Extracellular Vesicles and Biomaterial Design: New Therapies for Cardiac Repair.

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Abstract:

There is increasing evidence that extracellular vesicles (EV) mediate the paracrine effects of stem cells. While they feature several attractive characteristics, they also raise issues related to delivery. For patients with a cardiac disease requiring a surgical procedure, direct intramyocardial administration of EV is straightforward but its efficacy may be limited by a fast wash-out, hence the interest of incorporating EV in a control-release polymer to optimize their residence time. For patients without surgical indication, the intravenous (IV) route is attractive because of its lack of invasiveness; however, the issue here is a whole-body distribution limiting the fraction of EV reaching the heart, hence the likely benefits of engineering them to increase their homing towards the target tissue.

Therapeutic potential of extracellular vesicles in cardiovascular diseases

Since the 2000s, therapeutic progress, in particular in the management of risk-factors and patient care, has permitted to reduce steadily the prevalence of myocardial infarctions and the related mortality [1]. However, improved survival rates after acute cardiovascular insults and rising life expectancy lead to an increased number of patients who develop heart failure (HF)[2]. For those who have exhausted conventional pharmacological treatments, mechanical assist devices and organ transplantation are not readily available options because of their complexity and the organ shortage worldwide. Over the past decades, scientists and clinicians from different fields have embarked on novel strategies for repairing, not to say regenerating, the functional tissue that has been lost[3].

In this context, the use of stem cells has emerged as a possible option for treating a wide variety of diseases for which unmet medical needs persist. Whereas the first postulated mechanism of action was that the grafted cells would be reparative by replacing the damaged ones of the diseased tissue, it soon became evident that it was unlikely to be the case since a functional benefit was often observed despite the lack of a sustained cell engraftment. This has raised an alternate mechanistic hypothesis based on **paracrine signaling** (see Glossary) whereby factors released by the transplanted cells harness endogenous repair pathways [4]. Many of these biologics are packaged in extracellular vesicles (EV; Box 1) which are gaining a growing interest because of their therapeutic potential in HF through mechanisms that can encompass systemic modulation of inflammation and/or direct site-specific effects.

The first use of EV for treating cardiac diseases goes back to several years when Brill *et al*. reported an improved revascularization of ischemic myocardium after injections of human platelet-derived microparticles [5]. Since then, there has been ample evidence that the EV released by mesenchymal stromal cells (MSC) or cardiac-committed cells (from adult or pluripotent stem cell sources) recapitulate the protective effects of their parental cells through the activation of signaling pathways in the recipient myocardium; this can translate into a stimulation of angiogenesis and a mitigation of inflammation, fibrosis and apoptosis while the re-induction of host cardiomyocyte proliferation remains much more debatable [6]. Put together, these events could account for the cardio-reparative effects of the cellular secretomes. This has led some investigators to move away from the transplantation of cells and to rather leverage their paracrine effects through the exclusive delivery of this

secretome which, from a clinical standpoint, features several advantages: its large-scale production is more akin to a pharma-type model; it can be cryofrozen without loss of efficacy and is thus available on-demand [7]; and it may not be immunogenic, depending on the source cells. For example, EV from dendritic cells can activate cognate T cells [8] and participate to rejection of allogeneic tissues and organs [9] whereas those derived from cardiovascular progenitor cells seem to be immunologically neutral [10]. However, the clinical use of these EV-enriched secretomes, although already implemented in the context of controlled trials, still raises translational issues, primarily the selection of the parental cells, the method and extent of purification of their conditioned medium and the characterization of the components of the final cargo. The discussion of these issues is beyond the scope of this review which will rather focus on another highly clinically relevant issue which is that of *delivery*. Here, from a clinical perspective, two distinct situations can be considered depending on whether the patient requires a surgical procedure or not as each of these settings has a direct impact on the delivery modalities (Figure 1).

This review will discuss the opportunity given by **biomaterials** for the controlled release of EV in the target tissue with a focus on their use in the specific context of heart repair. These novel approaches relying on engineering technologies could potentiate the therapeutic effects of EVs. Although these effects could be provided by EVs from plasma or adipose tissue, this review will concentrate on EVs collected from cell culture media which in the context of heart diseases have been the most extensively studied.

Surgical applications: Direct intra-myocardial delivery

One-shot uncontrolled delivery

Anytime the heart is directly accessible, the most straightforward approach is obviously the direct intramyocardial (IM) delivery of EV and this would expectedly be the method of choice in patients requiring an open-chest operation for a valvular or coronary procedure. Furthermore, because repeat dosing may potentiate the therapeutic effects of cells or their secreted factors [11], a direct access to the heart could also provide the opportunity of delivering an epicardial reservoir connected by an indwelling catheter to a subcutaneous pocket which can be periodically refilled with cells or cell products [12]. So far, however, this technique has only been tested experimentally and both its clinical feasibility and safety still need to be validated.

Currently, the direct IM injection of EV is the procedure which has been the most commonly used in preclinical studies, as shown in Table 1 which non exhaustively illustrates the diversity of parental cells used for heart repair. Its advantages are that it allows choosing precisely the injection site and to not disrupt the surrounding vasculature [13]. Of note, the high mortality rate associated with repeated open-chest procedures in rodents results in that most of these studies have entailed the EV injection immediately after the ischemic insult. This timing is clearly not relevant to chronic HF but the issue can be addressed by transcutaneous echo-guided IM injections which, because of their limited invasiveness, yield an excellent survival record[14,15].

However, the efficacy of IM injections is hampered by a varying degree of mechanical leakage of the injectate, particularly if the heart is beating. This issue can be partly overcome by some tips and tricks such as use of a screw needle or occlusion of the needle track entry site by glue or sutures[16,17]. However, these maneuvers still do not allow to accurately control the distribution of the secretome and therefore delivery supports are eagerly needed to provide its controlled release and expectedly optimize its therapeutic benefits through a prolonged exposure time. This objective can be reached by functionalization of biomaterials.

Basic principles of time-controlled delivery systems

Since decades time-controlled delivery systems are used to optimize the resulting concentrations of active agents at their sites of action in the living body, assuring improved therapeutic efficacies and safeties of many drug treatments[18,19]. Often, the active agent is physically trapped within a macromolecular network, avoiding its immediate release upon administration[20]. Once in contact with aqueous body fluids, the drug or EV “have to find its way” out of the polymeric matrix. Different physicochemical phenomena can be involved in the control of the resulting release rate, in particular diffusion, dissolution, degradation and swelling[21]. The relative importance of these processes strongly depends on the type of active agent and polymer as well as on the exact composition of the system. While the size of the drug molecule/EV can play a major role in the resulting release kinetics, the underlying physicochemical principles are the same. For example, diffusion can be decisive for the transport of liposomes in hyaluronic acid based hydrogels (liposomes are artificial vesicles and exhibit sizes which are in a similar range as those of EV)[22].

Diffusional mass transport in controlled delivery systems is generally caused by the thermal motion of molecules in a liquid. The diffusing compound is dissolved in this liquid (e.g. is present in the form of individual molecules or ions) and diffuses from regions of higher concentration towards regions of lower concentration. In the case of a controlled drug delivery system, the region of higher concentration is the system itself or parts of it and the region of lower concentration is the surrounding environment (e.g., heart tissue). The rate at which this spontaneous diffusional mass transport occurs depends on the difference in drug concentration, the distance to be overcome and the mobility of the active agent in the delivery system[23]. If a polymeric network is used to trap the drug or EV, the mobility of the latter often depends on its size and eventually on the size of the meshes of the macromolecular network as well as on potential interactions between the diffusing compound and the polymer. Furthermore, the macromolecular network might dissolve with time: If the polymer chains are only physically entangled, and if they are water-soluble, they slowly disentangle from the network. Consequently, the latter shrinks and finally disappears[24]. Certain polymers are also degraded with time into smaller fragments, which dissolve and diffuse away[25]. In both cases (polymer dissolution and polymer degradation), the consequence for the embedded drug or EV is that it is released because it is no more trapped. Another phenomenon that might be used to control the release of a compound, which is trapped within a macromolecular matrix is swelling: In this case, the polymer takes up substantial amounts of water upon contact with aqueous body fluids. This generally leads to increased mobilities of the macromolecules and of the drug molecules, which more rapidly diffuse out of the system[26].

The controlled drug delivery system can be either pre-formed (e.g., a patch), or might be formed upon injection of a liquid into the living organism[27]. In the latter case, a specific triggering mechanism induces the phase transition “liquid to solid” or “liquid to semi-solid”. For example, a change in temperature (from room temperature to body temperature) can induce such a phase transition in certain polymer solutions, which become gels. Other polymer-water mixtures are semi-solid gels at rest, and liquify upon exposure to mechanical stress, e.g. shearing (temporarily destroying the three-dimensional macromolecular network). Thus, the system can be injected as a liquid because it is sheared when it passes through the needle of a syringe, and becomes a (semi-)solid gel at the side of administration under rest. These types of systems are also called *in-situ* forming gels.

A variety of biomaterials can be used to effectively trap EV and control their release rates, as described in more detail in the following.

General characteristics of EV-functionalized biomaterials

To avoid rapid EV wash-out, their possible off-target effects and, at the end, a loss of efficacy, different biomaterials have been developed to encapsulate them and ensure their controlled release in the target myocardium. This approach has actually leveraged the already well-established ability of scaffolds to control spatially and temporally the distribution of stem cells or stem cell-derived biologics such as growth factors or miRNAs [28]. For a complete review of biomaterials see the the review of Sepantafar *et al.*[29].

Biomaterials can be broadly categorized as natural (alginate, collagen, hyaluronic acid, chitosan, fibrin, decellularized extracellular-matrix) or synthetic (polyethylene glycol, polyurethane, N-isopropylacrylamide, to name a few). Both have advantages and drawbacks: natural materials are more biomimetic and biocompatible; conversely, synthetic materials are more easily tunable and show a higher batch-to-batch reproducibility[30,31].

Several other parameters described in Figure 2 such as mechanical (stiffness, **viscoelasticity**), structural (porosity, surface topography) and biological properties (biocompatibility, signaling cues) are specific for a given material and govern the release rate of the encapsulated active compounds as well as the interactions between the implanted biomaterial and its microenvironment. Regarding these interactions, the major concern also shared by scaffolds for cell-based therapy is that the biomaterial must not impair the biologics integrity (this will be further examined in the part "*methodological challenges*"). Parameters depicted in Figure 2 also impact more practical aspects that must not be neglected in the perspective of clinical applications such as product manufacturing, sterilization, storage, stability and administration modalities. The latter depend on the form of the biomaterial. If it features **shear thinning** properties or is able to gel *in situ* following a thermal or ionic stimulus, it can be intramyocardially injected[32,33]. Alternatively, biomaterials can be epicardially delivered as a patch provided that they are endowed with mechanical characteristics compatible with manipulations and eventually suturing [34]. Beginning at the design stage of the biomaterial, it is thus important to define its administration as well as processing modalities since the latter will strongly impact the cost, risk and feasibility of the procedure. Indeed, if the EV are embedded within the biomaterial

extemporaneously, i.e., right before its use, the method (dispersion, soaking or mixing) must be adaptable to the clinics, that is, simple, fast and safe enough to guarantee sterility.

Some studies have even shown that biomaterials are efficient for cardiac repair when administered alone. This is the case for an extracellular matrix-based hydrogel derived from decellularized porcine myocardium (Ventrigel®) which has been investigated in pre-clinical and clinical studies and displayed an attenuation of negative cardiac remodeling [35,36] However, successful outcomes of biomaterial-alone-based therapies have been inconsistent, as exemplified by the injectable calcium alginate hydrogel Algisyl® which only yielded mixed functional results[37,38]. These suboptimal results encourage to functionalize biomaterials with EV to protect the latter from rapid wash-out and clearance [39] and take advantage of the distinct and respective bioactivities of the cellular secretome and its vehicle.

Applications of EV-loaded biomaterials

EV-functionalized biomaterials have thus been actively studied during the last years for both cardiac and non-cardiac applications (Table 2).

For cardiac applications, materials used are mostly natural with the exceptions of an hybrid hydrogel composed of gelatin and synthetic nanoclays (Laponite®)[40]. This secretome-loaded injectable hydrogel is charged and structured in a way that allows to modulate the release of embedded EV through electrostatic interactions and to impart a **thixotropic** behavior of the gel (the viscosity of a “thixotropic” system decreases with time upon stress). In a rat model of myocardial infarction, this EV-loaded biomaterial successfully increased angiogenesis and heart function while reducing infarct size. The importance of using an hydrogel as a delivery vehicle is evidenced by the finding of better post-injury cardiac function parameters in animals injected with the secretome-loaded nanocomposite hydrogel compared with those receiving injections of the secretome solution alone. In keeping with these data, mesenchymal stromal cell-derived EV encapsulated in an alginate hydrogel feature a longer retention time than EV injected in a saline solution and this extended EV release was paralleled by an improvement in post-infarction functional and histological markers of cardiac recovery.[41] The ability of a collagen patch loaded with induced pluripotent stem cells-derived EV to preserve infarcted rat hearts from declining myocardial function was also documented, with the caveat that in this study the presumed

benefits of the patch-based approach could not be conclusively established because of the lack of a true control entailing injections of EV alone[34].

Among the various materials that can be considered as platforms for EV controlled delivery, hyaluronic acid (HA), presented in Box 2, is particularly attractive because of its bioactivity, which has been widely demonstrated, and tunability as its physical properties or half-life can be adjusted by straightforward modifications of molar mass or chemical functionalization[42,43]. In fact, a HA-based hydrogel without any additional therapeutic product has yet demonstrated robust regenerative abilities in a chronic **myocardial infarction model**[44]. Extracellular vesicles embedded in a combination of lyophilized polymers of adamantane- and β-cyclodextrin-modified HA were also shown more efficient that if they were simply injected in suspension in a myocardial infarction model[45]. Like in the studies mentioned above, these benefits were reflected by an increase in peri-infarct vascularization, decrease of adverse remodeling and improvement of function.

Methodological challenges

The use of a biomaterial as a vehicle for the cell-derived secretome, or more specifically its exosomal fraction, requires extensive characterization related to both the support material (mechanical properties, degradability) and the loaded EV (structure, bioactivity, spatial distribution), as illustrated in Figure 3.

A key and yet unsettled issue is the optimal period during which EV should be released for inducing a physiologically relevant cardio-protective effect. Literature reports indicate period varying from 2 days to 3 weeks. Regardless of the duration, a thorough assessment of the suitability of a given biomaterial to serve as an EV vehicle requires the use of tools allowing to both reliably quantify the number of EV released over time and ensure that their bioactivity has not been altered. *In vitro,* quantification of release kinetics can be achieved by a variety of techniques such as **Nanoparticle Tracking Analysis** (NTA), **Resistive Pulse Sensing** (qNano), protein content assays (BicinChoninic Acid assay BCA and Bradford assays) or flow cytometry on EV labeled with organic fluorescent dyes (DiD, DiR, PKH26) [46]. EV released from a chitosan hydrogel were also monitored by **bioluminescence imaging** (BLI) following the parental cell transfection with a Gaussia luciferase lactadherin fusion protein report system [47]. A cautionary note should be expressed about the interpretation of NTA and qNano results which yield data on number and size distribution of particles which are

not necessarily EV. Some of these particles can represent material end-degradation products, thereby making mandatory control experiments with the biomaterial alone to reflect the background noise. Furthermore, these methods do not detect EV smaller than 60 nm, which may represent a large proportion of the secretome [48]. Data collected from these techniques can also be confounded by aggregation of EV, a phenomenon which has been highlighted in studies of the impact of isolation or storage on EV and is well-known in "synthetic vesicles" or liposomes that share important physicochemical features with EV[49–51]. This aggregation can be confirmed by imaging single particles with electron microscopy (EM) and, at best, by cryo-EM which can more accurately resolve lipid bilayers [52]. Care should also be taken in the interpretation of protein content assays which yield substantial differences among commonly available methods [53].

Even if *in vitro* studies are essential, physiological conditions *in vivo* are likely to heavily impact the release of EV, especially if natural polymers derived from ECM are used as they are more sensitive to native enzymatic activities. To confirm the sustained release of EV in the myocardium, Liu *et al.* imaged hearts 0, 4 and 7 days after the implantation of a patch loaded with DiL-labeled EV using a custom laser light sheet illumination platform [34]. The same strategy but a different dye (lipophilic PKH26) and fluorescence microscopy were used by Han *et al.* [54] for up to 21 days, while Lv *et al.* [41] compared the biodistribution of DiR-labeled EV in the heart and the other organs (lungs, liver, kidney, spleen) in a quantitative manner. In this study, the fluorescent signal emitted in the heart by EV embedded in an alginate hydrogel was significantly higher at 7 days in comparison with injections of free EV. This observation was paralleled by decreased cardiac cell apoptosis and inflammation, increase in angiogenesis and improved heart function, thereby identifying biomaterial-supported EV retention as a factor of better outcomes, even though these data need to be interpreted with caution because of the challenges of EV tracking *in vivo*. Namely, commonly used dyes are known for their prolonged half-life so that they can persist in tissues even if EV have already been degraded. Their aggregation might also induce a false signal, being similar to that generated by EV. Other techniques such as BLI or radiolabeling are more reliable but are limited by their availability and costs. Radiolabeling is possible with EV and is attractive because of its accurate live imaging but the relatively short half-life of the commonly used isotopes limits their use for long term biodistribution studies [55].

Even if EV are not subject to the engraftment and survival issues encountered with stem cells, their therapeutic potency is likely to rely on the preservation of their structural and biological integrity. Therefore, asides from the optimal duration of EV release, it is critical to assess the functional properties of the released EV which are likely to change over time. Surprisingly, only a few studies have characterized the dynamic profile of EV after their incorporation in a support material. Reports on MSC-derived EV released from a chitosan hydrogel demonstrated their stability through microRNA quantitation and dynamics of EV uptake by human umbilical vein endothelial cells (HUVEC)[47]. Rat CPC-derived EV bioactivity was also evaluated directly after their release from an extracellular matrix hydrogel by assessing their protective effect on H_2O_2-induced apoptosis of human CPC and stimulation of protein kinase-like endoplasmic reticulum kinase (pERK) expression in human coronary artery endothelial cells. The phosphorylation of ERK was actually reduced after 1 week of encapsulation, which could be explained by the lower amount of EV released after the first days as well as by EV degradation[39]. In another study, the bioactivity of CPC-EV released from a supramolecular ureidopyrimidinone hydrogel was checked through their ability to activate ERK signaling in endothelial cells. Results showed that this bioactivity was fully preserved after one week, but decreased after two weeks in comparison with fresh EV [56]. Thus, different end points are available but it is likely that in addition to standard measurements of the RNA and protein content of the EV, the most convincing evidence for the persistence of their bioactivity comes from potency tests like those which evaluate their pro-survival or angiogenic potential [57]. Of note, these assessments can be challenging because of the gradual release of EV which may render analytical procedures increasingly difficult to interpret given the small amount of EV collected at late time points. However, this characterization is even more crucial for biomaterials made of synthetic polymers because they require the use of strong organic solvents or toxic photo-initiators for fabrication and/or cross-linking; this results in the release of toxic monomers during their degradation, hence the importance of ensuring that this event does not impair EV bioactivity [58]. Of note, while this bioactivity can be tested *in vitro* by potency tests like those mentioned above, it is by far more challenging to assess EV function *in vivo* following their controlled release from a given biomaterial in myocardial tissue and it can then be acceptable to rather rely on surrogate markers of efficacy like functional end points and/or histological patterns of tissue damage in comparison with EV suspensions. In these studies, it

is critically important to include the appropriate controls, i.e., the EV-free biomaterial (and, at best, biomaterial-free EV suspensions) since the immune response triggered by the material can, by itself, exert cardio-protective effects [59].

These biomaterials are overall aiming at the same goal, i.e., the controlled release and the protection of EV in the myocardium to assure a prolonged therapeutic effect. Another approach, however, is to increase the cellular uptake of EV and improve their intracellular delivery by no longer using the biomaterial as a delivery platform, but rather as a specific tissue-targeting coating. For example, polysaccharide-based amphiphilic self-assembled nanogels (with ethylenediamine-modified cholesteryl pullulan) are able to coat EV thanks to hydrophobic interactions. The resulting nanogel/EV hybrid system was drastically more internalized and had more pronounced effects (neuron-like differentiation of human adipose derived stem cells) on cells than vesicles alone [60].

Non-surgical applications: catheter-based and intravenous delivery

Most patients suffering from heart failure do not however require a surgical procedure and are therefore not eligible to a direct-vision delivery of the secretome or its EV fraction. To address this issue, a flexible shape-memory patch has been developed which can be introduced in a folded form through a minimally invasive keyhole access and is then deployed over the surface of the heart [61]. Although this device has been shown not to compromise the viability of the loaded cells, its application to secretome delivery remains unsettled and consequently, for medically treated patients, the intravascular route looks the most straightforward. In this context, the only study which has entailed EV delivery through an intracoronary catheter in a pig model has shown a limited efficacy compared with endomyocardial injections as only the latter allowed a reduction in infarct size and a better preservation of function compared to the placebo group, both findings consistent with a higher myocardial retention of exosomes [62]. One possible explanation is the nanoscale size of EV which facilitates their quick wash-out in the bloodstream and an attendant low retention in the tissue in contrast to cells which can extravasate and thus better engraft, possibly through an “active vascular expulsion” mechanism[63,64]. However, even though in this study, direct IM injections were the most efficacious, their efficacy is still hindered by the squeezing of the myocardial fibers triggered by heart beats and which tend to expel part

of the injectate out of the target tissue akin to the well documented wash-out of cells, notwithstanding the invasiveness of endomyocardial cathether-based procedures [65].

These limitations highlight the potential interest of intravenous (IV) EV administrations.

Intravenous injection of unmodified EV for cardiac repair

IV injections of EV are clinically attractive since they are easy to implement, do not require dedicated facilities or highly trained staff and are much less invasive, which allows repeated dosing, the benefits of which have been previously documented [66].

Indeed, a positive outcome of IV delivered EV-enriched conditioned media has now been demonstrated across a wide variety of preclinical models of acute myocardial infarction, and nonischemic cardiomyopathies such as those associated with Duchenne muscular dystrophy or induced by chemotherapy (Table 3). Therapeutic benefits have also been reported in non-cardiac disease models such as brain injury or bronchopulmonary dysplasia, to name a few[67,68]. Conversely, in a porcine model of chronic myocardial ischemia, a comparative study failed to show any benefit of the IV delivery of MSC-derived EV compared to a direct intramyocardial injection but this negative outcome is difficult to interpret because of the small sample size (4 animals) and a possibly too low dosing.[69]

In the specific context of cardiac diseases, these results are intriguing since biodistribution studies have documented that only a limited amount of the injectate may reach the heart (Box 3). Importantly, asides from dosing, the cell source is an important factor influencing EV biodistribution patterns and calls attention to the interest of deriving them from cells phenotypically matched to those of the target tissue as EV seem to feature an organotropism which could facilitate their homing toward tissues sharing the same lineage as their parental cells [70]. Clearly, the cell source has a major influence on the therapeutic efficacy of the derived EV, as exemplified by the failure of fibroblast-derived EV to improve function compared with EV originating from cardiac cells [71,72] but more work still needs to be done to identify the most effective parental cells for a given target disease and ensure that privileging organotropism of the secreted EV will not compromise their therapeutic efficacy.

However, concerns about off-target effects and persisting uncertainties regarding EV organotropism have been a major incentive to develop techniques aimed at improving the cardiac targeting of EV with the premise that even though their primary mechanism of

action could be a shift of endogenous immune/inflammatory cells towards a tissue-reparative phenotype, a greater therapeutic benefit might still be achieved by increasing their direct homing to the target organ [73,74].

Improved cardiac targeting of EV

These techniques can be broadly divided into 3 main categories: genetic modification of the parental cells, direct engineering of the EV (i.e., modification of their surface, content or structure) and non-invasive physical techniques.

Genetic modification of parental cells. The first strategy developed is the genetic modification of the parental cells to endow their secreted EV with targeting capacities [75]. These genetic modifications allow restructuring transmembrane proteins to fuse with peptides or specific ligands. For specific heart targeting, lentivirus packaging of a recombinant plasmid has been used to modify the outer portion of lysosome-associated membrane protein 2 (Lamp2b), an abundant protein at the surface of EV, by its fusion with a cardiac-targeting peptide (APWHLSSQYSRT) [76] or a cardiomyocyte-specific peptide (WLSEAGPVVTVRALRGTGSW) [77]. The resulting EV were more efficiently internalized by cardiomyocytes *in vitro* and displayed improved cardiac retention in comparison with non-targeted EV *in vivo*. A similar pattern of improvement was shown after transfection of cardiac progenitor cells with CXCR4 and IV infusion of the resulting CXCR4-expressing EV [84]. *In vivo*, these EV improved heart function and reduced infarct size compared with their untreated counterparts in a murine model of ischemia/reperfusion while companion *ex vivo* experiments documented their more efficient delivery in Langendorff-perfused hearts. Thus, these genetic modifications can generate a wide array of tailored EV but their complex development and the lack of stability of fused peptides render this approach challenging and time-consuming [78].

Direct engineering of EV. Direct engineering of already isolated EV thus appears as a promising alternative. Peptides can be added on the surface of EV by several techniques such as click chemistry or integrin binding. Click chemistry or copper-catalyzed azide-alkyne cycloaddition permits to conjugate small molecules to EV's surfaces thanks to the formation of a triazole linkage between functionalized amine groups found on exosomal proteins (the

alkyne moiety) and an azide group [79]. Targeting a specific tissue using this technique has been reported with EV conjugated with a glioma-targeting peptide (neuropilin-1) [80]. This kind of reaction can thus allow the functionalization of EV with a cardiac-targeting peptide. Alternatively, the natural affinity between integrins and specific ligands can be leveraged to conjugate peptides to the surface of EV. For now, this approach has only been studied for an opposite objective with α3β1 integrin-binding peptide (LXY30) linked to EV derived from ovarian tumor cells [81]. The goal was actually to reduce EV uptake by a specific cell type. However, this work yet showed the possibility of influencing EV targeting by integrin-binding peptides. Identification of peptides that can anchor to EV could benefit from phage display, as demonstrated by docking of the peptide CP05 to EV via CD63, a tetraspanin enriched on the surface of EV, and its subsequent therapeutic benefits in a dystrophin-deficient mouse model [82].

Another technique of surface functionalization is also made possible by the lipid bilayer membrane structure of EV which allows the embedding of phospholipid agents. Once integrated, these agents act as an anchor for specific ligands or fluorescent molecules [83]. This method, easy to implement, has been developed for cardiac applications in ischemia/reperfusion models by coupling an ischemia-homing peptide to a modified glycerol-phospholipid-PEG conjugate (DMPE-PEG). The IV injection of EV secreted by cardiosphere-derived cells and modified by this technique was then shown to localize in greater amounts in the injured myocardium[84]. The same approach has been successfully used by adding the ischemia-homing peptide to another phospholipid agent (dioleoylphosphatidyl-ethanolamine N-hydroxysuccinimide or DOPE) in an ischemia-reperfusion-induced cardiomyopathy model [85]. This conjugation of EV with the homing peptide reduced cardiac fibrosis, increased angiogenesis and overall improved heart function compared with the control (PBS and scramble peptide-conjugated EV) groups. The phosphatidylserine binding domains of lactadherin which is exposed on EV surface was also exploited for the fusion with anti-EGFR nanobodies, which resulted in an enhanced uptake of EVs by EGFR-overexpressing tumor cells cells [86]. This approach may be applicable for fusion with other moieties endowed with organ-specific targeting properties.

Instead of peptide signaling, targeting can also be mediated by the **glycome** of EV. The glycosylation pattern is a crucial regulator of membrane-to-membrane interactions. Modified glycosylation by an enzyme that removes the terminal residue of sialic acid which

is involved in EV recognition by cells, results in an alteration of EV biodistribution and more specifically an increased EV accumulation in lungs [87]. Aside from surface modification, adjustment of EV content may also improve their organ-specific targeting. For example, in a doxorubicin-induced cardiotoxicity model, *in vivo* biodistribution of EV was altered by their loading with a siRNA against clathrin heavy chain which is involved in EV endocytosis by macrophages: EV uptake by macrophages in the spleen and liver was subsequently reduced [88].

Physical approaches. The third strategy for driving EV towards a given tissue is based on physical approaches with the premise that they can overcome difficulties raised by the stabilization of biological components. A technique, previously investigated for cell-based therapy but potentially applicable to EV, is magnetic targeting [89]. The proof of principle has been brought by experiments whereby loading iron-oxide nanoparticles into microvesicles allowed to manipulate their spatio-temporal distribution by a magnetic field gradient [90]. However, the drawback of this technique is that it still involves modifications of EV and the subsequent potential to alter their content and impair their function. This contrasts with the ultrasound-targeted microbubble destruction approach. This technique is based on the cavitation effect within the microvasculature of target tissues and could thus non-invasively enhance EV infiltration in these areas by increasing vessel permeability. Even if it has not been studied yet in a myocardial disease model, *in vivo* studies have shown an improved delivery of EV in the normal heart when their IV injection was combined with this ultrasound heart-targeted microbubble destruction [91]. So far targeted delivery of nanaoparticles has only yielded limited clinical success. However, the use of nanoparticle systems has primarily pertained to cancer therapeutics (reviewed in [92,93]) and the associated physiological and manufacturing challenges may not be directly relevant to delivery of EV whose therapeutic benefits might actually benefit from leveraging the convergence of nanotechnology and disease-specific pathogenesis.

Concluding Remarks

In this review, we have appraised standard delivery methods of EV as well as more innovative solutions to potentiate their cardioprotective effects. Indeed, no single delivery

strategy will apply to all clinical circumstances (patient requiring a surgery or not). However, for each situation, optimizations are under way and may be summed up as the functionalization of biomaterials for the controlled release of EV for direct delivery in the heart and EV engineering for cardiac targeting if delivery is systemic. Nonetheless, whereas the aim of these strategies is to enhance EV beneficial effects, the potential loss or alterations of EV bioactivity have to be taken in account (see Outstanding Questions). The characterization of EV is therefore essential. EV-based therapies may have benefits over stem cell transplantation with regard to production and storage, but this advantage could be curtailed by the complexity brought by these optimizations. Hence the importance of keeping the final product's clinical applicability in mind during its developmental phase (see Clinician's Corner).

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Glossary

Anthracycline: cancer drug that inhibits DNA and RNA synthesis thanks its intercalating function and the blockade of topoisomerase II. However, this chemotherapy is known for its cardiotoxicity.

Bioluminescence imaging (BLI): optical imaging based on detection of visible light produced by catalyzed reactions of a substrate by an enzyme considered as a molecular reporter

Glycome: entire repertoire of glycans (complex oligosaccharides) in every scale of living unity (protein, cell, tissue, organism) which depicts the cellular memory and governs cellular behaviors.

Hyaluronic acid (HA): linear and anionic glycosaminoglycan component of the extracellular matrix found in all tissues.

Left Ventricular Ejection Fraction (LVEF): (in %), parameter that evaluates the cardiac function. It is calculated with the following equation

$$LVEF\ (\%) = 100 \times (\text{end diastolic volume} - \text{end systolic volume}) / (\text{end diastolic volume})$$

Left Ventricular Fractional Shortening (LVFS): as the LVEF it evaluates the cardiac function. It reflects the percentage of contraction of the left ventricle.

Myocardial infarction model: experimental model that mimics infarct of the myocardium, it is most often realized by the ligation of the coronary artery of the left ventricle. The ischemia can be definitive or temporary if the blood flow is restored after a certain amount of time (it is then called ischemia-reperfusion).

Nanoparticle tracking analysis (NTA): Technology that visualizes nanoparticles and analyses their Brownian motion in liquids by following them individually. This method allows to extract the particle size distribution.

Paracrine signaling: form of cell communication where an emitting cell influence nearby cells and exert their actions via several mechanisms. Secreted molecules from emitting cells called paracrine factors interact with the target cell by direct contact (receptor/ligand interaction), internalization or fusion with the recipient cell.

Resistive Pulse Sensing: as NTA, it visualizes and analyses individual nanoparticles in liquids but by an electrical based technology.

Shear thinning: property of a fluid that has its viscosity decreased when the shear rate is increased contrary to a Newtonian fluid which possesses a viscosity independent from the shear rate. This property is very interesting for an easy injection of a gel through a needle.

Viscoelasticity: property of materials that exhibit both viscous (resistance to flow) and elastic (ability to recover its initial shape after a force has been applied) characteristics when undergoing deformation.

Thixotropy: property of a fluid that has its viscosity decreased when a stress is applied but recovers progressively its initial state when the stress is removed.

Box 1 – Extracellular vesicles, a key player in paracrine signaling.

EV encompass a heterogeneous population of particles bounded by a lipidic bilayer membrane. They are divided in 3 main families depending on their origin : exosomes, with a diameter from 50 to 100 nm, which are formed by exocytosis of multivesicular bodies (intermediates in endolysosomal transport formed by the invagination and budding of the endosomal membrane into its own lumen), microvesicles which have a bigger size (100 to 1000 nm) and are formed by budding of the plasma membrane and finally apoptotic bodies (1 to 5 µm) which are released from dying cells.[94] Their size overlap challenges the discrimination between families of EV. Exosomes and microparticles have been extensively studied because of their roles in intercellular communication in both physiologic and pathologic situations. Indeed, EV contain nucleic acids (mRNA, miRNA, DNA and ssDNA), proteins, lipid rafts and other molecules that can be actively internalized by a target cell [95]. The packaging of this bioactive cargo within the vesicle protects it from proteases, nucleases and the immune system [96]. Many methods have been developed to isolate EV and their sub-fractions. However, because of the persisting uncertainties regarding the specificity of fraction-associated markers and the potential co-purification of nonvesicular compounds [97] which may also have a therapeutic interest, the word "EV" should not be interpreted in a too restrictive fashion, hence the broader "EV-enriched secretome" terminology that we have selected to use throughout this manuscript. Major efforts are ongoing to facilitate the clinical translation of EV-based treatments [98,99]. A comprehensive summary of the major characteristics of EV (nature, biogenesis, function, preparation) can be found in this snapshot[97].

Box 2 – Hyaluronic acid, a valuable medical biopolymer

Hyaluronic acid or hyaluronan is a native component of the extracellular matrix. and is already widely used in different biomedical applications (i.e., rheumatology, ophthalmology, wound healing) because of its mucoadhesive, anti-inflammatory, and angiogenic properties. This diversity of applications can be explained by the possibilities offered by the structure of this anionic macromolecule. Indeed, its linear deed structure is composed of D-glucuronic acid and N-acetyl-D-glucosamine linked via glycosidic bonds which allows chemical techniques to extend the chain length (from 5 to 20 000 kDa) and therefore modulate its

stability in physiologic conditions as well as its viscosity [43] [100]. This polymer has clinically attractive features as an EV platform for heart repair because of its long-established safe use in humans, biocompatibility and suitability for fine-tuning its physical and chemical characteristics. Yet, this does not exclude other biocompatible polymers as equally relevant candidates.

Box 3 – Biodistribution of EV

Different labelling methods have been optimized in order to track EV *in vivo* such as molecular imaging, bioluminescence imaging or nuclear imaging [101]. EV seem to reach many different organs such as the lungs, spleen, pancreas, heart or kidney depending on the labeling technique, the route of administration, the cell source or the model studied. However the majority is routed to the liver after an IV injection [102]. Clearance of EV from the circulating blood occurs rapidly and seems to be at least partly mediated by the innate immune system [103]. One possible mechanism of action could be that following their predominant uptake by macrophages and liver sequestration, EV would act like cells through a systemic modulation of inflammation [104]. EV-modified endogenous inflammatory/ immune cells might then convey tissue-protective signals to the target organ.

Box 4 - Clinician's Corner

- Extracellular vesicles (EV) play a major role in intercellular communication by transferring a biologically rich cargo into recipient cells, thereby modulating their function. This mechanism of action is increasingly thought to underlie the cardio-reparative effects of stem cells.
- In the clinic, the practical advantages of delivering EV instead of their parental cells include a manufacturing process more akin to that of a biological medication, the possibility of cryopreservation and thus of an off-the-shelf use and the likely lack of immunogenicity. However, the EV-induced therapeutic benefit is highly dependent on the efficiency of their delivery.
- For patients requiring a surgical procedure, direct intramyocardial injections of EV under visual control is a straightforward approach. However, concerns about a rapid wash-out and the attendant loss of a treatment effect highlight the interest of

incorporating EV into shielding biomaterials. This would allow their controlled release in a time-dependent manner and the attendant prolongation of their exposure time to the target tissue.

- For patients not requiring surgery, an intravascular route should be considered. While a catheter-based endomyocardial administration might be one option, IV infusions are more attractive in the clinic because of their simplicity, lack of invasiveness, possibility of repeated dosing and user-friendly management.
- Despite the persisting challenges, among which the understanding of the precise mechanism(s) of action of EV remain(s) prominent, the clinical use of EV for treating different diseases, including heart failure, is now a realistic perspective. It should benefit from leveraging the large amount of data accumulated in the fields of stem cells, nanotechnologies and biomaterials to combine them for generating cost-effective GMP-compliant composite EV-biomaterial products.

618 **Table 1 – EV-based therapies for cardiac repair administered by IM injections in myocardial**

619 **infarction models.**

Origin	Model	Timing of injection	Dose	Outcomes	Ref
hCPC	Rat acute myocardial infarction model	60 min post ischemia	30 or 300 µg of protein	Less cardiomyocyte apoptosis, enhanced angiogenesis, and improved LVEF	[105]
	Mouse chronic myocardial infarction model	3 weeks after myocardial infarction	1×10^{10} particles	Improved LVEF, reduced infarct size	[106]
hBM-MSC	Rat acute myocardial infarction model	30 min post ischemia	80 µg of protein released by $2x10^6$ cells	Improved neoangiogenesis, reduced infarct size	[107]
Rat BM-MSC	Rat acute myocardial infarction model	After ischemia	20 µg of protein	Reduced fibrosis and inflammation, preserved LVEF	[108]
ESC	Mouse acute myocardial infarction model	Immediately after ischemia	10 µg of protein	Improved neovascularization, cardiomyocyte survival, LVEF and LVFS, reduced fibrosis	[109]
hCDC	Mouse acute and chronic myocardial infarction model	Immediately after ischemia or 3 weeks after	2.8×10^9 particles	Improved LVEF and angiogenesis, less cardiomyocytes apoptosis	[110]
	Pig acute and chronic myocardial infarction model	30 min after reperfusion or 4 weeks after	16.5×10^{11} particles	Improved LVEF, increased vessel density, reduced scarring, fibrosis and cardiomyocytes	[62]

				hypertrophy	

620 CDC: cardiosphere-derived cell; ESC: embryonic stem cell; hBM-MSC: human bone marrow
621 mesenchymal stromal cell; hCPC: human cardiac progenitor; **LVEF**: Left ventricular ejection
622 fraction; **LVFS**: Left ventricular fractional shortening
623

624 **Table 2 - EV-functionalized biomaterials for cardiac repair and other diseases**

Model	Delivery platform		Loading		Outcomes	Ref
	• Form • Time of release *in vitro*	Material	Type *	Origin		
Myocardial infarction rat model	**Injectable hydrogel 2 days**	**Methacrylated gelatin and Laponite®**	**Secre-tome**	**hASCs**	**Increased angiogenesis, LVEF and LVFS, decreased scarring**	[40]
	Injectable hydrogel 10 days	**Alginate**	**EV**	**BM-MSC**	**Increased angiogenesis, LVEF and LVFS, decreased inflammation, apoptosis and infarct size**	[41]
	Injectable hydrogel 21 days	**Modified hyaluronic acid**	**EV**	**EPC**	**Improved hemodynamic function and angiogenesis**	[45]
	Injectable hydrogel 21 days	**PA-GHRPS peptide + pro-gelator-NapFF peptides**	**Exo**	**hUC-MSC**	**Improved angiogenesis, LVEF and LVFS, reduced fibrosis, inflammation and apoptosis**	[54]
	Hydrogel patch 21 days	**Collagen type I within a gelfoam mesh**	**EV**	**iPSC derived CM**	**Promoted recovery of contractile function, reduced cardiomyocytes**	[34]

					hypertrophy and infarct size	
Ischemia-reperfusion infarction mouse model	**Injectable nanofibrous hydrogel Not specified**	**Heparin Binding Peptide Amphiphile**	**Secretome**	**BM-MSC**	**Preserved haemodynamic function**	[111]
Ischemic hindlimb rat model	Nanoparticles 14 days	Synthetic polymer (mE2N-PLA-PMDA2)	Secre-tome	EPC	Improved neoangiogenesis and hindlimb blood flow	[112]
Ischemic hindlimb mouse model	*In situ* hydrogel 3 days	Chitosan	Exo	Placenta-derived MSC	Improved angiogenesis, reduced necrosis and fibrosis	[47]
Calvarial bone defect rat model	Porous scaffold 4 days	Tricalcium phosphate (β-TCP)	Exo	iPS-MSCs	Dose-dependent increased bone formation, enhanced osteogenesis	[113]
	In situ hydrogel 14 days	Hydroxyapatite, hyaluronic acid-alginate	Exo	hUC-MSC	Increased osteogenesis and angiogenesis	[114]
	HyStem-HP hydrogel#	Thiolated hyaluronic acid, thiolated heparin and thiolated gelatin	EV	Marrow stromal/ stem cell	Increased bone formation	[115]
Articular cartilage defect rabbit model	*In situ* hydrogel 14 days	Modified hyaluronic acid	Exo	hiPSC-MSCs	Integration with native cartilage matrix, increased formation of hyaline cartilage-like tissue	[116]

Diabetes-induced kidney injury mouse model	Injectable nanofibrous hydrogel#	Peptide E2(SL)6E2GRGDS	Secre-tome	hESCs	Decreased protein permeability (albumin from glomerular epithelial cells)	[117]
Full-thickness excisional wound model	Patch 5 days	Alginate	Exo	ADSCs	Reduced wound healing time and scarring	[118]
Spinal cord injury rat model	Adhesive hydrogel 7 days	Modified hyaluronic acid	Exo	hPAM-MSC	Improved nerve recovery and urinary tissue preservation	[119]
No *in vivo* studies	Injectable hydrogel 7 days	Porcine derived decellularized ECM	EV	hCPCs	NA	[39]
	Injectable hydrogel 4 days	Supramolecular Ureido-pyrimidinone	EV			[56]

Footnotes:

bold : cardiac applications of functionalized biomaterials

*: The terminology (Secretome, Exosome (Exo), Extracellular vesicle (EV)) is the one used in the corresponding papers.

#: no more specifications

ADSC: adipose tissue-derived stem cells; CDC: cardiosphere-derived cell; ECM: extracellular matrix; EPC: endothelial progenitor cell; Exo: exosomes, hASC: human adipose stromal cell; hBM-MSC: human bone marrow mesenchymal stromal cell; hCPC: human cardiac progenitor; hESC: human embryonic stem cell; hPAM-MSC: human placenta amniotic membrane mesenchymal stromal cell; hUC-MSC: human umbilical cord mesenchymal stromal cell; iPSC: induced pluripotent stem cell.

637

638 **Table 3 – EV-based therapies for cardiac repair administered by IV injections**

Cell of origin	Model	Timing of injection	Dose	Outcomes	Ref
hMSC	Chronic myocardial ischemia swine model	2 weeks after ischemia	50 µg of protein	Insignificant effects on myocardial perfusion and cardiac function compared to IM injections	[69]
	Myocardial ischemia-reperfusion porcine model	IV: 5 min before onset of reperfusion IC bolus: after reperfusion	2 mg of protein (IV) + 8 mg of protein (IC)	Improved cardiac performances, reduced infarct size and apoptosis	[120]
	Myocardial ischemia-reperfusion mouse model	5 min before reperfusion	1, 4 or 16 µg of protein/kg	Reduced infarct size and inflammation, improved cardiac function	[121]
hCPC	Myocardial ischemia-reperfusion rat model	3h after reperfusion	2×10^{11} particles	Increased angiogenesis and LVEF, reduced scar size	[122]
	Dox/Trz induced cardiotoxicity rat model	3 injections every 5 days during dox/Trz treatment	3×10^{10} particles	Reduced fibrosis, inflammation, oxidative stress, improved LVEF and LVFS	[123]
hCSC	Dox induced dilated cardiomyopathy mouse model	7 days after dox injection (5 mg/kg)	3×10^{10} particles	Reduced apoptosis, fibrosis, improved LVEF and LVFS	[124]

ESC		3 injections every 2 days during dox treatment	Conditioned media from 5×10^5 cells	Decreased apoptosis, fibrosis, myofibrillar loss and cytoplasmic vacuolization	[125]
hCDC	Mdx dystrophic mouse model	1 injection	2×10^9 particles	Improved LVEF, reduced fibrosis, increased cardiomyogenesis	[126]

639

640 *Footnotes:*

641 Dox: Doxorubicin; hMSC: human mesenchymal stromal cells, hAFS: human amniotic fluid-
642 derived stem cells; hCDC: human cardiosphere-derived cell; hCPC: human cardiac progenitor
643 cells; hCSC: human cardiac stem cell; Trz: Trastuzumab; ESC: embryonic stem cell; IC:
644 intracoronary; IV: intravenous; LVEF: Left ventricular ejection fraction; LVFS: Left ventricular
645 fractional shortening.

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Figure Legends

Figure 1, Key Figure: Optimization of extracellular vesicle delivery strategies.

EV represent an heterogeneous population of particles secreted by cells including exosomes, microvesicles, and apoptotic bodies which have shown beneficial effects on damaged hearts. To potentiate their cardioprotective potential, their administration needs to be tailored to the patient's clinical condition. For patients requiring open-chest surgery (left panel), EV can be delivered in a controlled fashion following incorporation into injectable or epicardial biomaterials. However, for patients who are not suitable for surgery (right panel), EV can be intravenously injected, but it is then likely important to engineer their surface or content to selectively increase their homing towards the target heart and thus limit their widespread biodistribution.

Figure 2: Main parameters and steps to consider for the development of an EV-functionalized biomaterial.

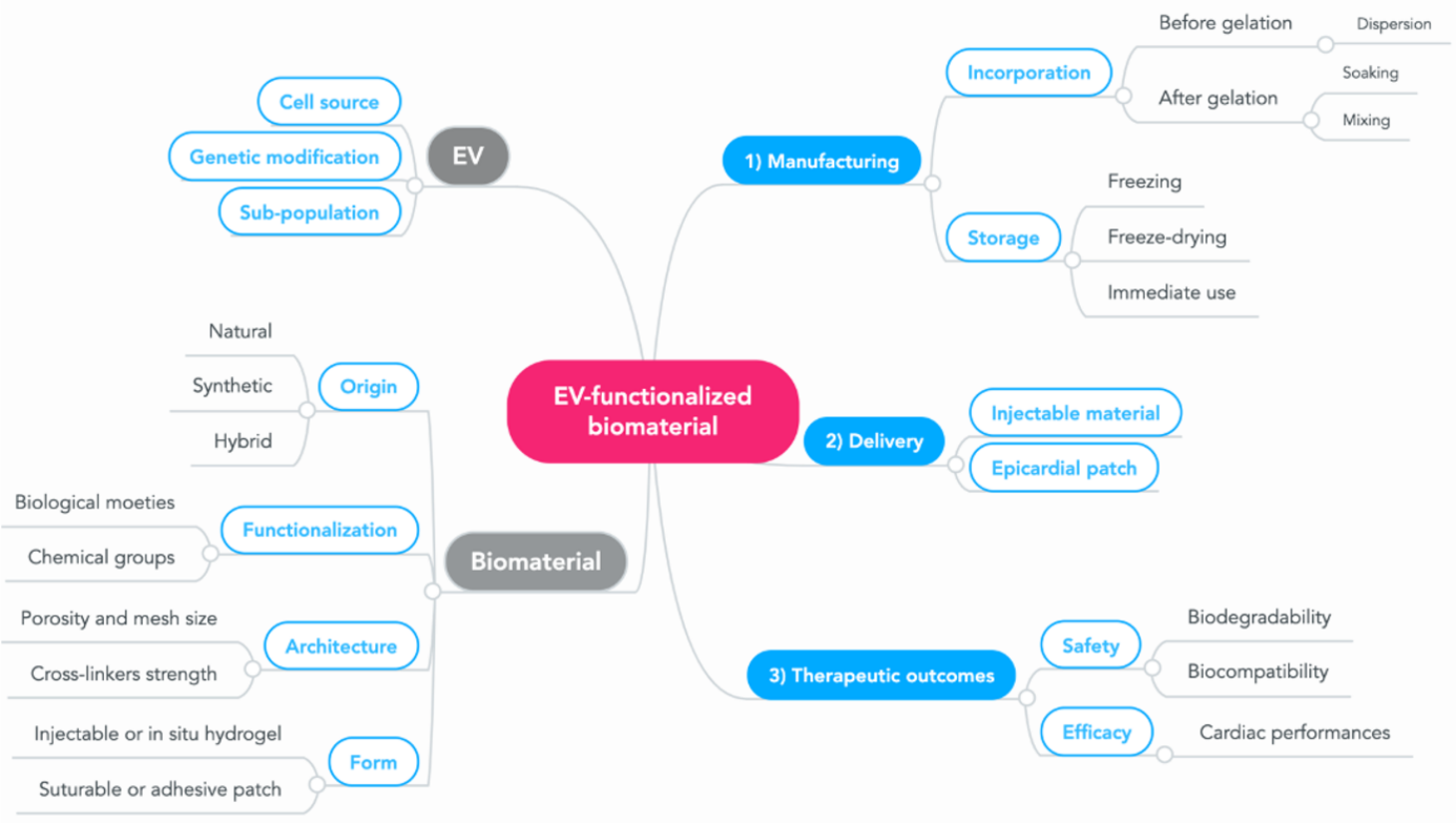
EV or the biomaterial parameters are listed on the left part of the figure. On the right, crucial steps and their possibilities are illustrated. During manufacturing, the method of incorporation of EV and storage will govern its availability and handling for the clinical practice. Then the final product delivery will be directly linked to the form and properties of the biomaterial (i.e its injectability or rigidity). At last, therapeutic outcomes can be summed up in 2 main aspects: the safety and the efficacy of the functionalized biomaterial.

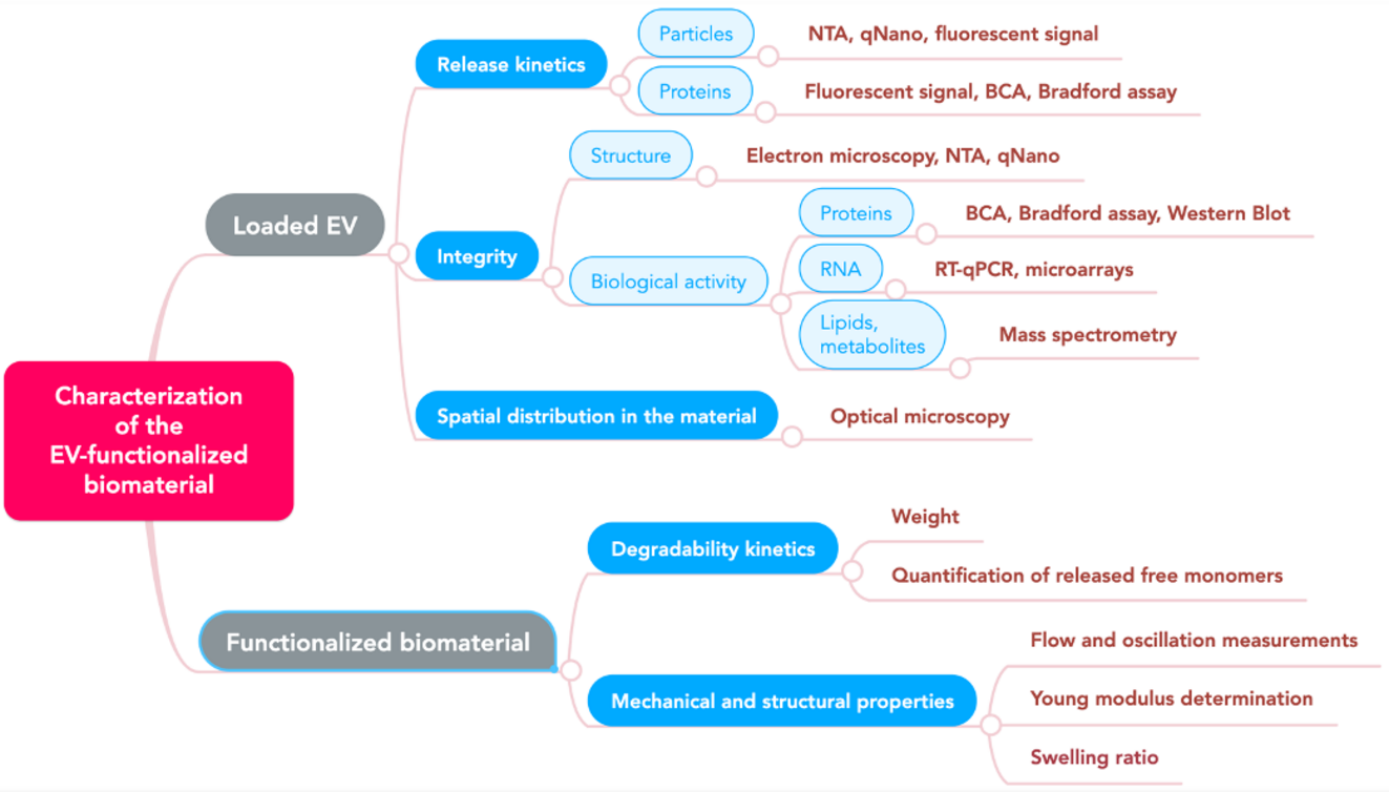
BCA: bicinchoninic acid assay; NTA: Nanoparticle tracking analysis.

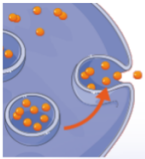
Figure 3: Parameters and characterization techniques of EV-functionalized biomaterials.

Firstly, the controlled release of EV from its biomaterial carrier needs to be measured by conducting EV release kinetics studies. Quantification of particles (single EV) or proteins can be performed by different methods listed on the figure. By loading EV within a biomaterial, the EV microenvironment will be modified or could be altered during the process. It is thus critical to ensure that the integrity of EV has been preserved, which can be achieved by techniques assessing their structural and biological properties. Conversely, depending on EV concentration, charge and/or size, the initial features of the scaffold can be changed. Some

of these features are crucial for further developments i.e injectability and residence time and should thus be assessed by rheology (flow and oscillation measurements, Young modulus) and degradability kinetics studies.


EV-functionalized biomaterial
EV
Cell source
Genetic modification
Sub-population
Biomaterial
Origin
Natural
Synthetic
Hybrid
Functionalization
Biological moeties
Chemical groups
Architecture
Porosity and mesh size
Cross-linkers strength
Form
Injectable or in situ hydrogel
Suturable or adhesive patch
1) Manufacturing
Incorporation
Before gelation
Dispersion
After gelation
Soaking
Mixing
Storage
Freezing
Freeze-drying
Immediate use
2) Delivery
Injectable material
Epicardial patch
3) Therapeutic outcomes
Safety
Biodegradability
Biocompatibility
Efficacy
Cardiac performances


Characterization of the EV-functionalized biomaterial
Loaded EV
Release kinetics
Particles
NTA, qNano, fluorescent signal
Proteins
Fluorescent signal, BCA, Bradford assay
Integrity
Structure
Electron microscopy, NTA, qNano
Biological activity
Proteins
BCA, Bradford assay, Western Blot
RNA
RT-qPCR, microarrays
Lipids, metabolites
Mass spectrometry
Spatial distribution in the material
Optical microscopy
Functionalized biomaterial
Degradability kinetics
Weight
Quantification of released free monomers
Mechanical and structural properties
Flow and oscillation measurements
Young modulus determination
Swelling ratio

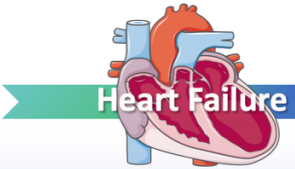


Exosomes

Microvesicles

Apoptic bodies

Extracellular vesicles (EV)

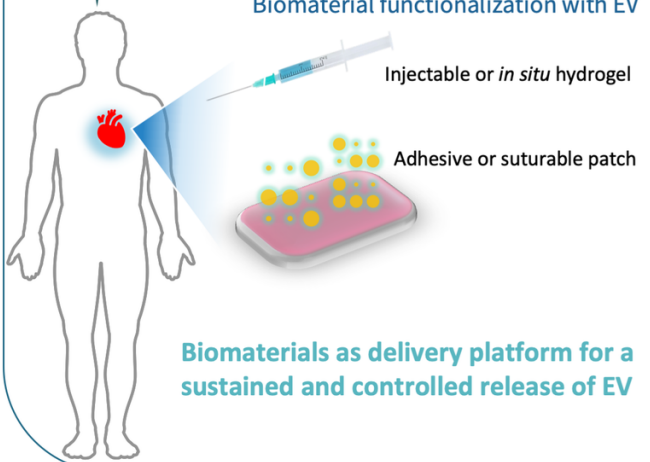


EV recapitulate most of the cardioprotective effects of stem cells

Optimization of extracellular vesicle delivery strategies

Surgery : Direct myocardial delivery

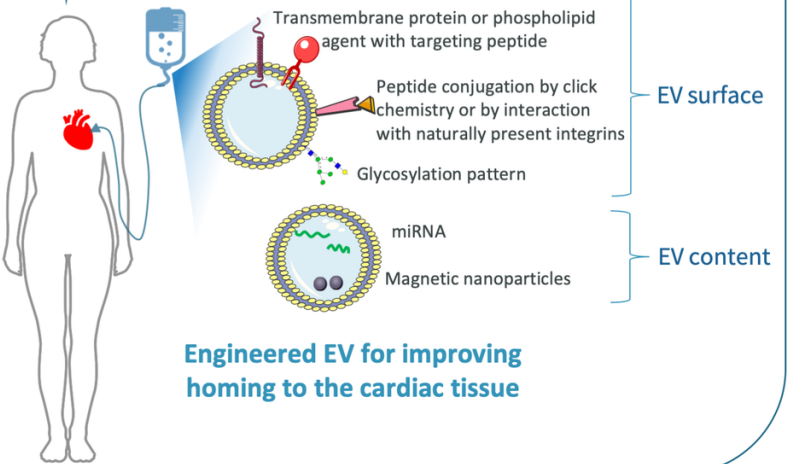
Biomaterial functionalization with EV



Biomaterials as delivery platform for a sustained and controlled release of EV

No surgery : EV infusion

Adjustment of



Engineered EV for improving homing to the cardiac tissue