Native and engineered extracellular vesicles for cardiovascular therapeutics

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Abstract

Extracellular vesicles (EVs) are a heterogeneous group of natural particles with relevance for the treatment of cardiovascular diseases. The endogenous properties of these vesicles allow them to survive in the extracellular space, bypass biological barriers and deliver their biologically active molecular cargo to recipient cells. Moreover, EVs can be engineered to enhance their stability, bioactivity, presentation and capacity for on target binding at both cell type and tissue levels. The therapeutic potential of native (i.e., EVs that were not modified via donor cell or direct modulation) and engineered (i.e. EVs that were modified either pre- or post-isolation or whose pharmacokinetics/presentation was altered using engineering methodologies EVs is still limitedly explored in the context of cardiovascular diseases. Efforts to tap into the therapeutic potential of EVs will require innovative approaches and a comprehensive integration of knowledge gathered from decades of molecular compound delivery. In this review, we outline the endogenous properties of EVs that make them natural delivery agents as well as those features that can be improved using bioengineering approaches. We also discuss the therapeutic applications of native and engineered EVs for cardiovascular applications and examine the opportunities and challenges that need to be addressed to advance this research area with an emphasis on clinical translation.

Key points

- EVs secreted from stem/progenitor cells as well as differentiated somatic cells have regenerative properties in the context of myocardial infarction, ischemic limb, chronic wounds and stroke.

- Despite the advantage of native EVs as delivery agents, their applicability in the cardiovascular context is hindered by intrinsic limitations, such as their undefined and heterogenous nature and limited tropism.

- Targeting, bioactivity, kinetics and biodistribution of EVs may be improved by engineering approaches using both pre- and post-isolation methodologies to functionalize and/or otherwise enrich EVs.

- Enhancing EVs is key to unlock their clinical potential for cardiovascular applications.

1-Introduction

Cardiovascular diseases have been the most prevalent cause of death and morbidity in the world for decades despite numerous breakthroughs and the discovery of novel therapies[1]. In the last 20 years, several therapeutic interventions have been initiated, including cell-based therapies; however, poor survival and/or engraftment of transplanted cells in the ischemic milieu of the cardiac tissue limited their clinical efficacy[2]. Mechanistically, the functional improvements observed with cell therapies are poorly understood; however, several pieces of experimental data indicate that they may act by paracrine action, mediated by the release of extracellular vesicles (EVs) and/or other factors[3,4]. Therefore, more recently, interest has been placed in cell-free therapies, in particular, those based in EVs, obviating the need of transplanting large number of cells whilst having a better-defined and less expensive product.

EVs are lipid bilayer-enclosed extracellular structures[5] secreted by virtually all cell types known, and include two major classes, namely exosomes and microvesicles[6]. Exosomes (30-150 nm), intraluminal vesicles formed via invagination of the membrane of multivesicular endosomes (MVEs), are released into the extracellular space upon fusion of MVEs with the cell membrane. Microvesicles (MVs) (50-1000 nm), a very heterogeneous class of EVs, are characterized by their origin and secretion via outward budding of the plasma membrane. Given the complexity involved in identifying their biogenesis, the size of the vesicles is the most widely used parameter to distinguish both types and, on that basis, we either have small EVs (sEVs) or medium/large EVs (m/lEVs)[7,8]. In this review, EVs represent sEV-enriched samples (many studies are not conclusive relatively to the biogenic origin of EVs), being the exception MV examples which are clearly defined in the text.

EVs carry proteins, RNAs and/or microRNAs (miRNAs), among other molecules, and they act as vehicles in cell-to-cell communication[9]. A large body of evidence suggests that EVs are involved in many physiological and pathological cardiovascular processes, including the regulation of angiogenesis[10,11], blood pressure[12,13], cardiomyocyte hypertrophy[14] and apoptosis/survival[15-17] and

cardiac fibrosis[18]. Given their ubiquitous presence in body fluids, EVs have been used as potential biomarkers of cardiovascular diseases[19]. Moreover, because EVs are an important component of the paracrine effect of stem cell-based therapies[20], they are candidates as a standalone therapy in the context of cardiovascular diseases. Pioneering work from the group of Lim suggested the therapeutic potential of EVs in protecting the heart from ischemic injury[20]. Shortly after, the group of Sahoo unravelled the pro-angiogenic potential of EVs in the setting of limb ischemia[10]. Ever since, a number of pre-clinical studies have reported the advantages of EVs for cardiovascular regeneration and protection[21-24]. Yet, several challenges need to be addressed before clinical translation of these therapies including (i) the development of platforms to monitor EVs (both the membrane and the cargo) *in vivo* to determine and optimize the EV dosage regimen, the route of administration, the biodistribution, potential toxicity, immunogenicity as well tumorigenesis, (ii) the characterization of EV cargo in order to use well-defined EV formulations and (iii) the development of strategies to modify the membrane of EVs in order to improve their accumulation in specific organs and tissues. To overcome these limitations, researchers developed pre- and/or post-isolation techniques capable of modulating the intrinsic properties of native EVs and modified their surface to enhance their targeting efficiency and track them *in vivo*. The hypothesis of this review is that the modulation of EVs by engineering approaches may unlock their clinical potential for cardiovascular applications.

In this review, we cover initially the EV biophysical properties (e.g., size, charge, membrane composition and cargo content). This will be followed by the presentation of the therapeutic potential of native EVs for cardiovascular applications. Then, we describe engineering strategies to modulate the content of EVs in order to favourably alter their bioactivity, biodistribution, delivery, targeting and intracellular trafficking. Finally, we present the studies performed using engineered EVs as cardiac therapies and discuss how this area can move forward.

2-EV properties

The biophysical properties of EVs are briefly described below. These properties may be modified using engineering tools, a topic that is covered in section 4. EV biogenesis is not covered in this review and readers are referred to recent reviews related to this topic[25,26].

2.1-EV size

EV size is one of the parameters adopted to categorize EVs[7,8]. Typically, exosomes are smaller (30-150 nm in diameter) than microvesicles and their size distribution more uniform. EV size is critical for their study and application due to most of the protocols used for isolation and characterization relying on EV density and/or diameter[27]. Moreover, EV size, precluded by their biogenic pathway, correlates with EV composition, as does the cell line of origin[28]. Finally, the efficiency in tissue biodistribution, cell internalization and intracellular trafficking of EVs are size-dependent[29-32]. Indeed, it has been shown that upon systemic administration, large and/or aggregated vesicles (>200 nm) may be trapped in the lung, liver and spleen, taken up by macrophages or unable to extravasate and interact with non-vascular cells and tissues[29,30]. Additionally, aggregation renders EVs less spherical, which may decrease their margination and extravasation from circulation[31]. For example, it has been shown that the vasculature has enhanced permeability after ischemic injury; however, only small-size particles (<200 nm) cross the endothelial barrier[33].

At a cellular level, particles with different sizes may elicit different uptake mechanisms. For example, particles with a diameter smaller than 100 nm may be taken up via clathrin- or caveolae-mediated endocytosis, while larger complexes may require macropinocytosis[34,35]. Therefore, larger aggregates are more likely to be directed towards lysosomal degradation or membrane recycling[32], while smaller vesicles may exhibit higher rates of effective intracellular delivery. In the cardiac setting, particularly for EVs administered systemically, this is of utmost importance as EVs are required to extravasate successfully to the cardiac tissue and then be efficiently taken up by the relevant cell types.

2.2-EV charge

Another important property of EVs is their surface charge. As part of EV membrane composition comes from the plasma membrane (being the remaining from other organelles including Golgi complex)[25,36], which is rich in phosphate groups, a global negative charge is the norm for EVs, just as in cells. However, the charge is also highly dependent on the sugar composition of the plasma membrane which is highly dependent in the expression level of sialyltransferase in the endoplasmic reticulum and Golgi apparatus[37]. Changes in surface charge can be used to infer stability of EVs in suspension, as low absolute values are thought to be typical of EVs more prone to aggregation due to lessened repulsion. This, however, must be balanced with the fact that closer to neutral nanoparticles are more stable in circulation, compared with highly charged ones[38]. Both EV size and surface charge are crucial in specifying the mechanism of interaction between the EV and a host of potential ligands, as well as their uptake by target cells[39]. Finally, the presence of contaminants (e.g. protein or lipid aggregates) in an EV sample may affect multiple functions and parameters. Given the heterogeneity in surface charge of these contaminants, aggregates may be formed between them and EVs. Therefore, proper purification protocols must be ensured. This, however, is aside from the scope of this review, and we direct the reader to other excellent papers on the matter[40,41].

2.3-EV membrane composition

Exosome membranes have higher content of cholesterol than donor cell membranes[42], making them less susceptible to the permeation of small solutes. In addition, exosome membranes contain higher levels of phosphatidylserine, glycosphingolipids and sphingomyelin and lower levels of phosphatidylcholine than the corresponding donor cells[42]. Moreover, exosomes have low protein to lipid ratios compared with, for example, microvesicles[43]. Indeed, high cholesterol and sphingolipid content makes exosomes more resistant to detergents and high temperatures than microvesicles[43-45]. Aside from their lipidic composition, exosomes are decorated with proteins and sugars which, on one

hand, contribute to exosome charge and maintenance of membrane structure and, on the other hand, mediate the interactions of exosomes with the target cells[25]. For example, tetraspanins are a class of membrane proteins abundantly present, in clusters, in exosomes and some of them, namely CD63, CD9 and CD81, are considered general exosome markers. Functionally, tetraspanins are involved in membrane fusion and cellular adhesion, and, as such, play a key role in exosome internalization. Other classes of proteins, such as chemokine receptors (e.g. CXC chemokine receptor type 4)[46], adhesion molecules[47-49] and proteoglycans (e.g. heparan sulfate)[50], have been shown to play a role in mediating EV interactions with the cell surface. When these protein and sugar-based components were deleted or masked, EV internalization[51] as well as EV biodistribution was affected[52,53]. EVs have their transmembrane proteins in an identical topology to that of the secreting cell, conferring them a degree of cellular identity and possible tropism[10,54].

2.4-EV lumen

From late 1990s, it has been proposed that EVs act as important players in intercellular communication, particularly in the context of immune responses and cancer[55,56]. This concept was further confirmed in 2007, after the discovery that exosomes contain microRNAs (miRNAs), as well as other types of RNAs, and can transfer their content to target cells ultimately affecting their activity[9]. Recent studies using high-resolution density gradient fractionation and direct immunoaffinity analyses further dissected EV composition[57]. EVs contain different proteins and RNAs in the lumen, including long and small non-coding RNAs, transfer RNA and ribosomal RNA[28,57-59]. Exosomes do not contain DNA, although it may be present in larger EVs or present in exosome-enriched samples due to the co-precipitation with histones[57]. Proteins, on the other hand, may be sorted into EVs via post-translational modifications, such as ubiquitination[60] and glycosylation[61] and these pathways can be hijacked in order to specifically target proteins onto EVs. Upon studying the interaction networks that can be functionally established between EV proteins, the

intravesicular protein content has been proposed as an organized "nanocosmos" and not cellular scraps[62].

3-Native EVs for cardiovascular applications

The first study describing the use of EVs as a potential therapeutic intervention for cardiovascular diseases was published in 2010[20]. In early 2000s, several groups have shown that upon MI, transplantation of different cell types, including mesenchymal stem cells (MSCs)[63] and hematopoietic progenitor/stem cells ($CD34^+$ cells)[64], improved heart repair. A few years later, it was shown that the positive effects of those stem/progenitor cells were mediated not by direct contribution of the engrafted cells but by paracrine factors[65,66], in particular EVs[10,20] secreted by the surviving cells (**Box 1**). Since these pioneer studies, several groups have demonstrated the regenerative properties of EVs secreted from stem/progenitor cells[67-70] as well as differentiated somatic cells[71,72] in the context of MI[69,70,72,73], ischemic limb[67], chronic wounds[71], among others. While there are several studies on cardiovascular diseases such as atherosclerosis[74] and stroke[75,76], most research with EVs has pertained to ischemic heart disease and MI.

EVs with cardiovascular efficacy have been isolated from different cell sources such as MSCs[77-83], putative cardiac progenitor cells[15,21,68-70,84-88], cells differentiated from pluripotent stem cells[20,72,89-93] and differentiated somatic cells[71,94-96]. The impact of allogenic EVs has been evaluated in mice[69,79,81,87], rats[70,78,85,86,88] and pigs[21,84], through intravenous[78,82,97] and intramyocardial[21,68-70,77,80,81,83,84,86,88] administration. The EV dosage regimen was highly variable as between 30 to 1300 μg[78,85] and 4 to 4000 μg[89,92] of EVs per kg of animal have been tested for rats (300 g was assumed as the weight of a rat) and mice (25 g was assumed for the weight of a mouse), respectively. Given the fact that EVs are less immunogenic than their cellular counterparts (with the possible exception of exosomes released from dendritic cells[98]), it is not surprising to see several studies where EVs isolated

193 from human cells have been tested into non-immunosuppressed animals such as mice[89,99], rats[80,85]
194 and pigs[21].

195 EV tracking studies indicate that the intramyocardial delivery of EVs yields higher EV
196 retention in the heart than intracoronary or intravenous administration routes[21]. The pre-clinical data
197 collected so far indicate that EVs, regardless of their origin, may improve the ejection fraction (EF)
198 (up to 1.3-fold increase[15,77,86,88] relatively to non-treated group) and reduce infarct size (up to 3-
199 fold[79,81,84,85] decrease relatively to non-treated group). The therapeutic effect of EVs and donor cells
200 has been evaluated/discussed and the results collected until now indicate that EVs are as effective as
201 the donor cells in the context of MI[21,80].

202 The therapeutic role of EVs in recipient cells has been mostly ascribed to the delivery of
203 proteins and/or non-coding RNAs, in particular miRNAs. For example, the cardiovascular protective
204 role of exosomes has been attributed to miRNA-19a-3p[100], miRNA-21[79,90], miRNA-24[16], miRNA-
205 22[81], miRNA-29a[18], miRNA-143[96], miRNA-146[69], miRNA-181b[84], miRNA-210[90], miRNA-222[96],
206 miRNA-294-3p[93], mir-126[67], among others. Some of the miRNAs have been identified in previous
207 studies of cell-based therapies (e.g. miRNA-19, miRNA-21, miRNA-24, miRNA-210)[101] to be
208 relevant for cardiovascular repair while others are novel. Another important component of EVs, also
209 associated with their bioactivity, are proteins such as platelet-derived growth factor D[78] and
210 pregnancy-associated plasma protein-A[15]. Some of these proteins are at the EV surface and therefore,
211 do not need to be delivered to the cytoplasm of the acceptor cell. For example, pregnancy-associated
212 plasma protein A (also known as pappalysin-1) is a protein highly expressed in exosomes of cardiac-
213 resident progenitor cells[15]. It has been shown that this protein mediates the cardioprotection and
214 angiogenesis of cardiac-resident progenitor cell-derived EVs by cleaving the insulin-like growth
215 factor binding protein-4 in insulin-like growth factor-1 which, in turn, activates the insulin growth
216 factor receptor, ultimately leading to the phosphorylation of Akt and ERK1/2 and subsequently to
217 decreased caspase activation and reduced cardiomyocyte apoptosis. In other cases, the proteins are in

the lumen of EVs and need to reach the acceptor´s cell cytoplasm to elicit a biological affect. For example, EVs secreted by stem/progenitor cells in mice under systemic inflammation conditions contain integrin-linked kinase that activate the NF-kB pathway[102]. The knockdown of integrin-linked kinase in inflamed exosomes attenuated their inflammatory response and enhanced the endothelial progenitor cell-derived exosome therapeutic activity in ischemic heart.

The mechanisms triggered by EVs depend on the source of EVs (as well as their content) and include: (i) an improvement in cardiomyocyte and endothelial cell survival[21,89,90,95,103] by the regulation of autophagy[104], activation of pro-survival signalling pathways (e.g. Akt, ERK, toll-like receptors)[95,105] and a decrease in oxidative stress[90], (ii) modulation of the inflammatory response[85,106] by influencing immune cell polarization (i.e. inducing a more reparative state rather than an inflammatory state)[84] and cytokine secretion[85] as well as increasing the activation of CD4-postive T cells[94] (iii) a decrease in scar content[21] and (iv) enhancement of angiogenesis[107,108]. For example, cardiosphere-derived EVs improved heart function in a mouse model of MI via miR-146, decreasing apoptosis and inflammatory response and increasing cardiomyocyte proliferation and angiogenesis[69]. It has also been shown that extracellular matrix-derived EVs carry miR-199a-3p, which, by regulating GATA-binding 4 acetylation, were able to rescue electric function in engineered and *in vivo* atria[109].

The above-mentioned studies underscore the potential of native EVs for cardiovascular therapy. However, their clinical potential has not been met yet and important limitations must be overcome before their establishment as an effective therapeutic tool (**Fig. 1**). These limitations may be surpassed by enhancing native EV using bioengineering approaches, as detailed in the following sections.

4-Engineering the EVs

Despite the therapeutic efficacy of native EVs to treat cardiovascular diseases, in recent years, technologies have been developed to modulate EV and thus to enhance their bioactivity, stability, targeting and presentation (by the development of EV-delivery systems) to the cardiovascular system

(**Fig. 2**). In the subsequent sections, we explore how EVs may be enhanced or modified and used for the treatment of cardiovascular diseases.

4.1-Tracking the EVs

Methods to track EVs *in vivo* and follow their biodistribution are very important to fully evaluate their cardiovascular therapeutic potential. Fluorescence[47,54,71,110,111], luminescence[112], positron-emission tomography (PET)/magnetic resonance imaging (MRI)[113] and single-photon emission computed tomography (SPECT)[114,115] imaging techniques have been used to monitor *in vivo* EVs. In most of the cases, the EVs were isolated and modified with chemical ligands[113,114]. In few cases, the EV-secreting cells were genetically modified to express a reporter. For example, human embryonic kidney 293T cells were engineered to express in their membrane a Gaussian luciferase fused to a biotin receptor domain[112]. The EVs could be monitored either *in vitro* or *in vivo* by luminescence or fluorescence (by the interaction with fluorescent streptavidin). Although fluorescence and luminescence imaging techniques are easy to operate and available in most laboratories, they do not offer high sensitivity and absolute quantification. In contrast, the methods that rely in PET/MRI or SPCET/computed tomography offer higher sensitivity and absolute quantification while allowing the acquisition of images with anatomical details. In general, the intravenous administration of labelled EVs (without any further modification besides the labelling) isolated from different cell sources indicate that less than 10% of the injected EVs accumulate into the non-injured heart[54,112-115]. Yet, the accumulation of EVs in the heart is influenced by the delivery route, concentration of EVs and the identity of the EV-secreting cell[54,112-114].

4.2-Modulation of EVs bioactivity

4.2.1-EV-secreting cell modulation

EV-secreting cells may be modulated by two different procedures: (i) by culturing them in stress-induced conditions (e.g. hypoxia[80,88,96], serum starvation[71,96], inflammation[116]) and (ii) by

transfecting them with exogenous compounds, such as nucleic acids, especially miRNAs[23,117,118], miRNA antagonists[119], Y-RNA[85], plasmid DNA[77,78,120,121] and small molecules[121] to enhance their bioactivity (**Table 1**). For example, EVs collected from cardiac progenitor cells cultured under hypoxia conditions increased the capacity of cardiac endothelial cells to form tube-like structures *in vitro*, reduced the expression of pro-fibrotic genes in cardiac fibroblasts cultured *in vitro* and improved function of the infarcted heart (in the acute phase, increased the fractional shortening from 30.6% to 36.4% and in the chronic phase increased from 27.6% to 34.2%) as compared to EVs collected from cells cultured under normoxia conditions[88]. The effect was mediated by several miRNAs including miRNA-292, miRNA-210, miRNA-103, miRNA-17, miRNA-199a, miRNA-20a and miRNA-15b. The modulation of EV-secreting cells might also be achieved by changing their culture medium. For example, EVs collected from adipose-derived stem cells (ADSC) cultured in endothelial differentiation medium showed an increase in miRNA-31 and the resulting EVs enhanced endothelial cell migration, tube formation and aortic ring outgrowth compared to EVs collected from ADSC grown in normal medium and thus not-enriched for miRNA-31[122]. Finally, as an example of EVs collected from cells that were modulated by external agents, EVs collected from mesenchymal stem cells transfected with miRNA-181a increased the pro-reparative state of peripheral blood mononuclear cells and, upon administration in infarcted mice hearts, the miR-181a enriched EVs increased EF (12% relatively to the baseline)[23].

Several cellular platforms have been developed for customizable enrichment of EVs with specific proteins[123] and RNAs[124,125] of interest; however, these platforms were not yet evaluated for cardiovascular applications. In addition, very few studies took advantage of the cellular machinery to engineer EVs with specific epitopes able to target the heart[126].

4.2.2-Post-isolation methods

Hijacking the cellular machinery to produce modulated EVs has the advantage of preserving the biophysical properties of EVs relatively intact, but also has drawbacks, namely overexpressing a given molecule in a cell may have unforeseen consequences for its biology, ultimately interfering with EV biogenesis. Modulation strategies based on post-isolation modification of EVs may be an alternative for effective control of EV loading, targeting, and delivery, regardless of their cell of origin. However, post-isolation methods may mask or impair endogenous EV properties and ultimately compromise EV bioactivity.

Membrane-permeabilizing strategies, such as electroporation (for both nucleic acids[127-129] and drugs[130]), heat-shock or freeze-thaw procedures[131,132], detergent treatment[130] and sonication[133], developed over the past decades to load cells with exogenous material, have been readily applied to the EV field with a varied degree of success. Additional strategies exploited the hydrophobicity of EV membranes in order to passively load compounds of interest into them[134,135] and the modification of the molecule of interest with cholesterol[136,137]. Recent work focused on the use of well-defined chemical formulations, some of which commercially available, designed with the specific purpose of transfecting EVs directly[138-140]. Thus far, these studies reported loading efficiencies up to 70% but the impact on the biophysical properties of EVs and the exact mechanism of action remains to be elucidated.

Several EV formulations have been enriched using transfection agents or membrane-permeabilizing strategies and evaluated in the context of cardiovascular applications to decrease cardiac fibrosis, modulate the inflammatory response and to increase angiogenesis (Table 1). For example, miRNA-21-5p has a critical role in the development of fibrosis after MI, regulating several gene targets including SMAD family member 7 (Smad7), sprout RTK signalling antagonist 1 and phosphatase and tensin homolog (PTEN)[141]. Human peripheral blood-derived EVs enriched for miR-21 inhibitors reduced fibrosis in a mouse model of MI as compared to non-modified EVs. In a separate example, EVs collected from cardiosphere-derived cells and enriched for miRNA-322 using

electroporation, reduced the infarcted area and fibrosis, and increased angiogenesis in a mouse model of MI as compared to non-modified EVs[142]. Together, these studies demonstrate the possibility to enrich EVs after their isolation and thus increase their bioactivity as compared to the non-modified EVs.

4.3-Modulation of EV biodistribution and targeting

Upon systemic administration of EVs in animal models, they are quickly cleared or trapped in the liver, spleen and lungs[134], with the EV half-life (minutes range), inherently dependent on the identity of the EV-secreting cell[143-145]. EV biodistribution is influenced by multiple factors, including the delivery route and dosage[54]. It is possible that EVs retained in several organs induce a systemic anti-inflammatory effect improving the regenerative capacity of the cardiovascular system, as already observed with cell-based therapies[4]. However, studies clearly indicate that increased EV efficacy is related with EV retention at the lesion area[21]. Therefore, in the last years, several strategies have been developed to control EV biodistribution and targeting to specific organs and tissues. One strategy to maximize the uptake is to increase EV stability in circulation and therefore, improve the likelihood of an interaction between the EVs and the cells/tissues to be targeted. Modifying EVs with polyethylene glycol (PEG), a strategy previously used for liposomes[146], enhanced their circulation time[45] and reduced their uptake by nonspecific cells. Another strategy relies on the modification of EV membrane with specific proteins[126] or peptides[22,110,111,127,147-151] that are able to interact with specific cellular receptors or extracellular matrix components expressed in the cardiovascular system (Table 2). Although it is known that tetraspanins present in EV membranes have preferential binding to specific cell lineages (e.g. Tspn8 binds to α4 and β4 integrin chains that are expressed by endothelial cells[152]) these might not be enough for selective and effective organ or tissue targeting.

In the case of heart diseases, the intramyocardial administration of EVs has been reported in pre-clinical studies; however, this route of administration is not always clinically desirable because

it involves a catheterization procedure, unless delivery is planned during cardiac surgeries[153]. The intravenous administration of EVs is a much simpler procedure and allows repeated applications; however, it is more prone to off-targeted binding, increasing the potential for unwanted effects. Moreover, the poor accumulation of EVs into the cardiac tissue (due to poor extravasation and lack of efficient and specific epitopes for recognition) remains an important barrier. Engineered EVs can eventually overcome these obstacles and deliver their therapeutic cargo into the injured heart. Two approaches have been used to modify the surface of EVs for targeting the heart. In one, the EV-secreting cell is genetically modified to express a peptide which is then incorporated in the membrane of the secreted EVs[148-150]. For example, EV-secreting cells were genetically modified with a lentivirus construct expressing a membrane protein (Lamp2b) fused with ischemic myocardium-targeting peptide CSTSMLKAC[148]. Although no absolute quantification was provided for the accumulation of EVs in the heart, the fluorescence imaging results indicate a higher accumulation of the peptide-modified EVs relatively to EVs without surface modification. As an alternative, many laboratories adopted the surface modification of EVs by chemical approaches[22,110], mainly by following two strategies: (i) physical incorporation of lipids modified with proteins (e.g. streptavidin)[126] or peptides[147] into the membrane of the EVs and (ii) chemical incorporation of linkers to functional groups (carboxylic[111] or amine[22,110] groups) present at the surface of EVs to which peptides are subsequently reacted by a copper-free click chemistry. These reactions can be performed in aqueous solutions, are rapid, selective and very efficient as compared to conventional bioconjugation schemes. In both strategies, the epitopes selected for targeting ischemic regions or a given cell type of interest were: (i) a cyclic RGD peptide with high affinity to integrin αvβ3 highly expressed in brain endothelial cells after an ischemic event[110], (ii) an ischemia-targeting peptide[22,126,147,148] and (iii) a cardiomyocyte-specific peptide[149]. According to one study, introduction of approximately 263 copies of the targeting peptide per exosome has been achieved[110]. Although in many reports, no absolute quantification of EV accumulation was performed, the results showed that the peptide-modifications

369 can increase the EVs tropism to ischemic regions, including the brain[110] or the heart[22,147-150], and
370 induce a higher therapeutic effect[22,147]. For example, the EF of mice hearts after MI, treated with EVs
371 modified with a myocardium-targeting peptide was approximately 46% while in mice treated with
372 scramble peptide-modified EVs was approximately 38%[22]. Similar improvements were reported by
373 others[147,148]. Both genetic modification of the EV-secreting cell and surface modification of EVs by
374 chemical approaches have pros and cons. The genetic approach may allow for a more standardized
375 product which is desirable to address regulatory expectations. However, this strategy has several
376 limitations including (i) changes in the biological activity of the EV as a consequence of the genetic
377 manipulation and (ii) difficulty to control the density of the targeting epitope in the surface of EVs as
378 well as to control their glycosylation state. The chemical approach may offer an effective control of
379 EV surface modification both in content (e.g. to include non-natural aminoacids to prevent peptide
380 degradation) and density (number of epitopes per surface area of EV) of the targeting epitope,
381 regardless of their cell of origin. The chemical approach may be performed during the purification
382 steps of EVs and thus amenable for clinical translation.
383
384 **4.4-Modulation of EV uptake and intracellular trafficking**
385 The internalization and intracellular trafficking of EVs can be studied using fluorescence
386 imaging techniques and labelled EVs[154,155]. The cellular internalization of EVs seems to be influenced
387 by both the interaction between EVs and the cell membrane[26,155] and the endocytic capacity of the
388 acceptor cell[71,156]. Internalization of EVs may be mediated either by non-specific interactions,
389 particularly endocytic processes such as macropinocytosis and micropinocytosis[51], or by specific
390 interactions, such as receptor-dependent pathways (in the case of peptide-modified EVs, see section
391 above). Little is known about the differences in the endocytic capacity of the cells, the impact of those
392 EV surface modifications in the intracellular trafficking and which EV surface modifications may
393 improve endolysosomal escape. Yet, these issues are critical because a large proportion of

internalized EVs are processed in the endolysosomal pathway and ultimately degraded in the lysosome[51,157]. Indeed, approximately 60% of internalized EVs colocalize with lysosomes after 48 h of contact[155].

Strategies have been proposed to enhance the endolysosomal escape of EVs. In one case, EVs have been coated with a combination of cationic lipids and pH-sensitive fusogenic peptides which enhanced the disruption of the endolysosomal membrane leading to the efficient cytosolic release of the EV cargo[158]. In another case, EVs have been coated with arginine-rich cell-penetrating peptides to induce active micropinocytosis and a more efficient release of EVs to the cell cytoplasm[159,160].

4.5-Modulation of EV delivery

Local administration of EVs at the injured site has been shown to increase the chance of cell targeting and uptake by the cells of interest. In some cases, EVs are washed out or taken up by non-relevant neighbouring cells. Interestingly, differential uptake of EVs by different cell types in an ischemic hind limb has been reported[67]. Local accumulation of EVs after few hours following intramyocardial, intramuscular or topical administration in an infarcted pig heart[21], an ischemic limb tissue[67] or in a mouse would healing model[71], respectively, has also been demonstrated. Several biomaterial-based strategies have been developed to engineer EV presentation by sustained the release of EVs in the injured site including hydrogels based in hyaluronic acid[71,161,162], alginate[163,164], chitosan[165,166], collagen[72] and amphiphilic peptides[167] (**Table 3**). Selection of hydrogel composition took in consideration its biomedical history, degradation, in situ jellification profile, mechanical and release properties. The EVs were incorporated in the hydrogels by several means. In the first case, EVs were mixed with a polymer solution without involving the reaction of both entities[72,161,162,164,166,167]. The solution was then injected in the tissue of interest and physically or chemically crosslinked in a few minutes retaining the EVs within the polymeric structure. In the second case, the EVs were mixed with a polymer solution for formation of a polymer-EV conjugate

and initiate the chemical crosslinking process[71]. Then, the solution was administered in the tissue of interest for further crosslinking and formation of a hydrogel. In the third case, the EVs were physically incorporated in the hydrogel after its polymerization[168]. In this case the hydrogel was already formed and the pores were large enough to allow the diffusion of EVs within the polymeric structure. It has been demonstrated that a 7 mm hydrogel patch could retain up to 3×10^{10} EVs and released the EVs for more than 7 days after implantation in the heart[72]. The hydrogel-releasing EVs significantly improved the activity of injured tissues relatively to EVs administered without a sustained release system. For example, in two similar studies, the EF of infarcted rat hearts treated with a hydrogel patch containing EVs was 40%[72] and 25% higher than control[161]. Importantly, the kinetics of EV release from the hydrogel seems to play an important role in their therapeutic effect[71]. For example, the slow release of EVs from hydrogels implanted in skin mouse wounds was not as effective as the coordinated release of EVs during skin regenerative process using remotely triggerable hydrogels[71].

5-Conclusions and perspectives

In the last decade, significant progress was made in understanding the biology of EVs as well as their application in the cardiovascular arena. We now have a better understanding of the composition of EVs[28,57], the role of EVs in the communication between cells of the same or different tissues in the body[14,17,47,96] and how the content of EVs secreted by stem/progenitor cells or other cardiac populations is affected by disease[17,102]. Regarding EV applicability, substantial advances have been made in the: (i) therapeutic effect of EVs in pre-clinical models of several cardiovascular diseases such as MI[21,84], skin wound healing[71,165], hindlimb ischemia[67] and stroke[110], (ii) how the therapeutic effect of EVs compares to the one obtained from the transplantation of stem/progenitor cells[80,161] and (iii) how to enhance the therapeutic effect of EVs by increasing the stability and targeting to a specific location, by enriching their therapeutic content, by improving their internalization and intracellular trafficking and controlling their spatial and temporal release from

444 within biomaterials (**Fig. 3**). As consequence of these progresses, there are 2 observational and 2
445 interventional clinical trials actively running. In the observational clinical studies, changes in
446 epicardial fat-contained EVs in patients with atrial fibrillation (NCT03478410) and the miRNA
447 expression profile in peripheral blood exosomes of patients with MI (NCT04127591) will be
448 investigated. In the interventional clinical studies, the therapeutic effect of exosomes on cutaneous
449 wound healing (NCT02565264) and in patients with acute ischemic stroke (NCT03384433) will be
450 evaluated.

451 Further translation of EVs as potential therapies will require further advances in the
452 implementation of good practices related to EV separation and characterization, which implies the
453 use of guidelines to track and organize data on EV separation and characterization, reference materials
454 for normalization or calibration and inter-laboratory validation and reproducibility studies[169]. Some
455 of these guidelines have been introduced in the last years by the scientific community[7,170]. In addition,
456 further technological progresses will be needed to overcome the challenges related to the purification
457 and characterization of EVs[28,57]. For further clinical translation of EVs in the cardiovascular area,
458 standard EV sources are needed to obtain EVs for therapeutic efficacy. EVs may be harvested from
459 autologous (i.e. donor biologic fluids or harvested cells) or exogenous sources (i.e. allogeneic
460 biological fluids or cell lines). Autologous EVs have the advantage of immuno-compatibility;
461 however, these EVs cannot be harvested on demand, may have reduced/unpredictable bioactivity
462 based on, for example, existing comorbidities and/or age of the donor and are much harder to
463 standardize as a clinical product. EVs produced from an exogenous source have the advantage of
464 being easier to standardize and store in larger quantities. Additionally, they may be standardized for
465 any given application and modified either by pre- or post-isolation modifications. However, for
466 clinical translation, further progresses are needed to optimize the administration route and the dosage
467 regimen. A clinical study in a human has shown that 10^{10}-10^{11} EVs was an effective therapeutic
468 dosage to treat one patient with graft-versus-host disease, with multiple administrations of increasing

amounts[171]. This dose was estimated based on therapeutic dosages of transplanted MSCs for similar purposes (~10^7-10^8 MSCs); however, other studies indicate that clinical application of EVs may require > 10^{14} particles per dose[172]. High concentrations of EVs will require the use of bioreactors for cell culture. For example, the culture of 10^8 MSCs for 2 weeks can generate approximately 10^{12} EV particles[173].

Overall, targeting technologies that increase EV accumulation in the cardiovascular system and thus decreasing the required dosage, and strategies to enrich EV content for specific biomolecules may be the key to unlocking their use in the clinical setting. This may be ensured by coupling tracking technologies, in order to mechanistically understand the biodistribution of EVs. Moreover, loading of EVs with exogenous molecules and controlling their *in vivo* delivery kinetics opens a number of opportunities to enhance EV bioactivity. Ultimately, engineered EVs represent a promising translational cell-free, robust and customizable platform to improve the outcomes in cardiovascular diseases.

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7-Author contributions

All authors contributed equally to all aspects of the article (researching data for article, substantial contribution to discussion of content, writing, review/editing of manuscript before submission).

8-Conflicts of Interest

The authors declare no competing interests.

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Glossary: Post isolation EV loading methods

Electroporation is a transfection method in which biological membranes are permeabilized upon exposure to an electrical pulse.

Heat-shock is a transformation technique reliant on heat to induce membrane permeabilization.

Sonication is the use of ultrasound technology to physically disrupt biological membranes and facilitate exogenous compound entry.

Passive loading is a strategy which relies on passive diffusion or complexation of a molecule with a cell or organelle. It may rely on a number of factors such as pH, osmotic pressure, electric charge or hydrophobicity.

Liposome fusion is the conjugation of a lipidic material with a liposome formulation.

Display Items

Box 1. Advantages and limitations of EVs in cardiovascular therapeutics

The use of EVs in cardiac regeneration was preceded by extensive research in cell therapies. As non-living entities, EVs overcame a major limitation of cell therapies, namely the poor survival after implantation. Of note, methods that were used for cell engineering can now be efficiently used to modulate EVs. Additionally, given the hostile environment of the injured myocardium, poor engraftment hindered clinical success. While EVs must contend with the low infiltration and endocytic rates of the heart, they are capable of overcoming the poor engraftment by being internalized directly by the recipient cells. Furthermore, adverse immune responses are associated with cell therapies, in particular when using allogeneic cells. This effect may be ameliorated, absent or even beneficially modulated by EVs, depending on their source and/or modulation strategy. The faster EV clearance, compared with their cellular counterparts, can be seen as a safeguard mechanism whilst an effective targeting of EVs to the tissue of interest pave the way to their therapeutic success. The limitations of EVs in cardiovascular therapeutics are related to the lack of tools for efficiently target the injured myocardium which limits their clinical use. Production and standardization of analytical parameters (i.e. storage, isolation and purification procedures) are some of the challenges that the field needs to overcome in order to translate EV therapies from the bench to the bedside. Since the physic-chemical properties of EVs are related with their cell/tissue/fluid of origin, the heterogeneity present in the source will be reflected in EV composition. Thus, methods capable of reducing the heterogeneity pre- as well as post-isolation are needed.

Figure 1. Limitations and challenges in the use of native EVs as cardiovascular therapies. While EVs have been shown to elicit functional responses on target cells, their potential is limited in several regards, including bioactivity, tracking, internalization and targeting. Therefore, several challenges should be addressed to harness the full potential of EVs. **(a) Bioactivity**. EVs must elicit a bioactive response in their target tissue/organ, which requires that EV cocktail of factors is potent enough to have therapeutic value. Yet, native EVs are limited by the nature of their content being intrinsically dependent on their donor cell features. **(b) Tracking**. The tracking of EVs is necessary to monitor their *in vivo* biodistribution and targeting. Yet, without modifications, EVs are only detectable after a biopsy is removed, limiting their monitorization. **(c) Targeting**. EVs should interact with specific cells and deliver their content. Although native EVs have some tropism, depending on the epitopes expressed by their donor cell, the process is limited. **(d) Internalization**. EVs should be taken up by target cells and deliver their cargo in the cell cytoplasm. Yet, native EVs have reduced endolysosomal escape and thus the ability to release their content in the cell cytoplasm.

Figure 2. Modulation of EVs for cardiovascular therapies. Several strategies have been used to overcome limitations inherent to native EVs. These modifications can be categorized, from a technical standpoint, depending on whether they were performed on the donor cell prior to EV secretion, or after the purification of EVs from the medium or fluid of origin. From a biological standpoint, these modifications may pertain to the membrane or lumen of the EVs. In the cardiovascular context, several approaches have been used to modulate EV bioactivity, cell internalization and targeting as well as to allow EV tracking. **(a) Bioactivity**. To enhance the therapeutic potential of EVs, functionalization of the membrane and the lumen have been used. **(b) Tracking.** To track *in vivo* EVs, these formulations have been labelled with fluorophores, luminescence reporters or radiotracers. **(c) Targeting.** To enhance the targeting efficacy of EVs, they have been modified with exogenous peptides, proteins or lipids. **(d) Internalization**. To enhance EV internalization and endolysosomal escape, the vesicles have been modified with cationic lipids, pH-sensitive peptides and cell-penetrating peptides.

Figure 3. Examples of EV modulation strategies for cardiac therapies. Both native or modulated EVs have been used as cardiac therapies. The modulated EVs were enriched in therapeutically relevant compounds such as miRNAs, proteins and small molecules, but also were decorated with surface epitopes that improved their cardiac targeting and pharmacokinetics. EVs have been administered in animal models either locally or systemically. In the local administration, biomaterial-based delivery systems have been used to control the release of EVs, as well as to act as a supporting scaffold for tissue regeneration. Therapies based in native or modulated EVs have translated into improved cardiac function mediated by a decrease in inflammation, cardiomyocyte death, fibrosis and infarct size, and increased angiogenesis.

Table 1. Modulation of EV bioactivity for cardiovascular applications.

Engineering	Source	EV type	Methodology	Animal model	*In vivo* outcome	Ref.
Modulation of EVs bioactivity: EV-secreting cell modulation	hAFSs	sEVs	Hypoxic preconditioning	Mouse MI	Improved cardiac repair	[174]
	hMSCs	sEVs + MVs	Hypoxic preconditioning	Rat MI	Improved angiogenesis and cardiac function and reduced infarct size	[80]
	rCPCs	sEVs	Hypoxic preconditioning	Rat MI	Improved cardiac function and reduced infarct size	[88]
	rCM	sEVs	Hypoxic preconditioning	Mouse MI	Improved angiogenesis	[96]
	hMNCs	sEVs	Hypoxic preconditioning	Mouse diabetic wounds	Improved angiogenesis and healing	[71]
	hASCs	MVs	Endothelial medium conditioning	Mouse subcutaneous Matrigel plug	Improved angiogenesis	[122]
	rMSCs	sEVs	GATA-4 overexpression	Rat MI	Restored cardiac contractile function and reduced infarct size	[77]
	hMSCs	sEVs	Akt overexpression	Chick allantoic membrane	Improved angiogenesis	[78]
	MsCs	sEVs	Fluorescent miR-181a overexpression	Mouse MI	Reduced infarct size, improved function, reduced inflammation	[23]
	hHEK293T cells	EVs	miRNA-146a overexpression	NA	NA	[117]
	hCDCs	sEVs	Y RNA overexpression	Rat MI	Reduced infarct size and CM apoptosis, reduced inflammation	[85]
Modulation of EVs bioactivity: post-isolation methods	hBlood	sEVs	EV loading with microRNA: ExoFect	Mouse MI	Reduced fibrosis	[141]
	mCPCs	sEVs	EV loading with microRNA: Electroporation	Mouse MI	Improved angiogenesis	[142]
	hMSCs	sEVs	EV loading with microRNA: Electroporation	Rat MI	Reduced fibrosis and improved cardiac function	[118]

AFCs (human amniotic fluid stem cells), ASCs (Adipose-derived stem cells), CSCs (Cardiac stromal cells), UMSCs (umbilical mesenchymal stem cells), MSCs (Mesenchymal stem cells), MsCs (mesenchymal stromal cells), CDCs (Cardiosphere-derived cells), CPCs (Cardiac progenitor cells), hMNCs (human mononuclear cells) and CM (cardiomyocytes). "h" stands for human, "r" for rat and "m" for mouse.

968 **Table 2. Modulation of EV biodistribution and targeting.**

Engineering	Source	EV type	Methodology	Model	Outcome	Ref.
Chemical modification of EV membrane with peptides/proteins	mMSCs	sEVs	Membrane modification with peptide (chemical conjugation)	Middle cerebral artery occlusion mice model	Suppression of the inflammatory response and cellular apoptosis in the lesion region	[110]
	mMSCs	sEVs	Membrane modification with peptide (chemical conjugation)	Mouse MI	Reduction of fibrosis and CM apoptosis and improvement of cardiac function	[22]
	hCDCs	sEVs	Membrane modification with peptide (chemical conjugation)	Rat MI	Reduction of fibrosis and CM apoptosis and improvement of cardiac function	[147]
	hCDCs	sEVs	Membrane modification with modified lipid	Rat MI	Improved targeting to the heart	[126]
EV-secreting cell is genetically or chemically modified to express a peptide/protein	mMSCs	sEVs	Membrane modification with peptide (genetic approach)	Mouse MI	Suppression of the inflammatory response, improved angiogenesis, reduction of myocardial apoptosis, improvement of cardiac function	[148]
	hHEK-293T	sEVs	Membrane modification with peptide (genetic approach)	Mouse MI	Improved targeting to the heart	[150]
	hCDCs	sEVs	Membrane modification with peptide (genetic approach)	Mouse MI	Improved targeting to the heart	[149]
	h562 cells	sEVs	Membrane modification with modified lipid	Vascular targeting in zebrafish	Improved targeting to the vasculature	[151]

969 MSCs (Mesenchymal stem cells), CDCs (Cardiosphere-derived cells), CPCs (Cardiac progenitor cells), hMNCs (human
970 mononuclear cells) and CM (cardiomyocytes). “h” stands for human, “r” for rat and “m” for mouse.
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Table 3. Modulation of EV delivery.

Source	EV type	Methodology	Model	Outcome	Ref.
rEPCs	sEVs	Injectable hyaluronic acid-based hydrogel	Rat MI	Improvement in angiogenesis and cardiac function, decrease in infarct size	[110]
rEPCs	sEVs	Injectable hyaluronic acid-based hydrogel	Rat MI	Improvement in hemodynamics, cardiac function and decrease in infarct size	[162]
hiPSC-derived CMs	sEVs	Encapsulation of EVs in collagen gel followed by its transplantation	Rat MI	Improvement in cardiac function, decrease in CM apoptosis, reduction in infarct size and cell hypertrophy	[72]
hMSCs	sEVs	Injectable peptide amphiphile hydrogel	Rat MI	Improvement in cardiac function, reducing inflammation, fibrosis and apoptosis, enhancement of angiogenesis	[167]
rMSCs	sEVs	Injectable alginate gel	Rat MI	Decrease in cardiac cell apoptosis, improvement in angiogenesis, improved cardiac function	[164]
hPRP	sEVs	Encapsulation of EVs in alginate gel followed by its transplantation	Skin wound healing diabetic rat model	Improved angiogenesis and re-epithelization	[163]
hMSC	sEVs	Encapsulation of EVs in chitosan gel followed by its transplantation	Skin wound healing diabetic rat model	Improved angiogenesis and re-epithelization	[165]
hMNCs	sEVs	Injectable hyaluronic acid-based hydrogel	Skin wound healing diabetic mouse model	Improved angiogenesis and re-epithelization	[71]
hMSCs	sEVs	Injectable chitosan-based gel	Mouse hindlimb ischemia model	Increased angiogenesis in ischemic hindlimbs and high limb salvage	[166]

MSCs (Mesenchymal stem cells), hMNs (human mononuclear cells), EPC (endothelial progenitor cell) and PRP (human platelet-rich plasma). "h" stands for human, "r" for rat and "m" for mouse.