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Extracellular vesicles and biomaterial design: new therapies for cardiac repair

3

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13

14

15 **Keywords:** biomaterials, cardiac repair, delivery, extracellular vesicles

16

17 **Abstract:**

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

27 **Therapeutic potential of extracellular vesicles in cardiovascular diseases**

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

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

48 The first use of EV for treating cardiac diseases goes back to several years when Brill
49 *et al.* reported an improved revascularization of ischemic myocardium after injections of
50 human platelet-derived microparticles [5]. Since then, there has been ample evidence that
51 the EV released by mesenchymal stromal cells (MSC) or cardiac-committed cells (from adult
52 or pluripotent stem cell sources) recapitulate the protective effects of their parental cells
53 through the activation of signaling pathways in the recipient myocardium; this can translate
54 into a stimulation of angiogenesis and a mitigation of inflammation, fibrosis and apoptosis
55 while the re-induction of host cardiomyocyte proliferation remains much more debatable
56 [6]. Put together, these events could account for the cardio-reparative effects of the cellular
57 secretomes. This has led some investigators to move away from the transplantation of cells
58 and to rather leverage their paracrine effects through the exclusive delivery of this

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

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

79

80 **Surgical applications: Direct intra-myocardial delivery**

81 *One-shot uncontrolled delivery*

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

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

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

108

109 *Basic principles of time-controlled delivery systems*

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

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

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

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

157

158 *General characteristics of EV-functionalized biomaterials*

159 To avoid rapid EV wash-out, their possible off-target effects and, at the end, a loss of
160 efficacy, different biomaterials have been developed to encapsulate them and ensure their
161 controlled release in the target myocardium. This approach has actually leveraged the
162 already well-established ability of scaffolds to control spatially and temporally the
163 distribution of stem cells or stem cell-derived biologics such as growth factors or miRNAs
164 [28]. For a complete review of biomaterials see the the review of Sepantafar *et al.*[29].

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

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

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

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

199

200 *Applications of EV-loaded biomaterials*

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

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

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

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

232

233 *Methodological challenges*

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

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

251 not necessarily EV. Some of these particles can represent material end-degradation
252 products, thereby making mandatory control experiments with the biomaterial alone to
253 reflect the background noise. Furthermore, these methods do not detect EV smaller than 60
254 nm, which may represent a large proportion of the secretome [48]. Data collected from
255 these techniques can also be confounded by aggregation of EV, a phenomenon which has
256 been highlighted in studies of the impact of isolation or storage on EV and is well-known in
257 “synthetic vesicles” or liposomes that share important physicochemical features with EV[49–
258 51]. This aggregation can be confirmed by imaging single particles with electron microscopy
259 (EM) and, at best, by cryo-EM which can more accurately resolve lipid bilayers [52]. Care
260 should also be taken in the interpretation of protein content assays which yield substantial
261 differences among commonly available methods [53].

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

282 Even if EV are not subject to the engraftment and survival issues encountered with
283 stem cells, their therapeutic potency is likely to rely on the preservation of their structural
284 and biological integrity. Therefore, besides from the optimal duration of EV release, it is
285 critical to assess the functional properties of the released EV which are likely to change over
286 time. Surprisingly, only a few studies have characterized the dynamic profile of EV after their
287 incorporation in a support material. Reports on MSC-derived EV released from a chitosan
288 hydrogel demonstrated their stability through microRNA quantitation and dynamics of EV
289 uptake by human umbilical vein endothelial cells (HUVEC)[47]. Rat CPC-derived EV
290 bioactivity was also evaluated directly after their release from an extracellular matrix
291 hydrogel by assessing their protective effect on H₂O₂-induced apoptosis of human CPC and
292 stimulation of protein kinase-like endoplasmic reticulum kinase (pERK) expression in human
293 coronary artery endothelial cells. The phosphorylation of ERK was actually reduced after 1
294 week of encapsulation, which could be explained by the lower amount of EV released after
295 the first days as well as by EV degradation[39]. In another study, the bioactivity of CPC-EV
296 released from a supramolecular ureidopyrimidinone hydrogel was checked through their
297 ability to activate ERK signaling in endothelial cells. Results showed that this bioactivity was
298 fully preserved after one week, but decreased after two weeks in comparison with fresh EV
299 [56]. Thus, different end points are available but it is likely that in addition to standard
300 measurements of the RNA and protein content of the EV, the most convincing evidence for
301 the persistence of their bioactivity comes from potency tests like those which evaluate their
302 pro-survival or angiogenic potential [57]. Of note, these assessments can be challenging
303 because of the gradual release of EV which may render analytical procedures increasingly
304 difficult to interpret given the small amount of EV collected at late time points. However,
305 this characterization is even more crucial for biomaterials made of synthetic polymers
306 because they require the use of strong organic solvents or toxic photo-initiators for
307 fabrication and/or cross-linking; this results in the release of toxic monomers during their
308 degradation, hence the importance of ensuring that this event does not impair EV bioactivity
309 [58]. Of note, while this bioactivity can be tested *in vitro* by potency tests like those
310 mentioned above, it is by far more challenging to assess EV function *in vivo* following their
311 controlled release from a given biomaterial in myocardial tissue and it can then be
312 acceptable to rather rely on surrogate markers of efficacy like functional end points and/or
313 histological patterns of tissue damage in comparison with EV suspensions. In these studies, it

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

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

326

327 **Non-surgical applications: catheter-based and intravenous delivery**

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

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

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

348

349 *Intravenous injection of unmodified EV for cardiac repair*

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

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

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

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

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

380

381 *Improved cardiac targeting of EV*

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

385

386 **Genetic modification of parental cells.** The first strategy developed is the genetic
387 modification of the parental cells to endow their secreted EV with targeting capacities [75].
388 These genetic modifications allow restructuring transmembrane proteins to fuse with
389 peptides or specific ligands. For specific heart targeting, lentivirus packaging of a
390 recombinant plasmid has been used to modify the outer portion of lysosome-associated
391 membrane protein 2 (Lamp2b), an abundant protein at the surface of EV, by its fusion with a
392 cardiac-targeting peptide (APWHLSSQYSRT) [76] or a cardiomyocyte-specific peptide
393 (WLSEAGPVVTVRALRGTGSW) [77]. The resulting EV were more efficiently internalized by
394 cardiomyocytes *in vitro* and displayed improved cardiac retention in comparison with non-
395 targeted EV *in vivo*. A similar pattern of improvement was shown after transfection of
396 cardiac progenitor cells with CXCR4 and IV infusion of the resulting CXCR4-expressing EV
397 [84]. *In vivo*, these EV improved heart function and reduced infarct size compared with their
398 untreated counterparts in a murine model of ischemia/reperfusion while companion *ex vivo*
399 experiments documented their more efficient delivery in Langendorff-perfused hearts. Thus,
400 these genetic modifications can generate a wide array of tailored EV but their complex
401 development and the lack of stability of fused peptides render this approach challenging and
402 time-consuming [78].

403

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

409 alkyne moiety) and an azide group [79]. Targeting a specific tissue using this technique has
410 been reported with EV conjugated with a glioma-targeting peptide (neuropilin-1) [80]. This
411 kind of reaction can thus allow the functionalization of EV with a cardiac-targeting peptide.
412 Alternatively, the natural affinity between integrins and specific ligands can be leveraged to
413 conjugate peptides to the surface of EV. For now, this approach has only been studied for an
414 opposite objective with $\alpha3\beta1$ integrin-binding peptide (LXY30) linked to EV derived from
415 ovarian tumor cells [81]. The goal was actually to reduce EV uptake by a specific cell type.
416 However, this work yet showed the possibility of influencing EV targeting by integrin-binding
417 peptides. Identification of peptides that can anchor to EV could benefit from phage display,
418 as demonstrated by docking of the peptide CP05 to EV via CD63, a tetraspanin enriched on
419 the surface of EV, and its subsequent therapeutic benefits in a dystrophin-deficient mouse
420 model [82].

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

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

441 is involved in EV recognition by cells, results in an alteration of EV biodistribution and more
442 specifically an increased EV accumulation in lungs [87]. Aside from surface modification,
443 adjustment of EV content may also improve their organ-specific targeting. For example, in a
444 doxorubicin-induced cardiotoxicity model, *in vivo* biodistribution of EV was altered by their
445 loading with a siRNA against clathrin heavy chain which is involved in EV endocytosis by
446 macrophages: EV uptake by macrophages in the spleen and liver was subsequently reduced
447 [88].

448

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

468

469 **Concluding Remarks**

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

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

483

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488

Glossary

489

490

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

494

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

497

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

501

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

504

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

507 $LVEF (\%) =$

508 $100 \times (\text{end diastolic volume} - \text{end systolic volume}) / (\text{end diastolic volume})$

509

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

512

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

517

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

521

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

526

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

529

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

533

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

537

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

540

541

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

543 EV encompass a heterogeneous population of particles bounded by a lipidic bilayer
544 membrane. They are divided in 3 main families depending on their origin : exosomes, with a
545 diameter from 50 to 100 nm, which are formed by exocytosis of multivesicular bodies
546 (intermediates in endolysosomal transport formed by the invagination and budding of the
547 endosomal membrane into its own lumen), microvesicles which have a bigger size (100 to
548 1000 nm) and are formed by budding of the plasma membrane and finally apoptotic bodies
549 (1 to 5 μm) which are released from dying cells.[94] Their size overlap challenges the
550 discrimination between families of EV. Exosomes and microparticles have been extensively
551 studied because of their roles in intercellular communication in both physiologic and
552 pathologic situations. Indeed, EV contain nucleic acids (mRNA, miRNA, DNA and ssDNA),
553 proteins, lipid rafts and other molecules that can be actively internalized by a target cell [95].
554 The packaging of this bioactive cargo within the vesicle protects it from proteases, nucleases
555 and the immune system [96]. Many methods have been developed to isolate EV and their
556 sub-fractions. However, because of the persisting uncertainties regarding the specificity of
557 fraction-associated markers and the potential co-purification of nonvesicular compounds
558 [97] which may also have a therapeutic interest, the word "EV" should not be interpreted in
559 a too restrictive fashion, hence the broader "EV-enriched secretome" terminology that we
560 have selected to use throughout this manuscript. Major efforts are ongoing to facilitate the
561 clinical translation of EV-based treatments [98,99]. A comprehensive summary of the major
562 characteristics of EV (nature, biogenesis, function, preparation) can be found in this
563 snapshot[97].

564

565 **Box 2 – Hyaluronic acid, a valuable medical biopolymer**

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

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

578

579 **Box 3 – Biodistribution of EV**

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

590

591 **Box 4 - Clinician's Corner**

592

- 593
- Extracellular vesicles (EV) play a major role in intercellular communication by
594 transferring a biologically rich cargo into recipient cells, thereby modulating their
595 function. This mechanism of action is increasingly thought to underlie the cardio-
596 reparative effects of stem cells.
 - In the clinic, the practical advantages of delivering EV instead of their parental cells
597 include a manufacturing process more akin to that of a biological medication, the
598 possibility of cryopreservation and thus of an off-the-shelf use and the likely lack of
599 immunogenicity. However, the EV-induced therapeutic benefit is highly dependent
600 on the efficiency of their delivery.
 - For patients requiring a surgical procedure, direct intramyocardial injections of EV
602 under visual control is a straightforward approach. However, concerns about a rapid
603 wash-out and the attendant loss of a treatment effect highlight the interest of
604

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

- 608 • For patients not requiring surgery, an intravascular route should be considered.
609 While a catheter-based endomyocardial administration might be one option, IV
610 infusions are more attractive in the clinic because of their simplicity, lack of
611 invasiveness, possibility of repeated dosing and user-friendly management.
- 612 • Despite the persisting challenges, among which the understanding of the precise
613 mechanism(s) of action of EV remain(s) prominent, the clinical use of EV for treating
614 different diseases, including heart failure, is now a realistic perspective. It should
615 benefit from leveraging the large amount of data accumulated in the fields of stem
616 cells, nanotechnologies and biomaterials to combine them for generating cost-
617 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 2×10^6 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
	<ul style="list-style-type: none"> Form Time of release <i>in vitro</i> 	Material	Type *	Origin		
Myocardial infarction rat model	Injectable hydrogel 2 days	Methacrylated gelatin and Laponite®	Secrete	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)	Secretome	EPC	Improved neoangiogenesis and hindlimb blood flow	[112]
Ischemic hindlimb mouse model	<i>In situ</i> 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]
	<i>In situ</i> 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	<i>In situ</i> 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	Secretome	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 <i>in vivo</i> studies	Injectable hydrogel 7 days	Porcine derived decellularized ECM	EV	hCPCs	NA	[39]
	Injectable hydrogel 4 days	Supramolecular Ureido-pyrimidinone	EV			[56]

625

626 *Footnotes:*

627 **bold** : cardiac applications of functionalized biomaterials

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

630 #: no more specifications

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

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.

646

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Figure Legends

955

956

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

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

967

968 **Figure 2: Main parameters and steps to consider for the development of an EV-** 969 **functionalized biomaterial.**

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

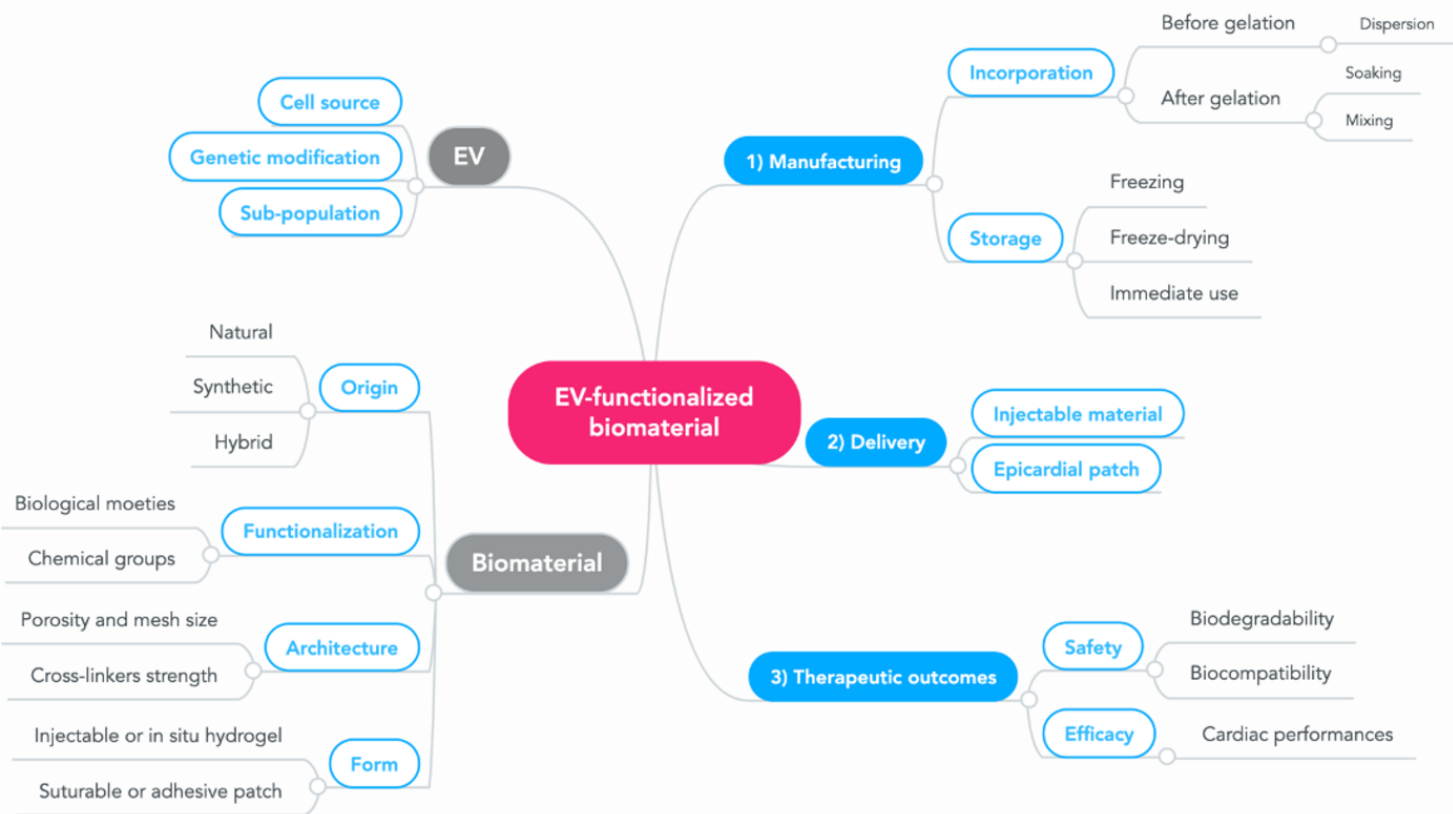
976 *BCA: bicinchoninic acid assay; NTA: Nanoparticle tracking analysis.*

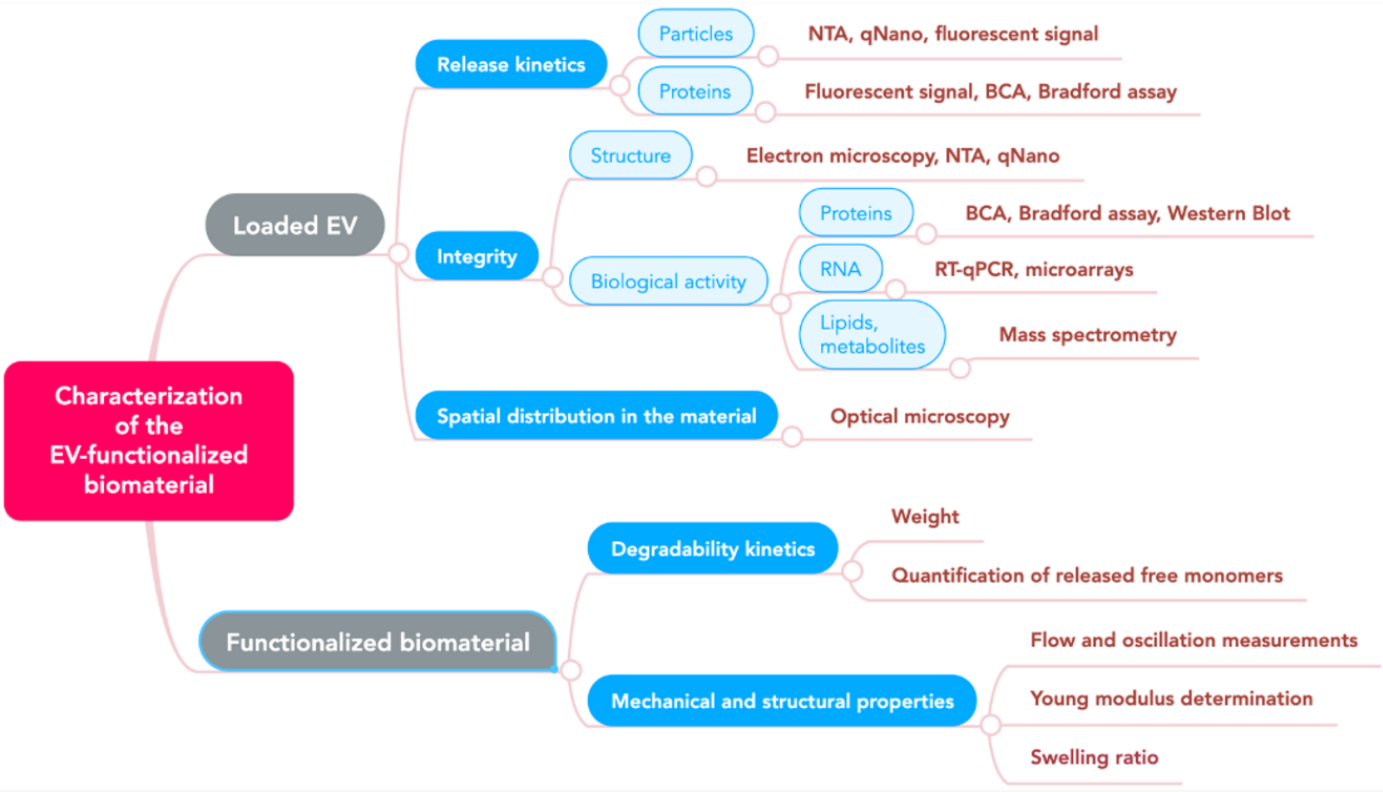
977

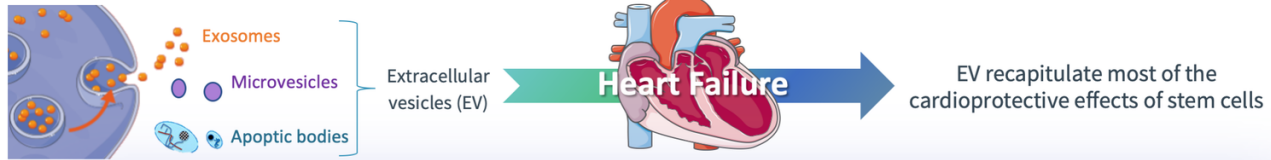
978 **Figure 3: Parameters and characterization techniques of EV-functionalized biomaterials.**

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

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







Optimization of extracellular vesicle delivery strategies

Surgery : Direct myocardial delivery



Biomaterial functionalization with EV

Injectable or *in situ* hydrogel

Adhesive or suturable patch

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

No surgery : EV infusion



Transmembrane protein or phospholipid agent with targeting peptide

Peptide conjugation by click chemistry or by interaction with naturally present integrins

Glycosylation pattern

miRNA

Magnetic nanoparticles

Engineered EV for improving homing to the cardiac tissue

Adjustment of

EV surface

EV content