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Milking the milk: Exploiting the full potential of milk constituents for nature-derived delivery systems

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ABSTRACT

Background: Nowadays, incrising global demand for milk and milk-derived products throughout the world has led to a massive growth of the dairy sector, and with it an increase in the associated environmental impacts. Milk surplus and by-products disposal represent a serious burden in this scope. Thus, it is essential to improve the valorization of this resource.

Scope and approach: In light of milk's natural delivery function, this review provides an up-to-date collection of the milk-derived delivery systems (MDDS). Special focus is given to the colloidal structures found naturally in bovine milk or reassembled architectures produced with materials derived from milk (milk fat globules, caseins, whey proteins and extracellular vesicles). Furthermore, this review takes a critical perspective on public health, environmental and regulatory issues related to milk production and consumption.

Key findings and conclusions: Milk has been shown to be a safe and low-cost source of materials with huge potential as delivery systems, applied both in nutrition and medicine. However, there are hurdles to overcome prior to their implementation. From a health perspective, testing carrier biocompatibility and considering milk intolerance and allergy is crucial. Dairy farming is associated with serious environmental burdens, which have been leading to increasing support for animal-free diets and products. The lack of standardized methods for producing these MDDS also hampers their translation. Thus, more research is still needed to surpass these problems and allow us to fully exploit milk's potential.

1. Introduction and motivation

Milk is a unique biological fluid secreted by the mammary glands of all mammals females, after parturition. Its primary function is to provide all nutritional and defensive requirements, essential to the normal growth of the neonate. A wide variety of compounds, such as oligosaccharides, immunoglobulins, subunits of distinct macromolecules, calcium, and phosphate, are efficiently supplied to the newborn by one of the most remarkable delivery systems designed by nature (Boland, Hill, Goulding, Fox, & O'Mahony, 2019).

Given its high nutritional value, apart from the progenitor's milk, humans have been consuming milk from other species as part of their daily diet for about 8000 years (Boland et al., 2019; Roy, Ye, Moughan, & Singh, 2020). Nowadays, besides drinking milk, several dairy products, such as cheese, butter, fermented products, milk powder, infant formula, among others, are widely consumed worldwide (Boland et al., 2019). Particularly in Europe, USA, Canada, Argentina, India, Australia and New Zealand, these products represent a significant fraction of the daily diet (Boland et al., 2019). In terms of production, according to FAO (2020), in 2019, 852 million tons of milk were produced globally, Europe being the second largest milk producer after India (FAO, 2020). From the 158.2 million tons of milk produced in Europe (in 2019) more than 96% was cow milk (Eurostat, 2021). In EU dairy industries, 79% of the whole milk is mainly used in cheese, butter and drinking milk production, the production of cheese being the highest portion of that percentage, accounting for 57.6 million tons of whole milk (Eurostat,

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2021). Despite the profit and jobs created by dairy industries some issues have been raised. It is widely known that these industries demand high water amounts for cattle consumption and land irrigation for pasture. Additionally, the limited availability of land for feed crop production, contamination of soil and water from cow excretions, the surge in antibiotic resistance caused by the excessive use of antibiotics in animal agriculture, and the animal welfare have all been prominent issues raised in relation to these industries. However, one of the most impactful sources of pollution from dairy industries is the discharge of untreated milk, or by-products of milk processing. It is estimated that 40,000 L of untreated milk whey can cause as much contamination as 250,000 people in their daily life (De Jesus, Ruth, Daniel, & Sharma, 2015). Some of these by-products have already been integrated in other products but this is still not enough to eliminate the huge negative impacts on the environment.

In what concerns scientific research, the unique chemical nature of milk has been a point of interest for the past 200 years, a trend that remains relevant to this day. There is an extensive literature describing in detail the composition and structure of milk (Meurant, 1995). Milk serves a vital natural function in delivering a diverse range of lipophilic, hydrophilic, and mineral nutrients, as well as bioactive proteins and peptides that play a critical role in supporting the early development of mammalian neonates. This intricate functionality is facilitated by a complex oil-in-water emulsion, characterized by unique structures such as milk fat globules (MFG), casein micelles (CM), whey proteins and small extracellular vesicles (sEVs) (Boland et al., 2019). The specialized nature of milk's composition and its capacity to deliver essential nutrients in a well-regulated manner make it a valuable subject of interest for researchers (Wiciński, Sawicka, Gębalski, Kubiak, & Malinowski, 2020). In particular, the colloidal nature of milk sharpened the curiosity of nanotechnology scientists, that are still facing toxicity, and scalability issues related to the development of synthetic nanocarriers. Trying to tackle this, bovine milk, generally considered safe for human consumption, has been providing multiple alternative ways to carry and deliver nutraceuticals and drugs, through their different native micro and nanostructures (Salim, Eason, & Boyd, 2022). Besides the natural delivery function, other advantages make milk a very attractive source of alternative delivery systems. In particular, cow's milk is particularly relevant in this area due to its high production scale, wide availability and low-cost, along with the need for valorization of the wasted surplus and by-products in dairy industries.

Therefore, this review provides a comprehensive overview of the developments of bovine milk-based delivery systems (MDDS), through the application of native structures or assembled micro and nano-architectures derived from its constituents. From a sustainability point of view, we decide to explore the potential of the milk constituents that can be easily obtained from by-products of the dairy industry. The focus will be on works developed with milk fat globules (MFG), proteins (caseins and whey proteins) and sEVs. A critical overview on the current barriers that may hamper the application of MDDS both in food and pharmaceutical contexts is also presented. Overall, this review brings together a set of works published in different fields, in the last years, and attempts to bring them to a new light, as potential approaches to take advantage of dairy by-products, reducing their negative effects.

2. Milk composition and structural aspects

Milk is a white, and heterogeneous fluid containing thousands of molecular species (Boland et al., 2019). Its composition is dynamic, since the abundance of each component varies widely among species, depending on the energy needs and growth rate of the newborn (Boland et al., 2019). Within the same species and even the same individual, the composition also varies, being strongly dependent on factors such as breed, type of feed, management of the animals, lactation stage, and season (Meurant, 1995).

In Table 1, average concentration values of each constituent of bovine milk are presented.

Table 1
General composition of bovine milk (Boland et al., 2019; Roy et al., 2020).

Constituents	Average Concentration (g/L of milk)
Water (86.6%)	–
Carbohydrates (5.0%)	–
Lactose	50
Oligosaccharides	0.05
Lipids (4.1%)	–
Triacylglycerols	30.7
Diacylglycerols	0.72
Monoacylglycerols	0.03
Free Fatty Acids	0.09
Phospholipids	0.36
Cholesterol	0.15
Protein (3.6%)	–
Caseins	26.3
Whey Proteins	6.3
Milk Fat Globule Membrane Proteins	0.4
Salts (0.7%)	–
Calcium	1.2
Potassium	1.5
Citrate	1.9
Chloride	1.0
Sodium	0.6

These components are organized in three main phases, as shown in Fig. 1. The fat is dispersed in an "oil-in-water" emulsion, forming the so-called milk fat globules (MFG), that present average diameters from 2 to 6 μm. Caseins, the major milk proteins, organize themselves into micelles that, together with the globular whey protein, form the colloidal phase. Casein micelles present sizes about 1/50 that of a fat globule (from 50 to 500 nm). Near this size range, it is also possible to find sEVs, namely, exosomes (Exo), with diameters between 30 and 150 nm. The remaining molecules are essentially present as true solution (Boland et al., 2019).

Due to these physicochemical properties, fractionation of the previously mentioned particulate milk components can be easily achieved. Through low-speed centrifugation, it is possible to easily separate the fat, since it rises to the liquid surface and forms a distinct cream layer (German, Argov-Argaman, & Boyd, 2019). Caseins can be isolated from the whey either by low-medium speed centrifugation, isoelectric precipitation, or even rennet-induced coagulation (Dalgleish & Corredig, 2012). The remaining whey can be centrifuged at high speeds allowing the extraction of sEVs and leaving in suspension whey proteins (β-lactoglobulin, α-lactalbumin, bovine serum albumin (BSA) and lactoferrin). Whey protein can still be purified by ultrafiltration, to obtain the so-called whey protein concentrates (WPC). Further purification by diafiltration or ion exchange can be carried out to obtain whey protein isolates (WPI) (Livney, 2010). The simplicity of these separation protocols along with the high yields obtained and their innate delivery function as part of milk open doors for their application in the nanotechnology field as an alternative to synthetic approaches.

In the following sections, we will focus on each constituent individually, highlighting their application as delivery systems.

3. Milk constituents as delivery systems

The natural function of milk encompasses the delivery of lipophilic, hydrophilic and mineral nutrients as well as different bioactive proteins and peptides that play a crucial role in the early days of mammalian neonates (Boland et al., 2019). Such diverse set of compounds can only be provided through a very complex oil-in-water emulsion, composed of self-assembled unique structures, like MFG, CM, whey proteins and sEVs (Roy et al., 2020). Translating this natural function to other fields has been the focus of work of many researchers in recent years. From

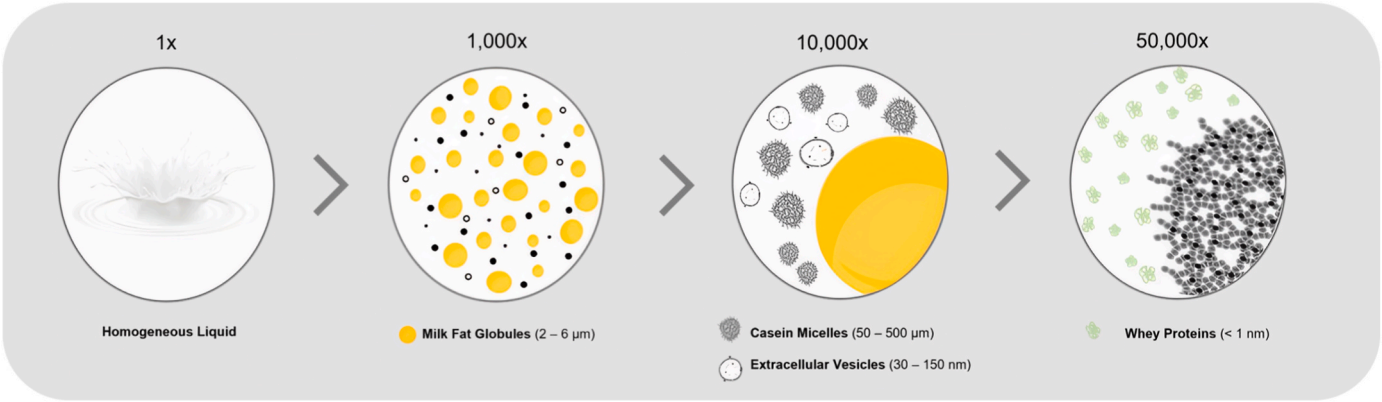

Fig. 1. Schematic representation of milk as a colloidal suspension, displaying milk fat globules, casein micelles, extracellular vesicles, and whey proteins at different magnifications.

nutritional supplementation to cancer therapy, milk components have been applied as nature-derived carriers of a huge variety of compounds, as it will be showed throughout this section.

3.1. Milk fat globules

As previously mentioned, lipids in milk are organized in globules known as MFG. This structure is composed by a core of triacylglycerides (TAG) surrounded by the milk fat globule membrane (MFGM), which ensures the stability of the oil-in-water emulsion (German et al., 2019). The assembly of MFG takes place in the endoplasmic reticulum of the mammary epithelial cell, where a first monolayer of phospholipids, derived from the ER membrane, coats the non-polar triacylglycerol core. Upon secretion from the cell, they are also covered with the plasma membrane, forming a unique trilayer structure, exclusively found in milk (Arranz & Corredig, 2017). Fig. 2 shows the representation of this structure. Besides preventing fat coalescence, this structure also retards the rate of lipid oxidation and its consequent degradation (German et al., 2019).

As a result of the secretion pathway, the composition of the MFGM mainly comprises polar lipids (namely, phospholipids (25%), cerebrosides (3%), cholesterol (2%)), and membrane-bound and associated proteins (Arranz & Corredig, 2017). Concerning the nonpolar triacylglycerol core, it is composed of more than 400 fatty acids types, with different chain length, position and number of unsaturation, which originates a huge number of different possible triglycerides combinations (Queiros, Viriato, Ribeiro, & Gigante, 2021). Around 65% of these fatty acids are saturated, being myristic (C14:0), palmitic (C16:0) and stearic (C18:0) acids the ones present in higher amounts (Queiros et al., 2021). As a consequent of such diversity, milk fat melting temperatures range from −40 °C to 40 °C, being the average melting point around 32 °C (Queiros et al., 2021). For delivery purposes, these two main components (MFGM and TAG core) can be fractioned and used separately, or as they naturally exist, as intact MFG. MFGM components can be obtained industrially from butter milk, usually a by-product of butter production (Davoodi et al., 2016).

Several authors have been using the phospholipid fractions of MFGM (commercially available) to produce liposomes, in substitution of egg or soy derived phospholipids (Thompson, Haisman, & Singh, 2006; Thompson & Singh, 2006). Phospholipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and sphingolipids, mainly sphingomyelin, are the most abundant in these milk fractions (Arranz & Corredig, 2017). Liposomes produced with MFGM lipids showed to have thicker bilayers, lower membrane permeability, and higher phase-transition temperature, than liposomes prepared with soy phospholipids (Thompson & Singh, 2006). Also, they were more

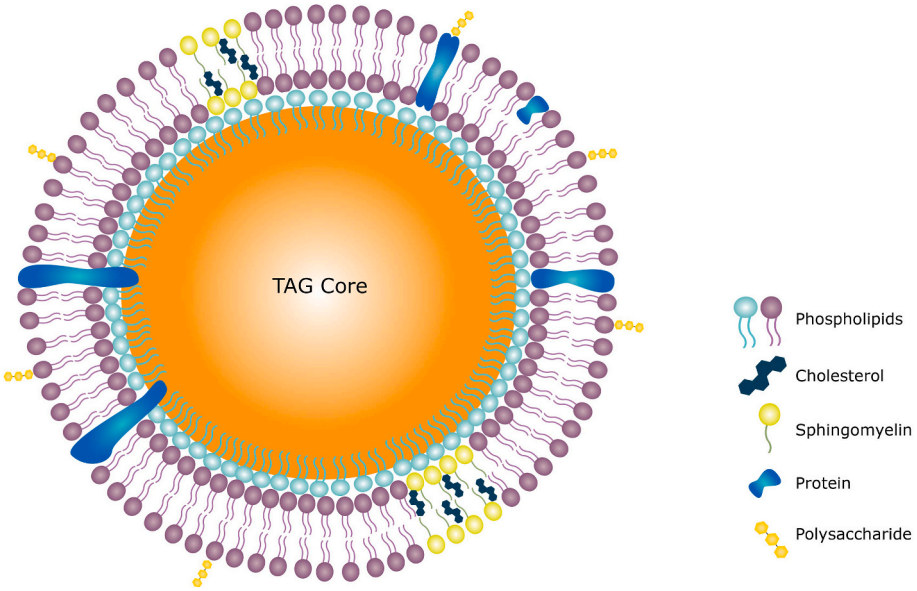

Fig. 2. Schematic representation of the structure of a MFG.

resistant to pH and temperature changes, and presented longer storage times (Thompson et al., 2006). These latter works show the great potential of MFGM lipids when assembled in liposomes, in terms of stability, when compared to other natural lipid sources. Taking advantage of these potential, Jash, Ubeyitogullari, and Rizvi (2020) used phospholipids isolated from butter milk (through sequential pure supercritical-CO_2) to load vitamins E and C. The purified isolate was then used to produce unilamellar liposomes, loaded with vitamins E and C, with diameters around 533 nm and encapsulation efficiencies of 77% and 65%, respectively, which in agreement with the versatility of liposomes to encapsulate both hydrophilic and hydrophobic molecules. Unlike those made with sunflower phosphatidylcholine, these liposomes were able to protect the loaded compounds and maintain their structural integrity when subjected to a heat treatment (90 °C, for 30 min) (Jash et al., 2020). Aiming to enable site-specific cargo-release in the intestine, Jash and Rizvi (2022) developed a green method to produce this type of liposomes coated with Eudragit™ S100. This pH-responsive and safe polymer allowed not only to improve thermal stability but also to protect the cargo and the vesicle itself until reaching the intestine, where it is destroyed by dissolution of the polymer and the entrapped compounds are released. The efficacy of the technology was proven by encapsulating vitamins E and C, as models of lipophilic and hydrophilic bioactives (Jash & Rizvi, 2022).

Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) have emerged as valuable tools in the field of nanotechnology, finding extensive applications in various domains. Zhang and coworkers were the first to describe the production of NLC based on milk fat. In this study, anhydrous milk fat (AMF) and Tween 80 were used to prepare NLC aiming to incorporate β-carotene, a lipophilic bioactive compound. Through a phase-inversion temperature method, the authors were able to produce a stable dispersion of particles smaller than 25 nm, which allowed the decrease of degradation rate of β-carotene (Zhang, Hayes, Chen, & Zhong, 2013). In the following years, other authors extended this idea to other types of nanoparticles, using exclusively edible ingredients derived from dairy products. In the case of de Souza Queirós, Viriato, Ribeiro, and Gigante (2020), fully hydrogenated anhydrous milk fat (FHAMF) was used to produce solid lipid microparticles by spray chilling microencapsulation. Later, de Souza Queirós et al. (2022) also reported the production of solid lipid nanocarriers (SLN) and NLC, using FHAMF and FHAMF:AMF matrices, stabilized by whey protein and sodium caseinate, following a high-energy production method. In both cases, the suspensions showed good stability for at least 90 days, showing promise in their future incorporation as delivery systems of poorly soluble bioactive compounds in functional food (de Souza Queirós et al., 2020, 2022). The potential utilization of milk-derived products for producing SLN and NLC presents a remarkable opportunity, heralding a significant paradigm shift. By leveraging milk-derived components, there is the potential to reduce reliance on synthetic materials and effectively manage dairy industry by-products, leading to a more sustainable and eco-friendly approach. The previously cited studies used MFGM extracts or commercially available anhydrous forms of milk fat. However, it is also possible to directly take advantage of the structure of MFG as they naturally exist in the milk. For instance, Alshehab, Reis, Day, and Nitin (2019) used intact MFG, from bovine (and ovine) milk, to evaluate the encapsulation and gastric stability of vitamin D3. MFG were extracted from raw milk by centrifugation, reconstituted in water (20% w/v), and their structural integrity was assessed by confocal microscopy. Sizes ranging from 5 to 10 μm were obtained for both types of milk (Alshehab et al., 2019). Despite the low values of encapsulation efficiency (between 14 and 27%), the encapsulated compound remained stable for 2 h, under acidic conditions (Alshehab et al., 2019). In another study, Alshehab and Nitin (2019) tested the feasibility of encapsulating hydrophobic bioactive compounds into the preformed complex lipid structures, like MFG. With this approach, a 3-fold increase in the encapsulation efficiency of curcumin was achieved when compared with the reported values in emulsions formed using edible oils (Alshehab & Nitin, 2019). In the same study the *in vitro* release profile showed that MFGs can sustain the release of curcumin in simulated gastric fluids. These studies demonstrate that even with minimal processing, milk components like MFG exhibit a high potential as delivery systems, particularly for hydrophobic compounds.

Overall, MFG can provide adequate source material to produce stable delivery systems, with different structures, sizes, and nature. From the MFGM-derived phospholipids it is possible to obtain liposomes with enhanced properties when compared to other natural lipid source liposomes. They present improved stability, heat resistance, compared to liposomes made from other sources (Jash et al., 2020; Jash & Rizvi, 2022; Thompson et al., 2006). The applicability of the liposomes produced with MFGM lipids is mainly found in food supplementation, with the encapsulation of both hydrophilic and hydrophobic molecules, with high efficiency. Taking advantage of AMF or FHAMF authors have been also able to produce SLN and NLC (de Souza Queirós et al., 2020, 2022; Queiros et al., 2021). Since these type of nanoparticles have been extensively used in nanotechnology, this approach could be transformative, presenting an opportunity to reduce dependence on synthetic materials and mitigate the disposal burden of dairy industry by-products. When simplicity is intended, MFG can be also applied as they naturally exist in milk, mainly to incorporate and stabilize hydrophobic compounds. However, this approach allows lower encapsulation efficiencies as well as less control on the structure itself (Alshehab et al., 2019; Alshehab & Nitin, 2019).

3.2. Proteins

Milk proteins are natural vehicles that present high nutritional value, excellent functional and sensory properties, while being widely available, and inexpensive (Livney, 2010; Poonia, 2017). They can be divided into two major groups: caseins (around 79% of total milk protein) and whey proteins (the remaining 19% of total milk protein) (Poonia, 2017). Besides their different abundance in milk, they also strongly differ in terms of structure and chemistry. However, both protein types present structural features and functionalities that make them suitable as nanocarriers. Additionally, peptides derived from casein and whey proteins have been described as active compounds with immunostimulant, antihypertensive, anticarcinogenic, antibacterial and antiviral activities (Pan et al., 2006).

3.2.1. Caseins

Caseins are the major milk proteins, and when in aqueous solutions, self-assemble into spherical colloidal particles known as casein micelles (CM) (Elzoghby, El-Fotoh, & Elgindy, 2011). These assemblies are formed by the association of four main casein types: αS1, αS2, β and κ-casein (1:4:1:4) (Elzoghby et al., 2011). The phosphorylated residues of caseins confer the ability to bind calcium phosphate. The presence of calcium phosphate nanoclusters along with protein-protein interactions (hydrophobic, hydrogen, and electrostatic binding) allow the stabilization of the micellar structure (Broyard & Gaucheron, 2015). Throughout the years, the proposed models of the CM structural organization have been evolving. Nowadays, the most accepted model describes CM as a structure with dense regions composed by casein and nanoclusters of calcium phosphate intercalated with water channels, stabilized by β-casein. Glycosylated forms of κ-casein are suggested to be present in the surface of the CM, conferring colloidal stability by electrostatic repulsion (negative charge) and steric hindrance (Broyard & Gaucheron, 2015). A schematic representation of the so-called sponge-like model of CM organization is shown in Fig. 3.

CM exhibit a pH-dependent behavior, with an isoelectric point of 4.6 (Elzoghby et al., 2011). These properties together ensure a good and controlled release of encapsulated compounds, taken up orally, in the gastrointestinal tract. The interior of CM is mostly hydrophobic while the surface, due to the presence of κ-casein, is hydrophilic (Broyard & Gaucheron, 2015). Such amphiphilic nature not only ensures their

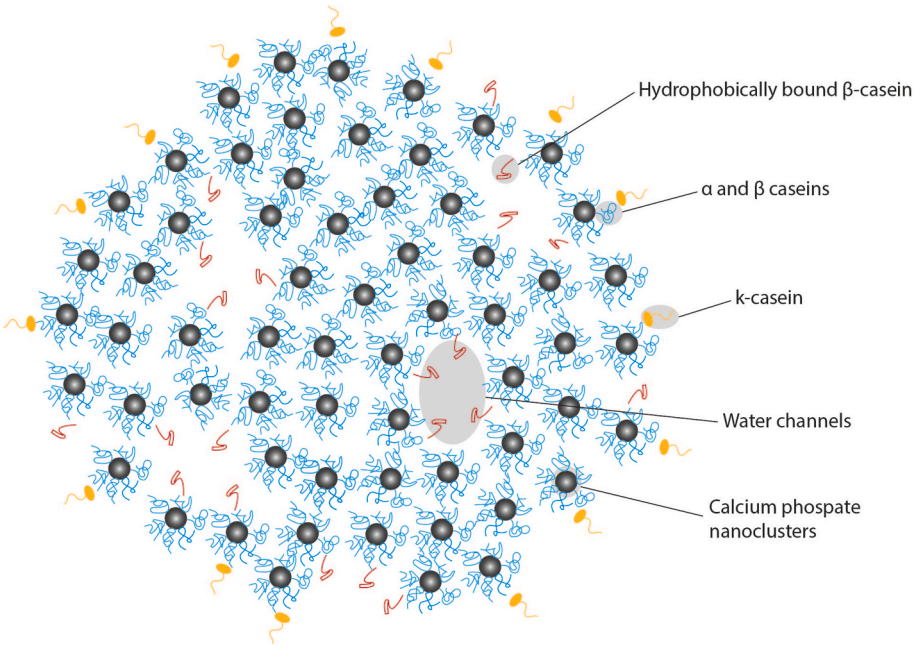

Fig. 3. Schematic representation of the structure of a casein micelle.

stability in aqueous environment but also allow CM to exhibit affinity for hydrophobic as well as hydrophilic molecules. Taking advantage of these features, several works have been showcasing the potential of casein as a delivery system, not only as native and re-assembled micelles but also as nanocomplexes, nanoparticles and even hydrogels. In this section, the most relevant and recent literature on this topic will be reviewed, aiming to highlight the potential and versatility of casein-based nanocarriers.

Semo, Kesselman, Danino, and Livney (2007) demonstrated the possibility of loading hydrophobic nutraceutical compounds within CM structure. The authors showed that vitamin D2 (the hydrophobic nutraceutical model compound) presented high affinity to casein and that the re-assembled CM could partially protect it from UV-light-induced degradation (Semo et al., 2007). Re-assembled CM and casein NPs also allowed the encapsulation of docosahexaenoic acid, an Ω-3 unsaturated fatty acid (Zimet, Rosenberg, & Livney, 2011). Casein not only allowed enhanced solubility and stability of the compound, but also protected it from oxidation (Zimet et al., 2011). Also, folic acid was loaded into casein nanoparticles (CNPs) and sizes around 150 nm were obtained (Penalva et al., 2015). In terms of release, it was found that casein was able to protect folic acid from the acidic gastric environment, allowing the release only under simulated intestinal conditions (Penalva et al., 2015).

Malekhosseini et al. (2019) proved that CM and CNPs can also incorporate hydrophilic molecules, such as EGCG and folic acid, with high efficiencies and good stability. When compared to CNPs, re-combined CM showed to be more effective in the protection of the compounds against heat-induced degradation (Malekhosseini et al., 2019). In the cancer therapy field, EGCG-loaded CM showed interesting results in the proliferation of HT-29 cancer cells (Haratifar, Meckling, & Corredig, 2014). An increase in the anti-proliferative activity of EGCG was observed, comparing to the free polyphenol (Haratifar et al., 2014). Sahu, Kasoju, and Bora (2008) studied the encapsulation of curcumin, another water-insoluble molecule, with anticancer activity. Produced micelles presented sizes around 160 nm, with strong binding between casein and curcumin. The binding did not appear to interfere with the anticancer efficacy of curcumin, as it was cytotoxic for HeLa cells (Sahu et al., 2008). In a different study, curcumin bonded to casein showed increased efficacy against HCT 116 cells, as well as improved antioxidant activity (Pan, Zhong, & Baek, 2013). Also aiming to improve anticancer therapy, crosslinked CM have been used to bind anticancer drugs, such as paclitaxel (Cuggino et al., 2021) and cisplatin (Zhen, Wang, Xie, Wu, & Jiang, 2013). The cisplatin-loaded CNPs exhibited great capability to penetrate cell membrane barriers, good passive tumor targetability and a tumor growth inhibition in H22 cells xenographic mice of 74% (1.5-fold higher than that of free drug). Penetration assays on tumor spheroids showed that CNPs can deliver the drug into the tumor, through a temperature- or energy-independent mechanism (Zhen et al., 2013). Doxorubicin efficacy was also increased when encapsulated in CNPs, against human pancreatic cell line (PANC-1) (Gandhi & Roy, 2019). Several other works have been exploring the use of CNPs or CM to increase stability and bioavailability of other chemoterapeutics (Elzoghby, Helmy, Samy, & Elgindy, 2013), anti-inflammatory drug (Zahariev, Marudova, Milenkova, Uzunova, & Pilicheva, 2021), antimicrobials (Yuan et al., 2021), a variety of bioactive natural compounds (Barick, Tripathi, Dutta, Shelar, & Hassan, 2021; Peñalva et al., 2019) and vitamins (Levinson, Ish-Shalom, Segal, & Livney, 2016; Loewen, Chan, & Li-Chan, 2018). Cohen et al. (2017) were able to take CM to human clinical trials, using vitamin D3 as a model lipophilic-bioactive. Vitamin D3 was administered either in CM in the absence or presence of fat or dissolved in milk fat. It was demonstrated that the bioavailability of vitamin D3 encapsulated in CM was not significantly different from that in fat, meaning that CM-encapsulation can represent a viable strategy for the enrichment of nonfat food. From a different point of view, Mohan, Jurat-Fuentes, and Harte (2013) decided to test the binding of vitamin A to unmodified CM, directly in commercial bovine milk. Indeed, even in a highly complex medium such as milk, the compound showed to strongly associate with the fraction containing caseins, whereas the whey proteins did not show any binding (Mohan et al., 2013). This way, with minor manipulation it was possible to achieve an efficient milk fortification, through CM binding.

Besides the above-mentioned structures, casein can also be cross-linked into hydrogels. These casein-based hydrogels, generally, present good water absorption and swelling capacities, and have been used not only in pharmaceutical, and food applications but also in biomaterial industries (Kaur et al., 2022).

A lot of work has been done in this scope and a variety of reviews exclusively focus on casein based nanosystems. Here, we thereby highlight some of the most relevant works that showcase interesting properties of these proteins and how they can be applied in a variety of

contexts, from nutrition to cancer treatment. Significant attention is directed towards the versatile nature of casein, enabling the efficient encapsulation of both hydrophilic and hydrophobic compounds. This occurs in the form of nanoparticles or micelles, within milk or after subsequent reassembly procedures.

3.2.2. Whey proteins

Milk whey contains a variety of globular and low molecular weight proteins that include α-lactalbumin (α-la), β-lactoglobulin (β-lg), bovine serum albumin (BSA), immunoglobulins, and lactoferrin (Ha, Rankin, Lee, & Lee, 2019). β-lg is the major whey protein in cow milk (50–60%) and presents in their structure two disulfide bridges and a free thiol residue (Ha et al., 2019). The second most abundant whey protein is α-la, that is a metaloprotein with 4 disulfide bridges (Livney, 2010). BSA, the larger globular protein, can be both present in milk whey as well as in blood serum. Its structure is mainly α-helical, with seventeen disulfide bridges and a free thiol (Livney, 2010). Lactoferrin is a glycoprotein that, *in vivo,* presents capacity to bind not only Fe^{3+} but also other metal ions such as copper, chromium, manganese and aluminum (Fox & McSweeney, 2013).

The amphiphilic nature of milk whey proteins has raised interest in the scientific community, namely for their application as natural stabilizers of poorly water-soluble compounds. Conventional synthetic stabilizers can present weak stabilizing effects and toxicological concerns for long-term treatment. Thus, the use of natural emulsifiers such as whey proteins has been tested as an alternative to conventional emulsifiers. He et al. (2011) tested different food-derived stabilizers, namely soybean protein isolate, WPI and β-lg, aiming to create a stable nanosuspension, using fenofibrate as drug model. Among the tested formulation, β-lg showed the best emulsifying capacity and cell viability results in Caco-2 cells. Moreover, *in vivo* studies in rats showed that any of the whey protein stabilized nanoemulsions could significantly enhance the oral absorption of fenofibrate. It is noteworthy to mention that, before the high-pressure homogenization that produces the nanosuspension itself, the proteins were subjected to thermal denaturation, to allow an exposure of the non-polar domains, leading to an increase in the stabilization capacity. A similar concept was also applied to the preparation of indomethacin nanosuspension, using denaturated WPI and β-lg (He et al., 2013). In this case, the nanosuspension was produced through nanoprecipitation–ultrasonication method. Carvedilol was also loaded into WPI nanosuspensions and its stabilization potential was compared with non-ionic linear copolymer-poloxamer 188 (PLX188) and anionic surfactant-sodium dodecyl sulfate (Geng et al., 2017). Prepared through antisolvent precipitation-ultrasonication technique, it was possible to achieve sizes between 275 and 640 nm. Besides the biocompatibility advantage of the WPI, the oral bioavailability of the loaded carvedilol was also significantly improved in Wistar rats compared to those of commercial formulations (Geng et al., 2017).He et al. (2011)

Li, Ma, and Cui (2014) prepared whey-protein-stabilized nanoemulsions as a potential delivery system for curcumin. Optimization revealed that to produce a nanoemulsion with small droplets (between 0.197 and 3.704 μm), a 5 g/100 mL protein concentration is required to fully cover the surface area of the formed droplets. The addition of ι-carrageenan did not seem to improve the physicochemical properties of the nanoemulsion, as whey proteins are notably good by themselves in stabilizing the curcumin nanoemulsions (Li et al., 2014). Also using curcumin, Li, Cui, Ngadi, and Ma (2015) were able to prove that β-lg and WPI nanoemulsions allow an increase in water solubility and pH stability of the compound. Additionally, it was found that both WPC-stabilized curcumin nanoemulsions and β-lg-curcumin complexes significantly improved the resistance to pepsin digestion and the permeation rate of curcumin in Caco-2 cell monolayer models (Li et al., 2015). Applying a similar concept to a more practical case, Tippetts, Martini, Brothersen, and McMahon (2012) designed four different oil-in-water vitamin D emulsions using not only whey protein but also sodium caseinate, calcium caseinate, and nonfat dry milk. These formulations were used to supplement milk and it was shown that all emulsions were efficient in the stabilization and retention of vitamin D3 in cheese curd in a model cheesemaking system (Tippetts et al., 2012). Other compounds have also been efficiently incorporated into whey protein stabilized nanoemulsions, namely, α-tocopherol, resveratrol and naringenin (Fang, Bao, Ni, Choijilsuren, & Liang, 2019), β-carotene (Cornacchia & Roos, 2011), fatty acids (Salminen, Herrmann, & Weiss, 2013), algal and menhaden oils (Djordjevic, McClements, & Decker, 2004), and folic acid (Assadpour, Jafari, & Maghsoudlou, 2017). This showcases the adaptability and broad potential of whey protein-based delivery systems for diverse bioactive molecules.

Even within more complex systems, such as SLN, whey protein continues to demonstrate its efficacy. et al. (2018) used WPI as emulsifier in SLN for the delivery of β-carotene. Not only the WPI allowed an increase in the stability of the suspensions of palmitic acid NPs, but also an increase in the oxidative stability of the compound (Mehrad, Ravanfar, Licker, Regenstein, & Abbaspourrad, 2018). Also, lactoferrin exhibited emulsifier capacity of tristearin NPs (Oliveira, de Figueiredo Furtado, & Cunha, 2019). Once again exploiting milk-derived components within established nanosystems such SLN, this strategy introduces a sustainable and environmentally aware approach, propelling nanotechnology research alignment with the principles of green and circular economy.

Another approach that has been explored with whey proteins is microencapsulation. It is known that WPI can bind and form complexes with various hydrophobic and amphiphilic compounds such as flavor compounds, fatty acids, and vitamins have also been widely reported (Flores, Singh, & Kong, 2014). However, Liu, Chen, Cheng, and Selomulya (2016) decided to explore the possibility of creating a dry powder containing WPI-curcumin complexes, generating curcumin-loaded WPI microparticles. The authors were able to successfully produce spray-dried WPI-curcumin microparticles with nearly 100% of curcumin retention. This protocol allowed an increase in solubility of around 11, 000-fold compared to the raw curcumin crystals (Liu et al., 2016). The strategy's value extends beyond enhanced solubility, as it also offers increased storage stability and cost-effective transportation advantages over liquid formulations. Testing other drying techniques, Parthasarathi and Anandharamakrishnan (2016) produced vitamin E microcapsules, with the same purpose of increasing the dissolution as well as the oral bioavailability of this hydrophilic compound. Spray drying, freeze-drying and spray freeze-drying techniques were employed, originating maximum encapsulation efficiencies of around 89%, 86% and 89%, respectively. The spray freeze-dried vitamin E microcapsules showed to be the most promising formulation, leading to an increase in oral bioavailability of 1.13-fold compared to spray dried, and 1.19-fold compared freeze-dried microcapsules (Parthasarathi & Anandharamakrishnan, 2016). Both studies could produce microparticles with sizes between 100 and 200 μm. Using a distinct method, Arroyo-Maya and McClements (2015) was able to produce WPI (and beet pectin) NPs enriched with anthocyanin-rich extract. Through pH and heat processing, efficiencies around 55% and sizes below 200 nm were achieved (Arroyo-Maya & McClements, 2015).

Aiming to enhance bioavailability and anticancer activity of lycopene, Jain et al. (2018) prepared nanoformulations of WPI, by pH adjustment and hydration of the biopolymer. With entrapment efficiencies ranging from 50 to 60%, WPI-NPs could enhance the oral bioavailability of lycopene by controlling its release and facilitating its absorption through lymphatic pathways (Jain et al., 2018). Other strategies have been carried out using whey protein NPs, namely for loading zinc (Shao, Shen, & Guo, 2018) or zinc derivatives (Mae, 2018), or studying the assembly dynamics of WPI interaction with daidzein (Lv, Fu, Zhang, & Wang, 2019). More recently, to improve pediatric drug delivery, Islam et al. (2019) developed a novel NPs based on food proteins to be loaded with lopinavir (antiretroviral) and fenretinide (anticancer agent). Composed of a zein hydrophobic core, covered by a shell

of whey protein, these NPs showed potential to prolong the gastrointestinal residence time of drugs, also not showing any immunogenicity, in *in vivo* studies (Islam et al., 2019). An increase in the oral bioavailability of lopinavir and fenretinide (4 and 7-fold, respectively) compared to those of free drugs in BALB/c mice was observed (Islam et al., 2019).

Owing to the mentioned functional and structural properties of whey proteins, namely the capacity to bind several bioactive compounds, and their emulsifying abilities, they represent very versatile delivery systems. As shown by the cited works, they stabilize, protect, and enhance the properties of various molecules both as liquid and powder formulations. Their application has been proving to be fruitful for nutritional purposes and has also started to show promising results in the field of nanomedicine.

3.3. Extracellular vesicles and exosomes

Extracellular vesicles (EVs) are vesicles secreted by cells into the extracellular space. These vesicles are divided into distinct categories, depending on their biogenesis and source: exosomes (Exo), apopotoc bodies, and ectosomes or shedding microvesicles (Sanwlani, Fonseka, Chitti, & Mathivanan, 2020). Specifically, Exo, or more broadly small EVs (sEVs), have been of great interest in the recent years for delivery purposes. These vesicles present an analogous structure to large unilamellar liposomes (a lipid bilayer that surrounds an aqueous compartment) and have sizes ranging from 30 to 150 nm (Sanwlani et al., 2020). Due to their biogenic nature (endosomal origin), the complexity of the bilayer is very close to the cell membrane. Besides the diversity in the lipid composition, several transmembrane proteins (*e.g.* CD9, CD63, CD81) and membrane-bound proteins can be found in the surface of Exo (Sanwlani et al., 2020). In Fig. 4, a schematic representation of an Exo is presented. In particular, milk exosomes (mExo) are produced by a heterogeneous group of cells residing in the source organism's mammary glands, which includes mammary epithelial cells, immune cells, stem cells, bacteria and adipocytes (Sanwlani et al., 2020). These vesicles carry a variety of biologically active compounds (*e.g.*, proteins, miRNA, DNA), which make them key players in the intracellular communication between the mother and her breastfed newborn (Munagala, Aqil, Jeyabalan, & Gupta, 2016).

Contrary to the other milk components presented so far, that have been mostly applied as nutraceutical delivery vehicles, research focusing on mExo have been exploring mainly the nanomedicine field, namely as enhancers of anticancer therapy. In this scope, Munagala et al. (2016) were the pioneers in the application of bovine mExo as vehicles of different chemopreventive compounds (withaferin A, anthocyanins and curcumin) and chemotherapeutic drugs (doxetaxel and paclitaxel). This work showed the high versatility of mExo as delivery systems of molecules with different lipophilicity. The drug-loaded mExo presented enhanced antiproliferative effect in cancer cell lines as well as enhanced anti-tumor activity in lung tumor xenograft mice, comparing to the free drugs. Increased stability, solubility and bioavailability achieved through the encapsulation of the anticancer compounds were the driving forces for such improvement in the treatment. Additionally, despite being from exogenous origin, mExo can be well tolerated by mice, since no toxicity issues or inflammatory response were observed (Munagala et al., 2016).

The potential of the oral delivery of paclitaxel-loaded mExo for lung cancer therapy was further explored by Agrawal et al. (2017). The produced formulation presented a loading of approximately 8%, achieved by passive incubation, and an increased mean size compared to the unloaded mExo (from 74 nm to 108 nm), showing also great stability in simulated-gastrointestinal fluids. When administered orally to mice bearing tumor lung xenografts (A549 cell line), paclitaxel-loaded mExo, at the higher dose (4 mg/kg b. wt.), induced a tumor growth inhibition of nearly 60% compared to vehicle treatment (tumor volume of 275 mm^3 versus 680 mm^3). Oral administration of exosomal paclitaxel induced remarkably lower systemic and immunologic adverse effects

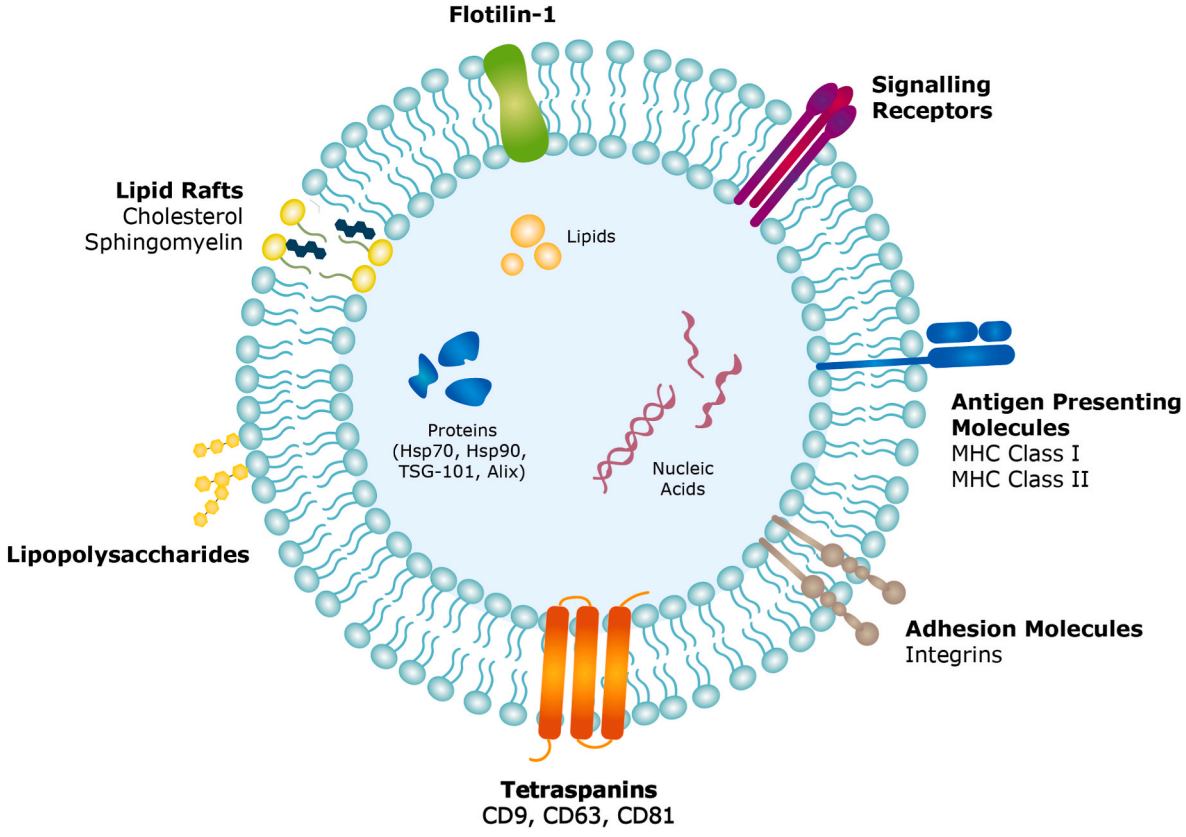

Fig. 4. Schematic representation of the structure of an exosome.

compared to the intravenous administration of the free drug (Agrawal et al., 2017). The encapsulation of celastrol in bovine mExo was also achieved, through passive incubation, resulting in a loading of 18–20% (Aqil et al., 2016). This formulation increased in 50% the anti-proliferative effect of celastrol against lung cancer cells (H1299 and A549 cell lines). *In vivo* studies, with athymic mice bearing lung tumor xenograft, showed a significant increase in anti-tumor activity (77% inhibition) compared to free celastrol (52% inhibition). While reports suggest systemic toxicity of celastrol in high doses and prolonged use, this study demonstrates that oral exosomal-celastrol administration in wild-type mice over two weeks exhibited good tolerance without adverse biochemical or hematological effects (Aqil et al., 2016). Aqil, Munagala, Jeyabalan, Agrawal, and Gupta (2017) showed the potential of anthocyanidins against drug-resistant (A2780/CP70, OVCA432 and OVCA433) ovarian cancer cell lines. In combination with cisplatin, this phytochemical resulted in significantly higher cell killing, requiring a 10 to 15-fold lower cisplatin dose than the IC50 of cisplatin alone. These anti-tumor effects were enhanced in ovarian cancer mouse model, through the oral administration of mExo loaded with anthocyanidins. *In vivo* (A2780 tumor xenografts), a significantly enhanced antitumor activity was observed with the combination of exosomal anthocyanidins and exosomal paclitaxel (Aqil, Jeyabalan, et al., 2017). Besides the improvement of the efficacy of anticancer compounds, interestingly, mExo have also shown anticancer activity by themselves (Munagala et al., 2017). Munagala et al. (2017) reported the such intrinsic anti-cancer activity of bovine mExo against several cell lines (A549, H1299, MDA-MB-231, MCF-7, PANC1, Mia PaCa2, PC3, DU145, HCT116, and OVCA432). The most relevant effect was noticed in the MCF-7 cell line, with a cell survival of 55%, when exposed to bare mExo. A recent study by Samuel et al. (2021) also demonstrated the induction of cell death in colorectal cancer cells (LIM1215 and SW620) through the use of milk sEVs both *in vitro* and *in vivo*. However, an interesting finding was the enhancement of the metastatic capacity of the primary tumor cells, possibly due to induced cellular senescence and epithelial-to-mesenchymal transition. To address this issue, the researchers proposed administering the milk-derived sEVs treatment after the surgical removal of the primary tumor, which demonstrated a significant decrease in metastatic events (Samuel et al., 2021).

The effect of anthocyanidins alone and in exosomal formulation against TNFα-induced NF-κB activity in lung (H1299) and breast (MCF-7) cancer cells was also evaluated (Munagala et al., 2017). Both free and loaded anthocyanidins were able to inhibit TNFα-induced NF-κB activity. However, exosomal anthocyanidins showed a significant enhancement of this effect, demostrating their anti-inflammatory potential (Munagala et al., 2017). Another natural chemical compound that has been used in this context is curcumin. Loaded in bovine mExo, curcumin exhibits enhanced stability and improved transport across the intestinal epithelial barrier (Carobolante, Mantaj, Ferrari, & Vllasaliu, 2020). Moreover, exosomal curcumin can also reach other tissues, such as the mammary tissues, in female rat models, at concentrations compatible with anticancer activity (González-Sarrías et al., 2022). Similar results have also been reported for exosomal resveratrol (González-Sarrías et al., 2022).

From another perspective, studies revealed that some phytochemicals, namely epicatechin gallate (ECG), have remarkable neuroprotective effects on neurons, with potential to reduce the incidence of diseases such as Parkinson's or Alzheimer's (Bae et al., 2020). However, its poor stability and poor intestinal permeability hamper its clinical application. Thus, Luo et a. (2021) designed an exosome-based delivery system for ECG and tested its neuroprotective effects on a rotenone-induced Parkinson's disease model *in vitro*. With a loading of nearly 26% (by sonication), mExo were able to successfully deliver EGC into SHSY5Y cells and enhance neuroprotective effects. The mechanisms involved in those effects may be related to the inhibition of SHSY5Y cell damage induced by rotenone through anti-apoptosis and anti-mitophagy (Luo et al., 2021).

Zhang et al. (2020) developed an innovative pH- and light-sensitive drug delivery system based on mExo to be applied in treatment of oral squamous carcinoma (HSC-3, SCC-9 and CAL-27 cell lines). By conjugating doxorubicin, anthracene endoperoxide and chlorin e6 (sonication), the authors achieved a significant decrease in cell viability when all system was applied and irradiated with an 808 nm laser. Also, *in vivo* assays, using mice bearing HSC-3 xenograft tumors, showed the potentiality of the exosomal formulation combining photochemistry activity and anticancer drugs (tumor growth from ~1.26 cm^3, for the free doxorrubicin, to ~0.05 cm^3 for the complete system) (Zhang et al., 2020).

The increasing knowledge on RNA-based therapies has been accompanied by the growing need for new delivery systems for these molecules. Despite the huge potential of both small interfering RNAs (siRNAs) or microRNAs (miRNAs) in the treatment of various diseases, their application has been hindered by thermodynamic issues (Yan et al., 2022). Due to their negative charge and their polymeric nature, they cannot cross cell membranes (Warren et al., 2021). Also, siRNAs and miRNAs, in a free state, suffer from enzymatic degradation by circulating RNAse (Warren et al., 2021). Interestingly, mExo, as natural carriers of miRNA, have the potential to protect these molecules and deliver them functional to cell. siRNA loaded in mExo could resist gastrointestinal conditions and diffuse across mucosal barriers (Warren et al., 2021). Aqil et al. (2019) loaded mExo with VEGF, EGFR, AKT, MAPK, SUR, BCL2, and KRAS siRNA through biochemical transfection and were able to show that those formulations could resist enzymatic degradation. Moreover, mExo-siKRAS exhibited anti-proliferative activity against KRASG12S mutant A549 lung cancer cells, while no effect was observed against H1299 lung cancer cells bearing a wild-type KRAS. *In vivo*, in A549 lung tumor xenograft-bearing nude mice, a reduction of 54% of tumor volume (compared to the control) was observed, with intraperitoneal administration of Exo-siKRAS (Aqil et al., 2019). Applied in the treatment of pancreatic cancer, bcl-2 siRNA loaded in mExo have also shown ability to induce inhibition of the growth of Panc28 cells, *in vitro* (Tao et al., 2020). *In vivo*, a significant inhibition of the tumor growth was observed in nude mice bearing Panc28 xenograph (Tao et al., 2020). Similarly to siRNA, different studies have demonstrated miRNA encapsulated within mExo can also reach the target cells and exert their function (del Pozo-Acebo et al., 2021; Yan et al., 2022).

In order to provide cell specificity to sEVs, a new field of work called exosomal engineering has emerged in recent years. This methodology focuses on surface modification for targeted delivery and anti-tumour efficacy (Li et al., 2020; Munagala et al., 2016). Pioneering work in this area includes Li's work on surface modification of doxorubicin-loaded exo with hyaluronan (Li et al., 2020). This molecule is a ligand of the CD44 receptor, that is overexpressed on the surface of various types of cancer cells (Li et al., 2020). Alternatively, folic acid decoration has also demonstrated the potential to deliver withaferin A to the tumor site and increase growth inhibition of lung tumor xenografts from 50% (WFA-mExo) to 74% (WFA-FA-Exo) (Munagala et al., 2016).

Overall, sEVs have been proving to be good alternatives to synthetic NPs, such as liposomes, allowing an improvement of stability, solubility, and bioavailability of a different variety of molecules, while presenting longer circulation times and cross-species tolerance with no adverse immune and inflammatory response. More interestingly, exosomal formulations can resist the harsh conditions of the gastrointestinal tract, without affecting the properties of the loaded compounds, which allows an oral administration, more convenient for the patients (Agrawal et al., 2017). In cancer therapy, mExo can show intrinsic antiproliferative effect and improvement in the efficacy of the treatments is achieved, while reducing dose-related toxicity (Aqil et al., 2016; Aqil, Jeyabalan, et al., 2017; Aqil, Munagala, et al., 2017; Munagala et al., 2016). Moreover, considering scalability and associated need for easy access to large volumes, cow milk/whey is still considered the most promising source of these nature-derived nanocarriers, when compared to other biological sources (Sedykh et al., 2020).

4. Current barriers to the clinical application of milk-derived nanocarriers

The presented studies clearly state the potential of milk-derived carriers in the improvement of the physicochemical properties of different types of molecules, both in nutrient and pharmaceutical applications. However, several obstacles, related to health, environmental impact, and regulation issues, are still in the way of their actual implementation.

4.1. Health-related concerns

Most of the formulations based on milk components are designed to be administered orally. For oral delivery applications, the biocompatibility of bovine milk is undeniable, with an exception for the fraction of the population that has milk allergies or intolerances. It has been estimated that around 68% of the adult population has lactose malabsorption (or hypolactasia) (Storhaug, Fosse, & Fadnes, 2017). In the case of lactose intolerance (or maldigestion), it strongly depends on ethnicity or traditional diet, varying between 15% of the population of northern Europe, and 100% of American Indians and Asians (Swagerty Jr et al., 2002). For people with these conditions, lactose should be avoided or ingested in limited amounts. In this context, although the cited studies of MDDS do not explicitly use lactose, part of the developed formulations may contain this disaccharide, even if in residual amounts. Therefore, it would be of interest to include, in the studies, a percentage of lactose content in the formulations, and, if of relevance, contraindicate their use for people with both lactose malabsorption and maldigestion. When whole milk or complete whey is employed, the possibility of using lactose-free milk/whey should also come into play.

For people with allergies to cow milk proteins, none of the presented strategies is a possibility. Milk allergies are frequently confused with the above-mentioned lactose maldigestion and malabsorption, but the severity of the conditions is completely different. While lactose-related problems are associated with an enzymatic deficiency, milk allergies involve adverse immune responses to milk proteins. It is most detected during infancy and may cause respiratory, gastrointestinal, and cardiovascular serious symptoms (Flom & Sicherer, 2019). However, in contrast with the harmfulness of the condition, the numbers are less impactful than the ones presented for lactose intolerance. Only 1.4–3.8% of young children are affected by cow milk allergies (Zepeda-Ortega et al., 2021). In addition, 70% of children who were previously allergic to cow's milk can tolerate it by their mid-teenage years (Skripak, Matsui, Mudd, & Wood, 2007). Overall, despite the generally lower toxicity of milk when compared to other synthetic delivery systems, allergies and intolerances should be carefully considered before the prescription of such products.

Lately, some studies have been raising questions about the general effects of milk (and milk-derived products) on health. It is noteworthy that humans are the only mammals who consume milk from an exogenous origin and keep consuming it even as adults(Pereira, 2014). While some studies state that dairy products represent an important source of macro and micronutrients, others claim that they might be associated with an increased risk of the development of cardiovascular diseases, diabetes, obesity, and cancer (Marangoni et al., 2019). In particular, the milk fat content encompasses a high fraction of saturated fatty acids that are commonly associated with the development of previously mentioned Western diseases (Haug, Høstmark, & Harstad, 2007). In this regard, the use of milk fat in delivery systems should be deeply studied to fully understand the role of the carrier itself in health, before any application, both in nutrition and pharmaceutical approaches. Nutritional guidelines and advisory boards recommend the consumption of low-fat milk (Marangoni et al., 2019). Thus, if supplementation of milk is intended using MFG or lipid NPs, the content of fat should remain within regulated values. In contrast to fat, milk proteins and peptides have been showing potential to improve the absorption of other nutrients, as well as multiple protective actions in human health, namely antimicrobial, antioxidant, antiviral, antifungal, antihypertensive, antithrombotic, and immunomodulatory roles (Pereira, 2014). In general, despite the controversy around this topic, milk can still be a part of a balanced diet with potential health benefits. Milk components can exert different effects and, therefore, their use should be dependent on the goal of the formulation as well as the problem that it intends to tackle, in a way that the carrier itself could be beneficial and not detrimental.

4.2. Environmental issues

The intensification of dairy farming has led to a migration of pasture-based feeding regimens to indoor regimens, accompanied by a decline in the cow's diet and quality of life. In fact, besides animal welfare issues, housing animals can result in an increased incidence of lameness, mastitis and aggression due to space constraints (Timlin et al., 2021). The increasing awareness regarding these problems has been arising and with it the public stigma associated with animal-derived products consumption, especially products derived from cattle production. Due to health, environmental or other personal reasons, animal-free diets, such as vegetarianism and veganism, have been gaining supporters. Therefore, depending on each person's beliefs and diet choices, the presented strategies, despite relying on sustainability, may be not appropriate.

Moreover, serious environmental problems are associated with intensive dairy farming. In the last decades, the high ecological footprint of milk production has received increasing attention. Indeed, besides the already mentioned disposal of milk surplus and by-products that represent a huge environmental burden, dairy industries are energetically expensive. In terms of cow's feeding, it has been estimated that for 50 kg of milk production, 7×10^6 kcal are needed, which represents a huge impact in water consumption, and land use (Boland et al., 2019). Also, methane produced during the fermentation process in the rumen represents a significant contribution to the greenhouse gas load. This gas possesses a warming potential 21 times greater than an equivalent amount of carbon dioxide over the course of a century (Boland et al., 2019). Not all ruminant livestock species are related to milk production, but, globally, they are responsible for about 28% of methane emissions associated with anthropogenic activities (Boland et al., 2019). Regarding milk, a lower, yet significant, percentage of emissions is accounted for. It is estimated that around 2.7% of the total global anthropogenic greenhouse emissions are originated from milk production, processing and transportation activities (Tricarico, Kebreab, & Wattiaux, 2020). Research efforts along with governmental regulations have been carried out to reduce this impact, without compromising the consumption needs.

With this in mind, the valorization of milk is even more relevant. As a motivation for this review, we claim that milk production must be carried out sustainably, with minimal waste. Thus, in our view, every milk component, derived from by-products and/or milk surplus, can have a second life, and be employed in the improvement of nutrition as well as medical treatments as source material for delivery systems.

4.3. Regulatory issues and translation

Another challenge regarding the implementation of MDDS is the inherent variability of bovine milk composition. In the field of nanomedicine and drug delivery systems, one of the requirements for approval is related to the consistency of the specifications of the formulation (Salim et al., 2022). Thus, variability in terms of composition and physicochemical properties poses a great obstacle in the general translation of nanosystems to the clinic.

Being milk a biological product, its composition and functionality suffer seasonal and regional variations, related to feed availability and quality, lactation stage, breed, genetics, and health (Meurant, 1995). Concerning the lactation stage, for instance, several studies have reported marked variations between colostrum and mature milk

composition. Colostrum is the milk produced in the first few days after parturition. As a consequence of its primordial biological role, it has amounts of oligosaccharides and decreased levels of lactose. Interestingly, it is also reported that colostrum contains less fat, but a higher number of fat globules, with reduced size which provides a higher surface area and, in turn, facilitates the digestive process (Meurant, 1995).

To a certain extent, the use of commercially available powder compounds overcomes the issue of natural variability since their composition is known and the concentration can be adjusted. Casein isolates, MFGM lipid extracts, AMF, FHAMF, WPI and WPC are some examples of commercially available milk components that were used in the reviewed studies. However, the challenge increases when raw bovine milk or whey is used. This poses a particularly relevant issue in the case of mExo, where the membrane composition and structure of the lipid bilayer are already defined since their synthesis. Additionally, another relevant limitation in the clinical transfer of these nanoplatforms, especially in the case of exosomes, is the establishment of a unified protocol for the extraction and isolation of these vesicles. The homogeneity and purity of the isolated exosomes are critical as they directly influence the biophysical properties of these nanovesicles. Since residual proteins can bind to the exosome membrane this may alter their surface charge and therefore their *in vivo* stability and biodistribution and tropism. Several approaches are currently used to isolate sEVs from milk, including sequential centrifugation, size exclusion chromatography, density gradient centrifugation or immunomagnetic bead precipitation (Sedykh et al., 2020). Among these, differential ultracentrifugation (dUC) is the physical separation technique of choice as it allows the processing of large volumes of milk, increasing the amount of sEVs that can be recovered. However, the possible co-precipitation of other constituents morphologically similar to nanovesicles, such as proteins, casein or fat-containing globules, limits the applicability of this methodology by directly affecting the purity of the vesicles isolated by this method (Théry et al., 2018). In this scope, research should be moving towards the development of standardized methods of isolation, purification, and characterization of the delivery system, to achieve consistency between studies and even within the same study.

Despite the obstacles in the translation, it is important to keep investigating and monitoring the general composition of milk aiming to understand how external and biological factors can influence each component. This way it would be possible to ultimately modulate the milk composition and ratio of constituents by changing those external factors. This would represent a huge step forward in the direction of standardization and implementation of MDDS in the market.

5. Conclusions and future perspectives

Milk is not only a valuable nutritional product but also a widely available source of different kinds of delivery systems. Each component plays a different role in the milk colloidal suspension and that function can be translated into new ways of improving the properties of bioactive compounds.

MFGM has been showing remarkable benefits in the development of immunity and cognition as well as in the prevention of a wide range of diseases. The uniqueness of this structure was employed for delivery purposes, in different forms. As intact MFG, they were mainly used as carriers of hydrophobic compounds, aiming to improve solubility and stability. With fat derived from MFG, both SLN and NLC have been produced. Using the phospholipids extracted from the MFGM, it has been possible to produce resistant and stable liposomes.

Milk proteins have high nutritional value and are known for their health benefits. Also, they are responsible for the delivery of amino acids, several ions, such as calcium, phosphate, iron and other minerals, and vitamins. In the case of caseins, the CM have potential to bind and interact with a great variety of molecules with different hydrophilicity. As a delivery system, through native or reassembled CM, CNPs, or casein nanocomplexes, it has been possible to encapsulate biologically active compounds, including pharmaceuticals, nutraceuticals, and nutrients, enhancing their stability and bioavailability. Whey proteins have been showing great emulsifier properties, being very useful in the stabilization of nanoemulsions.

Although exosomes are the most recently explored native nanocarrier present in milk, they have great potential. Their application has mainly been in cancer therapy, as a natural alternative to synthetic liposomes. Other areas could also be explored, having, however, in mind that the amount of this structure in milk is very much lower than the MFG, CM or whey proteins.

Despite the amount of work that researchers have been putting into the development of milk-derived nanocarriers, their translation into the clinic and the market remains hampered by several concerns. More research is required to establish standardized protocols not only for the isolation of the components but also in the preparation of the formulations. Ensuring reproducibility is crucial to allow a smoother transition to the market. Scale-up should also be a concern in this scenario. Concerning health safety, more *in vivo* experiments should be performed, in short and long terms. Milk allergies and intolerances should be mentioned and taken into consideration in this type of work, as well. As far as the environmental issues are concerned, it can be pointed out that the presented strategies can take advantage of the milk surplus and by-products, reducing their impact on the ecosystems caused by their disposal. This does not require exclusive production.

Overall, despite the challenges that lay ahead, it can be said that the potential of MDDS is undeniable. The versatility in terms of application and type of molecule that can be incorporated into the carrier is clearly stated. The complexities discussed earlier highlight the need for close collaboration between the dairy industry, pharmaceuticals, and food industries to realize the potential of MDDS while fostering a circular economy. By leveraging surplus products and by-products from the dairy industry, such as excess milk and components not suitable for direct consumption, collaboration with other sectors can lead to sustainable solutions.

CRediT authorship contribution statement

Filipa A. Soares: Investigation, Writing - original draft; Beatriz Salinas: Writing - review & editing; Salette Reis: Writing - review & editing; Cláudia Nunes: Supervision; Writing - review & editing.

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Declaration of competing interest

The authors declare no conflicts of interest.

Data availability

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