fluorescence spectroscopy	(Hosseini et al., 2015)
	4.25	6.60×10^{7}	0.23				
Bovine serum albumin	7.40	2.63×10^{5}		ΔG°: − 30.91 kJ mol^{-1} ΔH°: − 1.65 kJ mol^{-1} ΔS°: 98.18 $Jmol^{-1}$ k^{-1}	Hydrophobic interactions and electrostatic attraction	Fluorescence spectroscopy	(Li et al., 2015)
					β-carotene interacts with both the C=O and C–N groups in the protein polypeptides	FT-IR	
Bovine serum albumin	7.40	-	3	-	-	Absorption (UV–vis)	(Reszczynska et al., 2015)
β-Lactoglobulin-carboxymethylcellulose	4.00	-	-	-	Hydrophobic interactions	Isothermal Titration Calorimetry	(Goltz et al., 2016)
Bovine serum albumin	7.40	4.67×10^{5}	-	-	-	Photooxidation – UV–vis	(Chang et al., 2016)
	-	-	-	ΔG°: − 25.30 kJ mol^{-1} ΔH°: 17.10 kJ mol^{-1} ΔS°: 142.20 $Jmol^{-1}$ k^{-1}	Hydrophobic interactions	Fluorescence spectroscopy	
α-Lactoalbumin	7.50	1.57×10^{3}	1.13	ΔG°: 0.66 kJ mol^{-1} ΔH°: − 3.24 kJ mol^{-1} ΔS°: − 13.12 $Jmol^{-1}$ k^{-1}	Hydrophobic interaction, hydrogen bonding and van der Waals interactions	Fluorescence spectroscopy	(Dumitraşcu et al., 2016)
					Hydrophobic interaction and aromatic interactions	Docking simulation	
β-Lactoglobulin	7.70	9.00×10^{7}	1.56	-	-	Fluorescence spectroscopy	(Aprodu et al., 2017)
					Hydrophobic interaction	Docking simulation	
Whey protein isolate	-	-	-	-	Hydrogen bonds	FT-IR	(Ursache et al., 2018)
Bovine serum albumin	7.40	1.19×10^{5}	0.97	ΔG°: − 28.36 kJ mol^{-1} ΔH°: 8.62 kJ mol^{-1} ΔS°: 126 $Jmol^{-1}$ k^{-1}	Hydrophobic interactions	Fluorescence spectroscopy	(Silva et al., 2018)
Unfolded bovine serum albumin		1.34×10^{2}	0.32	ΔG°: − 12.13 kJ mol^{-1} ΔH°: 32.20 kJ mol^{-1} ΔS°: 148.60 $Jmol^{-1}$ k^{-1}			
β-Casein micelle	7.00	2.44×10^{2}	0.54	ΔG°: − 13.62 kJ mol^{-1} ΔH°: 57.87 kJ mol^{-1} ΔS°: 239.70 $Jmol^{-1}$ k^{-1}			
Whey protein nanoparticles	-	-	-	-	Hydrophobic interactions	FT-IR	(Salem et al., 2018)

Table 1 (continued)

Protein	pH	Binding constant M^{-1} (25 °C)	*n* (25 °C)	Thermodynamic parameters	Types of interactions	Technique	References
Casein	6.00	1.21×10^4	0.77	-	van der Waals forces and	Fluorescence spectroscopy	(Allahdad et al., 2019)
	7.00	0.40×10^4	0.69	ΔG°: − 24.52 kJ mol^{-1} ΔH°: − 158.62 kJ mol^{-1} ΔS°: − 450 KJmol^{-1} k^{-1}			
				–			
	8.00	3.30×10^4	0.89				
	9.00	17.76×10^4	1.02	ΔG°: − 30.47 kJ mol^{-1} ΔH°: − 60.17 kJ mol^{-1} ΔS°: − 100 KJmol^{-1} k^{-1}	hydrogen bonds		
αs1-Casein	7.00	1.57×10^4	0.87	ΔG°: − 33.05 kJ mol^{-1}	Hydrophobic interactions	Docking simulation	
β-Casein		0.04×10^4	0.59	− 31.79 kJ mol^{-1}			
K-Casein		3.01×10^4	0.87	− 35.56 kJ mol^{-1}			
Whey protein isolate	4.00	0.29×10^4	0.71		Hydrophobic interactions	Fluorescence spectroscopy	(Allahdad et al., 2019)
	5.00	0.67×10^4	0.75				
	6.00	0.46×10^4	0.75				
	7.00	0.52×10^4	0.79	ΔG°: − 22.21 kJ mol^{-1} ΔH°: 153.61 kJ mol^{-1} ΔS°: 590 Jmol^{-1} k^{-1}			
	8.00	0.75×10^4	0.84				
	9.00	1.53×10^4	0.87				
α-Lactoalbumin	7.00	1.80×10^4	0.88	–	Hydrophobic interactions	Docking simulation	
β-Lactoglobulin		1309.65×10^4	1.44				
Bovine serum albumin		4.51×10^4	0.84				
Casein	-	-	-	-	van der Waals interactions	Raman spectra	(Allahdad et al., 2020)
Whey protein isolate					Hydrophobic interaction		
Casein	-	1.23×10^5	-	-	-	Photodegradation	
Whey protein isolate		0.91×10^5					
Casein	-	0.04×10^5	-	-	-	Fluorescence spectroscopy	
Whey protein isolate		0.05×10^5					
Whey protein isolate fibrils	-	-	-	-	Hydrophobic interaction and hydrogen bonds	FT-IR	(Zhang et al., 2021a, b)
					hydrophobic interaction	Fluorescence spectroscopy	

Standard Gibbs free energy change (ΔG°), standard enthalpy change (ΔH°), and standard entropic change (ΔS°)

FT-IR Fourier transform infrared spectroscopy

3 molecules of denatured BSA are needed to carry a molecule of BC. Small aggregates can be formed when BSA is denatured due to exposure to hydrophobic groups. BC showed preferential binding to BSA site I, known to be a large hydrophobic cavity that agrees with the hydrophobic characteristic of the BC molecule and with the data obtained Allahdad et al. (2019) by molecular docking. When denatured the BSA molecule, the site I was lost, and a possible rearrangement of the denatured BSA was necessary to carry BC (Silva et al., 2018).

As observed for β-lg-BC, the BSA-BC complex was predominantly formed by hydrophobic interactions (Allahdad et al., 2019; Aprodu et al., 2017; Chang et al., 2016; Li et al., 2015; Silva et al., 2018). For BSA-BC, in addition to hydrophobic interactions, electrostatic interactions were shown to play an essential role in complex formation, and BC was found to interact with both the C = O and C–N groups in the protein polypeptides (Li et al., 2015).

ΔG° values between − 25 and − 31 kJ mol^{-1} were obtained in studies between BSA and BC (Chang et al.,

2016; Li et al., 2015; Silva et al., 2018). Negative ΔG values suggest that the chemical balance BSA + BC ⇄ BSA-BC favored the complex formation. In both studies, the term entropic was the one that most contributed to constructing BSA-BC complex. This entropic gain is justified by increasing the degree of translational freedom of water molecules released from solvation layers of BSA and BC molecules.

For denatured BSA, hydrophobic interactions also prevail in the complex formation, and the enthalpic and entropic effect was more significant compared to the native BSA, demonstrating that the denatured BSA aggregation process provided a more substantial release of water molecules from the solvation layer of BSA (Silva et al., 2018).

α-Lactoalbumin

α-la, at pH 7.0, presented a binding constant of 1.80×10^4 M^{-1} (Allahdad et al., 2019) and at pH 7.5 $Kb = 1.57 \times 10^3$ M^{-1} (Dumitraşcu et al., 2016). Both studies suggested the occurrence of hydrophobic interactions, hydrogen bonds, and van der Waals interactions. Through molecular docking, it was proposed that BC interacts in the cleft region between α- and β-domains of apo α-la, binding preferentially in the aromatic cluster II (Allahdad et al., 2019). We suggest that the different values of the binding constant found in the studies may be related to the solvents used to prepare the BC solution (ethanol and hexane) and the amount of solvent added to the protein solution. Fluorescence spectroscopy is a very sensitive technique (Strasburg & Ludescher, 1995), and minimal changes in the protein structure by the solvent can influence the final results obtained. Furthermore, the amount of BC aggregates present in the solution may differ due to the solvents used. These structural changes in the protein and the presence of BC aggregates in an aqueous solution were suggested by Allahdad et al. (2019) by determining the size of milk proteins in the absence and presence of BC dissolved in ethanol.

Whey Protein Isolate

BC demonstrated to adopt a distorted conformation around carbons bonds of milk proteins. The hydrophobic interactions predominated in the formation of the WPI-BC complex, in addition to hydrogen bonds (Allahdad et al., 2019, 2020; Ursache et al., 2018). Binding constants of 0.91×10^5 M^{-1} and 0.05×10^5 M^{-1} were obtained from photodegradation and fluorescence (Allahdad et al., 2020). Again, higher binding constant are obtained by techniques that consider interactions at sites other than those close to tryptophan and tyrosine residues. The larger binding constant for photodegradation suggests other interaction sites for BC, whose affinity is greater than the close interaction sites for tryptophan and tyrosine. Values relative to 5×10^3 M^{-1}, at pH 7.0, were also found by Allahdad et al. (2019). Lower binding constant were obtained at lower pH values, 4 to 7. WPI is composed of several proteins, mainly β-lg and α-la, so the effects of pH on these proteins will reflect the behavior of WPI to interact with BC. At pH < 7, the closure of the hydrophobic cavity of the β-barrel by the EF loop prevents BC access to the central calyx of the β-Ig. At pHs far from the isoelectric point (pH 4.2) of α-la, the protein has a more open conformation with exposed hydrophobic fractions. At higher pHs, it is in the form of a monomer that can facilitate access to BC. However, when analyzing on increasing ionic strength, they found that the interaction parameters are influenced by β-lg (Allahdad et al., 2019).

An interesting observation was that the concentration of WPI influenced the solubility of BC. At concentrations of 1.5 mg/mL, there was an increase of up to 3.6 times the solubility in an aqueous system containing 20% ethanol. However, when increasing the protein concentration, it was found that the solubility decreased, suggesting the occurrence of protein–protein interactions. WPI increased BC retention in a buffer/ethanol solution (80/20) by up to 12%. It is suggested that the binding cavity of β-Ig, the most abundant protein in WPI, protects BC's rings and polyene chain from light and water molecules, thus protecting BC from photooxidation. The addition of WPI to the BC solution also increased its hydrogen donating capacity. WPI fibrils obtained by heating (80 °C) for several hours (5–24 h) under acidic conditions and low ionic strength have also been shown to interact with BC. The hydrophobic interactions and hydrogen bonds are the main ones involved. Long-term heat treatment increased the hydrophobic region of the WPI fibrils, increasing the interaction with BC. The WPI fibril-BC complex demonstrated an increase in gastro-intestinal digestion, antioxidant capacity, a reduction in the total color difference, and increased sedimentation stability (Zhang et al., 2021a, b).

WPI nanoparticles prepared using the pH cycle method at an aggregation pH value of 6 and 22 h aging in the presence of calcium ions were used to trap BC and zinc ions. Like WPI, WPI nanoparticles have also been shown to interact with BC mainly through hydrophobic interactions (Salem et al., 2018).

Casein and Its Fractions

In addition to whey proteins, casein and its fractions have also been proposed to interact with BC. Allahdad et al. (2020, 2018) suggested that the formation of the casein-BC complex occurs mainly through van der Waals forces and hydrogen bonds. BC in the aggregate form as possible to interact on the casein surfaces, contrary to what was observed for β-Ig. The larger hydrodynamic radius of aggregated BC restricts its insertion into the hydrophobic pocket

of β-Ig, the predominant protein in the WPI. However, the aggregated BC has lower antioxidant efficiency, and consequently, the aggregated BC-casein complex also presented lower oxidizing power. This demonstrates that knowledge about the chemical structure of BC, its functionality, and the formation of the proteina-BC complex is of grear relevance to use milk proteins as carries for BC (Allahdad et al., 2020).

pH has also been shown to influence casein's interactions with BC significantly. Changes in protein structure due to changes in pH can make the protein more accessible to the ligand. At alkaline pH, a higher binding constant was obtained due to the looser structure of casein (the occurrence of electrostatic repulsion), facilitating the access of BC to the hydrophobic domains. The presence of salt also influences the formation of the casein-BC complex. The presence of high salt concentrations (NaCl) neutralizes the surface charges of casein, promoting its aggregation and preventing BC access to the hydrophobic segments of casein. The formation of the casein-BC complex is an exothermic, enthalpy-driven process. The study was also carried out with casein fractions. In this study, the hydrophobicity of the protein fractions and the capacity of self-associated micelle formation were shown to influence complexation with BC. Despite having lower hydrophobicity compared to β-casein (β- > k- > αs_1- > αs_2-casein), k-casein and αs_1-casein were stronger carriers for BC. αs_1 has a lower degree of association and condensation, making the groups more reactive available to BC (Allahdad et al., 2018). The complex formed by β-casein micelles and BC presented a binding constant of the order of 10^2 (Silva et al., 2018), as found by Allahdad et al. (2018).

Considering that each β-casein micelle contains between 15 to 60 β-casein monomers and that the interaction stoichiometry was 0.54, the authors suggested that each β-casein micelle can carry 7 to 30 BC molecules. That demonstrates the enormous potential of β-casein as a nanocarrier for BC. The formation of the complex was entropically directed, being driven by the hydrophobic effect, that is, by the release of water molecules from the solvation layer of the hydrophobic sites. By analyzing the photostability of BC, it was found that the β-casein micelles reduced by 4 times the degradation constants of BC compared to the 3 times reduction for β-casein monomers. The hydrophobic core of β-casein micelles is better able to transport and stabilize BC in its interior (Silva et al., 2018).

Unfortunately, not all thermodynamic parameters (n, k, ΔG°, ΔH°, and ΔS°) were determined in the works. Considering the studies that determined all thermodynamic parameters and described the studied pH, we assembled an image representing each parameter's effect on the formation of the complex between milk proteins and BC.

When analyzing Fig. 2, we verify that BSA was the protein with the highest binding constant for BC (Kb ≈ 10^4–10^5 M^{-1}) (Gu et al., 2021; Rodrigues et al., 2020). The BSA may store, transport, and deliver the BC at a moderate binding constant at the target location. An interaction constant of the order of 10^5 M^{-1} was also reported in the study of the interaction between lysozyme, bromelain, HSA, and BC (Li et al., 2015, 2018; Magalhães et al., 2021).

Although BSA demonstrates a higher binding constant for BC than milk proteins, the value was much lower than that obtained by papain (order of 10^8 M^{-1}) (Li et al., 2018). After denaturing the BSA, the binding sites for BC decreased, thus reducing the stoichiometry of complex formation. It concludes that BSA as a nanocarrier for BC in environments and/or matrices that will undergo heating may not be attractive. The lowest Gibbs free energy was shown for α-la and WPI, suggesting a low favoring of the formation of the α-la-BC and WPI-BC complex under the conditions analyzed.

Similarly, the complex formed by BSA-, denatured BSA-, β-casein-, and WPI-BC were entropically driven, favored by the release of water molecules from the solvation layer hydrophobic sites. On the other hand, casein-BC was enthalpically driven, an exothermic process, demonstrating that despite all the milk proteins studied showing potential to carry BC, the mechanisms involved in this process vary, more or less, from protein to protein. Therefore, an essential factor to consider for practical application. Given the facts, we suggest that although BSA is the most promising protein among those analyzed in Fig. 2, it has not been shown to have suitable application in systems subjected to heating.

Further studies on the interaction between BC and milk proteins are relevant for further clarification of the mechanisms involved in the complex formation. Possible structural modifications of proteins due to different pH, temperature, and ionic strength should be analyzed as conditions found in food processing and the human body. In addition to the thermodynamic process, studies on the kinetics of complex formation allow an understanding of the dynamics involved in the complex formation, providing further clarification.

Some Techniques Involved in the Study of the Interaction

From Table 1, we can see that the main methods involved in the interaction study are the spectroscopy of fluorescence, isothermal titration calorimetry, UV–visible absorption spectroscopy, Fourier transform infrared spectroscopy, and docking molecular.

Fluorescence Spectroscopy

The fluorescence quenching methodology for interactions studs between protein and ligand has recently become popular. In milk proteins, the intrinsic fluorescence is associated

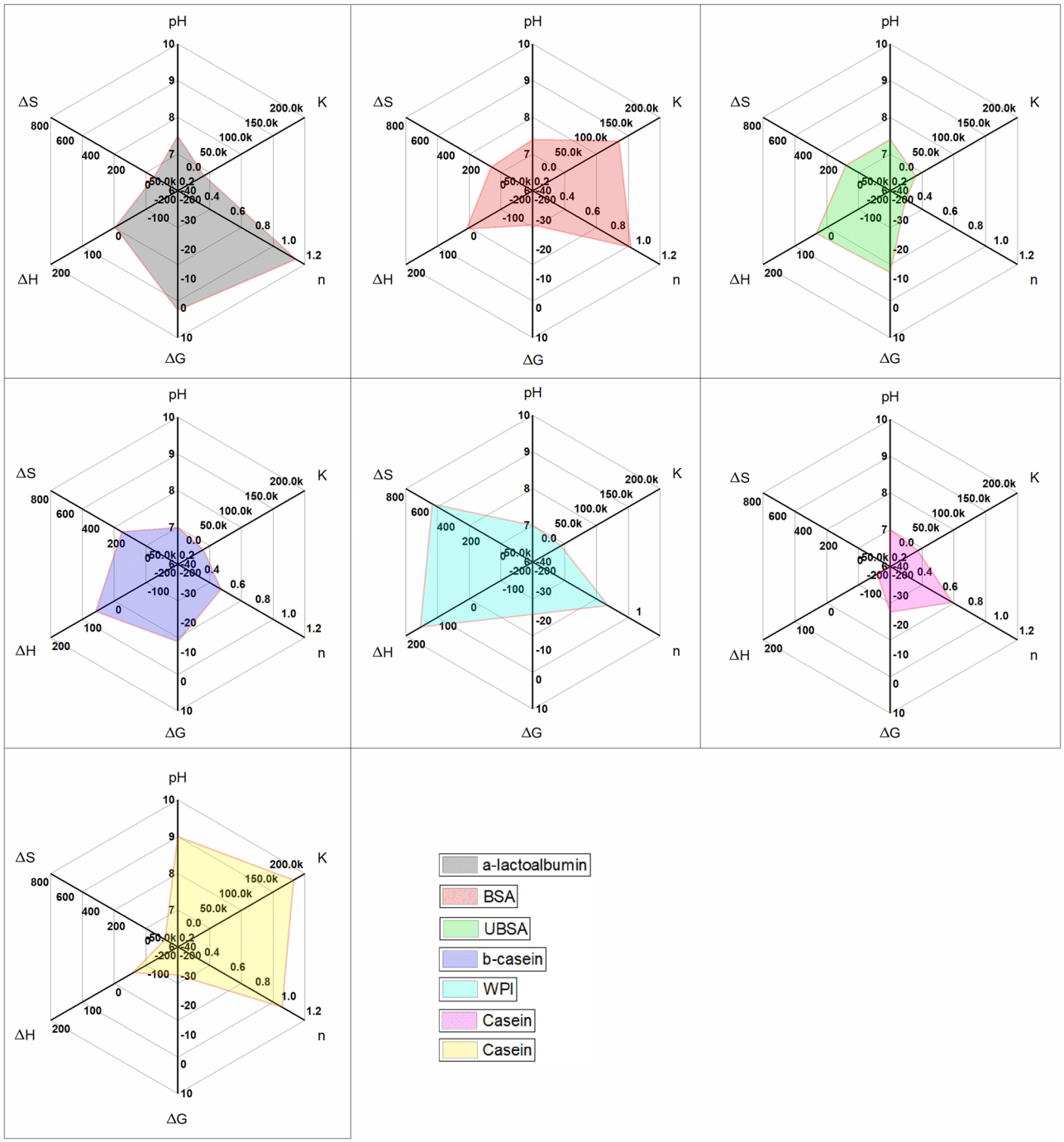

Fig. 2 Radar chart for thermodynamic parameters binding between proteins and β-carotene. a-lactoglobulin: α-lactoglobulin, b-casein: β-casein, UBSA: desnatured BSA

with tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe), highlighting the Trp. The lactoferrin showed 13 Trp residues. However, only seven residues (Trp 8, Trp 16, Trp 22, Trp 24, Trp 361, Trp 467, and Trp 549) are responsible for the intrinsic fluorescence of lactoferrin. The others have their intrinsic fluorescence influenced by neighboring amino acid residues. β-lg has two tryptophan residues (Trp 19 and Trp 61), with the significant fluorescence component being Trp19. α-la has four tryptophan residues (Trp 26, Trp 60, Trp 104, and Trp 118). BSA has two tryptophan residues, Trp 134 and Trp 212, located at the hydrophilic protein surface and hydrophobic pocket of the protein, respectively. β-casein and k-casein have only one Trp residue, Trp 143 and Trp 76, respectively. α_{S1}-casein contains Trp 164 and Trp 199, and

α_{S2}-casein contains Trp 109 and Trp 193 (Abdollahi et al., 2021; Lelis et al., 2020; McSweeney & Fox, 2009; Stănciuc et al., 2013). The reduction in the intrinsic fluorescence can be associated with the collision between milk proteins and BC (dynamic quenching) or by the complexation of milk protein-BC (static quenching). A high bimolecular quenching rate constant can prove the fluorescence quenching of the protein by ligand binding compared to a collisional quenching mechanism. After confirmation of binding, the binding constant and stoichiometry are determined (Lakowicz, 2006; van de Weert & Stella, 2011). Conducting the experiment at various temperatures allows for the determinations of the other thermodynamic parameters as Gibb free energy (ΔG°), enthalpy variation (ΔH°), and entropy variation (ΔS°).

However, fluorescence spectroscopy is liable to error, and care should be taken with the experimental approach. Among the possible pitfalls of the analysis are the inner-filter effect and collisional quenching. The inner-filter impact can occur when the compounds presented in the solution (e.g., ligand) absorb light at the excitation or emission wavelength. However, when the fluorescence comes precisely from the middle of the cuvette, the inner filter effect can be estimated and the measured fluorescence correct. Other ways to avoid the inner-filter effect are reducing cuvette thickness or selecting emission or excitation wavelengths that minimize ligand absorption, but this is not always possible. Another issue that should be considered is the ratio of concentration protein to a ligand that needs at least a factor 10 of the dissociation constant (van de Weert & Stella, 2011).

Isothermal Titration Calorimetry

The isothermal titration calorimetry (ITC) is based on fundamental characteristics of molecular complex formation, e.g., heat uptake or release. Titration of ligand to the protein-containing cell will saturate the protein binding sites and lower the free ligand concentration. The formation of a specific amount of ligand–protein complex is monitored for heat release. However, as in fluorescence, the ITC has limitations: low throughput (high time required for complete titration, about 2.5 h or more); very high and very low-affinity processes cannot be studied, besides the large amount of material that can be required (Samuelsen et al., 2020; Vuignier et al., 2010). Through the ITC, the enthalpy (ΔH°) of binding is determined, providing information about the interactions (hydrophobic, H-bonding, and electrostatic) between protein and ligand. If the determination of K (binding constant) is possible, where the c-value ($c = K[\text{protein}]$) can be between 10 and 1000, it also is possible to determine ΔG° and ΔS°. This fact makes a limitation to the technique because the protein and ligand solubility in the desired concentration may not be achieved (Rühmann et al., 2015).

Absorption Spectroscopy

In the UV–visible absorption spectroscopy is possible to detect interactions between small molecules and proteins based on changes in the absorption spectrum. Changes in the protein absorbance due to the successive addition of ligand can suggest perturbation in the secondary structure of the protein and/or in the vicinity of aromatic amino acid residues. Besides this, the shift in the absorption spectrum suggests a change in the hydrophobic properties of proteins when ligand binding (Siddiqui et al., 2021). The extinction coefficients of protein alone and protein complexes with the ligand can determine the binding constant through absorbance and molar absorption. Effect on the spectral properties of the ligand (e.g., BC) also can indicate interactions between protein and ligand. Besides this, the technical is a straightforward and helpful tool. Red shift/blue shift and hyperchromic/hypochromic effects change the macromolecule's conformations (Allahdad et al., 2020; Hussain et al., 2021; Salim et al., 2021; Siddiqui et al., 2021).

Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FT-IR) studies structural and conformational changes in proteins. This technique provides information related to functional groups present in the molecule, characterizing the secondary structural changes in protein before and after binding with the ligand. IR spectrum reflects amide bonds in the protein associated with the secondary structure. During the interaction process with the ligand, the position of the bond region can change, suggesting conformational changes in protein (Salim et al., 2021; Siddiqui et al., 2021).

Molecular Docking

The molecular docking illustrates the protein binding site for a small molecule, helping understand the interactions between small molecules and protein, forming a stable complex. Before wet-lab experiments, molecular docking can predict the binding mode between ligand and protein, reducing labor and cost before an in vitro experimental screening. It is possible to predict the binding way, site, affinity of binding, and the energetic parameter between a ligand and protein through molecular docking. Besides these, the amino acid residues involved during the binding also can be determined (Hussain et al., 2021; Salim et al., 2021; Siddiqui et al., 2021).

Other techniques such as equilibrium dialysis, ultrafiltration, parallel artificial membrane permeability assay, size-exclusion chromatography, high-performance affinity chromatography, capillary electrophoresis, and surface plasmon resonance can also be used in the interaction studies.

However, they are less usual due to the limitations for the interaction studies between proteins and/or ligands and high cost (Rühmann et al., 2015; Vuignier et al., 2010).

Encapsulation of β-Carotene Using Milk Proteins

The most outstanding methodology for BC encapsulation using milk protein was emulsion (Table 2). The emulsions of oil–water (O/W) are recognized as delivery systems for different compounds as bioactive compounds. For an emulsion to be a suitable delivery system, it must be physically stable without creaming, droplet aggregation, and changes in droplet dimension and prevent the degradation of bioactive compounds (Guan et al., 2016; McClements, 2010). The emulsions can also be submissive to the other process as ultrasonic and press high to improve their stability (Chen et al., 2018, 2020a, b; Chuyen et al., 2019; Niu et al., 2020).

When using WPI for nanoencapsulation of BC, the authors found that the emulsions formed (size) depended on the concentration of the emulsifier (WPI). However, this dependence was limited up to a concentration of 0.5 wt%. For concentrations up to 3 wt%, there was no difference. A greater amount of emulsifier can stabilize a larger interfacial area (oil–water) of the droplets, in addition to allowing a faster coverage of the droplets, preventing coalescence. However, above 0.5 wt% WPI, the non-reduction in size can be justified by limiting the maximum disruptive energy generated by the homogenizer. It is concluded that the mean diameter of the droplets formed by the procedure is sufficiently stabilized by a concentration of 0.5 wt% of emulsifier (WPI). Another factor influencing the droplet size was the homogenization pressure used in double channel micro fluidization. A reduction from 170 to 136 nm was observed for 62–131 Mpa (Luo et al., 2017).

Dextran conjugated with WPI was also used in Fan et al. (2017). Polysaccharides such as dextran have been shown to reduce the size of nanoemulsions formed by WPI by up to 10 mn (Yi et al., 2018). A relatively high magnitude of the zeta potential of the droplets (approximately − 65 mV), together with the droplet size, was able to keep them stable for 14 days at 4 °C, 25 °C, and 55 °C. However, the results demonstrate that nanoemulsified BC is unstable to chemical degradation when incubated at 55 °C, after 14 days of storage (Luo et al., 2017). When using WPI associated with pullulan, low encapsulation efficiency was obtained; however, the stability BC after 10 days was 35% higher than free BC. After baking (160 °C/20 min) had 9.72 mg of BC in 100 g (Niu et al., 2020). López-Monterrubio et al. (2021) associated whey protein hydrolysate with hydrolysate-high methoxyl pectin, and no significant variations in L*, a*, and b* values after 30 days of storage were observed. The increase in size observed due to the variation from pH 4.25 to 8 can be explained by the electrostatic repulsion between whey protein hydrolysate and high methoxyl pectin (HMP), which causes progressive dissociation of the soluble complex biopolymers, coexisting in solution as individual macromolecules. Associating epicatechin and catechin with WPI also improved the resistance to color changes up to 55 °C and pH 6.5 (Chen et al., 2020a, b). WPI has also been shown to reduce the degradation rate constant after UV radiation at 254, 302, or 365 nm for 8 h. In this study, eugenol and lecithin were added in the oil phase, suggesting that they have reacted with the compounds generated by UV radiation, improving the stability of BC. No changes in hydrodynamic diameters were observed after radiation for 8 h (Guan et al., 2016). WPI, WPI-dextran, and WPI-dextran-RES nanoemulsions containing BC were stable about heating and NaCl presence. However, better performance was obtained for WPI-dextran and WPI-dextran-RES to the stability of the nanoemulsions storage for 30 days in different temperatures and pH variations. Adding dextran to WPI provides steric hindrance, preventing flocculation of nanoemulsions (Yi et al., 2018). Dextran conjugated with WPI was also used in Fan et al. (2017) study. The presence of polysaccharides such as dextran has been shown to reduce the size of nanoemulsions formed by WPI by up to 10 mn, just as observed by Yi et al. (2018). However, the zeta potential of the nanoemulsion formed only by WPI (− 45.7 mV) was lower than that formed when the nanoemulsion was formed by WPI-dextran conjugates (− 13.0 to − 28.7 mV). Both size and zeta potential were dependent on the molecular weight of the dextran used. By analyzing the influence of pH on the stability of nanoemulsions containing BC, it was found that emulsions formed only by WPI were not stable between pH 3 and 6. However, the addition of dextran made the emulsions stable between pH 2 and 8. This demonstrates that the polysaccharide can provide enough steric repulsion to prevent coalescence and aggregation of nanoemulsions, even at pH where there is weak electrostatic repulsion by whey proteins. An increase in the size of the emulsions after 30 days of storage at 25 °C and 50 °C was observed for both formed emulsions, but the increase was smaller than 40 nm. The greatest increase was observed at 50 °C compared to 25 °C. Despite this, no phase separation, creaming, or flocculation was observed (Yi et al., 2018).

Polyphenols such as lotus seedpod proanthocyanidin, epicatechin, and catechin were conjugated with WPI to nanoencapsulate BC. The addition of polyphenol lotus seedpod allowed greater electrostatic repulsion between them and possibly its aggregation and sedimentation stability, improving the stability of encapsulated BC. However, the efficiency in creating stable nanoemulsions is conditioned to pH values above or below the isoelectric point of the WPI (Chen et al., 2020a, b). The effect of pH on the stability of nanoemulsions was also demonstrated for β-carotene emulsions stabilized with arabinoxylan hydrolysates-soy protein isolate

Table 2 Nanoencapsulation of β-carotene by milk proteins

Wall for encapsulation	Encapsulation methodology	Size (nm)	EE (%)	Results	References
α-Lactalbumin α-Lactalbumin-catechin conjugates	Emulsion	α-Lactalbumin: 158.8 α-Lactalbumin-catechin conjugates: 162.7	-	Size: increase less than 10% at 25 °C and 10–30% at 50 °C after 30 days of storage Retention BC: ↓ of 11% and 22% for BC, α-lactalbumin-catechin conjugates and α-lactalbumin, respectively at 25 °C; ↓ of 43% and 70% for BC, α-lactalbumin-catechin conjugates and α-lactalbumin, respectively, at 50 °C	(Yi et al., 2016)
α-Lactalbumin-(-)-epigallocatechin-3-gallate complex	Emulsion	190	-	Increase of 17% and 19% the integral transmission for β-lg-(-)-epigallocatechin-3-gallate complex and α-la-(-)-epigallocatechin-3-gallate complex Maintenance of thermal stability (25 °C, 37 °C, and 55 °C) and against UV light	(Wei et al., 2015)
Sodium caseinate (SC) Whey protein isolate (WPI)	Emulsion	SC: 77.8 WPI: 89.7	SC: 99.1 WPI: 98.8	↑ BC reduction power up to ~ 60% (SC) and 40% (WPI) compared to free BC; ↑DPPH radical scavenging activity of 318% (SC) and 336% (WPI) compared to free BC ↑ hydroxyl radical scavenging activity of 183% (SC) and 164% (WPI) compared to free BC; ↓ 41.9% (SC) and 47.5% (WPI) in the mean effective dose for cellular antioxidant activity	(Yi et al., 2015a)
α-Lactalbumin	Emulsion	199	-	↑ 7 days the lag phase of hexanal formation compared to Tween 20	(Liu et al., 2016a, b)
WPI WPI – dextran conjugates	Emulsion	WPI: 165.5 WPI – dextran conjugates: 155.6 to 156.8	-	Retention of approximately 75% and 40% of BC at 25 °C and 50 °C, respectively	(Fan et al., 2017)
WPI	Emulsion	0.75% WPI: 116 2.0% WPI: 48	-	BC nanoemulsions were able to adsorb on a microgel made from oxidized potato starch polymers	(Wang et al., 2015)
α-Lactalbumin (micelles)	Self-assembly	23	-	↑ 2000% in solubility in aqueous solution compared to free BC; ↑ ~ 3900% in stability to UV light (254 nm) and heat (60 °C) after 24 h ↑300% in cellular antioxidant activity	(Du et al., 2019)
WPI WPI-lotus seedpod proanthocyanidin (LSPC); WPI-epicatechin (EC); WPI- catechin (CC);	Emulsion	~ 150	-	↑ resulting negative charge of the WPI-lotus seedpod proanthocyanidin nanoemulsions compared to the others; Stability of BC in nanoemulsion of WPI at 25 °C, pH 6.5 Coating the WPI nanoemulsion with 0.1% de epicatechin and catechin was most resistant to color changes at 55 °C, pH 6.5 BC retention after 3 days storage at 55 °C (better results): pH 3.0 = LSPC 0.01%, 0.01% and EC 0.1% pH 4.0 = EC 0.1% pH 6.5 = LSPC 0.1% pH 8.0 = CC 0.1% BC retention after 7 days storage at 55 °C (better results): pH 3.0 = LSPC 0.01%, EC 0.1% and CC 0.1% pH 4.0 = LSPC 0.1% pH 6.5 = LSPC 0.1% pH 8.0 = CC 0.1% and EC 0.1%	(Chen et al., 2020a, b)

Table 2 (continued)

Wall for encapsulation	Encapsulation methodology	Size (nm)	EE (%)	Results	References
Whey protein hydrolysate-high methoxyl pectin	Emulsion	95.42 to 127.97	27.02 to 91.99	Creaming rate 25% BC = 2.74% 50% BC = 3.03% 75% BC = 3.72%; 25% and 50% showed an increase in size of at most 7% after 30 days of storage and 75% BC increase by approximately 30% 75% BC showed BC concentration after 30 days was 58% less than 25% and 50% BC Antioxidant activity after 30 days: 25% BC = 80.39% 50% BC = 84.33% 75% BC = 46.37% Size variation due to pH increase from 4.25 to 8.0: 25% BC = ↑ 44% 50% BC = ↑40% 75% BC = ↑20% 25% and 50% showed no significant variations in L*, a* and b* values over the 30 days of storage	(López-Monterrubio et al., 2021)
Nanoparticles of chondroitin sulfate and bovine serum albumin	Blend	75–150	-	Loading of BC takes place preferably at the outskirts of the NPs	(Papagiannopoulos et al., 2019)
WPI	Emulsion	168–185	-	Bioaccessibility and degradation were dependent on the type of oil using emulsion formation. Palm oil was shown to be the best carrier oil test	(Zhou et al., 2018)
Sodium caseinate	Emulsion	180	-	After intestinal digestion, BC stability, release, and overall bioaccessibility were 87%, 74%, and 55%, respectively	(Tan et al., 2020)
WPI WPI–dextran WPI–dextran-RES	Emulsion	186.5 173.4 192.6	-	The size stability of the nanoemulsions was maintained up to 90 °C/30 min; WPI size in the presence of 200 mM NaCl was stable up to 65 °C/30 min; WPI–dextran, WPI–dextran-RES: stable up to 90 °C/30 min even in the presence of 200 mM NaCl WPI: size increase at pH between 3 and 6; WPI–dextran, WPI–dextran-RES: size stability at pH between 2 and 7; WPI–dextran and WPI–dextran-RES: Diameter = ↓ ~ 20% and 14% after 30 days at 25 °C and 50 °C, respectively, compared to WPI WPI–dextran and WPI–dextran-RE: ↑ 10.8% and 70.1%, respectively, in BC retention during 8 h of storage under UV light, compared to WPI WPI–dextran and WPI–dextran-RE: ↑ 6.9% and 25.2%, respectively, in BC retention during 30 days of storage at 25 °C, compared to WPI WPI–dextran and WPI–dextran-RE: ↑ 8.9% and 35.7%, respectively, in BC retention during 30 days of storage at 50 °C in the dark, compared to WPI	(Yi et al., 2018)

Table 2 (continued)

Wall for encapsulation	Encapsulation methodology	Size (nm)	EE (%)	Results	References
Bovine serum albumin	Emulsion	199	-	BC retention: from 89 to 98%, between pH 1 to 10 from 98 to 66%, between 30 °C to 90 °C ↓BC retention from 5 to 50% between 1 and 10 h irradiation ↓BC retention up to 15% after up to 120 min reaction with NaClO ↓BC retention up to 55% after up to 35 days storage at 25 °C	(Sheng et al., 2018)
WPI	Emulsion	143. 5 and 153.6	-	↓kinetics of BC degradation up to 83% after up to 30 days of storage at 21 °C ↓degradation rate constant up to 93% up to 80 °C Pronounced ↓in degradation rate constant after UV radiation at 254, 302, or 365 nm for up to 8 h Compared to control: BC dissolved in ethyl acetate	(Guan et al., 2016)
WPI and pullulan	Emulsion followed by electrospraying	150	31.9	↑35% in BC stability after 10 days compared to free BC ↑87% antioxidant activity compared to free BC	(Niu et al., 2020)
Sodium caseinate (SC) WPI	Emulsion	180.8 175.7	-	WPI: ↑ 30% droplet size after heating (60 °C/4 h) ↑ 2700% droplet size after freezing (-20 °C/24 h) SC: without effect after heating (60 °C/4 h) without effect after *freezing* (− 20 °C/24 h) WPI and SC: low stability in pH 1, 3, and 5; droplet size between 175 nm and 250 at pH 7, 9, 11, and 13 WPI and SC: greater instability to aggregation in the presence of $CaCl_2$ compared to NaCl WPI was more stable to $CaCl_2$ compared to SC; ↑ stability to aggregation in the combined use of a protein with Tween 20 in $CaCl_2$ solution ↓ 50% (WPI) and 65% (SC) BC retention after 8 weeks of storage at room temperature; ↑ BC retention compared to Tween 20	(Jo et al., 2019)
Casein micelles-WPI	Reformation	90.4	-	The loading of the casein micelles with BC did not change the protein and mineral equilibria	(Moeller et al., 2018)
WPI	Emulsion	182.7	-	BC Bioaccessibility after in vitro digestion: 30%	(Park et al., 2018)
Sodium caseinate	Emulsion	30	-	Stable under alkaline pH, heat (90 °C), and ion strength (500 mM NaCl) Aggregation under gastric condition (stomach and intestine) Free fatty acid released (%) up to 65% after 120 min lipid digestibility	(Zhang et al., 2017a)
WPI	Emulsion	136 to 170	-	Increase of 1.1, 1.3, and 6.5 in ΔE* at 4 °C, 25 °C, and 55 °C, respectively, after 14 days of storage	(Luo et al., 2017)
β-lactoglobulin β-lactoglobulin-catechin conjugates	Emulsion	161.7 167.6	-	Z-average diameter (nm) after 30 days of storage ↑ < 5 at 4 °C ↑5–15 at 25 °C ↑25–35 at 50 °C BC retention: β-lg: ↓ 10.2%, 25.5% and 72.2% at 4, 25 and 50 °C β-lg-catechin: ↓ 6%, 13.2% and 51.4% at 4, 25 and 50 °C	(Yi et al., 2015b)

WPI whey protein isolate, *BC* β-carotene, *SC* sodium caseinate, *LSPC* lotus seedpod proanthocyanidin, *EC* epicatechin, *CC* catechin, *NP* nanoparticles

conjugates, ovalbumin-dextran conjugates, low methoxy pectin, and low methoxy pectin modified with soy peptide, corn peptide, and whey protein peptide (Li et al., 2022; Luo et al., 2021; Sun et al., 2021).

Given the results presented, we can verify that although WPI demonstrates potential as a wall material for nanoencapsulation BC, adding polysaccharides and polyphenols has helped form and stabilize nanoemulsions formed by WPI. The addition of polysaccharides or polyphenols possibly contributes to increased steric repulsion and, depending on pH, electrostatic repulsion. We believe that studies involving the occurrence of possible interactions between WPI and polysaccharides and polyphenols are interesting to understand the mechanisms involved, as well as the effect of pH and temperature on the interaction, to obtain a wall material with better properties for nanoencapsulate BC and to identify the best conditions for the nanoencapsulation process.

WPI comprises proteins such as β-lg, α-la, bovine serum albumin, and lactoferrin. These proteins in isolated form were also used for nanoencapsulation BC.

Despite using so much α-la, β-lg, lactoferrin, and sodium caseinate complexed with (−)-epigallocatechin-3-gallate (EGCG to encapsulate BC, only α-la (199.0 nm) and β-lg (190.3 nm) showed droplet size less than 200 nm. Zeta potentials of − 32.7 mV and − 40.6 mV were obtained to α-la and β-lg complexed with EGCG, respectively, suggesting the occurrence of repulsive forces between nanoemulsions. The β-lg-EGCG complex was shown to stabilize better the nanoemulsions containing BC compared to the α-lactalbumin-EGCG complex. We suggest this greater stability effect for nanoemulsions formed by β-lg-EGCG due to the higher negative charge, which contributed to the electrostatic repulsion of nanoemulsions. Table 1 shows a higher binding constant for β-lg-BC than α-la-BC. The nanoemulsions containing BC were thermally and UV stable. The protein-EGCG complex has free radical scavenging capacity, and/or the complexes form thick layers on the surface of the drop, acting as a physical barrier protecting BC from oxidation (Wei et al., 2015). A similar size (199 nm) of nanoemulsion containing BC formed by α-la was obtained by Liu et al. (2016a, b). In this study, α-la was demonstrated to be better than Tween 20 in retarding the formation of hexanal, a by-product of lipid oxidation, due to its ability to chelate prooxidant metals and inhibit lipid oxidation. Increased stability of BC to UV light and heat (60 °C) was also obtained using lyophilized α-la micelles. These data demonstrate the enormous potential of α-la in improving BC's thermal and light stability. (Du et al., 2019). According to Lavelli and Sereikaitė (2022a, b) in dry systems, the steady state should occur over a wide range of water activity and glass transition temperature, addressing the complexity of the relationship between moisture and stability. The α-la in micelle form has a hydrophilic corona and hydrophobic core incorporating BC inside, thus increasing the solubility of BC significantly, which is highly relevant to its application in aqueous systems, such as in food (Du et al., 2019).

A slight increase in nanoemulsion size was seen for α-la-catechin (162.7 nm) conjugates compared to using only α-la (158.8 nm) as the wall material. However, the zeta potential was more negative (− 61.9 mV) for the conjugate than only α-lactalbumin (− 57.5 mV). The size and zeta potential of nanoparticles are essential for stability during storage, and there was no difference between α-la and α-la-catechin conjugates in the stability of nanoemulsions containing BC after 30 days of storage at 25 °C and 50 °C. We believe that the increase in the size of the nanoemulsion for α-la-catechin was compensated by the increase in its negative charge. An increase of less than 10% in size was observed at 25 °C and 10–30% at 50 °C. The most significant effect at 50 °C is related to the greater movement of molecules, increasing gravitational flocculation. The similarities in the data found between α-la and α-la-catechin conjugates suggest that catechin does not assist α-la in stabilizing nanoemulsions. However, the conjugated greater retention capacity to BC after 30 days was associated with more significant antioxidant activity formed by the conjugate (Yi et al., 2016).

Sodium caseinate and WPI were highly efficient in encapsulation BC, 99.1% and 98.8%, respectively, with reduced size, 77.8 nm and 89.7 nm, respectively. The nanoemulsions were stable to the freeze-drying procedure, showing similar mean particle diameters, even after resuspension in water (Yi et al., 2015b). However, the heating process (60 °C/4 h) and freezing (− 20 °C/24 h) affect the nanoemulsions containing BC formed by WPI. Silva et al. (2018) demonstrated that heating (denaturing) the BSA (present in the WPI) reduced the interaction constant for BC compared to its native form. Nanoemulsions formed by sodium caseinate show stability after heating and freezing processes. This effect is due to the disordered structure of sodium caseinate, responsible for its thermal stability (Jo et al., 2019). The stability of the nanoemulsion in the presence of salt ($CaCl_2$) was improved when it combined protein and Tween 20. This result, together with the previous study (WPI-dextran), demonstrates that the combined use of milk proteins with polysaccharides, polyphenols, and surfactants present better results in the formation and stability of nanoemulsions containing BC. We believe that steric hindrance is a critical factor in preventing nanoemulsion coalescence. Furthermore, these compounds can hinder the electrostatic attraction of proteins when the pH of the medium is close to the isoelectric point of the protein. The impact of polyphenols on the stability of nanoemulsions is also related to the electrostatic interactions between polyphenols and proteins, pH-dependent interactions (Chen et al., 2020a, b; Jo et al., 2019; Sheng et al., 2018; Yi et al., 2018).

The effect of pH was demonstrated in Tan et al. (2020) study. Caseinate also formed nanoemulsion containing BC, but the nanoemulsion was not stable after simulated gastrointestinal tract (GIT) conditions (size from 180 to 15,600 nm), demonstrating the occurrence of flocculation. Dissociation of some flocs was seen when the pH was adjusted to attain the SI-initial phase. However, some irreversible flocculation was observed after gastric digestion. After intestinal digestion, the stability of BC was 87%. The caseinate inhibits lipid digestion; consequently, more fraction of the BC is trapped inside the non-digested oil phase in these emulsions. The release under the same conditions was 74%, probably due to their reduced lipid digestion degree. The bioaccessibility of BC was 62%, suggesting that part of BC was trapped within the non-digested lipid phase and was therefore not incorporated into the mixed micelles. The overall bioaccessibility was 55%, critical when the BC used is highly unstable to chemical degradation (Tan et al., 2020). When using WPI as wall material, BC bioaccessibility was 30% after in vitro digestion (Park et al., 2018). Other works also demonstrated that WPI and sodium caseinate nanoemulsions showed low stability in acid pH and gastric condition (Jo et al., 2019; Park et al., 2018; Sheng et al., 2018; Zhang et al., 2017b). Between pH 5 and 10, BC nanoemulsions were stable to heating (90 °C) and ionic strength (up to 500 mM NaCl). These results demonstrate that electrostatic forces overcome hydrophobic and van der Waals forces involved in nanoemulsion flocculation. Furthermore, the concentration of NaCl was not sufficient to shield the effect of the electrostatic repulsion caused by the protein adsorbed on the surface of the nanoemulsion, unlike what was demonstrated for nanoemulsions containing BC stabilized by low methoxy pectin and BC nanoemulsion stability by potato protein (Hu & Zhang, 2022; Luo et al., 2021; Zhang et al., 2017b). When using the potato protein and chitosan complex, there was an improvement in the stability of the emulsion containing BC, demonstrating a possible inhibition of the electrostatic shielding of salt ions (Hu & Zhang, 2022).

One of BC's potential uses is as a natural antioxidant. Lyophilized nanoemulsions containing BC using sodium caseinate and whey isolate protein increased by approximately 60% and 40%, respectively, the reducing power of BC, compared to its free form. Free BC is poorly soluble in an aqueous solution, limiting its interaction with aqueous metal ions. Furthermore, the amino acids in milk proteins may have reduced ferric ions. An increase in DPPH radical scavenging activity and hydroxyl radical scavenging activity demonstrates that BC-containing nanoemulsions can be an extremely potent antioxidant in aqueous food systems. By analyzing the antioxidant effect of BC nanoencapsulation with SC and WPI on human liver cells (HepG2), it was found that the mean effective dose was 41.9% and 47.5% lower for BC nanoencapsulation with SC and WPI, respectively. The small diameter of the formed nanoemulsions improves absorption, justifying the more significant cellular antioxidant activity (Yi et al., 2015a). López-Monterrubio et al. (2021) found that the antioxidant activity of BC in nanoemulsions prepared with WPI hydrolysate-high methoxyl pectin depended on the BC concentration in the nanoemulsions. These nanoemulsions showed antioxidant activity up to 84% after 30 days. The wall material was also the protein conjugated with polysaccharides in this study. Free BC and nanoencapsulated in WPI-pullulan were applied in flour to manufacture a meal replacement bar, and its antioxidant activity was determined. The antioxidant activity of the bars increased with the addition of BC compared to the control. However, the antioxidant activity of nanoencapsulated BC was about 1.8 times higher than BC added in free form. Soon the nanoencapsulation maintained the antioxidant activity of BC even after heating at 160 °C/20 min (Niu et al., 2020). The authors show that the greater antioxidant capacity of nanoencapsulated BC is related to the protection that WPI-pullulan exerts on BC.

An increase (300%) in cellular antioxidant activity was also shown when BC was nanoencapsulated with α-la (micelles) (Du et al., 2019). α-la and α-la-catechin conjugates demonstrated greater capacity to protect BC, with emphasis on α-la-catechin conjugates. This fact is associated with eliminating hydroxyl radicals and acting as strong ligands and reducers of metal ions such as Fe^{3+}. Catechin has a significant antioxidant activity that helps reduce the conjugate's power, showing solid synergistic effects with α-la in eliminating the hydroxyl radical. The presence of catechin increases the number of hydroxyl groups contributing to antioxidant activity (Yi et al., 2016). BSA was another protein that was shown to protect BC against strong oxidants (NaClO) in the aqueous phase (Sheng et al., 2018). These data demonstrate the enormous potential of milk proteins associated, or not, with other molecules in maintaining/increasing the antioxidant activity of BC, an extremely relevant factor for the food and pharmaceutical industry.

The type of oil also influenced the stability of BC. Palm oil was the best oil used in forming nanoemulsions compared to corn oil, coconut oil, and fish oil. In addition to forming smaller nanostructures, palm oil provided the formation of nanoemulsions with more negative zeta potential, contributing to its stability. Furthermore, it is believed that strong interactions between palm oil and β-Ig (a protein present in WPI used as a wall material) protected the nanoemulsions from pepsin degradation in the gastric digest. Although palm oil and corn oil showed an effect in the in vitro bioaccessibility of BC, palm oil retained a more significant amount of BC after 42 days at 25 °C and 55 °C (Zhou et al., 2018).

We found that milk proteins have enormous potential as carriers for BC in different approaches from the topics

discussed. The first approach discussed (interaction studies) involves simpler systems and high purity BC, targeted at the naturally occurring levels of bioactive compounds. The encapsulation approach (nanoemulsions) uses compounds other than protein and BC. New challenges involve the use of unrefined sources of BC (Lavelli & Sereikaitė, 2022a).

Conclusions and Outlook

This review article highlighted studies that used milk proteins as natural carriers for BC or as a wall material for encapsulation of BC. The chemical balance favored the formation of milk protein-BC complexes, and hydrophobic interactions were dominant, although hydrogen and van der Waals bonds were suggested. Variations in temperature and pH were demonstrated to affect the formation of complexes.

Milk proteins also showed potential as a wall material for forming of BC-containing nanostructures. Emulsification was the most used methodology for nanoencapsulation BC by milk proteins. The concentration of the wall material and the type of oil used influenced the nanoencapsulation process. The combination of milk proteins with other compounds, such as polysaccharides and polyphenols, has favored forming and stabilizing of the formed nanostructures.

More studies to improve nanoencapsulation methodologies are needed to be applied in the food and pharmaceutical industry. Further studies combining milk proteins with other proteins and polysaccharides may help improve nanoencapsulation performance.

Author Contribution Carini A. Lelis: Investigation, validation, data curation, and writing—original draft. Diego Galvan: Data curation, review and editing. Carlos A. Conte-Junior: Resources, review and editing, supervision, project administration, funding acquisition.

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Declarations

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