31-5-2023

Therapeutic Potential of Milk-derived Extracellular Vesicles as Drug Delivery System

GSLS Writing Assignment

Bart Boogaard - 6158374

SUPERVISOR: DR. M.J.C. VAN HERWIJNEN

Plain Language Summary

Besides the nutritional value of milk, other components can have additional health benefits. Milk-derived extracellular vesicles (mEVs) are natural carriers that deliver biological cargo to recipient cells in the consumer of human or animal milk. Although the function of mEVs is dependent on their exact content, mEVs have been associated with positive effects in a number of disease models, including for cancer and immunoregulatory diseases. This capacity to deliver cargo in combination with positive effects in disease and health sparked interest in the application of mEVs as a drug carriers. Drug-loaded mEVs could be applied in treatment of a number of different diseases. Additional benefits include possible oral administration since mEVs were found to be partially resistant to damage caused by digestive juices when passing through the gastro-intestinal tract and can be taken up by the intestine. Moreover, mEVs can be modified to target disease cells, increasing the specificity of potential mEV treatments. However, before mEV drug delivery systems can be developed, a number of aspects need to be considered. In this review, the natural function and distribution throughout the body of mEVs were discussed. Also, the different techniques necessary in obtaining mEVs from milk and loading them with drugs were reviewed. Lastly, research into different diseases using mEV carriers was described in combination with further requirements for the development of clinical applications for mEVs. Although, mEVs are promising possible drug carriers, practical issues in isolation and characterisation and knowledge gaps in mEV function and biodistribution need to be overcome before clinical application can be realised.

Abstract

In recent years, milk-derived extracellular vesicles (mEVs) have been extensively studied in the context of drug delivery in a multitude of disease models. mEVs present in milk are derived from mammary cells and are hypothesized to comprise exosomes, microvesicles, and apoptotic bodies. mEVs effectively deliver their content, consisting of different biological components, including protein, RNA, and lipids, to recipient cells. The function of mEVs is dependent on their exact content, but several beneficial effects have been attributed to mEVs, including immunoregulatory and anti-tumour effects. This, in combination with the delivery potential of mEVs, makes them a suitable candidate for the development of novel delivery systems for therapeutics. Moreover, mEVs have been found to resist degradation in the gastro-intestinal tract and blood circulation, hinting towards the possibility of oral and intravenous administration. These properties seem to persist cross-species, meaning that mEVs can be derived from a cost-effective and widely available source in the form of bovine milk. In addition, it has been shown that modification of mEV lipid bilayers enables specific targeting of disease tissue, adding to the therapeutic potential of mEVs. Although the development of mEV drug carriers is promising, considerations need to be made before therapeutic applications can be realised. This review aims to provide an overview of the current state of knowledge on mEV characteristics in relation to their potential as drug delivery systems and to discuss practical considerations in the process of developing such a system. To this end, the current methods for isolation and drug loading were evaluated and coupled to the biodistribution of mEVs. Finally, recent developments in mEV therapies for cancer and other diseases were discussed, as well as considerations for future perspectives. It was concluded that improvements in scalability and standardisation of isolation and characterisation methods need to made in order for clinical application to be realised. Moreover, mEV functions, their mechanism of action, and biodistribution need to be elucidated for the development of a safe drug delivery system. Lastly, methods used in industrial processing, isolation, loading, and administration can reduce mEV integrity, but further *in vivo* studies are required to determine the degree of reduction in functionality.

Abbreviations

Abbreviation:	**Full Description:**
Anthos	Anthocyanidins
BBB	Blood-brain-barrier
BCA	Bicinchoninic acid assays
CUR	Curcumin
DLS	Dynamic light scattering
DNMT	DNA methyltransferase
DOX	Doxorubicin
dUC	Differential ultracentrifugation
ECG	Epicatechin gallate
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EV	Extracellular vesicle
FA	Folic acid
FC	Flow cytometry
HA	Hyaluronic acid
IBD	Inflammatory bowel disease
iRGD	Neuropilin receptor agonist peptide
IV	Intravenous injection
LNP	Lipid nanoparticle
mEV	Milk-derived extracellular vesicle
MFG	Milk fat globule
MRPS	Microfluidic resistive pulse sensing
MS	Mass spectrometry
NTA	Nanoparticle tracking analysis
PAC	Paclitaxel
PDT	Photodynamic therapy
SEC	Size exclusion chromatography
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor β
TLR4	Toll-like receptor 4
TRPS	Tunable resistive pulse sensing
UHT	Ultra heat treatment
WFA	Withaferin A
qPCR	Quantitative polymerase chain reaction

1. Introduction

The nutritional value of milk has long been established as well as the immunological benefits to infants.[1] However, over the past decade interest has shifted towards a more recently identified component in milk, extracellular vesicles (EVs). EV is a collective term used for lipid bilayer structures secreted by cells that lack a functional nucleus.[2] EVs play an important role in intercellular communication by transporting RNA, proteins, lipids, and other molecules between cells.[3] EVs can be subdivided into three categories: exosomes, microvesicles, and apoptotic bodies, each of which is characterised by different secretion and biogenesis pathways. The smaller exosomes have a diameter of 30–150 nm and originate from the multivesicular endosome by inward budding of the endosomal membrane (*Figure 1*).[4] On the other hand, microvesicle size is determined at 50–1.000 nm, while apoptotic bodies measure 500-2.000 nm in diameter. The latter both derive from the plasma membrane by outward budding, by vesicle shedding and apoptotic blebbing, respectively.[4] These different biogenesis pathways result in a difference in mEV membrane composition and content and thus a difference in nature of information transfer between cells.[5] The terms EV and exosome are used interchangeably in literature. However, in this review both will be referred to as EV for consistency.

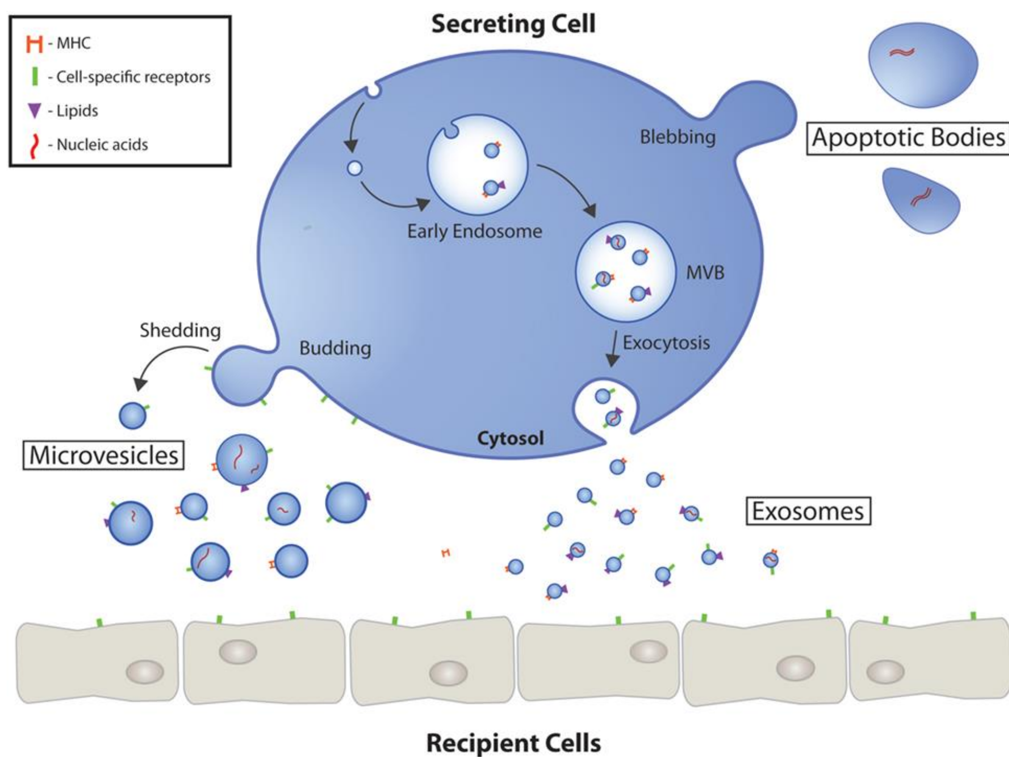

***Figure 1**: An overview of the biogenesis of extracellular vesicles. Microvesicles are formed by outward budding of the plasma membrane, similar to apoptotic bodies, which originate from outward blebbing. In contrast, exosomes derive from inward budding of the endosomal membrane, resulting in the formation of exosomes in the multi-vesicular endosome (MVB). Exosomes are secreted upon fusion of the endosome with the plasma membrane. (Gustafson et al.)*[4]

EVs are found in virtually every bodily fluid, including urine, blood, saliva, cerebrospinal fluids, bronchoalveolar fluid, semen, and, more relevantly, breast milk.[6] Milk-derived EVs (mEVs) have been associated with several health benefits, including systemic and local immunomodulatory effects, for example in the gastro-intestinal tract.[7] Interestingly, a number of these positive effects seem to persist in cross-species milk consumption.[3] Gastro-intestinal tract-related benefits of human and animal mEVs can be partially attributed to the route of administration and the specific properties of mEVs. mEVs are largely resistant to the harsh conditions of the gastro-intestinal tract, which enables them to maintain functionality when orally administered.[8,9] These characteristics of mEVs sparked interest in the possibility of a new oral drug delivery system with potential advantages over existing systems.

Besides protection and increased solubility of cargo, additional desired properties of mEVs for drug delivery applications include their ability to overcome physiological barriers, such as the blood-brain-barrier (BBB), and remain in circulation for several hours.[10,11] Furthermore, mEVs can be modified for tissue targeting purposes, increasing specificity of drug delivery.[12] In combination with the relative ease of drug-loading, a straightforward and specific drug delivery can be achieved.[13] Moreover, due to the natural origin of mEVs, biocompatibility is increased resulting in improved internalisation and reduced toxicity and immunogenicity.[14] The combination of these described aspects with the inherent health benefits attributed to mEVs make them an interesting prospect in the development of new drug delivery systems. The practical advantages of relatively cost-effective isolation of mEVs from commercially available cow's milk only strengthen this claim.[6]

Over the past years, extensive research has been performed into the different aspects required for clinical application of mEVs as drug carriers. *In vivo* experiments using mEVs or drug-loaded mEVs have shown efficacy in a number of diseases, including diabetes and different types of cancer.[15–17] However, there are still several issues to overcome before clinical application and large scale production of mEV therapies can be realised. This review aims to discuss the therapeutic potential of mEVs as drug carriers by evaluating the different aspects important to understanding the production and application of mEVs. Background knowledge on the isolation, characterisation, content, function, biodistribution, and loading of mEVs is vital in grasping the potential and clinical relevance of mEV drug delivery systems.

2. Isolation, Characterisation & Industrial Processing

A crucial first step in mEV research and application is mEV isolation from milk. Since different isolation methods result in variations in mEV samples, it is important to characterise purified samples. Moreover, the source of mEVs and the process of industrialisation can influence mEV morphology and content. So, in order to interpret research outcomes of mEV studies, consideration of isolation, characterisation, and industrial processing procedures is vital.

2.1. Isolation

Milk is composed of a wide range of particles that have a similar categorical size to mEVs, including casein micelles.[5] These pose a challenge in mEV isolation since most suitable separation methods are based on size.[18] The intended use and thus acceptable level of contamination by other similar-sized particles has to be taken into consideration when selecting the method of isolation.[19] Buschmann et al. provided an extensive overview of EV isolation methods, the most relevant of which will be discussed further (*Table 1*).[18]

Table 1: Overview of EV isolation methods.

Method	**Principle**	**Advantages**	**Disadvantages**
Tangential flow filtration	Size	Highly scalable, automatable, gentle, choice of membrane, disposable devices	Unable to remove EV-sized impurities, non-specific interactions with membranes
Ultrafiltration	Size	Rapid, scalable, choice of membrane, no dedicated major equipment, disposable devices	Clogging, sample loss, EV damage, unable to remove EV-sized impurities
Size-exclusion chromatography	Size	Gentle, scalable, good separation, removes soluble proteins and small molecules	Sample dilution might require post-separation concentration, low yield, limited sample capacity, unable to remove EV-sized impurities
Bind-elute chromatography	Size/affinity	Rapid, scalable, gentle, one-step elution, removes soluble proteins and small molecules	Low yield, limited sample capacity
Ion-exchange chromatography	Charge	Rapid, scalable, one-step elution	Low specificity for EVs, separation conditions need to be optimized, might require post-separation buffer exchange and concentration
Affinity chromatography	Affinity	Rapid, scalable	Unclear purity, elution might damage vesicles
(Immuno)affinity	Affinity	Rapid, high purity, no specialized equipment, specific capture of engineered EVs	Costly, low throughput, low yield, unclear scalability, *a priori* knowledge of surface markers is necessary, affinity reagents need to be removed without damaging EVs
Differential ultracentrifugation (dUC)	Sedimentation	Inexpensive, easy to use	Low throughput, low scalability, needs specialized equipment, unable to remove EV-sized impurities, potential EV damage and aggregation
Density gradient centrifugation (DGC)	Density	Commonly used method, inexpensive, high purity, often used in combination with other methods	Low throughput, low scalability, needs specialized equipment, low yield, lengthy and cumbersome procedure
EV Precipitation	Solubility	Highly scalable, rapid, no specialized equipment, easy to use, inexpensive	Low purity, co-separates soluble non-EV material, precipitation reagent needs to be removed

(adjusted from Buschmann et al.)[18]

The most commonly used isolation methods are centrifugation-based approaches, which separate EVs from other particles by mass or density.[20] Preparative centrifugation for EV isolation can be subdivided into two approaches: differential ultracentrifugation (dUC) and density gradient centrifugation (DGC). The most frequently applied method is dUC.[19] In dUC, multiple sequential centrifugations are performed and after

each step, the pelleted or supernatant fraction containing EVs is used in the next step.[11] Eventually, only the fraction of EVs and similarly sized particles remains. dUC is a cheap and easy-to-use technique, but the degree of contamination is relatively high. Hence, additional separation methods can be applied to improve sample purity. On the other hand, DGC uses a stepped density gradient medium from bottom to top of a centrifugation tube, containing either sucrose or iodixanol. This gradient allows for particle separation based on density.[19] DGC improves the quality of purified EV yields compared to dUC, but is time-consuming and unlikely to be used on an industrial scale due to its low throughput.[18]

An additional method to reduce contamination applied prior to dUC is isoelectric precipitation of caseins. Isoelectric precipitation is based on the principle that a reduction in pH leads to a decrease in solubility of caseins.[21] Reduction in pH is often achieved by the addition of acetic acid or hydrochloric acid. Lowering the pH to 4.6, the isoelectric point of caseins, will cause caseins to aggregate and precipitate, allowing for separation from mEVs by simple centrifugation.[22] Although this improves separation, it is important to note that acidic conditions were also found to have detrimental effects on mEV integrity. A detected reduction in WB, CD9, and CD81 membrane proteins after casein precipitation indicates that mEV bilayer structures are not immune to acidic damage.[22] In contrast, internal proteins were found to be unaffected.

To increase purity after dUC or as a separate isolation method, chromatography techniques can be applied. Chromatography methods are based on the interaction of particles with the stationary phase of a column.[20] This interaction results in a delay of the particles moving through the column based on their specific properties and thus in separation. Size exclusion chromatography (SEC) is used most often in this context.[19] Since SEC can effectively increase sample purity and is highly scalable, it is a promising method of mEV isolation on an industrial level. However, sample yield remains relatively low.[18]

Other size-based isolation methods include ultrafiltration, tangential flow filtration, and the more recently developed flow field fractionation.[18,19] The main disadvantage of size-based techniques is that there is no way to distinguish between mEVs and particles of similar size. To this end, several isolation methods using different properties of mEVs have been developed, such as immunoaffinity capture and mEV precipitation.[18,20] Even microfluidic techniques automating and combining different isolation methods have been suggested.[19] However, as of yet, the standard in mEV separation remains dUC, potentially supplemented with additional methods, such as casein precipitation and SEC, to improve sample purity.

2.2. Characterisation

Different isolation techniques can result in mEV samples with varying compositions and properties.[13] Therefore, it is important to characterise these properties before application. Size, concentration, morphology, purity, and content are of importance in mEV research, and to determine these characteristics a number of different techniques have been developed. A comprehensive summary of characterisation methods has been made by Bushmann et al. (*Table 2)*, the most relevant of which will be elaborated on.[18]

Table 2: Overview of EV characterisation methods.

Method	Principle	Advantages	Disadvantages
Scanning electron microscopy (SEM)	Capturing emitted electrons	Low sample volumes, single-EV analysis, surface topography	Little informative on surface structure and membrane features, risk of agglomeration and dehydration during sample preparation
Transmission electron microscopy (TEM)	Detecting diffracted electrons	Low sample volumes, single EV analysis, visualizing membranous makeup and intra-vesicular structures	Potentially biased by sample preparation, toxic chemicals
Cryogenic electron microscopy (Cry-EM)	Detecting scattered electrons at extremely low temperature	Most native EV morphology, single EV analysis, low sample volume	Specialized sample preparation with dedicated equipment

Method	**Principle**	**Advantages**	**Disadvantages**
Atomic force microscopy (AFM)	Scanning interaction forces between sample and detector tip	Label-free sample preparation, single-EV analysis, three-dimensional topography	Morphological changes by immobilization and by tip in contact mode possible, specialized equipment
Tunable resistive pulse sensing (TRPS)	Measuring resistance pulses	No sample preparation required, fast	Challenging for samples with unknown size ranges, risk of pore clogging, not EV-specific
Microfluidic resistive pulse sensing (MRPS)	Measuring resistance pulses	No sample preparation required, quick, low sample volumes	Challenging for samples with unknown size ranges, risk of pore clogging, not EV-specific
Dynamic light scattering (DLS)	Detecting fluctuations in light intensities scattered by moving particles	No sample preparation required, quick	Bulk measurement, approximate size distribution, not EV-specific, heavily hampered by polydisperse samples, limited resolution
Nanoparticle tracking analysis (NTA)	Recording the displacement of particles that scatter light	Single particle analysis, fast, no sample preparation required, fluorescent labelling possible to increase specificity	Not EV specific, slightly hampered by polydisperse samples, limited resolution
Flow cytometry (FC)	Detecting scattered light	Fast, single EV analysis and multiparametric measurement possible	Limited resolution, confounded by swarming effect and refractory index
Bead-based flow cytometry	Detecting scattered light	Quick, multiparametric measurements	Bulk analysis only, risk of aggregation, limited information on size and concentration
Nanoflow cytometry	Detecting scattered light	Single-EV analysis, improved resolution, multiparametric measurement possible	Confounded by refractory index
Imaging flow cytometry	Detecting scattered light with subsequent microscopic imaging	Single-EV analysis, improved resolution, multiparametric measurement possible	Dedicated equipment
Single-particle interferometric reflectance imaging sensing	Recording interferometric reflectance	Single-EV analysis, multiparametric measurement	Dedicated equipment
Immunoblotting	Detecting antibody-labelled signals	Established method, simple contamination check	Bulk analysis, high sample volume, requires pure preparations
Enzyme-linked immunosorbent assay (ELISA)	Detecting antibody-labelled signals	Established method, commercial kits available	Bulk analysis, cross-reactivity possible
Mass spectrometry (MS)	Separation of ionized molecules by their mass to charge ratio	High resolution, high-throughput, comprehensive data output	Bulk analysis, time consuming, requires pure preparations, sophisticated data analysis
Next-generation sequencing (NGS)	Transcript identification with single-nucleotide resolution	High sensitivity, high-throughput, comprehensive data output	Bulk analysis, time consuming, requires pure preparations, sophisticated data analysis
Quantitative polymerase chain reaction (qPCR)	Real-time detection of sequences selected *a priori*	Established protocols, high sensitivity	Bulk analysis, requires pure preparations and *a priori* knowledge

(adjusted from Buschmann et al.)[18]

mEV size and concentration are crucial for drug carrier applications, since they provide information necessary for drug loading and formulation. Tunable resistive pulse sensing (TRPS) is a technique that is used to estimate this. In TRPS, particles pass through a pore, thereby temporarily interrupting an electric current, resulting in a detectable resistance pulse.[23] The size of the pulse correlates with the size of the particle, allowing for size estimation. In addition, by evaluating the frequency at which the pore is blocked, the concentration of particles in the sample can be determined. Nevertheless, no distinction can be made between mEVs and other particles. Via this method, TRPS is able to determine size, concentration, and surface charge, or zeta potential, of particles within the detection range, which is dependent on the pore

size.[18] This detection range can be lowered by the application of microfluidic resistive pulse sensing (MRPS), which uses a small sample volume in an automated microfluidic approach.[24] This allows for detection of smaller particles, but is not suitable for zeta potential determination. Alternatively, nanoparticle tracking analysis (NTA) and dynamic light scattering (DLS) can be used for size and concentration estimation. Both techniques rely on the detection of light scattering by Brownian motion of particles.[25] DLS analysis results in a size distribution of particles, giving an indication of mEV size useful in bulk analyses.[18] On the other hand, NTA is able to track single particles, allowing for estimation of concentration, but thereby reducing the sensitivity. NTA can also be used to analyse fluorescently labelled particles, increasing the selectivity of mEV detection. Since each described technique has its own benefits and limitations, all are used in mEV characterisation in different drug delivery studies depending on the exact requirements for characterisation.

mEV morphology can be altered due to processing and isolation procedures. Therefore, it is useful to determine impurities, such as caseins, and changes in morphology and integrity using electron microscopy (EM).[14] With EM techniques, mEVs can be imaged using the detection of electrons fired at the sample. In scanning EM (SEM), emitted electron detection provides a topographic picture of the particle surface, while the goal of transmission EM (TEM) is to clarify particle content, allowing for distinction between mEV and other particles.[26] These techniques are the most widely used in mEV morphology research, but require specialised equipment, thus making application more complex. Other microscopy methods that can be advantageous over SEM and TEM in certain circumstances include cryo-EM and atomic force microscopy.[18] However, they are less commonly described in mEV drug delivery-related research.

For quality assurance, it can be useful to perform flow cytometry (FC) and western blotting or enzyme-linked immunosorbent assay (ELISA) analysis. FC enables the determination of overall dimensions and selective protein cargo of single particles, thus allowing for characterisation of particles in the sample and estimation of sample purity.[18] Since mEVs are relatively small, the detected signal could partially overlap with background noise, making the identification of mEVs more difficult. Therefore, additional experiments to indicate and quantify the presence of mEVs in the sample, using western blotting or ELISA techniques, can be conducted.[20]

Lastly, the native content of mEVs can influence drug delivery and therapeutic effects. This will be discussed more in depth in Chapter 3. Mostly, content analysis focusses on protein and RNA concentration and composition. Protein content can be examined using straightforward protein quantification methods, such as bicinchoninic acid assays (BCA), or more elaborate proteomic analysis, for instance mass spectrometry (MS).[14,27] Similarly, for RNA cargo characterization capillary gel electrophoresis or quantitative polymerase chain reaction (qPCR) analyses can be applied.[19] Although protein and RNA content are the most studied cargo, lipidomics and metabolomics using MS can provide insight into other types of content present in mEVs.[28] These and the aforementioned characterisation methods are vital in determining possible effects of isolation and industrial processing techniques on mEV properties.

2.3. Industrial Processing

Commercially available bovine milk is an abundant and cost-effective source of mEVs and therefore attractive for drug delivery applications. However, in order to create a product safe for consumption, raw milk is subjected to several industrial processes. To sterilise milk, pasteurisation and ultra-heat treatment (UHT) methods can be used. In the pasteurisation process, milk is heated to 72 °C–78 °C for at least 15 s, while in UHT the temperature is much higher (135 °C) and the time of processing is shorter (<1 s).[29] Both methods of sterilisation were found to affect mEV integrity and molecular composition in a study by Kleinjan et al.[29] FC analysis revealed that while mEV numbers after isolation of UHT processed milk were reduced, pasteurised sample mEV counts were comparable to raw milk. However, further cryo-EM imaging showed that both pasteurisation and UHT led to morphologic changes, including a loss of spherical shape and reduced membrane intensity. Moreover, protein and RNA analysis indicated changes in protein and RNA profiles. This confirmed previous findings by Howard et al., where a reduction in miRNAs after milk processing was shown.[30] In the Kleinjan et al. study, no additional detrimental effects on mEVs were observed in homogenised samples. Homogenisation is a technique used to reduce milk fat globule (MFG)

size. Other studies imply that factors influencing mEV numbers and content are microwaving and of milk at 4 °C, but more research is required to study the impact of this and previously described industrial processes on mEV functionality in relation to drug delivery.[31,32]

3. mEV Content, Function & Therapeutic Potential

mEV content is highly dependent on the cell type of origin and the status of the cell. mEVs secreted from different cell types can be found in breast milk, including mEVs derived from mammary epithelial cells, immune cells, and stem cells.[3] mEV content is not only variable between species, but also between individuals of the same species, as milk is highly dynamic in composition during lactation.[5] Although the general function of mEVs is to protect their cargo from degradation and facilitate intercellular communication, the effect of individual mEVs in health or disease is largely dependent on their content.

3.1. mEV Content & Function

Native cargo of mEVs consists of a number of different components, including proteins, RNA, lipids, metabolites, oligosaccharides, and DNA fragments.[28,33] Lipids, proteins, and RNA are among the most studied and most important for mEV function in relation to drug delivery, but in recent years focus has also shifted towards metabolites. Lipids are vital to the structural integrity of mEVs. Lipid composition of mEVs differs from the membrane of the cell type of origin.[28] As is characteristic of EVs, mEVs are enriched in sphingolipids and glycerophospholipids with saturated fatty acids, which aid in the stability of mEVs by increasing membrane rigidity.[34] However, lipids also contribute to mEV functions relevant for clinical application, including immune regulation, mainly via phospholipid precursors present in the mEV bilayer.[35] Although there are differences in lipid composition between mEVs derived from human and animal milk, the similarity and level of conservation is relatively high. For example, Blans et al. compared human mEVs to bovine mEVs and found that both were enriched in phosphatidylserine and sphingomyelin, while phosphatidylcholine was less abundant when compared to MFG composition.[36] Another property relevant for drug delivery is the possibility of lipid bilayer modification for tissue targeting, incorporation of targeting protein or peptides, as is further elaborated on in chapter 6.3.[37]

The mEV lipid bilayer also contains several membrane protein including CD9, CD63 and CD81 tetraspanins important for structural integrity, while other tetraspanins, such as CD40, CD54, CD80, CD86 were found to be less abundant compared to other EVs.[38] Moreover, a multitude of proteins associated with synthesis and budding of mEVs, including ADP-ribosylation factor and testilin were detected. Also, mEV-specific proteins related to mammary tissue and milk, lactadherin and lactoferrin, have been identified. All combined, human mEV proteomics analysis indicated the presence of 1963 proteins.[27] Out of all these proteins, the most relevant for drug delivery are proteins associated with mEV function. For instance polymeric-Ig receptor precursor, immunoglobulins and immunosuppressive cytokines, such as transforming growth factor β (TGF-β), serving a purpose in the mEV immunomodulation.[39] In addition, protein found to regulate cell proliferation were found to be present in human mEVs.[28] Interestingly, mEV protein function seems to be preserved cross-species as well, as is illustrated by the presence of identical proteins or proteins with a similar function in both bovine and human mEVs.[40]

Similar findings were published about the miRNA content of mEVs after transcriptomic analyses.[41] miRNAs regulate gene expression by hybridising with mRNAs, marking them for degradation and reducing translation of specific mRNA molecules. The broad range of miRNAs found in mEVs indicates their involvement in the regulation of numerous local and systemic processes, for example immunomodulation, cell proliferation, and differentiation.[42–44] miRNA content seems to be preserved cross-species, as is indicated by the 5 most abundant miRNAs found in human mEVs, which were also found to be present in similar concentrations in bovine mEVs.[45] The most prevalent miRNA in human mEVs, miRNA-148a, is a regulator of DNA methyltransferase (DNMT).[46] miRNA-148a can enact epigenetic changes via inhibition of DNMT and is identical in human and bovine mEVs, thus enabling cross-species regulation. Besides miRNAs, the presence of gene regulatory long non-coding and circular RNAs has been shown in mEVs.[5]

3.2. Intrinsic Therapeutic Potential of mEVs

The different components of mEVs have been found to be involved in numerous biological processes in health and disease.[3] Some of these effects have implications for the intrinsic therapeutic potential of mEVs. mEV treatment has been associated with beneficial effects in a number of disease models, including inflammatory bowel disease (IBD) and different types of cancer.[39,47,48] Therefore, in relation to drug delivery, the focus mainly lies on mEV immunomodulatory and tumour-specific anti-proliferative properties.

Regulation of immune responses is an important aspect in treatment of many diseases. In the case of IBD, pathogenesis is characterised by active adaptive and innate intestinal immune responses leading to symptoms, including abdominal pain, diarrhoea, and rectal bleeding.[39] TGF-β, a protein present in mEVs, was found to have an immunomodulatory effect in IBD disease models via gene expression regulation.[39] TGF-β influences gene expression by activating transcription factors SMAD2 and SMAD3.[7] In turn, SMAD2 and SMAD3 stimulate expression of *FOXP3*, which is associated with regulatory T-cell differentiation. These regulatory T-cells modulate inflammation and thus alleviate IBD symptoms. mEV miRNAs also contribute to immune modulation via this pathway by regulating gene expression of *FOXP3* via epigenetic modifications. miRNA-148 induces demethylation of CpG regions important in the transcription of *FOXP3*, thus stimulating regulatory T-cell differentiation.[7] Other examples of immune regulation via gene expression are miR-22-3p, which inhibits pro-inflammatory *NF-kB* expression, and let-7b, a regulator of *Toll-like receptor 4* (*TLR4*).[49,50] Similarly, several proteins and metabolites have been identified that exert immune regulatory functions.[28] In addition to beneficial effects in immune-related diseases, the ability of mEVs to inhibit pro-inflammatory signalling can also aid in the treatment of cancer.

The anti-proliferative effect associated with mEV treatment of tumours could be an additional benefit in the therapeutic application of mEVs. mEVs are found to have intrinsic anti-tumour properties, as was shown by Fonseka et al.[47] In this study, a neuroblastoma cell line was treated with bovine mEVs and proliferation was examined using a MTS metabolic assay. Results showed a significant reduction in proliferation when cells were treated with mEVs compared to negative controls. Proteomic analysis revealed that this inhibition was related to a reduction in expression of proteins involved in the Wnt signalling pathway. Furthermore, mEV-derived miRNA-2478 was found to inhibit Rap1a expression, thereby decreasing melanogenesis via the Akt-GSK3β signalling pathway in an *in vitro* melanoma model in a study by Bae et al..[48] Tumour inhibitory effects were confirmed in studies into the application of mEVs as drug delivery systems, where non-drug-loaded mEVs were shown to reduce tumour proliferation *in vitro* as well as *in vivo*.[11] Interestingly, this anti-proliferative effect was shown to be tumour-specific. The effects of mEVs on an *in vitro* colonic model of healthy and tumour cells were compared by Reif et al., and while the proliferation of the tumour cell line was reduced, the non-tumour cell line remained unaffected.[51] These findings suggest that mEV drug carriers potentially have a dual effect in the treatment of cancer. However, caution is warranted since Samuel et al., although confirming previous findings in an *in vivo* breast and colon tumour model, showed that mEV treatment resulted in an enhancement of tumour metastasis.[52] Therefore, it is crucial for future mEV drug delivery applications to obtain more information about the effect of mEVs on different types of tumours and to further elucidate their role in biological processes involved.

4. Biodistribution

mEV function and therapeutic potential are influenced by their bioavailability in different tissues, which is dependent on biodistribution. Therefore, biodistribution is an important aspect to consider in the development of mEV drug delivery systems. The route of administration is an important factor to consider when studying biodistribution. The natural route of entry for mEVs is oral administration, but intravenous (IV) injection and other administration methods have also been investigated.

4.1. Intestinal Uptake after Oral Administration

The oral route of drug administration holds several advantages over alternatives in the treatment of gastrointestinal-associated conditions, as well as systemic diseases. Oral administration is a non-invasive, low-cost, and self-administrable way of treatment.[53] Nevertheless, oral drug delivery faces a number of obstacles. The harsh conditions in the gastro-intestinal tract can lead to damage or degradation of different types of drugs.[53] Moreover, the intestinal barriers of passing the mucosal layer and being internalised by epithelial cells need to be overcome in order for drugs to reach blood circulation and have systemic effects. As a result, drug carriers suitable for oral administration, like mEVs, have certain properties that aid them in facing these challenges.

The gastro-intestinal tract consists of a number of different organs, each containing their own digestive juice which aids in degradation of nutrients (or drugs) passing through. In order to investigate the effect of these juices on mEVs, an *ex vivo* model resembling the digestive environment was developed by Tong et al.[54] Bovine and human mEV integrity were studied in this system and compared to liposomes. mEVs were found to be more stable than liposomes, reporting a 12% and 45% loss for bovine and human mEVs, respectively. Moreover, functional integrity preservation in the gastro-intestinal tract of bovine mEVs was shown in an epithelial tight junction protection model. In addition, oral administration of bovine mEVs in an *in vivo* murine model revealed that mEVs were able to reach the colon in 6 h, while they passed the small intestine after 1 h. This indicated that especially bovine mEVs were relatively resistant to gastro-intestinal tract conditions, as was confirmed by other studies.[9] In one of these studies by Samuel et al., it was also found that a naturally occurring component of milk, calcium, further increased mEV stability in low pH conditions.[52] Although a relatively high stability of mEVs was shown, further studies are required to determine the exact effects of the gastro-intestinal environment on mEV functionality.

The next challenge in uptake of mEVs is passing the mucosal layer and epithelial barrier. Warren et al. studied the penetration of mEVs in an *in vitro* intestinal mucosal model and concluded that unmodified mEVs were able to penetrate the intestinal mucus layer, overcoming the first barrier.[55] In addition, modification of mEVs by PEGylation resulted in an even higher penetration coefficient. Besides penetration, functionality of mEV cargo was confirmed using a siRNA reporting system. It was shown that target genes in intestinal epithelial cells were knocked down by mEV-delivered siRNA. *In vivo* experiments indicating the mEV presence in liver and other organs after oral administration, confirmed this.[52] Although the exact mechanism of intestinal uptake is yet to be elucidated, transendocytosis or paracellular translocation pathways have been hypothesised to facilitate mEV crossing of the epithelial barrier.[56] A number of transporters, including peptide transporters, amino acid transporters, glucose transporters, but most notably neonatal Fc receptor, were shown to be involved in mEV endocytosis.[6,57]

4.2. Biodistribution of Different Administration Methods

Already in the first study on biodistribution of bovine mEVs in a female athymic nude mice model by Munagala et al. it became clear that biodistribution of mEVs is dependent on the route of administration.[11] Fluorescently labelled mEVs were given to mice either via oral gavage or IV injection. After *ex vivo* examination of several organs (brain, colon, kidney, liver, lung, ovaries, pancreas, and spleen) orally administered mEVs were found to be uniformly distributed, while IV-injected mEVs were located predominantly in the liver (*Figure 2*). The resence of mEVs in brain tissue indicated that mEVs were able to cross the BBB as well. This was confirmed in a follow up study by Zhou et al.[58] Furthermore, Munagala et al.

reported no changes in toxicity profile parameters, except for an increase in anti-inflammatory cytokine GM-CSF. A subsequent study that assessed the toxicity of mEVs was in line with these findings.[57] Munagala et al. also investigated the presence of fluorescently labelled mEVs in blood over time after oral gavage.[11] It was concluded that mEV blood levels peaked at 24 h after administration. This provided some insight into the circulation time of mEVs and was supported by findings by Manca et al., who compared the fluorescent signal of mEV-encapsulated miRNA *ex vivo* after IV and oral administration.[10] Maximum liver mEV levels were detected after 3 h for IV, while the signal of orally administered mEVs was highest after 24 h. Different observations were made by Khanam et al., who investigated the biodistribution and bioavailability of direct fluorescently labelled mEVs.[59] Plasma levels peaked 6 h after oral administration, while after 24 h the signal was reduced to background. Moreover, a significantly higher estimated bioavailability of 45% was determined compared to the previously reported 25% by Manca et al.[10,59] Differences in labelling provide a possible explanation since detection of fluorescently labelled miRNA likely resulted in a loss of signal.

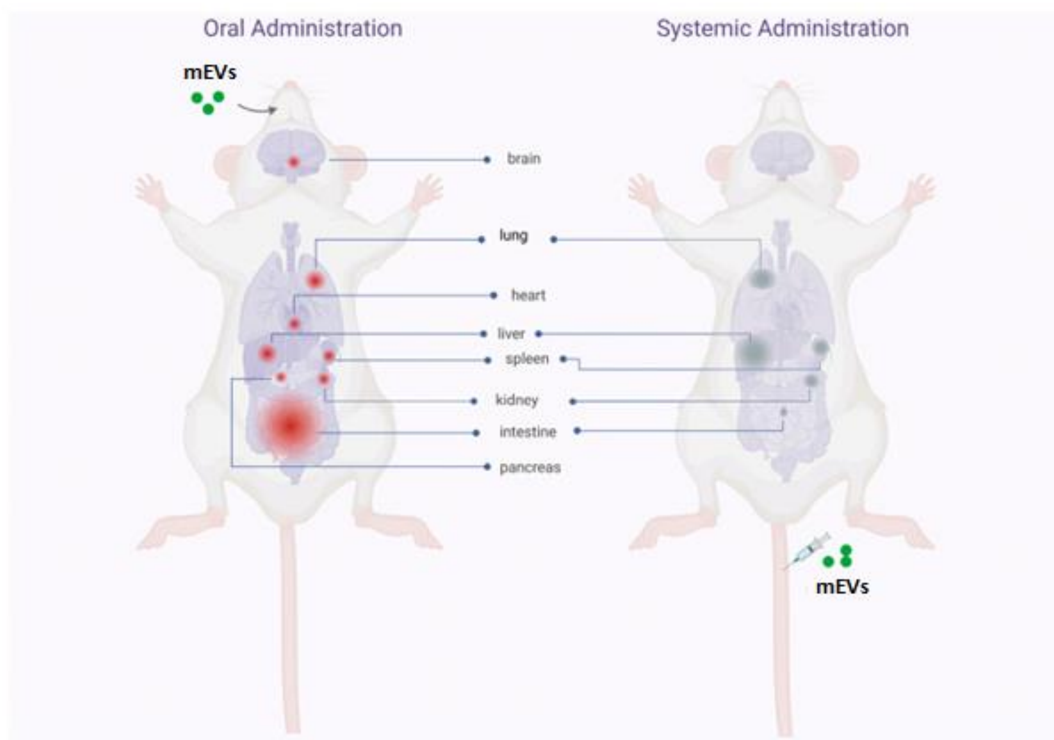

Figure 2*: Biodistribution patterns of milk-derived extracellular vesicles (mEVs) after oral and intravenous (IV) administration. mEVs are more evenly distributed throughout bodily organs, while IV-injected mEVs predominantly accumulate in the liver (adjusted from Donoso-Meneses et al.).*[56]

Biodistribution of mEVs can be influenced by targeting modifications. The inclusion of tumour-specific molecules can lead to an increased presence of mEVs in tumour tissue, as was found by several studies, including one by Betker et al.[6,60] Betker et al. also investigated the difference in biodistribution and bioavailability of orally administered mEVs compared to liposome nanocarriers. mEV presence in blood was still detectable after 360 min, while liposome signal diminished after 120 min. Moreover, detected mEV levels *ex vivo* were found to be significantly higher in all examined organs (heart, kidney, liver, lung, and spleen).

The most studied routes of administration of mEVs are oral and IV, but a number of other methods have also been considered. Depending on the target tissue and the type of disease intraperitoneal or sublingual administration can hold advantages over oral and IV administration.[61,62] In a study in a diabetic murine model using blood-glucose lowering drug liraglutide, orally administered liraglutide-mEVs were unable to exert a hypoglycaemic effect, while sublingually administered liraglutide-mEVs were.[62] Moreover, intranasal administration of EVs derived from other sources, such as mesenchymal stem cells, has also been effectively

applied in *in vivo* neurological disease models.[63] However, in the field of mEVs more research is needed to further elucidate the possible advantages of these alternative methods of administration.

All in all, current biodistribution studies indicate that oral administration of mEVs results in a more homogenous distribution in organs and increased circulation time compared to IV administration. Also, in comparison to liposomes, orally administered mEVs were found to have increased bioavailability. Other methods of administration might also have advantages in drug delivery, but more data is required to make accurate comparisons.

5. Loading

Efficient mEV drug loading without affecting drug delivery capacities is vital to the development of mEV-based therapeutics. For mEV applications, *in vitro* loading techniques, where drugs are encapsulated by isolated mEVs after production, are preferred to *in vivo* methods, where encapsulation occurs in the cells of origin.[64] *In vitro* loading techniques that have been effectively applied in the production of mEV drug carriers are incubation, physical mEV treatments, including electroporation, sonication, extrusion, and surfactant treatment, and chemical transfection (*Figure 3*).[13]

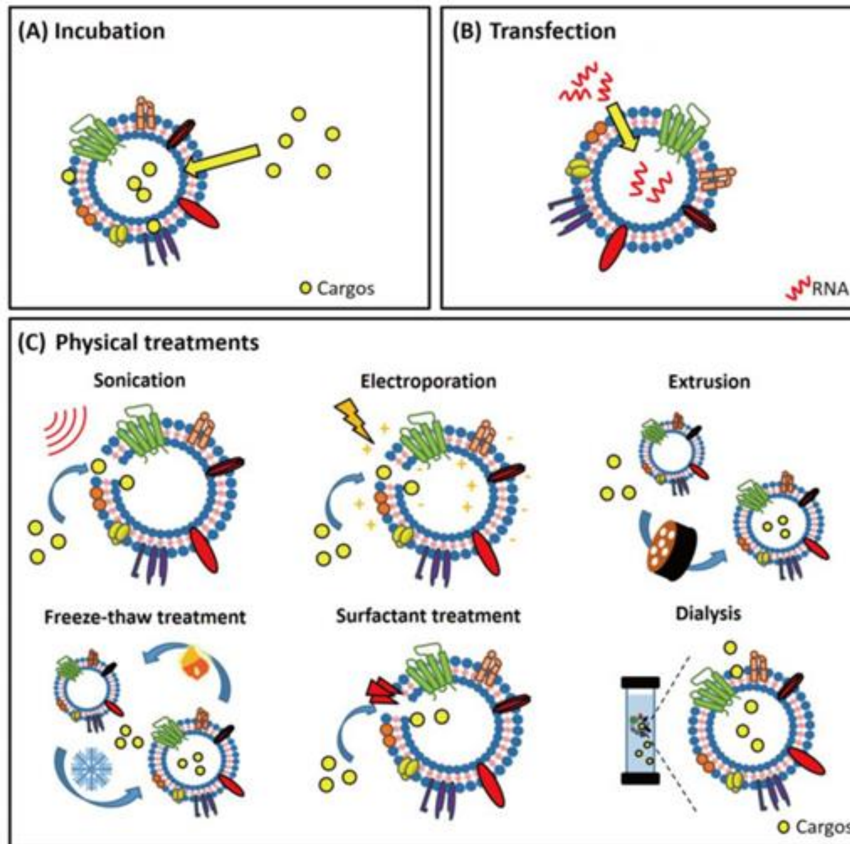

***Figure 3**: Overview of different milk-derived extracellular vesicles (mEV) loading methods. A) Diffusion of cargo over the mEV membrane after incubation leads to mEV loading. B) Treatment with a chemical transfectant for RNA loading or C) physical treatments, including sonication, electroporation, extrusion, freeze-thawing, surfactant treatment, and dialysis increase membrane permeability and loading efficiency.*

The simplest method of drug loading is incubation of mEVs with drugs. This has been applied for the loading of multiple drugs, including withaferin A (WFA), paclitaxel (PAC), curcumin (CUR), anthocyanidins (Anthos), and doxorubicin (DOX).[11,16,37,60] To this end, drugs are dissolved in a solution suitable for hydrophilic drug loading, often consisting of ethanol and acetonitrile. This allows for straightforward drug loading at room temperature without causing major damage to mEVs.[13] However, the process has a limited loading efficiency and is hardly quantifiable since incubation-based loading is influenced by a number of different factors, such as pH. Despite this, due to its relative ease and low costs, incubation remains a widely used mEV loading method.

To improve loading efficiency, physical treatments that create micropores in lipid mEV bilayers can be utilised. The current standard for mEV loading is electroporation, which creates micropores by the application of short, high-voltage electric pulses. Although membrane integrity and stability can be affected, electroporation significantly increases drug loading and has been shown to enable efficient mEV drug loading of PAC, CUR and small RNAs.[15,65–67] Moreover, when DOX loading was compared in mEVs after

incubation, sonication and electroporation methods, loading efficiency was found to be highest in electroporation-loaded mEVs.[68] Sonication, is another physical treatment applying additional mechanical shear force using sound at a certain frequency to disrupt membrane integrity, allowing for the loading of mEVs.[64] Although sonication is used in some instances, the relatively low loading efficiency in combination with reduction in membrane integrity and stability limit its applicability in research.[66] Furthermore, other physical treatments include freeze thawing, extrusion, dialysis and treatment with surfactants, such as saponin.[13,64] Interestingly, a study by Ahmed et al. not only revealed that saponin treatment was beneficial in terms of DOX loading efficiency compared to incubation and sonication methods, results also indicated a difference between mEVs derived from different animal species.[69] It was found that for all loading methods, goat-mEVs yielded higher DOX concentrations than mEVs derived from cow or buffalo, suggesting that the origin of mEV also influences drug loading. Despite the positive results shown for saponin loading, its applicability in research and clinic is still limited by its negative effects on mEV integrity and cargo.[13] In addition, a purification step is required to remove the homolytically active saponin before it can be safely administered.

Lastly, a method often applied for mEV loading of RNA-based therapeutics is the direct transfection method, using chemical transfection agents such as lipofectamine. In chemical transfection, RNA is mixed with lipofectamine to form liposomes encapsulating the RNA molecules. Next, the liposomes are co-incubated with mEVs to allow for transfection. As a consequence, liposome and mEV fuse, thereby forming a chimeric structure.[64] Chemical transfection is not the most commonly applied mEV loading technique due to damage caused by the transfection procedure and treatment with lipofectamine.[64]

6. Therapeutic Applications: Drug Delivery

Solubility, degradation, and cellular uptake can be limiting factors in efficient drug delivery. Several chemotherapeutic, biological, and RNA drugs could therefore benefit from a mEV carrier. The advantageous properties of mEV carriers in the protection, biodistribution and biocompatibility of their cargo have led to the utilisation of mEVs in drug delivery studies involving different diseases and therapeutics over recent years (*Table 3*).

Table 3: Overview of mEV drug delivery systems used in studies using *in vitro* and *in vivo disease models*

Disease:	Therapeutics:	Publications:
Cancer	Withaferin A (WFA)	Munagala et al. (2016)[11]
	Paclitaxel (PAC)	Munagala et al. (2016)[11] Agrawal et al. (2017)[16] Aqil et al. (2017)[60] Chen et al. (2022)[65]
	Curcumin (CUR)	Munagala et al. (2016)[11] Aqil et al. (2017)[60] Vashisht et al. (2017)[70] Carobolante et al. (2020)[71] González-Sarrías (2022)[66]
	Anthocyanidins (Anthos)	Munagala et al. (2016)[11] Aqil et al. (2017)[61] Munagala et al. (2017)[72] Mudd et al. (2020)[73] Chen et al. (2021)[74] Ahmed et al. (2022)[69]
	Doxorubicin (DOX)	Li et al. (2020)[37] Fonseka et al. (2021)[47] Pullan et al. (2022)[12] Mukhopadhya et al. (2023)[68]
	Photodynamic Therapy (PDT)	Huis in ’t Veld et al. (2022)[75] Guo et al. (2023)[76]
	miRNAs	Zhang et al. (2017)[77] Li et al. (2020)[15]
	siRNAs	Matsuda et al. (2018)[78] Aqil et al. (2019)[67] Tao et al. (2020)[79] Roerig et al. (2022)[80]
	Astaxanthin	Cui et al. (2022)[81]
	Oxaliplatin	Go et al. (2022)[82]
Diabetes	Liraglutide	Shi et al. (2022)[83] Xu et al. (2022)[62]
	Insulin	Wu et al. (2022)[57]
	miRNAs	Yan (2022)[17]
Parkinson’s disease	Epicatechin gallate (ECG)	Luo et al. (2021)[84]
Intestinal bacterial infections	α-mangostin	Qu et al. (2022)[85]

6.1. First mEV Drug Delivery Study

The first evidence of an effective mEV drug delivery system for chemotherapeutic drugs in an *in vivo* cancer model was reported by Munagala et al.[11] Munagala et al. loaded bovine mEVs with a variety of different low-solubility chemotherapeutic agents, including WFA and PAC by incubation. Both *in vitro* and *in vivo*, a significantly greater reduction in tumour growth was observed after treatment with mEV-WFA and mEV-PAC compared to non-carrier-loaded drugs. A similar trend in anti-inflammatory effects was observed, while no signs of adverse toxicity were detected. It was also shown that these anti-proliferative effects *in vivo* were

increased when folic acid (FA) molecules were incorporated in the mEV-WFA membrane, showing the possibility for targeted treatment of tumour cells. Interestingly, results indicated that anti-cancer effects could partially be attributed to the intrinsic therapeutic potential of mEVs since treatment with only mEVs resulted in a reduction in tumour size and pro-inflammatory signalling. In the seven years since this study was published, the number of studies involving a multitude of different anti-cancer drugs using mEV drug carriers has grown exponentially.[5] Moreover, the possibilities of targeted mEV tumour treatment have exceeded FA alone, with a number of different possible membrane modifications having been examined.

6.2. mEV Drug Delivery in Cancer

In an expanded study on mEV-PAC delivery, tumour growth reduction and lower immunologic and systemic toxicity after mEV-PAC treatment compared to non-encapsulated PAC were confirmed in lung cancer xenograft mice.[16] Other known anti-cancer drug mEV encapsulation studies yielded similar results, for example when loading polyphenol curcumin (CUR) in mEVs for treatment of cervical tumour xenografts.[60] More recently, the potential of mEV-CUR in combination with another anticancer dietary polyphenol, resveratrol, in treating breast cancer, was examined. Encapsulation of CUR and resveratrol by mEVs resulted in a significant reduction in xenograft tumour growth, possibly even overcoming ABC-mediated chemotherapeutic resistance.[66] Similarly, the potential of an mEV-therapeutic involving anthocyanidins (Anthos) to overcome chemotherapeutic resistance in ovarian tumours was investigated in OVCA432 cells.[61] It was shown that where mEV-PAC had severe side effects against cisplatin-resistant OVCA432 cells, mEV-Anthos was able to exert antiproliferative effects and decrease the multi-tumour resistance-associated P-glycoprotein level without inducing toxic and proinflammatory effects. mEV-Anthos was already found to have antiproliferative effects in multiple *in vitro* tumour models and specifically reduced tumour growth in *in vivo* lung cancer models.[72] Furthermore, a study into the chemopreventative ability of mEV-Anthos in *Bacteriodes fragilis*-induced colorectal cancer adds to the potential of mEV-Anthos to combat cancer.[73]

The beneficial effects of mEV drug delivery in cancer treatment have also been described for RNA-drugs, which can directly target gene expression of oncogenes. mEV-encapsulated tumour suppressor miRNAs were found able of inducing gene expression changes and reducing tumour growth *in vitro*.[15,77] Furthermore, mEV-siRNAs could knock down *VEGF*, *EGFR*, *AKT*, *MAPK*, and *KRAS in vitro* and significantly inhibit tumour growth in KRAS-expressing xenografts.[67] miRNA and siRNA approaches seem to be promising prospects in the development of new anti-cancer therapeutics. As was confirmed by a study into mEV-BCL2-siRNA. mEV-BCL2-siRNA not only inhibited tumour growth but also drastically reduced tumour cell migration and invasion in a digestive system tumour murine model.[79]

6.3. Targeted mEV Drug Delivery

In the treatment of cancer, specificity for tumour cells is often required. Recent studies into the combination of photodynamic therapy (PDT) with an mEV-drug delivery system show a tissue-specific approach to mEV cancer treatment. PDT uses a photosensitizer that can induce cell death when exited by light at a certain wavelength.[86] By limiting the light to tumour sites, a certain degree of specificity can be obtained, as was shown in an *in vivo* model by Guo et al.[76] Further tumour targeting can be realised by modification of the mEV lipid bilayer by inclusion of targeting molecules, as was shown by Munagala et al.[11] Similar to FA, hyaluronic acid (HA), a CD44 tumour marker-specific ligand, was found to have targeting potential (*Table 4*). HA when coupled to mEVs via DSPE-PEG$_{2000}$ was investigated for miRNA-204 as well as chemotherapeutic DOX.[15,37] Both studies showed an increase in uptake and tumour inhibition of HA-mEV-drugs compared to mEV-drugs. This suggests that the specific targeting of HA increases the efficacy of drug-loaded mEVs. Furthermore, in a study by Cui et al. cytokine expression was shown to be reduced when HA-mEVs were loaded with Astaxanthin, a known anti-inflammatory low solubility carotenoid, further confirming the targeting potential of HA.[81] HA is not the only modification used for targeting purposes in mEV delivery, inclusion of other targeting moieties, such as neuropilin receptor agonist peptide (iRGD) have also been investigated.[6] The ability of iRGD-mEV-PAC to induce cell death in lung carcinoma cells and penetrate 3D structures was shown by Chen et al. and similar results were observed when iRGD was combined with a hypoxia-responsive lipid for mEV-DOX targeting in triple negative breast cancer.[12,65] Moreover, EGFR-specific

GE11 peptide coated on mEVs containing known anti-cancer drug oxaliplatin was able to specifically target EGFR expressing colorectal xenograft tumours *in vivo*.[82] These mEV modifications allow for targeted delivery of drugs to specific tissues, further expanding the potential of mEV delivery in cancer treatment.

Table 4: Targeting modifications of mEV drug carriers in literature

Modification:	Target receptor:	Publication:	Used therapeutic:
Folic acid (FA)	Folate receptor	Munagala et al. (2016)[11]	Withaferin A
Hyaluronic acid (HA)	CD44 receptor	Li et al. (2020)[37] Li et al.(2022)[15] Cui et al. (2022)[81]	Doxorubicin miRNA-204 Astaxanthin
iRGD peptide	Neuropilin receptor	Pullan et al (2022)[12] Chen et al. (2022)[65]	Doxorubicin Paclitaxel
GE11 peptide	EGFR receptor	Go et al. (2022)[82]	Oxaliplatin

6.4. mEV Drug Delivery in Other Diseases

Besides the potential of mEVs in therapeutic applications for cancer, they also hold potential for treatment of several other diseases, including impaired diabetic wound healing, which has been associated with a decrease in expression of miRNA-31-5p.[17] Yan et al. created mEVs containing miRNA-31-5p and studied uptake, degradation and endothelial function *in vitro*. It was concluded that miR-31-5p mEV-loaded mimics were taken up by cells more efficiently and improved endothelial function. Subsequent *in vivo* experiments added to this by showing enhanced wound healing in diabetic mice. Also, blood glucose levels can be altered using this system. Orally administered mEVs containing insulin were shown to have an increased hypoglycaemic effect relative to commonly used subcutaneous injections of insulin in type I diabetic mice, as were liraglutide-mEVs.[57,62,83] In addition, neurological diseases could benefit from mEV carriers. Delivery of epicatechin gallate by mEVs in a Parkinson's disease model showed neuroprotective effects.[84] Even beneficial effects against intestinal bacterial infections by α-mangostin-loaded mEVs have been described.[85] All these possibilities for delivery of different drugs for a multitude of disease show the versatility of mEV drug delivery systems.

7. Discussion

mEVs have been proposed as promising candidates in the development of new drug delivery systems. Benefits related to their composition and natural origin include increased solubility and protection from degradation of encapsulated drugs.[9] Moreover, high biocompatibility and intrinsic positive effects have been attributed to mEVs.[7,54] Immunomodulatory functions as well as anti-tumour effects have been described after mEV treatment in a number of studies.[39,46] Since many of these effect persist cross-species, potential mEV drug carriers could be derived from a commercially available and cost-effective source in the form of bovine milk.[3] Although the processing, isolation and characterisation of milk were found influence mEV properties, mEVs have already been widely studied in the context of drug delivery.[11,18] Biodistribution studies revealed that orally administered mEVs were relatively stable in the gastro-intestinal tract, were able to cross the intestinal barrier and the BBB, distributed evenly throughout organs and could be found in blood circulation several hours after administration.[11,54,70] This has major implications for the oral application of mEVs as drug carriers as has been shown in multiple disease models.[5,16] Several mEV-loaded chemotherapeutic agents and miRNA drugs were found to be significantly more effective in the treatment of *in vitro* and *in vivo* cancer models.[15,16,72] In other studies, it was also found that lipid bilayer modification allowed for targeted drug delivery enhancing specificity of potential therapies.[65] All in all, this makes mEV a promising prospect for therapeutic drug carrier applications.

Before further steps toward the clinic can be taken, considerations need to be made about the source and industrial processing of milk. Although Ahmed et al. found that the anti-cancer effect of DOX-loaded mEVs was enhanced in mEVs isolated from goat milk compared to buffalo and cow-derived counterparts, more comparative studies are required to clarify differences between species.[69] Furthermore, pasteurisation and UHT of raw milk were found to affect membrane integrity and mEV numbers by Kleinjan et al., possibly influencing mEV functionality.[29] However, as of yet, the exact effect on functionality has been insufficiently studied. Since consumption of raw milk is illegal in some countries, this aspect could have implications for mEV drug delivery systems.[29]

Industrial processing is not the only area lacking functionality studies. Although mEVs are found to be relatively stable in the acidic conditions of the gastro-intestinal tract, reductions in membrane integrity and mEV numbers have been observed.[9] Moreover, acidic conditions used in the casein precipitation isolation method also affect mEV morphology.[21] Similarly, physical treatments applied in drug loading, such as electroporation, sonication, and most notably saponin treatment, can damage mEV lipid bilayers.[13] Premature drug release due to reduced membrane integrity and stability could lead to serious side effects, especially when loading chemotherapeutic agents. Despite *in vivo* drug delivery studies showing the ability of mEVs to effectively deliver cargo after being subjected to the aforementioned conditions and treatments without significant side effects, it remains largely unclear to what extent it affects the drug delivery capacity of mEVs.[16,52,69] Before clinical applications can be realised, the effect on mEV functionality needs to be elucidated further since it heavily influences dosage considerations.

Other practical issues in developing mEV drug carriers include scalability and standardisation of isolation and characterisation methods. dUC, the current standard in mEV isolation, has limited scalability (*Table 1.*).[18] More scalable techniques, such as ultrafiltration and chromatography-based methods, have other drawbacks, including low yield, sample loss, and clogging.[19] In addition, isolated mEV samples are heterogeneous and contaminated to a certain degree due to mEV origin and isolation procedures, while current characterisation methods are insufficient to control for this.[3] Therefore, standardisation of mEV sample preparation and potency assays are required to reduce variations and control for functionality, especially considering their inherent heterogeneity.[3] Similarly, storage protocols should be standardised. Agrawal et al. and Munagala et al. noted that storage of isolated mEVs at -80 °C had minimal effect on mEV activity and morphology after approximately a month, while Leiferman reported a reduction in mEV-sized particles of 49% after a similar period of time when milk was stored at 4 °C.[11,16,32] Besides this, little is known about the stability of mEVs in different storage conditions, so further examination of mEV storage is required.

Moreover, safety considerations need to be made when isolating mEVs. Especially, the possibility of potentially dangerous contamination needs to be diminished. Due to similarities in size and density, most currently applied isolation methods are unable to distinguish between mEVs, virus particles, and bacterial EVs.[87] Standardised methods to determine possible viral and bacterial contamination are to be developed before further steps can be taken towards clinical application. Since affinity-based methods are best suited for distinguishing between contaminants and mEVs, development of such a technique will likely involve this approach.

In terms of safety, remarks can be made about the current knowledge on mEV content, function, and biodistribution. The complexity herein lies in the fact that the exact function and effects of mEV content are not fully elucidated, and existing studies are known to be contradictory. For example, TGF-β was found to have immunomodulatory effects acting through epigenetic modifications via *FOXP3* expression, but TGF-β was also found to play a role in epithelial–mesenchymal transition and stimulation of breast-tumour progression.[7,88] Moreover, although several studies have stated the anti-proliferative effects of mEVs in cancer, Samuel et al. found evidence of promotion of metastasis in xenograft mice after mEV treatment.[52] When considering biodistribution studies, Munagala et al., Manca et al. and Khanam et al. all concluded different plasma circulation times of mEVs after oral administration and maximum detected mEV signal in peripheral organs also varied.[10,11,59] All studies used comparable murine models, which are the current standard in extracellular biodistribution studies, but variability in other aspects of methodology partly explain differences in outcome.[89] Therefore, standardisation is advised in future studies, since accurate biodistribution profiles and mEV half-life are required for potential therapeutic applications. Besides distribution, the pathway of intestinal uptake is poorly understood. Although it is believed that transendocytosis and paracellular translocation might be involved, the exact mechanism and factors involved in epithelial cell internalisation are to be clarified.[3,56] Before clinical implementation can be realised, light needs to be shed on these and other mEV properties. At later stages, other considerations, such as possible allergies to components in mEV formulations, for example, milk and lactose allergies, should be taken into account.

Although gaps in knowledge remain about the exact effects and biodistribution of mEVs and practical issues need to be overcome in order for mEVs to be applied as drug carriers, recent developments in the field show the potential of this method for drug delivery. Over the past years, the path towards the application of mEVs in drug delivery has become increasingly shorter, and if this trend is continued, further steps towards the clinic can be taken.

References

1. Palmeira, P. & Carneiro-Sampaio, M. Immunology of breast milk. *Rev Assoc Med Bras (1992)* **62**, 584–593 (2016).
2. Théry, C. *et al.* Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* **7**, (2018).
3. Sanwlani, R., Fonseka, P., Chitti, S. V. & Mathivanan, S. Milk-Derived Extracellular Vesicles in Inter-Organism, Cross-Species Communication and Drug Delivery. *Proteomes* **8**, (2020).
4. Gustafson, D., Veitch, S. & Fish, J. E. Extracellular Vesicles as Protagonists of Diabetic Cardiovascular Pathology. *Front Cardiovasc Med* **4**, 71 (2017).
5. Mecocci, S., Trabalza-Marinucci, M. & Cappelli, K. Extracellular Vesicles from Animal Milk: Great Potentialities and Critical Issues. *Animals (Basel)* **12**, (2022).
6. Betker, J. L., Angle, B. M., Graner, M. W. & Anchordoquy, T. J. The Potential of Exosomes from Cow Milk for Oral Delivery. *J Pharm Sci* **108**, 1496 (2019).
7. Melnik, B. C., John, S. M., Carrera-Bastos, P. & Schmitz, G. Milk: a postnatal imprinting system stabilizing FoxP3 expression and regulatory T cell differentiation. *Clin Transl Allergy* **6**, (2016).
8. Tong, L. *et al.* Milk-derived extracellular vesicles alleviate ulcerative colitis by regulating the gut immunity and reshaping the gut microbiota. *Theranostics* **11**, 8570 (2021).
9. López de Las Hazas, M. C. *et al.* Dietary bovine milk miRNAs transported in extracellular vesicles are partially stable during GI digestion, are bioavailable and reach target tissues but need a minimum dose to impact on gene expression. *Eur J Nutr* **61**, 1043–1056 (2022).
10. Manca, S. *et al.* Milk exosomes are bioavailable and distinct microRNA cargos have unique tissue distribution patterns. *Sci Rep* **8**, 11321 (2018).
11. Munagala, R., Aqil, F., Jeyabalan, J. & Gupta, R. C. Bovine milk-derived exosomes for drug delivery. *Cancer Lett* **371**, 48–61 (2016).
12. Pullan, J. *et al.* Modified Bovine Milk Exosomes for Doxorubicin Delivery to Triple-Negative Breast Cancer Cells. *ACS Appl Bio Mater* **5**, (2022).
13. Fu, S., Wang, Y., Xia, X. & Zheng, J. C. Exosome engineering: Current progress in cargo loading and targeted delivery. *NanoImpact* **20**, 100261 (2020).
14. Wang, Z., Mo, H., He, Z., Chen, A. & Cheng, P. Extracellular vesicles as an emerging drug delivery system for cancer treatment: Current strategies and recent advances. *Biomedicine & Pharmacotherapy* **153**, 113480 (2022).
15. Li, D. *et al.* Hyaluronic Acid-Coated Bovine Milk Exosomes for Achieving Tumor-Specific Intracellular Delivery of miRNA-204. *Cells* **11**, (2022).
16. Agrawal, A. K. *et al.* Milk-derived exosomes for oral delivery of paclitaxel. *Nanomedicine* **13**, 1627–1636 (2017).
17. Yan, C. *et al.* Milk exosomes-mediated miR-31-5p delivery accelerates diabetic wound healing through promoting angiogenesis. *Drug Deliv* **29**, 214–228 (2022).
18. Buschmann, D., Mussack, V. & Byrd, J. B. Separation, characterization, and standardization of extracellular vesicles for drug delivery applications. *Adv Drug Deliv Rev* **174**, 348 (2021).

19. Li, P., Kaslan, M., Lee, S. H., Yao, J. & Gao, Z. Progress in Exosome Isolation Techniques. *Theranostics* **7**, 789 (2017).

20. Sidhom, K., Obi, P. O. & Saleem, A. A Review of Exosomal Isolation Methods: Is Size Exclusion Chromatography the Best Option? *Int J Mol Sci* **21**, 1–19 (2020).

21. Yamauchi, M. *et al.* Efficient method for isolation of exosomes from raw bovine milk. *Drug Dev Ind Pharm* **45**, 359–364 (2019).

22. Rahman, M. M. *et al.* Acidification effects on isolation of extracellular vesicles from bovine milk. *PLoS One* **14**, (2019).

23. Vogel, R. *et al.* Quantitative sizing of nano/microparticles with a tunable elastomeric pore sensor. *Anal Chem* **83**, 3499–3506 (2011).

24. Fraikin, J. L., Teesalu, T., McKenney, C. M., Ruoslahti, E. & Cleland, A. N. A high-throughput label-free nanoparticle analyser. *Nat Nanotechnol* **6**, 308–313 (2011).

25. Anderson, W., Kozak, D., Coleman, V. A., Jämting, Å. K. & Trau, M. A comparative study of submicron particle sizing platforms: accuracy, precision and resolution analysis of polydisperse particle size distributions. *J Colloid Interface Sci* **405**, 322–330 (2013).

26. Mukhopadhya, A., Santoro, J., Moran, B., Useckaite, Z. & O'Driscoll, L. Optimisation and comparison of orthogonal methods for separation and characterisation of extracellular vesicles to investigate how representative infant milk formula is of milk. *Food Chem* **353**, (2021).

27. Van Herwijnen, M. J. C. *et al.* Comprehensive Proteomic Analysis of Human Milk-derived Extracellular Vesicles Unveils a Novel Functional Proteome Distinct from Other Milk Components. *Mol Cell Proteomics* **15**, 3412–3423 (2016).

28. Buratta, S. *et al.* Protein and Lipid Content of Milk Extracellular Vesicles: A Comparative Overview. *Life* **13**, (2023).

29. Kleinjan, M. *et al.* Regular Industrial Processing of Bovine Milk Impacts the Integrity and Molecular Composition of Extracellular Vesicles. *J Nutr* **151**, 1416–1425 (2021).

30. Howard, K. M. *et al.* Loss of miRNAs during processing and storage of cow's (Bos taurus) milk. *J Agric Food Chem* **63**, 588–592 (2015).

31. Zhao, Z., Yu, S., Xu, M. & Li, P. Effects of microwave on extracellular vesicles and microRNA in milk. *J Dairy Sci* **101**, 2932–2940 (2018).

32. Leiferman, A., Shu, J., Upadhyaya, B., Cui, J. & Zempleni, J. Storage of Extracellular Vesicles in Human Milk, and MicroRNA Profiles in Human Milk Exosomes and Infant Formulas. *J Pediatr Gastroenterol Nutr* **69**, 235 (2019).

33. Malkin, E. Z. & Bratman, S. V. Bioactive DNA from extracellular vesicles and particles. *Cell Death Dis* **11**, (2020).

34. Buratta, S. *et al.* Extracellular vesicles released by fibroblasts undergoing H-Ras induced senescence show changes in lipid profile. *PLoS One* **12**, (2017).

35. O'Donnell, V. B., Rossjohn, J. & Wakelam, M. J. O. Phospholipid signaling in innate immune cells. *J Clin Invest* **128**, 2670–2679 (2018).

36. Blans, K. *et al.* Pellet-free isolation of human and bovine milk extracellular vesicles by size-exclusion chromatography. *J Extracell Vesicles* **6**, (2017).

37. Li, D. *et al.* Hyaluronan decoration of milk exosomes directs tumor-specific delivery of doxorubicin. *Carbohydr Res* **493**, (2020).

38. Admyre, C. *et al.* Exosomes with immune modulatory features are present in human breast milk. *J Immunol* **179**, 1969–1978 (2007).

39. Ihara, S., Hirata, Y. & Koike, K. TGF-β in inflammatory bowel disease: a key regulator of immune cells, epithelium, and the intestinal microbiota. *J Gastroenterol* **52**, 777–787 (2017).

40. Reinhardt, T. A., Lippolis, J. D., Nonnecke, B. J. & Sacco, R. E. Bovine milk exosome proteome. *J Proteomics* **75**, 1486–1492 (2012).

41. Zhou, Q. *et al.* Immune-related microRNAs are abundant in breast milk exosomes. *Int J Biol Sci* **8**, 118–123 (2012).

42. Dull, K. *et al.* miR-146a modulates TLR1/2 and 4 induced inflammation and links it with proliferation and lipid production via the indirect regulation of GNG7 in human SZ95 sebocytes. *Sci Rep* **11**, (2021).

43. Zhang, W. *et al.* miR-26a attenuates colitis and colitis-associated cancer by targeting the multiple intestinal inflammatory pathways. *Mol Ther Nucleic Acids* **24**, 264–273 (2021).

44. Kim, C., Ye, Z., Weyand, C. M. & Goronzy, J. J. miR-181a-regulated pathways in T-cell differentiation and aging. *Immun Ageing* **18**, (2021).

45. Golan-Gerstl, R. & Reif, S. Extracellular vesicles in human milk. *Curr Opin Clin Nutr Metab Care* **25**, 209–215 (2022).

46. Melnik, B. C. & Schmitz, G. DNA methyltransferase 1-targeting miRNA-148a of dairy milk: a potential bioactive modifier of the human epigenome. *Functional Foods in Health and Disease* **7**, 671–687 (2017).

47. Fonseka, P. *et al.* Temporal Quantitative Proteomics Analysis of Neuroblastoma Cells Treated with Bovine Milk-Derived Extracellular Vesicles Highlights the Anti-Proliferative Properties of Milk-Derived Extracellular Vesicles. *Cells* **10**, 750 (2021).

48. Bae, I. S. & Kim, S. H. Milk Exosome-Derived MicroRNA-2478 Suppresses Melanogenesis through the Akt-GSK3β Pathway. *Cells* **10**, (2021).

49. Takata, A. *et al.* MicroRNA-22 and microRNA-140 suppress NF-κB activity by regulating the expression of NF-κB coactivators. *Biochem Biophys Res Commun* **411**, 826–831 (2011).

50. Guo, Z. *et al.* Let-7b ameliorates Crohn's disease-associated adherent-invasive E coli induced intestinal inflammation via modulating Toll-Like Receptor 4 expression in intestinal epithelial cells. *Biochem Pharmacol* **156**, 196–203 (2018).

51. Reif, S., Elbaum Shiff, Y. & Golan-Gerstl, R. Milk-derived exosomes (MDEs) have a different biological effect on normal fetal colon epithelial cells compared to colon tumor cells in a miRNA-dependent manner. *J Transl Med* **17**, (2019).

52. Samuel, M. *et al.* Oral administration of bovine milk-derived extracellular vesicles induces senescence in the primary tumor but accelerates cancer metastasis. *Nat Commun* **12**, (2021).

53. Hua, S. Advances in Oral Drug Delivery for Regional Targeting in the Gastrointestinal Tract - Influence of Physiological, Pathophysiological and Pharmaceutical Factors. *Front Pharmacol* **11**, (2020).

54. Tong, L. *et al.* Milk-derived extracellular vesicles protect intestinal barrier integrity in the gut-liver axis. *Sci Adv* **9**, eade5041 (2023).

55. Warren, M. R. *et al.* Milk Exosomes with Enhanced Mucus Penetrability for Oral Delivery of siRNA. *Biomater Sci* **9**, 4260 (2021).

56. Donoso-Meneses, D., Figueroa-Valdés, A. I., Khoury, M. & Alcayaga-Miranda, F. Oral Administration as a Potential Alternative for the Delivery of Small Extracellular Vesicles. *Pharmaceutics* **15**, 716 (2023).

57. Wu, L. *et al.* Milk-derived exosomes exhibit versatile effects for improved oral drug delivery. *Acta Pharm Sin B* **12**, 2029–2042 (2022).

58. Zhou, F. *et al.* Small Extracellular Vesicles in Milk Cross the Blood-Brain Barrier in Murine Cerebral Cortex Endothelial Cells and Promote Dendritic Complexity in the Hippocampus and Brain Function in C57BL/6J Mice. *Front Nutr* **9**, (2022).

59. Khanam, A., Ngu, A. & Zempleni, J. Bioavailability of orally administered small extracellular vesicles from bovine milk in C57BL/6J mice. *Int J Pharm* **639**, (2023).

60. Aqil, F., Munagala, R., Jeyabalan, J., Agrawal, A. K. & Gupta, R. Exosomes for the Enhanced Tissue Bioavailability and Efficacy of Curcumin. *AAPS J* **19**, 1691–1702 (2017).

61. Aqil, F. *et al.* Exosomal delivery of berry anthocyanidins for the management of ovarian cancer. *Food Funct* **8**, 4100–4107 (2017).

62. Xu, M. *et al.* Liraglutide-Loaded Milk Exosomes Lower Blood Glucose When Given by Sublingual Route. *ChemMedChem* **17**, (2022).

63. Herman, S., Fishel, I. & Offen, D. Intranasal delivery of mesenchymal stem cells-derived extracellular vesicles for the treatment of neurological diseases. *Stem Cells* **39**, 1589–1600 (2021).

64. Kimiz-Gebologlu, I. & Oncel, S. S. Exosomes: Large-scale production, isolation, drug loading efficiency, and biodistribution and uptake. *J Control Release* **347**, 533–543 (2022).

65. Chen, J. *et al.* Targeted Therapy of Lung Adenocarcinoma by the Nanoplatform Based on Milk Exosomes Loaded with Paclitaxel. *J Biomed Nanotechnol* **18**, 1075–1083 (2022).

66. González-Sarrías, A. *et al.* Milk-Derived Exosomes as Nanocarriers to Deliver Curcumin and Resveratrol in Breast Tissue and Enhance Their Anticancer Activity. *Int J Mol Sci* **23**, (2022).

67. Aqil, F. *et al.* Milk exosomes - Natural nanoparticles for siRNA delivery. *Cancer Lett* **449**, 186–195 (2019).

68. Mukhopadhya, A., Tsiapalis, D., McNamee, N., Talbot, B. & O'Driscoll, L. Doxorubicin Loading into Milk and Mesenchymal Stem Cells' Extracellular Vesicles as Drug Delivery Vehicles. *Pharmaceutics* **15**, 718 (2023).

69. Ahmed, F., Tamma, M., Pathigadapa, U., Reddanna, P. & Yenuganti, V. R. Drug Loading and Functional Efficacy of Cow, Buffalo, and Goat Milk-Derived Exosomes: A Comparative Study. *Mol Pharm* **19**, 763–774 (2022).

70. Vashisht, M., Rani, P., Onteru, S. K. & Singh, D. Curcumin Encapsulated in Milk Exosomes Resists Human Digestion and Possesses Enhanced Intestinal Permeability in Vitro. *Appl Biochem Biotechnol* **183**, 993–1007 (2017).

71. Carobolante, G., Mantaj, J., Ferrari, E. & Vllasaliu, D. Cow Milk and Intestinal Epithelial Cell-derived Extracellular Vesicles as Systems for Enhancing Oral Drug Delivery. *Pharmaceutics* **12**, (2020).

72. Munagala, R. *et al.* Exosomal formulation of anthocyanidins against multiple cancer types. *Cancer Lett* **393**, 94 (2017).

73. Mudd, A. M. *et al.* Chemoprevention of colorectal cancer by Anthocyanidins and mitigation of metabolic shifts induced by dysbiosis of the gut microbiome. *Cancer Prev Res (Phila)* **13**, 41 (2020).

74. Chen, C. *et al.* Active cargo loading into extracellular vesicles: Highlights the heterogeneous encapsulation behaviour. *J Extracell Vesicles* **10**, (2021).

75. Huis in 't Veld, R. V. *et al.* M1-derived extracellular vesicles enhance photodynamic therapy and promote immunological memory in preclinical models of colon cancer. *J Nanobiotechnology* **20**, (2022).

76. Guo, R. *et al.* Chlorin e6-loaded goat milk-derived extracellular vesicles for Cerenkov luminescence-induced photodynamic therapy. *Eur J Nucl Med Mol Imaging* **50**, 508–524 (2023).

77. Zhang, D., Lee, H., Zhu, Z., Minhas, J. K. & Jin, Y. Enrichment of selective miRNAs in exosomes and delivery of exosomal miRNAs in vitro and in vivo. *Am J Physiol Lung Cell Mol Physiol* **312**, L110–L121 (2017).

78. Matsuda, A. & Patel, T. Milk-derived Extracellular Vesicles for Therapeutic Delivery of Small Interfering RNAs. *Methods Mol Biol* **1740**, 187–197 (2018).

79. Tao, H. *et al.* Exosomes-coated bcl-2 siRNA inhibits the growth of digestive system tumors both in vitro and in vivo. *Int J Biol Macromol* **161**, 470–480 (2020).

80. Roerig, J. *et al.* Synergistic siRNA Loading of Extracellular Vesicles Enables Functional Delivery into Cells. *Small Methods* **6**, (2022).

81. Cui, W. *et al.* Engineering Milk-Derived Exosome for Enhancing Cellular Astaxanthin Delivery. *J Agric Food Chem* **70**, 10794–10806 (2022).

82. Go, G., Park, H. J., Lee, J. H., Yun, C. W. & Lee, S. H. Inhibitory Effect of Oxaliplatin-loaded Engineered Milk Extracellular Vesicles on Tumor Progression. *Anticancer Res* **42**, 857–866 (2022).

83. Shi, Y. *et al.* Construction and Evaluation of Liraglutide Delivery System based on Milk Exosomes: A New Idea for Oral Peptide Delivery. *Curr Pharm Biotechnol* **23**, 1072–1079 (2022).

84. Luo, S. *et al.* Enhanced Neuroprotective Effects of Epicatechin Gallate Encapsulated by Bovine Milk-Derived Exosomes against Parkinson's Disease through Antiapoptosis and Antimitophagy. *J Agric Food Chem* **69**, 5134–5143 (2021).

85. Qu, S. *et al.* Milk Exosomes Facilitate Oral Delivery of Drugs against Intestinal Bacterial Infections. *J Agric Food Chem* **70**, 16069–16079 (2022).

86. Gustalik, J., Aebisher, D. & Bartusik-Aebisher, D. Photodynamic therapy in breast cancer treatment. *J Appl Biomed* **20**, 98–105 (2022).

87. McNamara, R. P. & Dittmer, D. P. Modern Techniques for the Isolation of Extracellular Vesicles and Viruses. *J Neuroimmune Pharmacol* **15**, 459 (2020).

88. Qin, W. *et al.* Exosomes in Human Breast Milk Promote EMT. *Clin Cancer Res* **22**, 4517–4524 (2016).

89. Kang, M., Jordan, V., Blenkiron, C. & Chamley, L. W. Biodistribution of extracellular vesicles following administration into animals: A systematic review. *J Extracell Vesicles* **10**, (2021).