RSC Pharmaceutics

ROYAL SOCIETY OF CHEMISTRY

REVIEW

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Cite this: *RSC Pharm.*, 2025, **2**, 1034

The potential of small extracellular vesicles for pancreatic cancer therapy

Richard Crow,[a] Oksana Kehoe[b,c] and Clare Hoskins *[a]

Extracellular vesicles come in various shapes and sizes and are released by most cell types. They have myriad roles in intercellular signalling in both physiological and pathological environments, carrying a range of lipids, proteins and nucleic acids. Their cargo is then unloaded at the target site inducing a change in their target cell. Cancers use these vesicles to their advantage for a wide range of outcomes such as immune evasion and chemoresistance leading to the reduced effect of chemotherapies and unfavourable patient outcomes. Pancreatic cancer has one of the worst outcomes of any cancer with surgery being the only cure. As surgery is only available in a small number of cases, targeted delivery of cargos directly to the tumour site is of high importance to efficiently target and destroy cancer cells with high effectiveness without the toxic off-target effects of chemotherapy drugs. Hijacking the body's postal system has gained interest in the last decade for the delivery of therapeutic drugs. The low immunogenicity and inherent biocompatibility of extracellular vesicles avoids the hurdles experienced by other nanoparticles such as toxicity. Various techniques for loading and functionalising extracellular vesicles have progressed to clinical trials, however, these therapies are yet to make it onto the market. This review seeks to be a call to action to the pancreatic cancer community, highlighting the potential of these biologic systems in the improvement of therapeutic outcomes of what is one of the deadliest cancers.

Received 23rd April 2025,
Accepted 3rd August 2025

DOI: 10.1039/d5pm00115c

rsc.li/RSCPharma

1. Introduction

With a 5-year survival rate of 11% in 2022,[1] pancreatic cancer (PC) has one of the highest mortality rates of any cancer, partly due to its lack of specific symptoms and the lack of distinct biomarkers hindering early diagnosis. Once diagnosed 50–55% of PC patients present with metastatic disease.[2] The most common form of PC is pancreatic ductal adenocarcinoma (PDAC) which makes up around 95% of all diagnosis. Depending on the degree of vascularisation; the localised pancreatic tumours are split into resectable, borderline resectable and unresectable. Typically, there is a multidisciplinary approach to treatment including chemotherapy, radiation, targeted therapy, or surgery depending on the disease progression. Various combinations of FOLFIRINOX (fluorouracil, irinotecan, leucovorin, oxaliplatin), gemcitabine, nab-paclitaxel and capecitabine are the common chemotherapy options, however, in unresectable PDAC these only increase the patient survival by 2–6 months.[3] Even with these therapies PDAC retains its high mortality rate. The tumour is made up of cancer associated fibroblasts (CAFs) and cancer stem cells (CSCs) regulate the tumour microenvironment (TME) allowing it to evade the host immune system and undergo uncontrolled and rapid proliferation. This hypovascular nature which PDAC possesses adds an extra hurdle when trying to deliver drug cargo into the TME, with the dense stoma acting as a barrier to penetration and hence protecting the cancer cells from drug treatment.[4] Novel approaches are needed to detect and target pancreatic tumours to increase early detection and survival rates. Obesity and type-2 diabetes are 2 of the main environmental influences in the development of PC along with other lifestyle choices such as alcohol, tobacco use and workplace chemicals.[5] However, there are also many genetic factors involved so a deeper understanding of PDAC pathology is required to explain differences in reactions to treatment and survival rate.[6]

Since the first identification of small extracellular vesicles (sEVs) in 1987 nearly 40 years ago,[7] a wide range of roles have been identified. A comprehensive history of extracellular vesicles (EVs) and their uses is reviewed by Yáñez-Mó *et al.*[8] Their roles in normal bodily function, such as cell–cell communication and physiological conditions are characterised by the transport of lipids, proteins, RNA and metabolites to the recipient cell, although their individual compositions are diverse.

[a]*Department of Pure and Applied Chemistry, University of Strathclyde, Technology Innovation Centre, 99 George Street, Glasgow, G1 1RD, UK.*
E-mail: clare.hoskins@strath.ac.uk; Tel: +44 (0)141 5482796
[b]*Centre for Regenerative Medicine Research, School of Medicine, Keele University, Keele, Staffordshire, ST5 5BG, UK*
[c]*Robert Jones and Agnes Hunt Orthopaedic Hospital, Oswestry, Shropshire, SY10 7AG, UK*

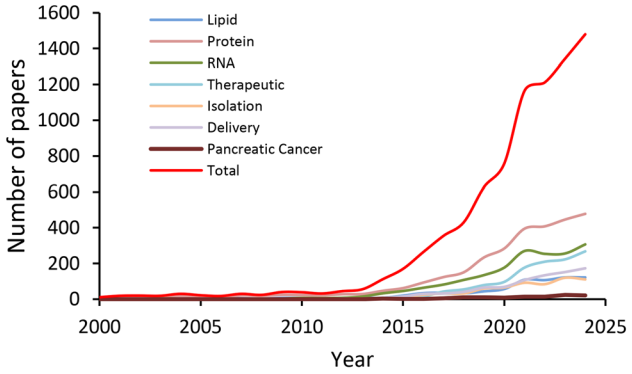

Fig. 1 Graph showing the output of papers containing sEVs and cargoes. "Small extracellular vesicles" and "lipid". "Small extracellular vesicles" and "protein". "Small extracellular vesicles" and "RNA". "Small extracellular vesicles" and "therapeutic". "Small extracellular vesicles" and "isolation". "Small extracellular vesicles" and "delivery". "Small extracellular vesicles" and "pancreatic ductal carcinoma". All searches taken from Web of Science™.

sEVs are also employed to chaperone specific cargo molecules during pathological conditions such as cancer, aiding in the advancement of tumour progression, metastasis, and in suppression of the immune system.[8–11] As additional roles of SEVs have been discovered, this has led to increased interest in their potential use as diagnostic markers and for therapeutic application (Fig. 1). One such potential application is in the treatment of PC, however, as detailed in the graph, relatively little work has been directed towards this cancer to date.

The systemic function of EVs mean they are found in most biological matrices: plasma, urine, semen, saliva, bronchial fluid, cerebrospinal fluid, lymph *etc.*[12] In cancer, EVs isolated from the cancerous cells possess an abundance of cancer associated antigens on their surfaces[13] which can be potentially detected using non-invasive liquid biopsies in mass screening for early detection. The small nano-size of extracellular vesicles allows them to permeate across tight inaccessible barriers in the body, such as the blood–brain barrier,[14] possessing low immunotoxicity[15] and high biocompatibility due to their naturally derived origins, reducing the hazards associated with synthetic nanoparticles. However, due to their ability to reside in systemic circulation for long periods, more work needs to be carried out in order to fully understand their long-term immunogenic effects.[15]

This review seeks to highlight the potential of sEVs for PC therapy, discussing the physiological challenges faced in the tumour microenvironment, the advantages of sEV based therapeutic delivery and how sEVs have demonstrated efficacy enhancement in PC therapy to date.

2. Pancreatic cancer

2.1. Tumour microenvironment

PDAC tumour cells are protected by an impenetrable, desmoplastic stroma rich in hyaluronic acid, and a range of CAFs. This physical barrier prevents efficient vascularisation which not only limits exposure to chemotherapy and immunotherapy penetration, but it also creates a hypoxic environment. This is why PDACs are characterised by limited infiltration of $CD8^+$ T cells. Low oxygen and nutrient concentrations lead PDAC to rearrange its metabolism potentially providing new targets for therapy, as cancer cells shift from oxidative phosphorylation to aerobic glycolysis, there is an increased scavenging for lipids and proteins. The immunosuppressive, hypoxic conditions of the tumour microenvironment, along with the physical barrier of the desmoplasia prevent effective drug or immunotherapy permeability.

Attempts to remove the stroma *via* deletion of the hedgehog pathway showed some promise in mice when combined with chemotherapy, however, in human have not been successful, with clinical trials being halted when a the combination of the Sonic hedgehog inhibitor (saridegib) plus gemcitabine resulted in an increased rate of progressive disease compared with the placebo and chemotherapy administered alone.[16] Large numbers of ongoing clinical trials are using various methods of increasing sensitivity of PDAC to the immune system by altering the TME. Evidence suggests that non-specific targeting of the extracellular matrix (ECM) is not effective (matrix metalloproteinase (MMP) inhibitors), which has led to a more specific approach using hyaluronan.[16] A high deposition of hyaluronan is associated with a poor prognosis and reducing it has been shown to increase vascular permeability and drug delivery.[16]

The importance of understanding the TME cannot be underestimated. Its complexity needs to be considered when determining future approaches and combination therapy targeting. Individual characterisation of the TME should aid in directing therapeutic decisions for more personalised oncology. Performing genomic, transcriptomic, and immune environment analysis on individual PDAC tumours is the future of personalised treatment due to the high degree of genetic heterogeneity of PDACs making singular therapies difficult. Targeted therapies have attempted various routes to target major signalling pathways, epigenetics, DNA repair deficiencies and immune responses with varying results in clinical settings.[17] KRAS mutations are found in >90% of all PDACs (TP53, SMAD4 and CDKN2A are the other major mutations)[18] and so provides a promising target using anti-KRAS therapies. KRAS PDACs are associated with chemoresistance, immunosuppressive properties and hypovascularity affecting the efficacy of treatments so combination therapies are being looked at. In addition, inhibitors for common KRAS mutations are yet to be determined, so downstream signalling pathways are targeted. A recent study used MEK1/2 and CDK4/6 inhibitors in mice to produce senescence-associated secretory phenotype (SASP) promoting tumour vascularisation as well as promoting $CD8^+$ T cells tumour infiltration.[19] Additionally, the use of anti-PD1 therapy showed areas of tumour destruction. However, it should be noted that the increase in $CD31^+$ cells may increase metastatic potential. The use of therapy-induced senescence could provide an effecting

multifaceted therapy alongside chemo and immunotherapies and increasing the vascularity of PDACs may be useful due to their hypovascularisation impeding delivery of therapeutic agents.

2.2. Nanotechnology for PC

Nanotechnology is a new frontier in cancer therapeutics with aims to improve the effectiveness of chemotherapy by directed delivery, increasing amount of the drug within the tumour whilst decreasing systemic toxicity.[20] It allows for site specific trafficking of therapeutics to target biomarkers reducing the off-target effects of traditional chemotherapy. This is of great importance for PC, where there is a lack of non-invasive tools for detection, which hinders advances in therapy. Only CA 19-9 is currently approved as a PDAC biomarker by the US Food & Drug Administration (FDA), but it is not recommended due to its poor sensitivity and specificity.[21] While there are various other biomarkers, few of these are specific to PC, different proteins and abundance are detected depending on the source, as well as potential contamination. The end goal will be to have biomarkers capable of differentiating between PC, other pancreatic disease, benign tumour and healthy tissue.

Currently two nanoformulations are approved for PC therapy, these are Abraxane and Onivyde. Abraxane is a formulation of paclitaxel bound to albumin, whilst Onivyde is a liposome formulation of fluoruracil and leucovorin. Both increase patient survivability of PC, but neither possess any biomarker specific targeting. Drug formulation into nanoparticles (NPs) such as liposomes or polymers has shown to increase their circulation times which was once believed to lead to passive targeting *via* the enhanced permeability and retention effect. Although, this phenomenon is contested greatly in solid tumours[22] where a study showed that active targeting was the preferential mechanism for intratumoral trafficking of nanotechnologies.[23] This has led researchers to believe that increased circulation times alone are the factor resulting in better tumour reduction, as the therapy has more time to exert its effect systemically, in a non-targeted fashion. We believe that this does not occur in pancreatic tumours where the TME is so dense and intratumoral pressures are high, that passive targeting is unlikely, therefore there is a greater urgency towards finding appropriate biomarkers.

sEVs set themselves apart from other nanocarriers or particles due to being non-toxic, having low immunogenicity and a natural targeting ability. They provide a stable environment for therapeutic drugs and can increase their stability. Additionally, due to their natural composition, sEVs possess longer circulation times compared to other nanoparticle systems. These systems are also exploiting their increased circulation times by coating themselves with sEVs in order to evade the immune system.[24] Unfortunately, there is no standardised method of isolation which leads to heterogenous populations and variations we don't know about. Furthermore, loading cargo into or onto sEVs can affect the integrity of sEVs making them visible to the immune system or lose their targeting ability.

3. sEVs

The proteome of sEVs is typically taken up by tetraspanins (CD9, CD37, CD63, CD81, CD82 and TSPAN8), ESCRT related proteins & their accessory proteins (HRS, TSG101, ALIX), integrins (Intergrin-α, -β, P-selectin), heat shock proteins (HSP-60, -70, 90, sHSP), Rab GTPases (Rab11, 27, 35) and immunoregulatory molecules (MHC Class I, II) with their abundance depending on the extracellular environment and state of the parent cell (Fig. 2).[11] No single sEV contains all these related proteins, they are designated depending on the sEVs purpose within the body and origin or make-up of the parent cell from which they were derived.

Tetraspanins are integral membrane proteins highly enriched on the sEVl membrane forming a transmembrane web of tetraspanin enriched microdomains (TEMDs) organising the plasma membrane and interactions occurring on it. Tetraspanin–tetraspanin and tetraspanin–partner interactions facilitate signalling pathways, trafficking, oligomerisation of proteins and aid in stability.[25] Recently, fresh understanding of the superfamilies conserved structure has been shown in the crystal structure of CD81,[26] CD53[27] and CD9.[28] The first reveal of CD81's full structure showed a the tetraspanin conserved 4 transmembrane domain cone-like structure as well as an open and closed conformation facilitating interactions with cholesterol and CD19.[26] These open and closed conformations allow for lateral interactions within the membrane and aid in TEMD formation. Tetraspanins are regularly used as sEV markers due to their role in membrane organisation and biogenesis of sEVs. CD63 is one of the most commonly used markers for sEVs and has been shown to play a role in biogenesis,[29] with CD9 and CD81 also employed. It is believed that the TEMDs are utilised biologically as areas of cargo sorting. CD151 has been shown to possess several important roles notably in tumour development and defective immunity. Cell adhesion has also been related to CD151 with its interactions

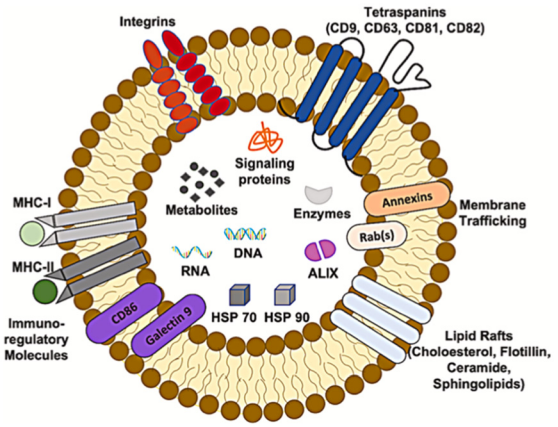

Fig. 2 Schematic illustration of the molecular composition of sEVs. It contains proteins, lipids, and nucleic acids in its structure. Reproduced from ref. 11 with permission from Elsevier, copyright 2022.

with integrins and has led to it being related to a range of functions in a range of human cancers.[30]

Lipids are integral components of sEVs from biogenesis to uptake and are abundant in their cell mimicking bilayer arranged structural membrane. However, the lipidomic profile of sEVs has had less attention than the proteome and RNA cargoes. The main families involved are sphingolipids (sphingomyelin, ceramide), cholesterol, phospholipids (phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol), and gangliosides. Making up part of the cargo of sEVs is RNA. Different patterns of non-coding RNA are present with microRNA (miRs) being the most abundant in human plasma derived sEVs which is from non-coding RNAs (ncRNA).[9] Other RNA included in ncRNA family ribosomal RNA (rRNA), long non-coding RNA (lncRNA), tRNA, small nuclear RNA (snRNA) and p-element-induced wimpy testis (PiWi)-interacting RNA as well as small interfering RNA (siRNA). They have been shown to play roles in sEVs cell–cell communication along with biological processes (angiogenesis, haematopoiesis) and cancer development modulating gene expression in recipient cells.[9] Approximately 2% of RNA encodes for proteins in humans. The majority of the remaining RNAs are ncRNAs involved in the regulation of cell development, differentiation, proliferation, cell death and metabolism. A subset of RNAs, miRNAs, regulate and influence most biological processes.[31] Novel biomarkers are sought after for determining tumorigenesis, prognosis and therapy response with various miRNAs showing up in a range of tumours. One of the main miRs is miR-21 affecting various tumours determining proliferation, migration and invasion.[32] The type of miRs in specific cancer cases, leads to issues in determining the most beneficial therapeutic route, as miRs such as miR-141 possess different roles in different cancers as well as some miRs for example miR-141 being overexpressed in one cancer type (colorectal) whilst being down-regulated in others (PC).[33] As well as being employed a potential biomarkers, miRs have also been shown to possess inherent anticancer effects. This makes sEVs an exciting delivery vector miR to disturb cancer cell signalling and suppress tumorigenesis & metastasis.[34,35]

3.1. sEV Biogenesis and secretion

The biogenesis of sEVs occurs at both membrane domains on the early endosome and from the plasma membrane. The endosomal route of biogenesis has garnered more attention,[36] but studies show a shared route of sEV biogenesis.[37] Internalised cargoes are commandeered into ILVs to form heterogenous intraluminal vesicle (ILV) subpopulations inside multivesicular bodies (MVBs) moving from early to late endosomes and either release *via* the plasma membrane[38] or degradation *via* the lysosome. Using Perfringolysin O to label cholesterol it was shown that only cholesterol enriched MVBs fused with the plasma membrane, releasing sEVs, from cultured B lymphocytes.[39] In addition, cholesterol may play a larger role in sEVs at endosomal membranes forming microdomains to order their cargos and biogenesis.[40] In contrast to this, the tetraspanin TSPN6 has been shown to negatively regulate sEV secretion and bring syntenin to lysosomal degradation in MCF-7 breast cancer cells.[41]

The endosomal sorting complexes required for transport (ESCRT) machinery is an evolutionary conserved process for membrane functions such as cytokinetic abscission, neuronal pruning, plasma membrane repair, nuclear envelope maintenance and autophagy. In addition, it is the most widely described mechanism for MVB and ILV formation.[42] The ubiquitinated, multi-subunit system of the ESCRT machinery consists of 4 ESCRT complexes (ESCRT-0, -I, -II, -III) and associated proteins (Vps4, ALIX and clathrin).[43,44] Each of these comprises of proteins to direct the subsequent complex and collected cargo to complete vesicle budding and sorting into MVBs40. ESCRT-0, -I and -II are known as the upstream ESCRTs. ESCRT-0, -I and -II all contain ubiquitin binding domains but the precise mechanisms of cargo sorting into MVBs is still unclear. It has been shown that a single ubiquitin moiety is sufficient to induce ESCRT protein sorting.[45] Evidence for ubiquitin independent incorporation has also been seen as non-ubiquitinated MHC-II are recovered in sEVs.[46] ESCRT-0 has been linked to sEV secretion using HRS-deficient dendritic cells (DCs) and tumour cells,[47] however, loss of ESCRT-0/-I both have an effect on sEV biogenesis potentially highlighting the more influential proteins in the ESCRT-dependant mechanism.[48] In addition, clathrin forms a flat coat around it. ESCRT-I and -II are mainly involved in the membrane deformation and budding but their full role is not completely understood. With ESCRT-III driving vesicle scission. The AAA-ATPase Vps4 is the final part which disassembles and recycles the ESCRT machinery.[43]

ALIX is a common component of sEV membranes due to its role in biogenesis, binding to ESCRT-III during ILV formation. ALIX-syndecan–syntenin interaction influences ILV sorting as well as being exploited in the tumour microenvironment (TME).[49,50] While ALIX-dependant biogenesis only uses parts of the ESCRT machinery, other ESCRT-independent routes of sEV biogenesis also occur. ESCRT not being the main form of sEV biogenesis has been established by removal of the essential VPS4 having no effect on sEV secretion of biomarkers such as CD63[36] and MVB formation even occurs in the absence of ESCRT machinery.[51] While tetraspanins are mostly used as sEV biomarkers, evidence shows that they are also involved in protein sorting. CD63 has been shown to be required in sorting both with and without the ESCRT machinery,[28] having an impact on sEV size[52] and is also part of an ESCRT-independent mechanism of MVB formation associating with LMP1.[53] CD81 has also been shown to act as a platform for compartmentalisation of proteins on membranes[54] as well as Tspan8 selectively recruiting proteins and mRNA in rat adenocarcinoma cells.[55] Complex lipids such as ceramide have been shown to be part of the ESCRT-independent mechanisms showing a separate biogenesis from the well documented ESCRT mechanism. Ceramide is produced from sphingomyelin through sphingomyelinase and forms lipid rafts *via* self-association.[56] Lipid-rafts are regions of the plasma membrane

which contribute to the initial membrane curvature for inward budding and are used by pathogens to gain entrance to cells.[57] However, lipid mediated pathways depend on the cell type. Lipid mediated and ESCRT-dependant pathways have been shown to co-exist in numerous biological processes. Different mechanisms can work at the same time within a single cell which leads to the heterogeneity of MVBs within a cell.

Movement of MVBs to the plasma membrane involves the cytoskeleton and associated molecular motors. Rab proteins have been shown to be majorly involved in various steps of intervesicular trafficking. This is the most abundant family of proteins in the Ras superfamily playing a crucial role in intracellular vesicle transport and endosomal recycling. They have an active GTP, and inactive GDP bound state. Various Rab proteins have been connected with sEV secretion including Rab27A/B, Rab 7, Rab 31 and Rab 35.

3.2. Transport, biodistribution and uptake

sEVs are ubiquitous throughout the body, with their effects experienced at a local and systemic level. sEVs have been detected in all manner of biological fluids and biodistribution studies are mostly carried out using heterologous sEVs. The route of administration, including for autologous sEVs, has shown to alter the kinetics and biodistribution. With cell or body fluid derived sEVs *via* oral administration, there is a wide biodistribution to most organs including liver, lungs, pancreas and colon. However, with intravenous injection most sEVs are found in the liver followed by spleen, lungs and GI tract.[58] Alongside the difference in biodistribution, IV injection results in faster clearance of sEVs. Contrary to this, intratumoral injection leads to longer residence within tumours. Size is also a factor affecting both the transport and biodistribution of sEVs with larger EVs accumulating in bones, lymph nodes and liver. All cells share the same non-specific uptake, but specific targeting of cells is cardinal in sEVs role in targeted therapy. There are various methods used to track and identify sEVs *in vivo* such as luminescence/fluorescence, radioisotopes and tomography imaging such as CT scans and MRI. Bioluminescence is the most sensitive detection method *in vivo* due to its high signal to noise ratio, as the luciferases used are not present in mammalian tissue as well their ability to emit within the far-red wavelengths which do not experience interference from the tissue.[59] Some studies have shown these signals to last up to 21 days.[60] While bioluminescence is very sensitive, due to the small size of the sEVs, the signal detected is quite weak. Various groups have tried to increase this signal intensity by attaching fluorescent proteins conjugated to the luciferases to create bioluminescence resonance energy transfer reporters. Other methods of EV tracking *in vivo* such as the use of organic vital dyes, radioisotopes and imaging tomography, all come with their own pros and cons as well as varied distribution profiles depending on the route of administration and the surface proteins present on the EVs as well as the clinical physiology being studied.[60]

The surface composition of sEVs is integral to their transport direction and biodistribution. The conservation of tropism between donor and recipient cell is a signature for recognition and sEV uptake in the recipient cell. Although, whether sEV targeting is direct or unpredictable, as well as the mechanism surface protein interactions play in their uptake requires further research. Specific protein signatures can be used to evade the host immune system with complex lipids also exerting an influence. It is possible that the sEVs undergo multiple cell-uptake and release cycles to penetrate layers of tissue such as the blood brain barrier[61] and the endothelium – potentially *via* transcytosis, to leave the bloodstream and affect the target cells.[62] Cell signalling once a sEV reaches the recipient cell can be induced by 3 main processes: direct interaction, fusion with the plasma membrane or internalisation (Fig. 3).[10] Direct interaction is the most efficient pathway where a transmembrane ligand on the sEVs surface binds directly with the receptors on the recipient cell such as the MHC I/II used by the immune system. This generates a downstream signal cascade, activating the target cell. Fusion with the plasma membrane releases the contents directly to cytosol. The process starts with hemi-fusion stalk formation between hydrophobic lipid bilayers of the sEV and plasma membrane leading to expansion and the formation of a consistent structure. Taking place directly on the plasma membrane, lipid-rafts, integrins and adhesion molecules also facilitate fusion. Dyes have been used to distinguish between endocytosis and fusion. pH could also play a large role in fusion with tumour

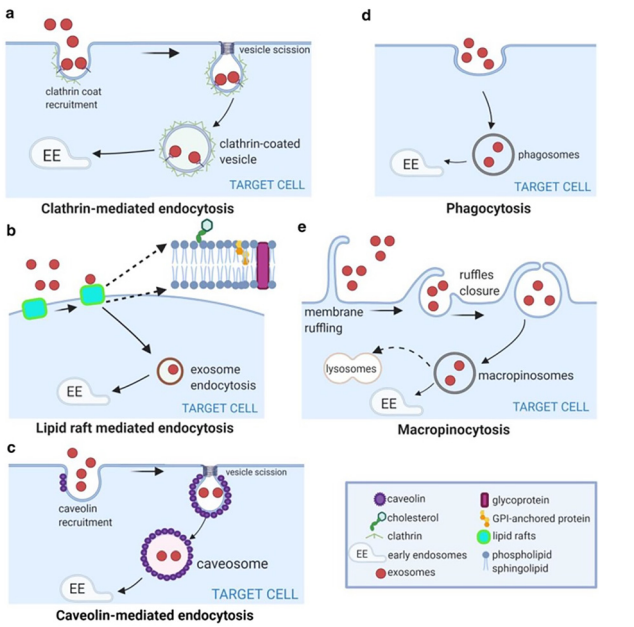

Fig. 3 Figure taken from "The exosome journey: from biogenesis to uptake and intracellular signalling". sEV (exosomes are classed as sEVs) internalisation: sEVS are internalised by the recipient cells and fuse with the intracellular compartments/endosomal pathway for cargo release. sEVs can be internalised by (a) clathrin-mediated endocytosis, (b) lipid-raft mediated, (c) caveolin-mediated endocytosis, (d) phagocytosis or (e) micropinocytosis. These pathways are not always mutually exclusive and can co-exist for the internalisation of a same set of sEVs. Reproduced from ref. 10 with permission from Biomed Central, copyright 2021.

cells. Internalisation has been suggested as the major mechanism for sEV uptake. The internalisation requires the sEV cargo to be released into the cytoplasm before lysosomal degradation and potential release from the target cell. A few different mechanisms have been elucidated: clathrin mediated endocytosis (CME), lipid raft-mediated, caveolin-mediated (CvME), phagocytosis, micropinocytosis.

CME involves various transmembrane receptors including clathrin, a triskelion scaffold, to coat sEVs until they are internalised where the clathrin is removed and the sEVs fuse with the endosome and is one of the major pathways for endocytosis. CvME has conflicting reports for a potential sEV uptake route. Mediated by integral proteins (Caveolins), they create small flask shaped membrane invaginations called caveolae enabling internalisation of caveosomes. Caveolin 1–3 are the main structural proteins of caveolae. Dynamin-2 is shared between CME and CvME and plays an important role forming a collar aiding in the scission of invaginations.[63] Inhibition has been shown to reduce sEV secretion. Micropinocytosis also uses inward budding of the plasma membrane but is dependent on actin and growth factors with the lysosome as its final destination.[64] Lipid-rafts are detergent-resistant membrane microdomains enriched in cholesterol, sphingolipids, and glycosylphospatidylinositol (GPI)-anchored proteins. They play a large role in endocytosis and it has been shown that inhibiting various complex lipids can either increase or decrease the uptake of sEVs.[65]

Phagocytosis is predominantly used by immune cells to engulf microbes and phagosomes are usually directed to lysosomes in the cell. However, this route can be taken by sEVs where the cell membrane deforms to engulf the sEVs.[66]

PC is characterised by a dense stroma, which often hinders drug penetration, leading to the poor prognosis. However, sEVs have been reported to penetrate the stromal barrier,[67] which may occur due to various mechanisms, these include: interacting with immune cells leading to immunosuppression; modulation of the ECM whereby the enzymes present in the inherent structure are capable of loosening the fibrous stromal network, enabling penetration or reprogramming the CAFs in order to modulate the ECM.[67]

3.3. Isolation and characterisation of sEVs

A wide variety of isolation techniques exist for sEVs but ultimately there may never be a standard method due to downstream use of the final product, source medium and the ability to integrate into a clinical setting. In addition, there is no one specific marker for each sEV population and the heterogenous subpopulations which evolve indicate more gaps in our knowledge of the function of sEVs.[68] Each isolation technique has its limits in yield, purity and maintaining sEVs integrity, so isolation technique is often determined depending on downstream utilisation.[69] Although complete isolation from extracellular components may be detrimental to sEV functionality,[70] full isolation of sEVs from other EVs and non-EV lipid particles is essential for biomarker and functional analysis. This requires pure sEVs free of other EVs (and interacting components of the extracellular milieu) which is also the case for determining roles in physiological and pathological conditions.

To first isolate sEVs an efficient method of cultivating them must be achieved. Liquid biopsy methods are preferential especially in cancers where a tumour biopsy is both invasive and can potentially cause metastasis leading to poor prognosis.[71] However, in the research setting cell cultures are the most widely used material (83% up until 2016).[72] Cell cultures are easily grown in a laboratory setting and can produce a high throughput source of sEVs usually from tumour cell lines. Mesenchymal stromal cells (MSCs) are being spotlighted, especially for drug delivery purposes, where hypoxic and pro-inflammatory preconditioning also increase yield of sEVs.[73] MSCs have been grown on beads in a 3D culture to potentially increase EV production 20-fold with the increase in culture area and MSC population.[74] However, some elements of cell culture such as the commonly used foetal bovine serum (FBS) can have detrimental effects on sEV population,[75] although efficient techniques for sEV depletion *via* ultrafiltration have been developed.[76] Ludwig *et al*. have optimised the production of tumour derived sEVs (TEX) by a reproducible mini-SEC, which can also be used for biological fluids, noting each tumour cell line had different culture requirements.[77] It has also been shown that in the tumour microenvironment, low pH, hypoxia and other stress can increase sEV release.[78] Splitting of the sEV isolation method is often carried out using 5 steps: centrifugation, chromatography, precipitation, filtration and immune-affinity based isolations.[79] Deciding which method to use depends on the downstream use of the sEVs produced, hence methods can be chosen from a scale of high purity, low yield to high yield, low purity.[80,81] A detailed account of sEV isolation techniques has been published by Welsh *et al*.[82] and is summarised in Table 1.

As with isolation, there is a wide range of techniques used for characterisation of sEVs, some overlapping with their isolation techniques, such as immunoaffinity microfluid chips where isolation and analysis can be carried out in tandem.[82] Both the characterisation and quantification of sEVs is necessary to understand their heterogeneity and composition towards the aim of more specific isolation and functional knowledge. Characterisation techniques are ever expanding and these have been described well by elsewhere.[81,101,102]

3.4. Functionalising and loading sEVs

The lipid bilayer of sEVs contains ligands and receptors in the same fashion as the source cell. This hydrophobic bilayer encloses a hydrophilic aqueous core. A robust knowledge of sEV biology is required to load and functionalise their surface a long with the cargo you are loading (drug, vaccine, RNA, *etc.*). Cargo can be loaded either *in vivo* (pre) or *in vitro* (post) with varying efficiency and stability.[11] During pre-loading cargo is sorted into the sEVs during biogenesis. This is preferred for high molecular weight RNAs such as mRNA and transmembrane proteins. As the understanding of sEV biogenesis is still limited the amount of cargo loading cannot be con-

Table 1 Comparison of common EV isolation methods and their advantages/disadvantages. Reproduced from ref. 83 with permission from Frontiers, copyright 2022

Technique	Principle	Advantages	Disadvantages	Ref.
Ultracentrifugation	Constituents of culture media or biological fluids possess varying physical properties allowing for separation at different centrifugation speeds	Straight forward Large sample volume Scalable Doesn't interfere with downstream analysis	Non-vesicular contamination Damage to EVs Aggregation Time consuming Expensive equipment	84–86
Density gradient Centrifugation	Buoyant density differences are utilised by laying a sample on a density gradient and centrifuging to separate fractions based on density. Fractions are then collected	High purity High specificity Doesn't affect sEV integrity	Time consuming Co-precipitation of lipoproteins from biological fluid Labour intensive	87 and 88
Ultrafiltration	Uses various sized filters to selectively isolate sEVs and pass through smaller contaminating proteins	High purity Rapid and easy to use Scalable Downstream compatibility	Size exclusion limit Loss of sEVs to membrane Varying membrane recovery rates	89 and 90
SEC	A porous gel allows small particles to be trapped inside and larger molecules to pass through the column. Individual fractions are taken and analysed for sEVs	High purity No damage to sEVs Scalable Quick and cheap	Variable yield Lipoprotein contamination	91–93
Immunoaffinity	Protein specific antibodies are chosen and attached to solid support to selectively capture sEVs presenting specific surface antigen	High purity and specificity No damage to sEVs Isolation of low-abundance biomarkers Adaptable for specific biomolecules	Known biomarkers leading to bias in population High cost of antibodies Aggregation Challenging to scale	94–96
Precipitation	Addition of reagent to a sample (*e.g.* PEG) aggregating sEV and precipitating out allowing the pellet to be centrifuged out	Simple and quick High yield Scalable No special equipment	Co-isolation Lack of specificity Variable recovery rate Additional purification required	97 and 98
Microfluidics	Channels are used to manipulate the fluid flow to isolate sEVs by physical properties or surface markers	High precision Low sample volume High throughput Label-free Potential automation	Technical expertise Device optimisation Cost Clogging	99 and 100

trolled so other techniques such as transfection, electroporation and co-incubation are commonly used. Post-loading is sEV loading after isolation. More control over the loaded vehicle gives better control of encapsulation efficiency (EE%) and loading capacity (LC%). Physical techniques include electroporation, incubation, sonication, extrusion, freeze–thaw cycles, surface treatments, hypotonic dialysis and pH gradients and chemical methods include transfection and *in situ* synthesis.[12,103] As with liposomes, hydrophobic drugs/cargo can be loaded into the lipid bilayer of the sEV membrane, whilst hydrophilic cargo can reside within the aqueous inner core.

Incubation is the most common method for drug delivery with the cargo diffusing into incubated sEVs along a concentration gradient. A wide variety of cargoes (nucleic acids, peptides, proteins, small molecules) can be loaded simply, cheaply and with minor damage to sEV integrity but this comes at a cost of loading efficiency. In addition, balancing the physiochemical properties of both sEV and intended cargo as well as the pH is important for optimising loading efficiency. The similar pre-loading method of co-incubation incubates cells and cargo so the cells can incorporate the cargo into their created sEVs. However, low loading efficiency is again an issue due to lack of control and manipulation over sEV biogenesis. Co-incubation is a similar process to the transfection of cells. A specifically designed vector such as a plasmid are transfected into cells to express the required cargo (protein, peptide, nucleic acid, *etc.*). As the cell produces sEVs these cargoes are then loaded into them and because of this drug loading isn't possible. As with the incubation methods, the lack of control brings about a low loading efficiency, but further harm and contamination can occur due to the transfection agent. A pH gradient can also be used to create a gradient inside and outside of sEVs. The usual internal pH of 9 allows for cargo loading with an external solution pH of 4.5. This led to an increased loading efficiency of 3 times greater with both size and zeta potential not changing after loading.[104]

Electroporation is another technique used in cargo loading of sEVs. Short, high-voltage electric pulses create micropores in the surface of the isolated sEVs allowing cargo to pass through the membrane. This process can be carefully opti-

Table 2 Techniques used for loading sEVs. Reproduced from ref. 11 with permission from Elsevier, copyright 2022

Loading mechanism	Loading technique	Advantages	Disadvantages
In vivo (pre-loading)	Co-incubation	Simple; low labour required; SEV integrity preservation	Low loading efficiency; difficult to manage; cargo can cause toxicity
	Transfection	Overexpression of desired molecules	Low loading efficiency: cause gene expression change in donor cells; transfection agents are toxic
	Electroporation	Simple	Low loading efficiency; require process optimization; SEV aggregation risk
In vitro (post-loading)	Incubation	Simple; inexpensive; SEV integrity preservation	pH and physicochemical qualities of cargoes can affect loading efficiency
	Sonication	High loading efficiency	SEV membrane damage; SEV aggregation risk
	Extrusion	High loading efficiency; uniform sEV size	SEV membrane damage; recombination of the exosomal surface structure
	Freeze–thaw	Simple	Low loading efficiency; SEV aggregation risk; repeated cycles cause inactivation of proteins
	Surface treatment	High loading efficiency	Surfactants can degrade or inactivate the cargo; need extra purification step
	Hypotonic dialysis	High loading efficiency	pH gradient cause degradation of proteins; need validation
	pH gradient	Simple; low labour required	pH gradient cause degradation of proteins; SEV aggregation risk
	In situ synthesis	SEV integrity preservation	Limitation of noble metals loading; complex operation process

mised by changing the condenser capacity, voltage, number of pulses, their length and interval duration. A range of cargoes such as drugs, nucleic acids and nanomaterials can be loaded, here the loading efficiency is affected by the reduction in membrane stability and integrity.[105,106] Sonication is a similar process where holes are made in the sEVs membrane using an ultrasonic probe, this process works similar to and has the same issues as electroporation. The main loading methods together with their advantages and limitations were summarised well by Kimiz-Gebologlu *et al.*[11] and are shown in Table 2.

Various other techniques can be used for both *in vitro* and *in vivo* loading. The choice of technique depends on the sample being loaded and if they're hydrophilic, hydrophilic or their size. While the main focus for therapeutics is on loading sEVs, unloading of cell internalised cargoes may also be necessary to both increase loading efficiency and reduce unknown effects. However, unloading sEVs may decrease stability as increased stability has been shown with loading.

4. sEVs in cancer therapeutics

sEVs have been shown to be capable of loading a host of drug cargos for cancer therapy inside their lipid membrane. These include drugs which are highly potent but are difficult to deliver due to their poor physicochemical properties (mostly lack of aqueous solubility) such as paclitaxel,[107] doxorubicin,[108] oxaliplatin,[109] camptothecin[110] and SN-38[111] to name a few. Studies have shown enhanced drug trafficking and efficacy on cancers including lung,[107] retinoblastoma,[108] colorectal[111] and breast[112] *etc*. Aside from drug molecules, sEVs have been shown to deliver other biologics such as siRNA[113,114] mRNA,[115] and other immunotherapies.[116] However, their ability to encapsulate the larger molecular cargo, is limited[117] and does not rival that of other technologies such as polymeric micelles.

Moradi *et al*. developed sEVs loaded with doxorubicin, targeted with an SP5-52 peptide, for site specific delivery to lung cancer. The sEVs were isolated from the serum of Balb/c mice and doxorubicin was incorporated within their membrane, these were surface engineered with a SP5-52 peptide for lung targeting.[118] The formulation was administered to lung tumour bearing mice and the study showed that those animals dosed with the drug alone (DOX) and the sEV loaded with drug (EXO–DOX) exhibited a body weight reduction, which can be attributed to the off-target drug effects. However, the peptide targeted sEV loaded with drug (EXO–SP–DOX) exhibited the least weight changes, which the authors concluded was an indication of successful treatment. Fig. 4 shows the accumulation of the drugs and sEVs within different organs in the body. Here the non-targetted sEVs EXO–DOX and targeted sEVs EXO–SP–DOX accumulated within the tumour to a greater extent for the mice dosed with free drug. Whilst in the targeted sEV formulation, less accumulated within the healthy lung tissues. The authors concluded that the SP5-52 conjugated sEVs with DOX incorporated within them, can safely and efficiently cause apoptosis in a direct and targeted manner against tumour cells.[118]

The main advantage to the use of sEVs over other nanotechnology platforms such a liposomes, polymeric micelles or metallic nanoparticles for cancer drug delivery is their innate biocompatibility and lack of immunogenicity, due to their naturally derived makeup.[119] In patients who are already ill, the ability to deliver drugs 'enveloped' safely inside a non-toxic carrier is paramount. Ideally, also being able to specifically target the tumour site. sEVs have been directed specifically to the tumour site using peptides,[118] antibodies[120] and aptamers.[121] As previously discussed, they can penetrate the dense

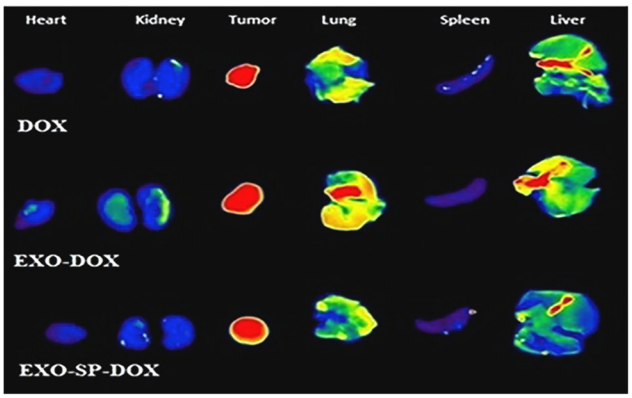

Fig. 4 Showing accumulation of DOX in 5 different organs and tumor in DOX, DOX encapsulated within a sEV (EXO–DOX), and DOX encapsulated within a sEV with an SP5-52 peptide for targeting (EXO–SP–DOX) after administration *via* tail vein into male Balb/c mice (4–6 weeks) bearing a lung tumour. Data obtained from fluorescent imaging. EXO–SP–DOX accumulated in tumor tissue and the amount of its accumulation in other tissues was lower than DOX alone. Reproduced from ref. 118 with permission from Elsevier, copyright 2024.

stromal barrier posed by PC tumours, to deliver their payload to the site of need. They have also been used as cloaking agents for other nanobased systems such as liposomes in order to make them appear more biologically acceptable to the body, in order to evade rapid clearance by the immune system.[122] sEV have currently entered clinical trials for various disorders including cancer.[123] One field less studied is for that of PC, where there are currently pre-clinical studies, but as of now, no clinical trials. However, there is exciting opportunities, particularly in their use as therapeutic carriers.

4.1. sEVs from the PC cell lines

The role of sEVs in PC is slowly shedding more light on the intricacies of the disease. As an exchange route for intercellular material, they regulate angiogenesis, cell proliferation, invasion, metastasis and chemoresistance. Within the stroma and TME the effect of each EV can differ depending on the parent cell. Developed from bone marrow derived mesenchymal stem cells (MSCs), CAFs make up around 80% of the stroma environment and are large contributors to chemoresistance in PDAC.[124] Gemcitabine (GEM) resistance is intrinsic to CAFs and when exposed to GEM, their sEV secretion increases. Secreted sEVs from GEM treated CAFs possess large amounts of mRNAs and miRNA including miR146a and Snail. PDAC epithelial cells readily take up these sEVs and therefore miR-146a and Snail, promoting both proliferation and chemoresistance.[125] Use of GW4869, a neutral sphingomyelinase inhibitor, has been shown to decrease sEV secretion. After the addition of GW4869 survival of GEM resistant CAFs was shown to decrease suppressing tumour growth and negating chemoresistance.[125] Along with determining chemoresistance within individual cells, sEVs can mediate the transfer of chemoresistance to other cells and cell lines. Using 3 different PC cell lines, sEVs were used to test the transfer of chemoresistance between cells. PANC-1, Mia PaCa-2 and BxPC-3 have varying levels of chemoresistance to GEM with PANC-1 being the most chemoresistant.[126] PANC-1 sEVs were shown to increase the chemoresistance of both Mia PaCa-2 and BxPC-3 to GEM. In this study EphA2 was determined to be a factor in the transfer of chemoresistance and is overexpressed on PANC-1 sEVs. EphA2 is thought to be a chemoresistant transfer factor and using EphA2-knockdown, PANC-1 sEVs no longer transmitted chemoresistance. In addition, sEV mediated transfer of this is potentially important as treatment of both Mia PaCa-2 and BxPC-3 did not promote chemoresistance.[126] Other proteins are overexpressed such as survivin, in the inhibitor of apoptosis family, which is overexpressed in KRAS-mutant PDACs such as Mia PaCa-2 and PANC-1. The PC cell line BxPC-3 has a BRAF mutation rather than a KRAS and sEVs still contain survivin but in smaller amounts. When BxPC-3 cells are treated with sEVs from Mia PaCa-2 or PANC-1 it was shown that paclitaxel had a reduced effect showing how surviving affects drug resistance and how sEVs can confer this resistance within a solid tumour.[127]

The mechanism with which sEVs confer drug resistance or other intercellular communication within a solid tumour is not well understood. There are a wide range of miRNAs that have been investigated for their role in gene and protein expression since Valadi *et al.* first noted sEV mediated transport of miRNAs between cells.[128] Regulatory factor X-associated protein (RFXAP) is a transcription factor for the MHCII gene widely expressed on immune cells. Down regulation of RFXAP leads to T-lymphocyte inactivation and immune evasion. sEVs derived from the PC cell lines BxPC-3 and SW1990 have been shown to down regulate RFXAP *via* miR-212-3p in immature dendritic cells (DCs).[129] These DCs then expressed 12 of the 84 PC-related miRNAs detected in PANC-1 sEVs. GEM resistance has also been shown to be transferred *via* sEVs through miR-210. GEM resistance was conferred from BxPC-3 resistant cells to BxPC-3 GEM sensitive and PANC-1 cells *via* sEVs in a dose dependant manner and similar changes were noted with miR-210 mimics.[130]

It isn't only chemoresistance, survival and immune regulation which is transferred in PDAC sEVs. A large portion of patients diagnosed with PDAC are also diagnosed with diabetes mellitus, however, the mechanism of this is still unclear. The potential role of miRNAs was investigated into PC induced β-cell dysfunction.[131] Glucose-stimulated insulin secretion (GSIS) was measured after pancreatic β-cells were treated with sEVs from PC cell lines BxPC-3 and SW1990. MiR-19a was investigated as a signalling molecule for the underlying mechanism and was shown to be a pivotal mediator in GSIS defect targeting ADCY1 and EPAC2. However, PANC-1 was also tested but didn't disrupt the GSIS as much.[131]

miRNAs can also be used against PC to reverse or halt proliferation and chemoresistance. Using bioinformatics it was suggested that miR-1231 can function as a tumour suppressor and an additional study showed the miR-1231 to be expressed in plasma sEV from PC patients.[132] It was also found that there was a correlation between the expression of miR-1231

and the TNM stage of PC with reduced miR-1231 potentially indicating metastasis. By forming tumours in mice using BxPC-3 cells, BM-MSc sEVs transfected with miR-1231 were injected and were shown to inhibit tumour growth; where miR-1231 inhibitor significantly increased the tumour growth.[133] miR-124 has also shown a similar phenomenon.[134]

While BxPC-3 is widely used in PC research there are anomalies in the reported protein concentration. It is widely reported, using western blot, that BxPC-3 sEVs are CD81 positive.[106,107] However, several studies show that CD81 isn't expressed.[135,136] This may be due to the use of Dulbecco's Modified Eagle's medium (DMEM) as a culture media as CD81 was shown to be present in a detectable quantity in DMEM + 10% FBS.[137] When studying the glycomic profile of sEVs, Roswell Park Memorial Institute (RPMI) 1640 was used for all cell lines. Both studies where BxPC-3 sEVs were lacking in CD81 had isolation procedures containing beads. In addition to this PANC-1 sEVs were shown to differentially express CD81 in hypoxic and normoxic conditions.[137]

The exciting potential for a naturally derived delivery system for delivery of therapeutics puts sEV at the top of the list for a new frontier in PC. Sadly, PC research is less well studied than other cancers due to its complexity of TME and low efficiency in ability to deliver cargo. However exciting new studies have shown that sEVs really may make a difference in this arena due to their ability to actively target cells as well and increasing circulation times, resulting in greater drug cargo reaching the TME in order to exert their intended antitumoral effects.[138]

4.2. sEVs for therapeutic delivery in PC

Although there are relatively small numbers of studies in the literature compared with the other cancer types, the data that does exist shows great promise. Geng *et al*. compared EVs isolated from four different cell lines (U937, THP-1, MIA PaCa-2 & MSCs) and evaluated their potential in drug delivery for PC therapy.[139] Each EV system was different and the sizes spanned from large EVs to sEVs (140 nm – 270 nm). Gemcitabine was loaded into each of the EVs and their cytotoxicity was tested *in vitro* against MIA PaCa-2 cells. The data showed that the large EVs possessed 14.2-fold greater GEM loading capacity compared to the sEVs, however, both the large EVs and sEVs formulations possessed similar cytotoxicity profiles, both of which outperformed gemcitabine alone. This interesting finding coupled with the knowledge that nanomaterials in the smaller size range may be more stable and less prone to macrophage detection – resulting in increased systemic circulation times *in vivo*, leads us to believe that the sEVs are more favourable for delivery purposes.[139]

sEVs have been reported for loading drug molecules, acting as biological cloaks and delivering the drugs to their site of need. Ahmadi *et al*. reported the loading of sEVs isolated from AsPC-1 cells with adenosine and a prodrug peptide conjugate of adenosine.[140] The authors concluded that the sEV formulations demonstrated a more rapid cellular internalisation and an enhanced level of apoptosis (56.9%) than the unencapsulated compounds, with the unloaded sEVs presenting no effect on apoptosis.[140] Li *et al*. isolated EVs from Panc-1 cell lines and loaded them with GEM.[141] The resultant EVs were capable of loading the drug up to 11.68% which released drugs (ExoGEM) in a sustained manner over 120 h. Upon cytotoxicity testing against Panc-1 cells, their 10 nM gemcitabine loaded sEV lead to a significant decrease in IC50 value compared to the drug alone. The empty sEVs with no gemcitabine loaded showed no cytotoxicity over the concentration ranges (3 μg & 30 μg) or timepoints tested (up to 72 h). Upon increasing the amount of GEM within the sEV, no significant improvement was observed. This highlights the ability of the sEVs to efficiently traffic the drug into cells at much lower concentrations than the drug requires alone, with superior anticancer activity. *In vivo* performance of the gemcitabine loaded sEVs showed their ability to escape phagocytosis, increase circulation times and enhance drug accumulation at the tumour site, with higher levels of GEM detected in the tumour. Xenograft mice (Panc-1) were treated with three doses of the ExoGEM (5 mg kg^{-1} and 10 mg kg^{-1}) at two-day intervals. The study showed that the EV loaded formulations were significantly better at retarding the tumour growth than the free drug, with 25% of the mice treated with 5 mg kg^{-1} and 50% of the mice treated with 10 mg kg^{-1} GEM sEVs possessing no tumour, with no recurrence showing after the end of the treatment regime (Fig. 5).[141] Other studies loading drugs into sEVs for pancreatic cancer therapy have shown similar results, whereby the sEV formulation was superior to the unformulated drug, although not all these were using sEVs derived from PC itself.[142–144]

sEVs have shown promise not only in the delivery of chemotherapeutics but also in the immunotherapy arena. Zhou *et al*. developed an sEV system to enhance immunotherapy efficacy and reverse M2 tumour immunosuppression associated macrophages.[145] Here, bone marrow mesenchymal stem cell derived sEVs were loaded with galectin-9 siRNA, and their surface was decorated with oxaliplatin, which is used as an immunogenic cell death (ICD)-trigger, yielding an average size

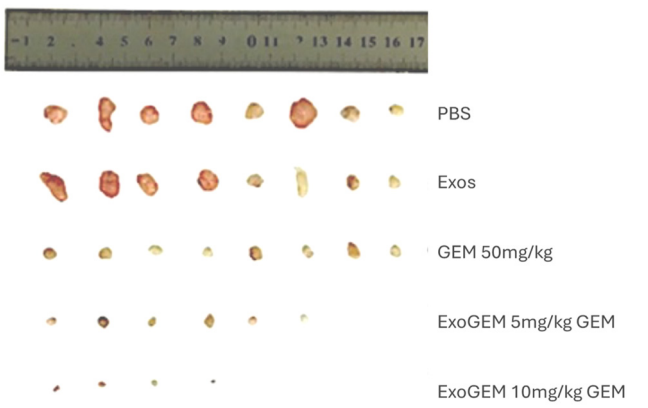

Fig. 5 Efficient therapeutic efficacy of ExoGEM against Panc-1 xenograft tumours in Balb/c nude mice after tail vein administration (three administrations with 2 days between). Tumours were excised at the end of day 30. Reproduced from ref. 141 with permission from Elsevier, copyright 2020.

of 140 nm. The sEVs were capable of loading siRNA at 0.5678 ± 0.0258 μg and oxaliplatin at 5.71 w/w%. Cell based studies in Panc-2 cells showed increased cellular uptake compared to the free drug which plateaued at around 6 h. The sEVs were shown to target PC efficiently after *in vivo* administration, in the healthy controls the sEVs were distributed to the liver and spleen. In Panc-02 xenografts, the combination therapy resulted in significant retardation of the tumour compared with gemcitabine, oxaliplatin alone and the empty carrier system. The authors concluded that the loaded sEVs developed offered a synergistic immune response in orthotopic mouse models through induction of ICD stimulus coupled with interfering in immune suppression.[145]

Other studies have reported using sEVs for delivery of immunotherapies, in the hope that this will enable these therapeutics which have so far been unsuccessful in PC treatment, to carry out their intended function.[146,147]

Aside from sEVs extracted from PC cell lines, studies have looked into the isolation of sEVs from tumour adjacent stromal fibroblast cells for their potential as delivery vehicles in therapeutics. Setua *et al*. investigated the use of sEVs isolated from stromal adjacent normal fibroblast cells (NAF) for the targeted delivery of ormeloxifene to pancreatic tumours.[67] They demonstrated that the sEVs isolated from NAF possessed significant levels of tumour suppressor miRs and were capable of encapsulating oremeloxifen with up to 84% loading efficiency (841 μg mL^{-1}). The sEVs were capable of internalisation into HPAF-II cells, where they were trafficked into sacks of cell organelles. The data showed that the formulations were capable of altering the expression of a range of proteins associated with desmoplasia (SHH, Gli and HAS 1), tumorigenesis/invasion (NFkB-p65, C-Myc), apoptosis (Bax, Bcl-2) and EMT (E-cadherin, N-cadherin, MMP2), with enhanced repression of the markers compared to the drug alone. The authors hypothesized that their novel loaded sEVs blocked the SHH signaling pathway *via* inhibition of SHH, Gli-1, and NFkB-p65 in AsPC1 and HPAF-II cells and inhibited stromal and tumour cell crosstalk. The authors concluded that the NAF derived sEVs were capable of efficient targeting of the pancreatic tumours, and that the ormeloxifene formulation was able to reduce tumour growth by modulating the key oncogenic mechanisms of the tumour microenvironment, which is promising for precision medicine.[67]

4.3. Stimuli responsive sEVs for PC treatment

Stimuli responsive sEVs are sEV based systems which have been developed to react to stimuli which are present or triggered within the microenvironment. Although there are limited reports for stimuli responsive systems in PC, it is expected that the growing number of studies using technologies such as photodynamic therapy,[148] photothermal therapy[149] and sonotherapy[150] in other cancers, will eventually translate over to PC. One such trailblazing study by Jang *et al*. reported the development of an sEV based system for image guided immunotherapy and photodynamic therapy for PC.[151] They isolated sEVs from MIA-PaCa2 cells, removed their biological contents and reassembled them loading with photosensitising agent chlorin e6. This inclusion of chlorin e6 not only allowed for image guidance after administration, but also generates reactive oxygen species when irradiated with a laser at 671 nm. After administration, it is also possible for the immune cells to recognise the antigens on the surface of the sEVs which lead to stimulation of the immune system acting as an immunotherapy. The study showed that combination therapy using the chlorin e6 loaded sEVs for combined photodynamic therapy and immunotherapy, resulted in reduced tumour volumes than the individual therapies alone. Immunohistochemistry studies demonstrated that loaded sEV contained many CD45- and CD8α-positive immune cells within the tumour tissue, indicating that immune system stimulation had occurred.[151]

5. Challenges in sEV scaleup

It is important to give a balanced view when discussing potential new technologies for drug delivery. Aside from their many benefits, there are some potential challenges in order for these sEV technologies to be translated into the clinic. A unified approach for isolation and characterisaton is required in order to be able to appropriately regulate the field. This is currently being evaluated and pushed forward by the International Society for Extracellular Vesicles. Better understanding on harvesting and isolation scale up is required. Currently this can be done in small scale bioreactors, but realistically, this technology gap requires improvement. Specific to PC, may be the identification of new, unknown biomarkers, which could be candidates for active targeting. Finally, a recent report has shown that tumour derived sEVs from PC, actually increase GEM resistance *via* enhancement of STAT3 expression by downregulating miR298, which results in cell growth enhancement, inhibiting cell death, which leads to drug resistance.[152] More studies are required to further understand this implication, and whether it may apply to all tumour derived sEVs in PC. As well, as knowledge within the field, there is an international skill shortage within this area, and in order to progress, upskilling of current pharmaceutists' as well a training the next generation of researchers is required.

6. Conclusions

The time to act for PC is now. The late diagnosis coupled with difficulty penetrating stromal barriers of existing therapies is hindering patient treatment success. Nanotechnology advances in pharmaceutical development have made some progress in this field, however, lack of ability to actively target and clearance is still a hinderance to the field. We believe sEVs may help to overcome these challenges and result in therapies which are overall more cost effective, delivering less active ingredient, more relevant, derived from PC, and more biologically acceptable, already produced in the body. Studies within

the field have highlighted their ability to load and traffic cargo of interest into the tumour sites, outperforming not only the free drugs themselves, but also other lipid-based nanoparticle formulations. Whilst their isolation and characterisation has been a technical hurdle for EVs in general, greater understanding, expertise and unified terminology has been realised. This does not mean it will be plain sailing from here, more challenges will be faced in scaleup and isolation of large quantities of sEVs for therapeutics, but these undoubtedly will be overcome with the growing interest and expertise in this field across all cancer types. However, of all the cancers, PC has been less studied, and the time is now, to really interrogate what these systems are capable of within our field. Patients of this terrible disease deserve better therapies, and sEV delivery of chemotherapy or immunotherapies may just be the answer. Time will tell.

Author contributions

RC, OK & CH wrote and the manuscript. All authors approved the manuscript before submission.

Conflicts of interest

There are no conflicts to declare.

Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

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