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Exosomes as Reconfigurable Therapeutic Systems.

Robert Steven Conlan[1,*], Simone Pisano[1,2], Marta I. Oliveira[3], Mauro Ferrari[4], Inês Mendes Pinto[3,*]

[1] Swansea University Medical School, UK.
[2] University of Trieste, Italy.
[3] International Iberian Nanotechnology Laboratory - INL, Portugal.
[4] Houston Methodist Research Institute, USA.

[*]Correspondence to Robert Steven Conlan (r.s.conlan@swansea.ac.uk) and Inês Mendes Pinto (inesmpinto@inl.int).

[Abstract]

Historically, small molecules, including steroid hormones and cytokines, have been attributed a role in paracrine and endocrine signaling, and now include a new player: biological nanoparticles, or 'exosomes'. Generated intracellularly, and defined simply as nano-particulate packages of signaling moieties, exosomes have emerged as vehicles for highly specialized local and distant intercellular communication. Exosomes are increasingly being recognized as contributing factors in many diseases, and their potential as biomarkers and in therapeutics is rapidly emerging. This review highlights recent advances in the exploitation of exosomes in diagnostic and therapeutic applications. We discuss various facets of nanoparticles, namely, the isolation and manipulation of exosomes, the construction of synthetic exosome-like particles in vivo, and their potential use in the treatment of various diseases.

Trends box

· Exosome diagnostics, although available, remain un-approved by regulatory agencies, and thus, might be used in parallel with existing approved tests.

· Exosome approaches to therapeutic interventions are far-reaching; from packaging of therapeutic agents, to driving immune responses. Applications range from oncology to regenerative medicine, and commercial GMP production at therapeutically relevant quantities is underway.

· Exosomes can trigger positive and negative immunomodulatory effects, as observed in early exosome clinical trials for advanced non-small cell lung cancer, thus potentially impacting on disease progression.

· The effects of MSC delivery to patients showing therapeutic benefit appear to be exosome-derived as exosomes purified from MSCs can promote similar effects to MSC based treatments.

· The potential for tumor-derived exosomes to control the establishment of organ-specific pre-metastatic niche has been demonstrated through their ability to program bone marrow-derived cells towards a pro-metastatic phenotype.

Exploring the Clinical Potential of Exosomes

Cellular communication pathways include direct cell-to-cell contact, inter-cellular molecular messengers and **exosomes**. These 30-130 nm membrane bound nanoparticles harboring biomolecular payloads, offer significant potential in both detecting and treating diseases. Efforts to capture the exosomes' potential are far reaching, from trans-species signaling to immune-system priming and drug delivery. Moreover, understanding any side-effects of their use in humans is essential. Whilst the complexities surrounding their therapeutic potential continue to be unraveled, the use of exosomes as diagnostic tools is underway, with prostate cancer exosome diagnostics being used alongside FDA approved tests. Clinical trials have evaluated the therapeutic potential of exosomes, and whilst demonstrating safety, have yet to show efficacy (Tables 1-3). Trials have largely involved using manipulated exosomes, rather than harnessing properties of native exosomes. For example, autologous dendritic cell-derived exosomes loaded with MAGE (Melanoma Associated Antigen) peptides are being used in a Phase II non-small cell lung cancer trial (clinicaltrials.gov/NCT01159288). In contrast, some biotechnology companies are producing exosomes from non-engineered stem cells, which necessitates research into the inherent mechanisms by which native exosomes target specific tissues and cell types, and an understanding of how tissues recognize such **nanoparticles**. The development of therapeutic exosomes will likely follow both paths, and require refined approaches for exosome isolation, programming of exosomes in vivo using genetic or epigenetic approaches, encapsulating therapeutics within purified exosomes, or the synthesis of semi-synthetic, more highly defined exosome-like therapeutic nano-vehicles (Figure 1). Using these approaches, it may become possible to tailor exosome-based therapeutics to treat disease. Here, we provide a review of exosome biogenesis and structure, before considering therapeutic and other applications. Finally, we consider the dynamic nature of exosomes and their interactions in vivo, before concluding how synthetic exosomes may overcome some of the limitations of native exosomes in therapeutic applications.

Exosome Structure and Composition

Exosomes have an aqueous core and lipophilic shell, and their **amphiphilic** properties enable them to compartmentalize and solubilize both native and introduced hydrophilic and hydrophobic materials (Box 1) [1]. Cargos include proteins, lipids, and RNAs that can be distinct from the cell's endocytic origin. The endocytic origin includes endosomal proteins involved in transport and fusion (annexins and flotillin), cell targeting (tetraspanins), multi-vesicular body (MVB) proteins (Alix and TSG101) [2], as well as various proteins linked to lipid metabolism [3]. Extracellular matrix and cell surface proteins

such as collagens, integrins, and galectins [4], cell surface receptors and signaling molecules, intracellular cytoskeletal components, as well as metabolic enzymes and G proteins are also present [5]. In contrast to their cells of origin, exosomes are enriched in cholesterol, ceramide, phosphoglycerides, and long and saturated fatty-acyl chains, all of which may provide structural stability [2]. Morphologically distinct MVBs within the same cell result in distinctive exosome populations with unique molecular and biophysical characteristics [6,7]. Differences and changes in exosome composition are widely observed in vitro and in vivo in response to localized environmental conditions. For instance, inflammatory signals including lipopolysaccharides, TNFα, and IFNγ strongly affect the composition of exosomes released by dendritic [8] or mesenchymal stem cells [9]. Hypoxia, observed in the core of large tumors can modify exosome composition of human endothelial [10] and patient-matched glioblastoma multiforme tumor [11] cells.

Exosomes: Function and Design

Exosomes satisfy a number of design criteria, including the ability to package small chemical and biomolecular agents, cross biological barriers such as the blood brain barrier [12]. Structural characteristics such as size, shape, and surface properties (surface charge/chemistry and ligand density) directly impact on their behavior in complex processes including protein **opsonization**, blood circulation, tissue penetration, cell interaction, and renal clearance [1]. For example, the size of nano-particles used in systematic delivery should be large enough to avoid rapid renal clearance, and small enough to evade **reticuloendothelial system (RES)** uptake, as determined in mice [13]. Moreover, for human cancer therapy such as Doxil® (PEGylated nano-liposomes), particles of 10–100 nm in diameter can take advantage of tumoral vascular alterations to passively reach the tumor site, through enhanced permeability and retention effects [14]. In addition, net surface charge determines the **colloidal stability** of nanoparticles and their interactions with biological systems [15]. Positively charged nanoparticles undergo fast protein opsonization, and are quickly cleared by RES from blood circulation by murine RAW264.7 macrophages, whereas neutral or slightly negative surfaces reduce RES clearance to provide more efficient delivery to tumor sites in SKOV-3 human ovarian cancer xenograft mouse models [16].

Exosomes in Health and Disease

In humans, exosomes play pivotal roles in normal physiological and pathological conditions (Figure 1) [29–31], through long range signaling via blood, cerebrospinal fluid, and breast milk to modulate target cell behavior [32,33]. Exosomes drive multiple biological processes including modulation of gene expression via RNA intercellular transfer [34] and immuno-suppressive or immuno-stimulating responses [35–37]. Moreover, these nanovesicles have been implicated in antigen presentation and T cell activation in different contexts, including in vivo bacterial infections [17]. The role of exosomes in angiogenesis has also been acknowledged and recent studies have suggested that exosomes present in human pericardial fluid can enhance therapeutic angiogenesis and vascular repair in ischemic mice [18]. This beneficial effect has also been observed for normal cardiovascular physiology, with mesenchymal stem cell (MSC)-derived exosomes conferring cardioprotection in mice, as demonstrated by reduced myocardial ischemia/reperfusion injury of such exosome-treated animals [19]. By contrast, human monocyte-derived exosomes have been found to induce programmed cell death of vascular smooth muscle cells under inflammatory conditions [20], an important process in human vascular diseases.

In cancer, tumor-derived exosomes have been shown to induce apoptosis of human pancreatic tumor cells [21] while inducing apoptosis of cytotoxic T lymphocytes as a tumor escape mechanism in an in vitro model of prostate cancer cell lines [22]. It has been further suggested that exosomes might favor or counteract tumor progression via dissemination of oncogenes or tumor suppressors between cells, in a context-dependent manner [23]. Moreover, in diseases such as pancreatic ductal adenocarcinoma (PDAC), tumor dissemination may involve the mechanistic contribution of exosomes to form pre-metastatic niches, as was determined in the liver of PDAC patients [24]. Of note, because exosomes can naturally cross the plasma membrane of recipient cells [25], their manipulation can lead to therapeutic molecule delivery in a selective manner, for example in mouse αv integrin-positive breast cancer cells [26]. Moreover, aside from being able to efficiently cross the blood-brain-barrier to induce potentially significant therapeutic effects [27] in contrast to most non-engineered small molecule drugs, exosomes can also be modified or functionalized with the addition of tumor target ligands, as has been proposed for folic acid to promote tumor size reduction in a human lung cancer mouse xenograft model [28].

As for pathogen infections, these biological nanoparticles might either function as clearing vehicles to expel invading bacteria from infected cells [29], or as vector-transmitted virulence factors to spread infections, as in the case of cutaneous leishmaniasis [30]. Due to the heterogeneity of induced biological effects on recipient cells, which appear to be triggered by distinct exosome subpopulations with unique molecular compositions [22], exosomes may open new avenues for exosome-based diagnostics and innovative therapeutic strategies.

Strategies for Therapeutic Deployment of Exosomes

Native Exosomes

Cells introduced into recipients to exploit native exosomes for disease treatment potentially offer the most direct route to exosome therapeutics. Exosomes from stimulated platelets have been shown to play a role in athero-thrombotic processes, impeding platelet aggregation and reducing levels of the platelet activator CD36, which might be considered as a potential therapeutic option of suppressing occlusive thrombosis [31]. Similarly, when secreted from mesenchymal stem cell (MSCs), exosomes appear to be significant effectors of MSC responses, rather than influencing the differentiation of MSCs in tissue repair [32]. For instance, MSC-derived exosomes have demonstrated cardiac and vascular benefits, including suppression of pulmonary hypertension (PH), vascular remodeling and inflammation in murine models of PH [32], as well as modulating angiogenesis in a human placental endothelial cell in vitro model [33]. Furthermore, exosomes derived from rat bone marrow MSCs have been reported to protect cardiomyocytes from ischemic injury in a rat model of myocardial infarction [34]. Human MSCs, isolated from bone marrow or adipose tissue appear to produce native exosomes with a wide range of effects, including regenerative capacities in cutaneous wound healing in a mouse model of skin incision [35] and skeletal muscle regeneration in mouse model of muscle injury [36]. However, the mechanism of action of these effects remains to be characterized, and caution must be exercised until specific effector molecule(s) contained within MSC-derived exosomes along with their mechanistic role are identified [37].

Modification of Content via Parent Cell Treatment

Treatment of cells to produce modified exosome content in order elicit a disease specific response has produced significant advances; this has been achieved either through exposure to various exogenous compounds such as cytokines, and gene transfection or stable genetic manipulation of the exosome-producing parent cell (Box 2). Specifically, treatment of human platelets with aspirin has resulted in a decrease in cargo protein levels in platelet-derived exosomes, without altering total levels of exosome numbers [38]. Furthermore, murine bone marrow derived dendritic cells treated with recombinant murine IL-10 protein, have produced exosomes harboring a significant immunosuppressive effect, as demonstrated in a delayed-type hypersensitivity mouse model leading to reduced inflammation, or in a murine collagen-induced arthritis model where the onset was reversed/repressed[39]. Another study documented that human vascular smooth muscle cells, engineered to carry fetuin-A (a calcification inhibitor), produced this factor which could be encapsulated in the cell-derived exosome MVs, which triggered vascular calcification under extracellular calcifying conditions in vitro [40]. Others initially reported that pulsing murine dendritic cells (DCs) with tumor peptides resulted in an increased ability of DC-derived exosomes to prime cytotoxic T cell immune responses against murine tumors [41]. Content can also be modified via cell transfection to deliver proteins and nucleic acids into exosomes; for example, upon transfection of synthetic double-strand miR-143 microRNA into human MSCs, exosome-formed miR-143 was shown to be secreted extracellularly from exosomes, and subsequently transferred into osteosarcoma cells, suppressing cell migration in vitro [42].

In addition, recent research has demonstrated that treatment of human ovarian cancer with sub-cytotoxic levels of decitabine -- a DNA methyltransferase (DNMT) epigenetic inhibitor (used to treat myelodysplastic syndromes and acute myeloid leukemia), --triggered cytokine release from tumor cells; this resulted in cytotoxic T cell recruitment and immune-cell mediated cancer cell death [43]. This process might likely to involve exosome signaling, but such a hypothesis remains to be demonstrated.

Modification of Content via Treatment of Isolated Exosomes

Loading exosomes with endogenous or exogenous content (Box 2) following exosome purification has been evaluated for effectiveness against Huntington's, Parkinson's and Alzheimer's diseases [44,45]. Insertion of siRNA targeting BACE1 in DC-derived exosomes resulted in targeted suppression of BACE1 mRNA and protein in wild-type neuronal cells in mouse brains [46]. In addition, peptide antigens were shown to enhance immune responses in mice where ovalbumin-loaded exosomes induced T-cell proliferation in vitro and in vivo [47]. Similarly, upon loading bone marrow-derived exosomes with lipid-derived molecules including α-galactosylceramide, NK cell and T-cell innate anti-tumoral immune responses were induced in vivo in a murine melanoma cancer model [48]. Another example is that of **vexosomes**, which are virus vector capsids associated with exosomes, which in the case of AAV have enabled efficient transgene expression in murine cerebellum Purkinje cells upon transduction [49]. Moreover, for drug delivery purposes, paclitaxel-encapsulated vesicles have been shown to be effectively delivered into multi-drug resistant murine lung carcinoma cells [50].

Negative Effects of Exosome Exposure

Whilst exosomes provide effective tools for transportation of anti-cancer drugs with lower toxicity [51], it is important to consider their function as immuno-stimulating or immuno-suppressing moieties [52]. Exosomes have been proposed to play a role in shaping the tumor microenvironment through their involvement in various biological processes including angiogenesis, immune escape, and triggering an epithelial-to- mesenchymal transition (EMT) leading to metastasis initiation [53]. For instance, melanoma-derived exosomes have been shown to transfer the C-Met oncoprotein to bone marrow progenitor cells, inducing vascular leakiness at pre-metastatic sites, and promoting metastasis in mouse tumor models [54]. They also appear to influence drug resistance; for instance, exosomes isolated from an adriamycin-resistant breast cancer cell lines have been found to transfer drug resistance to drug-sensitive human breast cancer cell lines upon delivery of miR-222 in vitro [55]. An association between exosomes and obesity-related insulin resistance has also been reported; in this study, adipocyte-derived exosomes were found to activate adipose-resident macrophages and secrete inflammatory cytokines (L-6 and TNF-alpha), leading to insulin resistance in a ob/ob obesity mouse model [56].

Exosomes as Potential Biomarkers

The opportunity to exploit exosomes as biomarkers is an area of intense investigation. Current trends are focused on specific internal micro RNA markers that can be detected by PCR, and surface protein markers. The first clinical exosome products to appear on the market are exemplified by the "ExoDx®Prostate (*IntelliScore)"* test for prostate cancer that analyses exosomes isolated from urine for RNA biomarkers, currently used in combination with conventional PSA diagnostic tests (http://www.exosomedx.com/prostate-cancer-0).

Biobanking

Being able to access an individual's exosome profile through a biobank, would further our approaches to personalized medicine through detailed clinical classification of complex diseases including cancers and metabolic disease [57]. To enable this, a standardized international approach to exosome isolation, characterization and storage needs to be established.

Benefits within Regulatory Frameworks

The European Union and United States regulatory agencies consider human exosome-based therapeutics as biological medicinal products, and, depending on pre- or post-isolation manipulations -- such as the genetic-manipulation or cell expansion of parent cells -- might be classified as advanced therapy medicinal products (ATMPs) [58]. When compared to MSC therapies, exosomes might be able to overcome safety concerns surrounding continued MSC proliferation, whilst having the same therapeutic effect. Presumably, exosomes might present a lower risk than cell-based therapeutic approaches. Nevertheless, the limited number of clinical trials, (Tables 1-3) reflects the difficulties associated with fully understanding molecular functions triggered by exosomes in target cell responses, and highlights the continued need for mechanism of action studies.

Future Outlook

To fully exploit the potential of exosomes, it will be critical to accurately define the modes of delivery to diseased tissues, either via systemic or localized delivery, or via localized assembly (Box 3). These considerations may include transit to target tissues and uptake, as well as the precise composition of exosomes or synthetic similars.

Exosome Dynamics in Circulation

Following release of exosomes into the circulation (or upon injection), these can move to sites that are distant from their point of origin/introduction, as observed from experiments using fluorescently labeled exosomes to monitor accumulation of siRNA delivery to the murine brain [46]. Indeed, one study used exosomes functionalized with a luciferase (gLuc)-lactadherin fusion protein to track

exosomes in vivo, upon intravenous injection in murine models; whole body imaging data revealed that exosomes exhibited very short half-lives following systemic administration, with less than 5% of administered exosomes remaining in the serum at 5 min post-injection [59]. This report proposed that this might be due to rapid clearance of exosomes from the circulation and/or to organ sequestration (e.g. by the liver) [59]. Similarly, miR-155 was shown to traffic from CD63-enriched B cell-derived exosomes that had been loaded with synthetic miR-155, into murine liver, adipose tissue, lung and muscle, but were absent from the thymus and heart, with plasma levels peaking at 5 min [60]. Consequently, the biodistribution of engineered exosomes containing different markers has indicated that these exosomes are short-lived in the circulation and in organs (5-40 mins) [59].

Organotropism and Mechanisms of Exosome Uptake

Signal transduction from exosomes to cells occurs through membrane fusion or endocytosis, where specific exosome surface molecules drive cell targeting and adhesion (Box 2). Many exosomes contain major histocompatibility complex (MHC) class I and class II molecules involved in antigen binding and presentation; however, the requirement for MHC components in targeting does not appear to be an absolute requirement, as $MHCI^{-/-}$ and wild type derived murine exosomes can both induce T cell responses [61]. In addition, integrins, annexins and tetraspanins are also present in these exosomes [2,62]. For example, based on proteomic analyses, specific α- and β-integrin combinations, such as α6β4 and α6β1 have been reported to direct human lung cancer cell exosomes to organ-specific sites in mouse models (i.e. lung), preparing an area for metastasis by fusing with specific tissue -resident cells (e.g. fibroblasts and epithelial cells) [63]. These integrins also induced S100 protein synthesis in recipient cells, thus promoting local pro-migratory and pro-inflammatory activity [63]. Thus, specific exosome membrane composition can lead to selective tropism as well as recognition by recipient cells [63].

Towards Synthetic Exosomes

Exosomes possess significant advantages over synthetic nano-vesicles, carrying multiple surface ligands in native conformations for cell targeting. The complexity of exosome structures, and the relatively low quantities produced by cells, offers challenges that may be overcome through the development of semi-synthetic or synthetic systems. The generation of exosome-mimetic vesicles is an exciting and important prospect for future therapeutic approaches. Synthetic nanovesicles, fabricated by forcing cells through microfluidic channels, have been assessed for augmented proliferation in murine skin fibroblasts [64] and as RNA carriers [65]. Since these nanovesicles are

generated directly from cells by extrusion, they are made up of cellular components including plasma membrane, membrane proteins, and intracellular biomolecules generated by the original cells.

Serial extrusion through filters with diminishing pore sizes has produced exosome-mimetic nanovesicles following the breakdown of monocytes or macrophages, enabling delivery of chemotherapeutics into malignant tumors following systemic administration in mice [66]. These cell-derived nanovesicles presumably have an almost identical targeting ability relative to parent cells, maintaining plasma membrane topology and constituent proteins; they also exhibit similar characteristics to naturally occurring exosomes, including size and membrane composition (e.g. the CD63 membrane marker) [66]. Moreover, hybrid exosomes, synthesized by fusing cell membranes with liposomes through freeze-thawing techniques, and subsequent cellular uptake studies of these vesicles have confirmed that their delivery function can be modified by changing the lipid composition [67].

Using a new de novo exosome synthesis system, a multi-component drug iNPG-pDox (injectable NanoParticle Generator with polymer-Doxorubicin conjugate), it may be possible to achieve long-term cures in preclinical murine models of triple-negative breast cancer with lung and liver metastases (human equivalent of 20+ years, disease-free) [68]. This drug's multi-stage nature essentially mimics MVB [69]. Specifically, it is designed so that its components act in a prescribed time sequence to overcome metastasis-associated biological barriers, and deliver chemotherapeutic agents preferentially to the perinuclear region of cancer cells, thus escaping the multi-drug resistance action of cell membrane-based efflux pumps [70]. A key finding for this effect was that doxorubicin-containing nanoparticles needed to be formed inside cancer cells, as it was not sufficient to inject them systemically in mouse models of metastatic breast cancer [70]. iNPG-pDox generates exosome-like nanoparticles once it preferentially reaches the metastatic microenvironment, and upon internalization by tumor cells, the nanoparticles are cleaved at the nuclei into doxycycline, avoiding excretion by drug efflux pumps [70]. Because of its similarity to physiological nanoparticles, iNPG-pDox can effectively deliver a mortal blow to cancer cells with high efficacy to otherwise untreatable cancer lesions [70].

Collectively, these different approaches provide a logical basis for the generation of reconfigurable semi-synthetic or synthetic exosomes, thereby increasing pharmaceutical acceptability through a controllable assembly process.

Concluding Remarks

The potential value of exosomes as therapeutic systems is increasingly promising, and for many, the safety and feasibility has been demonstrated in clinical trials. Nevertheless, it is clear that further

robust studies are necessary to deliver clinical outcomes. Future steps include the fine-tuning of therapeutic payloads, as well as targeting behavior and bioinspired redesign. We anticipate that these steps may open exciting new avenues for therapeutic application and facilitate clinical translation of these multi-tasked sentinels (see Outstanding Questions and Box 4). Ultimately, bridging nature with synthetic biology and biophysics will uphold the natural evolvability of exosomes into reconfigurable therapeutic systems.

Outstanding questions box

- Characterization exosome content requires multiple analytical approaches; there is a significant challenge in relating the highly complex content of an individual exosome, or the heterogeneous content among exosomes, to the biological effect. Is full characterization required? Will regulatory agencies deem that unnecessary, and approve clinical use in a similar way to cell-based therapeutics based on functionality?
- Recently, cancer cells treated with non-cytotoxic doses of decitabine (therapeutic epigenome modulator) have resulted in cytokine release, triggering immune-cell attraction, and subsequent cancer cell cytotoxicity. It is likely that this 'holy grail' approach to cancer cell drug-initiated self-killing might also involve the release of modified exosomes, but this is a question that remains to be explored.
- Do specific/clinically active exosomes exhibit target cell specificity and can we identify the responsible cell surface features? An increased understanding of the contribution of the biophysical properties of exosomes toward target cell recognition and reception (rather than simplistic receptor-driven principles), will likely inform our ability to modulate the properties of exosomes.
- Polarized trafficking of exosomes through regulated cytoskeletal remodeling might enable a directed release of exosomes; it may also establish a concentration gradient corridor for their intercellular delivery. What will be the most direct approaches to determine this?
- Where does the convergence lie between natural and synthetic exosomes? Might we consider the reconfiguration of autologous or allogenic exosomes, and/or the synthesis of more complex liposome-like structures?

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Box 1. Biogenesis of the Exosome

Exosomes were independently discovered in 1983 by P. Stahl [71] and R. Johnstone [72], when maturing red blood cells from rat and sheep were shown to jettison ~30-100nm diameter globules into the extracellular matrix. Receptor-bound transferrin was sequestered into nano-vesicles that formed inside bigger **multi-vesicular endosomes (MVE**), some of which fused with the cell membrane and discharged the transferrin containing nano-vesicles outside the cell [71]. Years later, those nano-vesicles were termed "exosomes" [73] in order to differentiate this new class of organelle from endosomal shuttles.

Exosomes are formed by invagination of the limiting membrane of "sorting" vacuolar endosomes towards the lumen of these compartments, thus forming intraluminal vesicles (ILVs); endosomes are then referred to as MVEs or multi-vesicular bodies (MVBs) (Figure I) [62,74]. Across species, MVB and ILV formation is driven by the highly conserved, thirty protein subunit, endosomal sorting complex required for transport (ESCRT) complexes 0-III ESCRT-0, -I, -II and -III) [75]. An alternate mechanism also exists where lipids generated in the limiting membrane of MVBs induce inward budding, thus forming ILVs in an ESCRT-independent manner [76]. Analysis of exosome content released during rat reticulocyte maturation has revealed the existence of three distinct sorting pathways for proteins and lipids into exosomes [77]. The first pathway relies on the protein Alix and either ESCRT complexes and/or lysobisphosphatidic acid, while the second and third pathways are associated with membrane lipid microdomains enriched in ceramides, and aggregating factors such as lectins, respectively [77].

The observation of the immunomodulatory function of B lymphocyte exosomes in triggering CD4+ specific T cell responses, revealed a role for exosomes as transporters of MHC class II-peptide complexes between immune cells [78]. Subsequently, exosome release from dendritic cells was demonstrated where exosomes expressing MHC class I, class II and T-cell costimulatory molecules were shown to suppress growth of established murine tumors in a T cell-dependent manner, paving the way for the clinical consideration of exosomes as cell-free 'vaccines' in cancer immunotherapy [41,79]. recently, exosomes were revealed to contain functional RNA (mRNA and microRNA (miRNA)) [80]. Moreover, when murine exosomes were transferred into human cells in vitro, murine proteins were detected in these cells, showing that mRNA shuttled via exosomes could be translated to

functional proteins[80]. Such exosomal RNA content was absent from the cytoplasm of donor cells, suggesting that protein production stemmed exclusively from extracellular signaling [80].

Box 2. Regulating Targeted Exosome Uptake

Exosome manipulation as a consequence of cell treatment with a specific targeting antigen has resulted in antigen display on the exosome surface. For instance, a study used exosomes carrying neuron-specific rabies viral glycoprotein (RVG) peptide on the membrane surface to "ship" opioid receptor mu (MOR) siRNA into mouse brains as a model to treat morphine addiction, further demonstrating the capacity of exosomes to cross the blood brain barrier [81]. Moreover, the expression, in two MHC type-distinct mouse cell lines of the target tumor antigen human MUC1 (hMUC1) -- a glycoprotein (mucin) overexpressed in different types of tumors-- produced exosomes which expressed that same peptide on their surface [82]. These exosomes were capable of stimulating immune cells and suppressing of hMUC1-expressing tumor growth in mice in a MUC1-specific and dose-related manner [82]. Another study showed that exosomes surface-armed with TRAIL (TNF-Related Apoptosis-Inducing Ligand) targeted different tumor types, inducing apoptosis and inhibiting cell growth in tumor-bearing mice [83]. Others have also reported that the display of exosomal membrane protein (Lamp2b) fused to αv integrin-specific iRGD peptide, could deliver Doxorubicin to αv integrin-positive breast cancer cells in mice [26]. To enhance this approach, glycosylation motifs have since been introduced at various positions in surface antigens in cell lines, arresting protease mediated degradation, and enhancing targeting-antigen stability, whilst retaining their ability to interact with target proteins [80]. As such, the concept of the exosome glycome has emerged [81], in which assumes that the glycome contributes significantly to exosome function, including the control of surface charge through modulation of silicate molecules on glycan structures in the cell membrane [1].

Other techniques have been developed to enrich exosome surfaces, including **Exosome Display**, a methodology that exploits the ability to express non-naturally occurring proteins on these vesicles [84]. This approach has proved to be resourceful for engineering exosome surfaces to display multimeric, trans-membrane or soluble antigens which are not normally present. For instance, exosome-surface localized lactadherin has been achieved by binding its C1C2 domain to exosome lipids; as such, antigens or extracellular domains of membrane proteins can be displayed on exosomes after fusion with the C1C2 domain, and this has been used in the fabrication of anti-tumor antibodies [84,85]. Similarly, **Click Chemistry** has proved efficient for the conjugation of small molecules, such as Azide-Fluor 545, to the exosome surface, showing no alteration of the size or association of small molecules with recipient cells, and ensuring a reliable functionalization method [86].

Box 3. Force-Generating Mechanisms of Exosome Secretion

The involvement of actin and actomyosin-mediated forces in exosome secretion in invasive cancer cells occurs from protrusive actin-rich structures called invadopodia that establish docking sites for MVBs [87–89]. By inhibiting actin polymerization at invadopodia sites, polarized delivery of MVBs to invadopodia and exosome secretion can become impaired [87,88].

The regulation of actin filament dynamics is a means of exosome secretion modulation in different cancer model systems. Cortactin, an activator of the actin polymerizing complex Arp2/3 has been shown to enhance MVBs docking at the plasma membrane with subsequent exosome release; this occurs via assembly and stabilization of actin filament branches in invadopodia-like structures [90]. Of note, cortactin knockdown or overexpression in head or neck squamous cancer cells in vitro has been shown to induce the release of exosomes from invadopodia without altering significantly exosome biogenesis [90,91]. Actomyosin-mediated contractility at the cell cortex has also been reported to facilitate actin-bound MVB movement toward the plasma membrane by a mechanism that depends on the pulling force of non-muscle myosin II on the sides of future docking sites for MVBs [87,92–94]. Such subcellular dynamics are dependent on interactions with cytoskeleton-based force generating systems (such as microtubules, actin and actomyosin), which is in turn are regulated by Rab proteins and their effectors [95]. At initial maturation stages, endosomes are transported along microtubules to the center of the cell by dynein/dynactin molecular motors[95], whereas translocation of MVBs to the plasma membrane requires kinesin-dependent movement towards microtubule plus ends [87], in coordination with actin and actomyosin cytoskeleton, GTPases and lipid second-messengers [87,95]. In polarized cells, such as lymphocytes, neurons and epithelia, specific lipid components, including cholesterol, sphingomyelin, and phosphoinositides domains of the plasma membrane harbor an increased capacity to recruit specific proteins that regulate the cytoskeleton, as well as those within signaling cascades that control vesicle tethering, membrane fusion and excision (Figure II) [96,97].

The docking and fusion of MVBs to specific points in the plasma membrane strongly depend on the structural organization and polarity of the cytoskeletal trafficking machinery, as well as on lipid composition and distribution; consequently, regulating these functions and properties may indeed lead to advantageous delivery of exosomes for signal confinement and enhanced uptake by recipient cells [95].

Box 4. Clinician's Corner

· Exosomes, 30-100nm extracellular vesicles produced by all cells in the body, are biological signaling systems that function to facilitate short and long range intercellular communication including the transfer of molecules (DNA, RNA, proteins, lipids) and therefore molecular information between cells.

· Exosomes are being evaluated for their potential as disease specific biomarkers, vectors for drug delivery, and therapeutic agents per se, with clinical trials currently being undertaken for these applications.

· GMP stem cells therapeutics have the potential to also provide excellent sources of therapeutic exosomes. For example, when cultured in specific media CTX cells from ReNeuron have been shown to preferentially enrich a set of microRNAs contained within their exosomes which demonstrate anti-cancer properties in pre-clinical models.

· Exosomes could be responsible for problems associated with lack of response to current therapeutic approaches. HER2 presenting exosomes have been isolated from HER2-expressing

breast cancer models, and inhibition of Trastusumab activity through the presence of these exosomes has been shown. By extension this could also lead to decrease in activity to the ADC Trastuzumab emtansine (Kadcyla®).

Glossary

Nanoparticles: particles with dimensions ranging from 1 to 100nm.

Multi-vesicular endosomes (MVE): Intra-luminal vesicles formed by inward budding of endosomal membranes as a function of the ESCRT machinery.

Amphiphilic: a term describing a chemical compound possessing both hydrophilic and lipophilic properties.

Opsonization: a process in which a supramolecular structure is functionalized or coated with a substance called an *opsonin*, marking the structure for selective cell or tissue recognition.

Reticuloendothelial system (RES): also known as macrophage system, is a part of the immune system that consists of the phagocytic cells located in reticular connective tissue.

Colloidal stability: a physical state that characterizes the relative ability of particles to remain dispersed in solution at equilibrium.

Vexosomes: endogenously enveloped adeno-associated virus (AAV) vectors into exosomes.

Exosome Display: a methodology enabling the manipulation of exosome protein content based on the identification of specific protein domains that mediate the distribution of proteins on exosomes.

Click Chemistry: organic chemistry methodology that uses highly reliable and selective reactions designed to rapidly join small modular units together in high yield, without offensive by-products.

Anisotropic: describes physical properties of a structure that exhibits different values depending on the direction from which they are measured.

Invadopodia: actin-rich protrusions of the plasma membrane; they are associated with degradation of the extracellular matrix in cancer invasiveness and metastasis.

Figure legends

Figure 1. The Multi-Faceted Exosome. Exosomes are composed of two major functional compartments, the external bilayer (yellow circle) and the internal 'cargo bay'. Exosomes can be broadly classified into two groups; native (produced internally by an organism) or modified externally (blue arrows), through cell growth in conditioning media, reprogramming of content and/or membrane composition by cell treatment with external agents (drugs, genetic modification), and loading of novel content (blue boxes). Exosomes, administered exogenously, or endogenously from implanted or endogenous cells, can have short or long distance effects targeting specific cells and inducing a positive molecular modulation (e.g. triggering cancer cell death) to the target (green) as a function of either exosome content or bilayer composition/biophysical effect. In addition exosomes have also been shown to activate immune responses to initiate immune-protective functions. Exosomes can also have a negative effect, including pacifying the immune system so as for diseased cells to evade recognition, and in triggering the establishment of niche environments for metastatic

cancer. Such exosomes as well as others simply originating from diseased cells, but without yet a defined function, will make effective biomarkers for disease identification and monitoring.

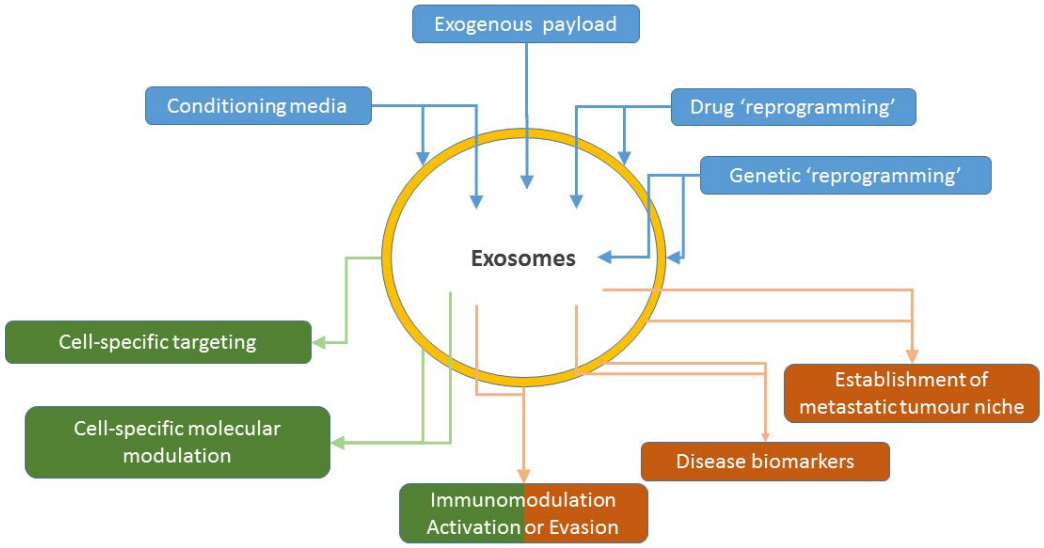

Figure 1. Multi-faceted exosome.

Figure I (in Box 1). The Formation of Exosomes. Inward budding of the plasma membrane originates the formation of early endosomes (not shown), which then mature into late endosomes or multi-vesicular bodies (MVBs). Intraluminal vesicles (ILVs) are generated by invagination of MVBs. MVBs release their content extracellularly after fusion with the plasma membrane, and secreted ILVs are now, designated exosomes. Inset: transmission electron microscopy image of neural stem cells releasing extracellular vesicles of various sizes. Scale bar corresponds to 1µm. "Courtesy of ReNeuron, UK".

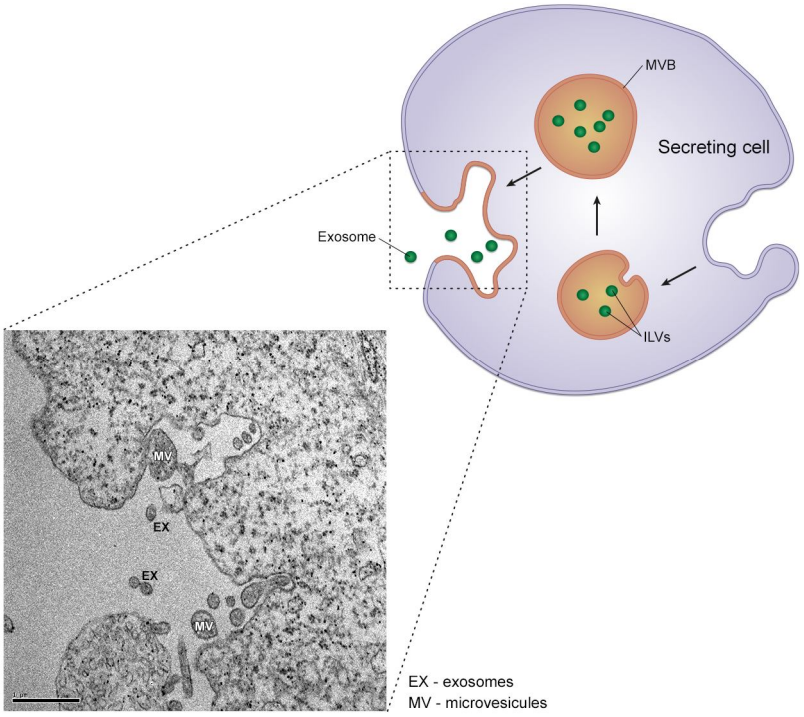

Figure I. The formation of exosomes.

Figure II (in Box 2). Cell Manipulation for Polarized Exosome Secretion. Exosome release normally occurs following the fusion of MVBs at random points in the cell membrane due to the **anisotropic** arrangement of the cytoskeleton along which MVBs transit (top). The establishment of actin-rich **invadopodia** targets MVBs, directing exosome release (bottom), which can be inhibited by blocking the function of cortactin. Direction of MVB and exosome movement/transport (arrows).

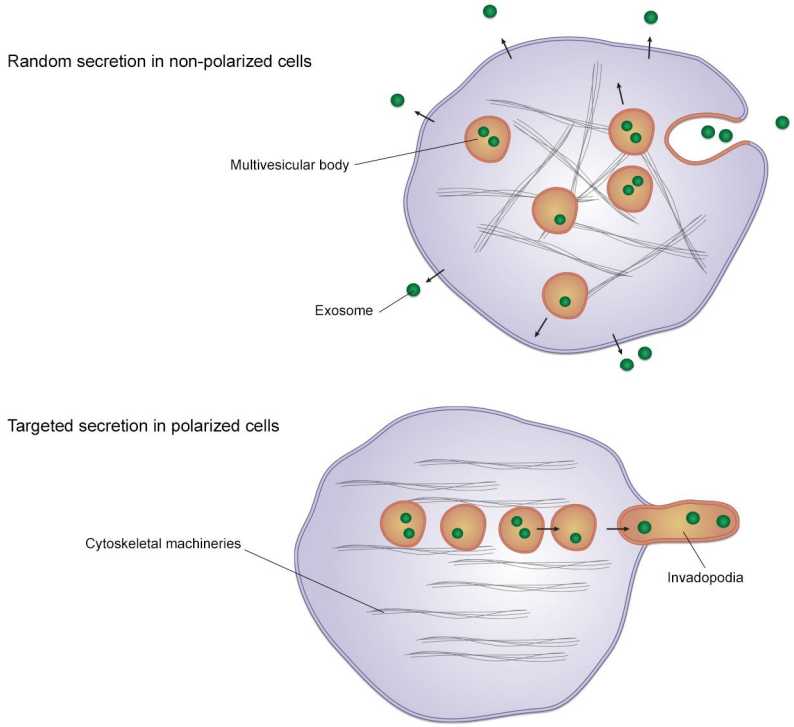

Figure II. Cell Manipulation for Polarized Exosomes Secretion.

Table 1. Current or Recently Completed Registered NIH Clinical Trials Involving Exosomes as Diagnostic Agents (clinicaltrials.gov).

Study Title	Disease	Study Design	Start Date	Reference
Circulating Exosome As Potential Prognostic And Predictive Biomarkers In Advanced Gastric Cancer Patients: A Prospective Observational Study ("EXO-PPP Study")	Gastric Cancer	Prospective Trial Observational Phase not provided (currently recruiting)	Jan 2013	NCT01779583
An Observational, Single-Institution Pilot/Feasibility Study of Exosome Testing as a Screening Modality for Human Papillomavirus-Positive Oropharyngeal Squamous Cell Carcinoma	Oropharyngeal Cancer	Prospective Trial Observational Phase not provided (currently recruiting)	Feb 2015	NCT02147418
LRRK2 and Other Novel Exosome Proteins in Parkinson's Disease	Parkinson's Disease	Prospective Trial Observational Phase not provided (ongoing, not recruiting)	Jan 2013	NCT01860118
Clinical Research for the Consistency Analysis of PD-L1 in Cancer Tissue and Plasma Exosome	Non-small cell lung cancer	Prospective Trial Interventional Phase not provided	Oct 2016	NCT02890849
Clinical Research for the Consistency Analysis of PD-L1 in Lung Cancer Tissue and Plasma Exosome Before and After Radiotherapy	Non-small cell lung cancer	Prospective Trial Interventional Phase not provided	Oct 2016	NCT02869685
Pilot Study With the Aim to Quantify a Stress Protein in the Blood and in the Urine for Early Diagnosis of Malignant Solid Tumors	Breast cancer, ovarian cancer, non-small cell lung cancer.	Interventional Phase not provided	Sep 2015	NCT02662621
Detection of Circulating Biomarkers of Immunogenic Cell Death After Radiotherapy and Chemotherapy: An Exploratory Study	Non Small Cell Lung Cancer	Interventional Phase not provided	Nov 2016	NCT02921854
Early Biomarkers of Tumor Response in High Dose Hypofractionated Radiotherapy Word, Package 3: Immune Response	Carcinoma, Hepatocellular Colorectal Neoplasms Melanoma Kidney Neoplasms	Interventional Phase not provided	Sep 2015	NCT02439008
Anaplastic Thyroid Cancer and Follicular Thyroid Cancer-derived Exosomal Analysis Via Treatment of Lovastatin and Vildagliptin and Pilot Prognostic Study Via Urine Exosomal Biological Markers in Thyroid Cancer Patients	Thyroid Cancer	Prospective Trial Observational Phase not provided	Aug 2016	NCT02862470

NCT - ClinicalTrials.gov registry number

Table 2. Current or Recently Completed Registered NIH Clinical Trials Involving Exosomes as Therapeutic Agents (clinicaltrials.gov).

Study Title	Disease	Study Design	Start Date	Reference
Effect of Plasma Derived Exosomes on Intractable Cutaneous Wound Healing: Prospective Trial	Ulcer	Prospective Trial Interventonal Phase 0	Sep 2015	NCT02565264
Phase I Clinical Trial Investigating the Ability of Plant Exosome to Deliver Curcumin to Normal and Malignant Colon Tissue	Colon Cancer	Clinical Trial Interventional Phase 1	Jan 2011	NCT01294072
Phase 1 Study of The Effect of Cell-Free Cord Blood Derived Microvesicles On β-cell Mass in Type 1 Diabetes Mellitus (T1DM) Patients	Diabetes Mellitus Type 1	Clinical Trial Interventional Phase 2 Phase 3	Apr 2014	NCT02138331
Preliminary Clinical Trial Investigating the Ability of Plant Exosomes to Abrogate Oral Mucositis Induced by Combined Chemotherapy and Radiation in Head and Neck Cancer Patients	Head and Neck cancer Oral Mucositis	Preliminary Clinical Trial Interventional Phase 1	Aug 2012	NCT01668849
Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived exosomes: results of the first phase I clinical trial	Melanoma	Clinical Trial Interventional Phase 1	Mar 2005	[99]
A phase I study of exosome immunotherapy in patients with advanced non-small cell lung cancer	Lung Cancer	Clinical Trial Interventional Phase 1	Feb 2005	[98]
Phase I clinical trial of autologous ascites derived exosomes combined with GM-CSF for colorectal cancer	Colorectal Cancer	Clinical Trial Interventional Phase 1	Apr 2008	[100]
Phase II Trial of a Vaccination With Tumor Antigen-loaded Dendritic Cell-derived Exosomes on Patients With Unresectable Non Small Cell Lung Cancer Responding to Induction Chemotherapy	Non Small Cell Lung Cancer	Clinical Trial Interventional Phase 2	Dec 2009	NCT01159288
Phase II Study of Tumor Cell-derived Microparticles Used as Vectors of Chemotherapeutic Drugs to Treat Malignant Ascites and Pleural Effusion	Malignant Pleural Effusion Malignant Ascites	Clinical Trial Interventional Phase 2	May 2013	NCT01854866

NCT - ClinicalTrials.gov registry number

Table 3. Current or Recently Completed Registered NIH Clinical Trials Involving Exosomes as Observational and Molecular Mechanisms (clinicaltrials.gov).

Study Title	Disease	Study Design	Start Date	Reference
Pilot Study of Exosomes Before and After BRAF Inhibitor Therapy in Patients With Advanced Unresectable or Metastatic BRAF Mutation-positive Melanoma	Metastatic Melanoma	Interventional Phase not provided	Dec 2014	NCT02310451
Isolation and Characterization of the Extracellular Vesicles Secreted by the Human Endometrium to the Endometrial Fluid	Healthy	Prospective Trial Observational Phase not provided	Apr 2016	NCT02797834

NCT - ClinicalTrials.gov registry number