HHS Public Access

Author manuscript

Nat Rev Mater. Author manuscript; available in PMC 2024 June 01.

Published in final edited form as:

Nat Rev Mater. 2023 June ; 8(6): 390–402. doi:10.1038/s41578-023-00551-3.

Extracellular vesicle-matrix interactions

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Abstract

The extracellular matrix in microenvironments harbors a variety of signals to control cellular functions and the materiality of tissues. Most efforts to synthetically reconstitute the matrix by biomaterial design have focused on decoupling cell-secreted and polymer-based cues. Cells package molecules into nanoscale lipid membrane-bound extracellular vesicles and secrete them. Thus, extracellular vesicles inherently interact with the meshwork of the extracellular matrix. In this Review, we discuss various aspects of extracellular vesicle-matrix interactions. Cells receive feedback from the extracellular matrix and leverage intracellular processes to control the biogenesis of extracellular vesicles. Once secreted, various biomolecular and biophysical factors determine whether extracellular vesicles are locally incorporated into the matrix or transported out of the matrix to be taken up by other cells or deposited into tissues at a distal location. These insights can be utilized to develop engineered biomaterials where EV release and retention can be precisely controlled in host tissue to elicit various biological and therapeutic outcomes.

Keywords

extracellular vesicle; extracellular matrix; biomaterials; nanoscale biophysics; nanotechnology

1. Introduction

The extracellular matrix (ECM) is a network structure consisting of various biomolecular and biophysical components essential to cellular functions, which represents the major acellular component of biological tissues. Tissues are active viscoelastic materials[1] that can change their properties depending on pathophysiological conditions. The ECM can determine the rheological properties of tissues both directly as constituents and indirectly by calibrating how cells generate contractile forces and tension via mechanotransduction[2,3],

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which can influence the ability of cells to remodel the ECM[4]. Understanding how the ECM is remodeled and how the materiality of tissue is dynamically controlled will necessitate biomaterial-based strategies to investigate the interplay between cell-secreted factors and polymer-based cues.

Previous studies with purified ECM proteins have highlighted roles of polymeric networks in determining rheological properties essential to tissue integrity, such as strain-stiffening[5]. To date, efforts to engineer synthetic ECMs to direct cellular functions have focused on controlling the crosslinking of polymeric networks to tune elasticity[6], viscoelasticity[7,8] and plasticity[9]. However, molecular profiling studies of decellularized tissues have shown the presence of soluble proteins tightly bound to fibrous ECM networks[10]. While cells can secrete soluble proteins directly, cells can also package molecules into nanoscale mediators and secrete them, especially in lipid membrane-bound vesicles, called extracellular vesicles (EVs). The presence of EVs in the ECM was documented several decades ago by electron microscopy studies in the context of vesicle-mediated mineralization[11,12]. However, ECM-bound vesicles were documented in other tissues only recently[13]. Recent studies with label-free third harmonic generation microscopy further showed the enrichment of EVs in tissue stromal regions, which consist of dense matrix fibers[14,15]. However, vesicles can also be found in blood[16] and lymph[17], suggesting that some secreted EVs from cells can transport out of the ECM[18] and end up at a distal location to be taken up by other cells[19] or deposited into tissues[20].

Here, we provide a comprehensive review on EV-ECM interactions. We review the current knowledge of different cell-secreted nanoscale mediators. We elaborate on the role of membrane trafficking in EV biogenesis and its regulatory mechanisms by the ECM as a key example of how cells leverage biological processes to produce and secrete nanoscale mediators. We examine biomolecular and biophysical determinants of EV-ECM polymer interactions. We highlight recent advances in interfacing EVs with engineered hydrogels as biologically inspired strategies to promote tissue regeneration by controlling transport or retention of EVs. Given the importance of sourcing EVs from cells, we also review the role of biomaterial design in controlling EV production from cells. Finally, we explore future areas of investigations into EVs as essential structural elements of hydrogel-based materials to better recapitulate mechanisms of health and disease, and to develop a novel class of biologically-inspired materials.

2. Diversity of cell-secreted nanoscale mediators

Cell-secreted EVs were previously classified into apoptotic bodies, ectosomes (also called microvesicles or microparticles), and exosomes based on their distinct biogenesis mechanisms[21] (Fig. 1). Apoptotic bodies are produced during apoptosis of cells by outward budding of the cell membrane[22,23]. Ectosomes are also produced by outward budding of the plasma membrane, but may or may not accompany apoptosis[24]. In contrast, exosomes are secreted when early endosomes become specialized into multivesicular bodies (MVBs) by inward budding of intraluminal vesicles (ILVs). MVBs then fuse with the plasma membrane to release ILVs as exosomes that express tetraspanins[25]. However, validating specific cell-

secreted EVs based on biogenesis pathways requires well-controlled investigations, such as employing live cell imaging techniques fused with genetic approaches[26].

From a practical point-of-view, EVs are classified into large (>200 nm) and small (<200 nm) EVs[27], since most investigators have been using differential centrifugation to separate large EVs (<10,000g) and small EVs (>100,000g), which may include a variety of EV subtypes in addition to apoptotic bodies, ectosomes, and exosomes. For instance, exophers are microscale large EVs that are isolated at ~1,000g and are known to help transport and eliminate defective mitochondria and protein aggregates[28]. Migrasomes (>500 nm) are large EVs that are produced from long membrane projections during cell migration on a rigid culture substrate[29,30]. Similarly, filopodia-derived vesicles (>200 nm) are formed by scission of filopodia[31]. Some of the recently reported small EV subtypes include arrestin-domain-containing protein 1-mediated microvesicles (ARMMs) that are formed by budding[32], and ECM-bound vesicles, which are known to be devoid of classical EV markers, tightly bound to the ECM after decellularization of tissues, and released only after enzyme-mediated digestion of the ECM[13].

Adding to the complexity, recent studies have also shown the presence of non-vesicular extracellular particles (NVEPs) that do not contain a lipid bilayer in the pellet after ultracentrifugation at 100,000g, which also contains small EVs. These NVEPs can be separated from small EVs by high-resolution iodixanol density gradient fractionation, followed by taking high density fractions[33]. The supernatant from the first ultracentrifugation can be subject to additional overnight ultracentrifugation at 100,000g to obtain smaller NVEPs (<50 nm)[34], called exomeres, which were first described by using the asymmetric-flow field-flow fractionation method[35]. After isolating exomeres, another round of ultracentrifugation at a higher speed (~360,000g) can be done overnight on the supernatant to obtain even smaller NVEPs (<30 nm), called supermeres[36]. While some NVEPs were shown to be released via a shared pathway as exosomes[33], the biogenesis pathway of NVEPs remains relatively unknown compared to that of EVs.

3. Mechanisms of EV biogenesis in the ECM

EV biogenesis is intricately linked to intracellular transport and secretory pathways, and physicochemical factors in the ECM that regulate these processes (Fig. 2).

3.1. Lipid membrane transport

The unique structural feature of EVs is that they encapsulate various cargo molecules in the lipid membrane, including proteins, nucleic acids, and various metabolites[37]. Thus, understanding the role of membrane turnover in the context of the ECM will help understand how EV biogenesis is regulated by the ECM. Lipid rafts are discrete, dynamic nanoscale domains in the external leaflet of the cell membrane, which are present in a metastable state, but become more stable by undergoing clustering in response to external signals, including those present in the ECM[38]. Some lipid raft domains undergo endocytosis[39], and the resulting vesicles fuse with early endosomes[40]. Lipid rafts are enriched with cholesterol and sphingolipids[41]. Importantly, cholesterol and ceramide, a simple sphingolipid, are essential for the formation of MVBs by recruiting the endosomal sorting complex required

for transport (ESCRT) machinery[42] and triggering the negative curvature of the MVB membrane to form ILVs in an ESCRT-independent manner[43], respectively. Both cholesterol and ceramide are highly hydrophobic, and intercalate between phospholipid acyl chains of the cell membrane in a competitive manner[44,45]. Loss of cholesterol increases membrane fluidity[46], but also promotes membrane-cytoskeleton interactions[47], thereby stiffening the cell membrane[48]. Thus, endocytosis of lipid rafts may result in temporary increase in the cell membrane tension. However, this increase can be counteracted when MVBs fuse to the cell membrane to release exosomes, the process that can restore the membrane pool and decrease the tension[49]. Similarly, MVB fusion or exocytosis could potentially serve as a homeostatic mechanism to counteract the loss of plasma membrane during outward budding when microvesicles or apoptotic bodies are formed.

3.2. Biophysical regulation by the ECM

Since cells pull on and sense the resistive force from the ECM[2,3], biophysical properties of the ECM can impact membrane trafficking[49,50], and hence EV biogenesis. Caveolae represent a subset of lipid rafts that contain the protein caveolin[51]. Previous studies showed the role of caveolae in mechanosensing, since they enable endothelial cells to be responsive to ECM rigidity[52,53] and shear flow[54,55], and protect cells from rupture by undergoing flattening and disassembly in response to acute mechanical stress independently of actin and ATP[56]. Interestingly, caveolin is known to be incorporated into MVBs and exosomes, and required for sorting of some ECM molecules into exosomal cargo, which can then be transported to distal tissues[20]. Conversely, cells reassemble caveolae in an actin-dependent manner in response to stress release[56], and also in a hydrogel matrix that recapitulates the physiological stiffness of soft tissue, where cells maintain low membrane tension[57]. Consistent with these observations, cells on a soft hydrogel matrix maintain the nanoscale assembly of short actin filaments, which permits MVBs to readily transport and fuse with the plasma membrane to release exosomes—in contrast, cells on a stiffer matrix form an extensive actin network, which serves as a physical barrier for MVB transport and exosome release[26].

3.3. Chemical regulation by the ECM

Chemical factors in the ECM can also impact EV biogenesis by modulating membrane trafficking. The ECM is the largest source of free calcium ions[58], which bind to lipid rafts to initiate calcium signaling and play essential roles in EV biogenesis, including MVB formation and fusion to the plasma membrane[59,60]. EV release can be enhanced by soluble extracellular mediators that elevate intracellular calcium, such as histamine[61,62]. In cancer and tissue injury, some tissues become rigid by increased ECM crosslinking[63], which by itself can impede EV production[26]. However, in these disease conditions, tissues undergo hypoxia, which decreases extracellular pH due to increased anaerobic metabolism[64,65]. Hypoxia has been shown to increase membrane trafficking by recruiting short actin filaments[66], to increase EV number, and to influence EV cargo content that induces pathogenic phenotypes[67–69]. Low extracellular pH not only enhances the secretion of caveolin-containing EVs but also makes EV membrane less fluid due to increased incorporation of sphingomyelin, another class of sphingolipid[70].

4. Biomolecular interactions between EVs and ECM network

The molecular basis of interactions between EVs and ECM polymers can be hypothesized based on biochemical compositions of EVs and the ECM, and chemical bonds that govern interactions between the molecules. EVs contain various protein and lipid molecules, some of which are known to interact with the ECM via covalent or hydrogen bonds (Fig. 3), although most of these interactions remain to be directly confirmed in the context of EV-ECM interactions.

4.1. Covalent bonds

In principle, covalent bonds can facilitate permanent interactions between EVs and the ECM. One way that covalent bonding can occur between EVs and matrix polymers is when proteins on EVs contain cysteines exposed to the extracellular space, which can form disulfide bonds with proteins in the ECM network. This interaction can be facilitated by an extracellular disulfide catalyst secreted by cells as exemplified by covalent incorporation of laminin, which is known to be present in some EVs[71], into the ECM[72]. Since EVs are enclosed by the lipid membrane, they can also form covalent bonds with matrix polymers through lipid-protein interactions. ECM-bound vesicles contain higher levels of oxidized phospholipids than vesicles in fluid[73]. Oxidized phospholipids that contain carbonyl moieties form Schiff bases by reacting with a primary amine group of lysine or arginine, while those that contain α,β-unsaturated carbonyl groups form Michael adducts by reacting with a thiol group of cysteine or basic residues of histidine[74]. Indeed, oxidized phospholipids were shown to modify collagen via lipoxidation throughout life, and hence associated with aging[75]. Thus, some covalent EV-ECM interactions may be subject to regulation by the redox state of their environments, which is altered in various pathological conditions where EVs have been implicated[76,77].

4.2. Hydrogen bonds

Hydrogen bonding is ubiquitous in nature and enables the formation of reversible interactions. One potential way for EVs to interact with ECM polymers via hydrogen bonds is through heparin binding domains, which are rich in basic amino acid residues, such as arginine and lysine, and are present in a number of ECM molecules, including fibronectin, vitronectin, collagen, and laminin[78]. Arginine contains the positively charged guanidinium group, which forms strong hydrogen bonding with negatively charged phosphate, sulfate, and carboxylate groups[79]. The same principle also applies to lysine, but its interaction with a negatively charged group is weaker than arginine because lysine forms one hydrogen bond, while arginine forms a cyclic structure with a negatively charged group by forming two hydrogen bonds. Thus, some ECM polymers with heparin binding domains may interact with either sulfated molecules on EVs, such as glypican[80], or phospholipids on the membrane of vesicles, such as phosphatidylserine, an acidic phospholipid, which is enriched in matrix-bound vesicles secreted from cells in cartilage[81]. Conversely, this process can be inhibited when ECM polymers themselves are phosphorylated by extracellular enzymes to become more acidic, as occurs in some tissues, such as bones[82]. In addition, EV membrane contains a number of receptors that can bind to the ECM where hydrogen bonding plays important roles, including integrin $\alpha_L\beta_2$ (LFA-1)[83,84], integrin $\alpha_4\beta_1$[85,86] and CD44[87,88].

5. Biophysical EV-ECM network interactions as a basis of effective EV transport

The ECM consists of a polymer network with meshes that enable the transport of liquid and solutes. The mesh size of the ECM ranges from nanometer to micrometer scales[89,90]. Unlike small molecules that transport freely through the meshes by diffusion, EVs are often larger and more likely confined in the nanoporous ECM ($r_{mesh}/r_{EV} \leq 1$) due to stronger steric hinderance by the polymer. Indeed, the ECM in the interstitium is known to impede the transport of larger (>100 nm) synthetic nanoparticles and drainage into the lymphatic system, thereby serving as a barrier for drug delivery[91]. Previous studies reported the presence of matrix remodeling enzymes, such as matrix metalloproteinases[92] and lysyl oxidases[93] in EVs, suggesting the potential of EVs in biochemically modulating the mesh size of the ECM. However, if each EV relies on the ability to degrade the ECM in order to transport, the energy cost of EV transport would be very high. Hence, some EVs may have evolved to rapidly transport in the nanoporous ECM with minimum energy cost by leveraging physical interactions with the network. Transport of EVs in the nanoporous ECM does not necessarily require energy, as long as mechanisms exist to temporarily reduce steric hinderance in the network, thereby restoring thermal motion of EVs. This notion is supported by the hopping diffusion model where trapped particles larger than the mesh size can escape at longer time scales by overcoming free energy barrier between the confinement cages[94]. Supporting this model, earlier studies show that synthetic nanoparticles exhibit subdiffusive behaviors with infrequent jumps in mucus[95,96], which is entangled polymers without covalent crosslinking. In context of ECM-based polymers, a number of studies over the past decades show that the cartilage matrix allows the transport of molecules larger than its pore size (~6 nm)[90], including nanoparticles[97], the process that is facilitated under mechanical loading due to convective flow[98,99]. Convective flow is also known to drive the transport of nanoparticles with a certain size range (20–50 nm) in the interstitial matrix by lymphatic drainage[91]. Recently, it was shown that EVs do not require actomyosin contractility, convective flow or matrix degradation to transport in the viscoelastic ECM[18]. Understanding the biophysical basis of EV-ECM polymer interactions will inform both fundamental mechanisms behind EV transport in the ECM and engineering strategies to release EVs from or retain EVs in hydrogels (Fig. 4).

5.1. EV biophysical properties

The rigidity of synthetic nanovesicles is known to impact their ability to transport in a confined space by deformation[100–103]. To date, several studies have reported a broad range of rigidity for EVs. The majority of studies used atomic force microscopy (AFM) to characterize nanoscale vesicle rigidity in terms of Young's modulus (E in Pa), which is defined by the response of a material to a force applied along a one-dimensional axis. Using the Hertz model of indentation[104], E of EVs has generally been reported to be within a megapascal (MPa) range, which varies depending on cell types and subpopulations. EVs from tissue preparations, including saliva[105], neuronal synapse[106], and blood plasma[107] show $E < 10$ MPa, while EVs secreted from cultured mammalian cells[18] and cancer cells[108,109] show $E > 20$ MPa. Within subpopulations, E was shown to be lower for larger EVs than smaller EVs and NVEPs from cancer cells[108]. Intriguingly, a previous study with

synthetic nanovesicles showed that there exists an optimum $E \sim 50$ MPa where vesicles show the fastest diffusivity through mucus[102]. This value is similar to E of $CD63^+$ EVs from mesenchymal stromal cells (MSCs) (~100 MPa), which were shown to transport in the crosslinked, viscoelastic ECM[18]. While the Hertz model has been widely used given its simplicity and independence of particle size, it requires the assumption that EVs are purely elastic and homogeneous in composition. Recently, a modified Canham-Helfrich model was used to account for membrane bending and pressurization from fluid in the vesicle lumen upon AFM probe indentation by measuring vesicle stiffness, size, and tether force[110]. From this model, bending rigidity (k_c in J), the energy to deform a membrane to a different curvature from its initial curvature[111], can be directly measured for nanoscale vesicles. Using this model, EVs from red blood cells (RBCs) was shown to be ~15 k_bT ($k_bT = 4.11 \times 10^{-21}$ J at room temperature)[112]. Like E, k_c is independent of EV geometry. However, a model is yet to be developed to enable the conversion between E and k_c for EVs, since the conversion is currently possible only for thin shell vesicles with a hollow lumen, whereas EVs are fluid-filled. Systematic studies are still needed to correlate between E or k_c of EVs from different sources and their diffusivity in the ECM.

The relationship between nanoscale particle rigidity and diffusivity raises an important question of what determines the rigidity of EVs. Synthetic phosphatidylcholine-based nanovesicles exhibit E of 2~10 MPa[113,114] and k_c of ~14 k_bT[110], the latter of which was also observed in microscale unilamellar vesicles[115,116]. The similarity of these values to E and k_c of EVs warrants further examinations into roles of natural lipid bilayer compositions and lumen fluid properties in determining the rigidity of EVs. Earlier studies with microscale unilamellar vesicles showed that at a constant temperature, the presence of *cis*-double bonds (unsaturated) in hydrocarbon tails of phospholipids introduces a structural kink, which decreases molecular packing, thereby increasing membrane fluidity and decreasing k_c[117,118]. These observations were confirmed with synthetic nanovesicles by AFM where liposomes with liquid-like, disordered membrane show lower k_c[119]. Culturing MSCs with polyunsaturated acids was shown to increase the content of phospholipids with unsaturated fatty acyl groups in EVs[120], suggesting the possibility that k_c of EVs could potentially be tuned *ex vivo*. In contrast, ECM-bound vesicles are enriched in phosphatidylglycerol[121], which was previously shown to increase k_c of synthetic vesicles[122]. In addition to phospholipids as a backbone, the bilayer in eukaryotic organisms contains other types of lipids, most notably cholesterol, which is abundant in EVs[123]. Cholesterol is known to decrease k_c of synthetic vesicles in the presence of sphingomyelin[124,125]. Indeed, sphingolipids are also enriched in EVs[34,121,126,127], and their content is higher than ECM-bound vesicles[32]. Together, lipid membrane compositions could potentially impact the ability of EVs to transport or remain within the nanoporous ECM by tuning their deformability.

In addition to lipids, the membrane of EV subpopulations consists of different transmembrane proteins[21,128]. It was shown that the rigidity of EVs from RBCs generally decreases with increased protein-to-lipid ratios[129], although this relationship will likely depend on how protein insertion impacts membrane order[114,130,131]. One important class of membrane proteins in natural vesicles is channel proteins that mediate membrane transport, since they regulate fluid content and properties in the vesicle lumen, which can impact

vesicle rigidity. To date, a diverse range of ion and water channel proteins have been identified in EVs[132]. Of these, the aquaporin family is one of the earliest channel proteins discovered in EVs in urine[133–135] and RBCs[136]. The amount of aquaporins in EVs is known to change depending on physiological demands by cells. For instance, more aquaporin-2 is packaged into EVs from the apical plasma membrane of the renal collecting ducts when there is an increased demand to retain water in the body[133], while RBCs secrete EVs with less aquaporin-1 under hypertonic conditions[136]. Interestingly, aquaporin-driven water flux was shown to maintain stability of plant-derived vesicles under hypertonic conditions[137], suggesting its role in resisting mechanical deformation. From a biophysical perspective, deformation of EVs would temporarily decrease the internal volume and hence increase the concentration of solutes in the lumen, thereby creating osmotic pressure and increasing vesicle rigidity[110]. A recent study showed that aquaporin-1 is essential for EVs to transport in the nanoporous ECM, and downregulating aquaporin-1 rigidifies EVs[18]. Thus, rapid water flux by aquaporins will likely help resist changes in osmotic pressure and rigidification of EVs upon deformation during the transport process.

5.2. ECM biophysical properties

The deformability of EVs alone is less likely sufficient to overcome steric hinderance by the matrix polymer, since extreme deformation of EVs would compromise their structures. Success of EV transport will also require the ability of the ECM polymer to undergo structural reorganization, which is determined in large part by polymer crosslinking. In general, a less permanent form of crosslinking, such as electrostatic and hydrogen bonds, results in a polymeric network that dissipates energy upon external force, leading to viscoelastic properties[138]. Since most tissues are viscoelastic[1], it is possible that EV transport occurs in tissues upon external load. Interestingly, a modeling study showed that in the absence of external force, a weakly crosslinked ECM polymer network can still rearrange if nanostructures in the polymer transiently bind to or interfere with the crosslinks of the polymer, thereby enabling nanoparticle transport in the ECM[139]. While this concept still remains to be directly tested for EVs in the ECM, a recent study supports this notion, since EVs but not synthetic nanoparticles can transport in ionically crosslinked hydrogels[18]. This raises an interesting possibility that EVs may be able to transport in viscoelastic hydrogels by influencing their crosslinks.

6. Interfacing EVs with engineered materials

EVs are dispersed and cleared by the liver after systemic injection *in vivo* in a solution form with half-life less than hours[140]. Analogous to controlled drug delivery[141], material-based strategies, especially engineered hydrogels, can be used to control either release or retention of EVs in a specific tissue of interest. From a macroscopic design point-of-view, implantation[142,143], injectable bulk hydrogels[144], *in situ* gelation[145–152] and microgels[153] have been employed to deliver hydrogels with EVs to the host. The majority of these strategies used EVs from MSCs as a means to restore damaged tissues, since they are known to contain cargo molecules with potential immunomodulatory and regenerative effects[154,155].

6.1. Controlled release of EVs to the host

6.1.1. Diffusion.—The ability to gradually release EVs from hydrogels will help control the extent at which EVs become available to occupy tissue over time in order to achieve therapeutic effects. The first important step to achieve this goal is to crosslink hydrogels from polymer solutions while EVs are present so that EVs can gradually diffuse from hydrogels over time (Fig. 5). However, EV transport is generally more sensitive to crosslinking than small molecule transport due to large particle-mesh size ratios. Thus, the choice of crosslinking strategies will determine both kinetics and maximum amount of EV release by diffusion. An earlier study showed a delayed release of EVs from alginate hydrogels with higher molecular weight[144]. The release might have been facilitated by the use of $CaCl_2$ as an ionic crosslinking agent, which results in a rapid but non-uniform gelation[156]. Viscoelastic hydrogels from purified alginate can release a significant fraction of EVs at an optimum elasticity when crosslinked with $CaSO_4$, which offers a slower, more uniform gelation, in part because EVs can control deformation via water flux[18]. In addition to partial or reversible crosslinking of hydrogels, temperature-sensitive crosslinking of hydrogels can be effective in achieving controlled EV release, while offering utility as injectable materials. A recent study loaded EVs in chitosan with glycerol-2-phosphate, which undergoes ionic crosslinking after injection at 37 °C, with an optimum porosity controlled by polymer concentration, EVs were shown to be gradually released and to promote corneal regeneration[147]. Another study used methylcellulose-based hydrogels with xylitol and polyethylene glycol (PEG) that undergo gelation at 37 °C via hydrogen bonds to control release EVs, while the release rate can be accelerated with lower temperature. This system can potentially be useful in some disease conditions, such as critical limb ischemia where temperature of damaged tissue is known to decrease due to reduced blood flow[157].

6.1.2. Erosion.—To ensure that EVs are more completely released from hydrogels in a localized manner, several studies have employed strategies to induce the erosion of the polymer backbone, which can be categorized based on degradation mechanisms (Fig. 5). The simplest strategy is to engineer polymer networks so that they can undergo hydrolytic degradation over time to gradually release EVs[150,153,158]. For example, cleavage of the ester bonds present in poly (lactic acid)-based 3D engineered scaffolds results in sustained release of EVs from human gingival MSCs to treat bone defects[158]. Similarly, clickable PEG-based hydrogels were used, where cleavage of the ester bonds in PEG-thiol derivatives leads to gradual swelling and sustained release of encapsulated EVs from MSCs over 4 weeks to treat an animal model of chronic liver failure[159]. In addition, aldehyde-containing oxidized sodium alginate hydrogels with a low degree of oxidation were used to achieve prolonged release of dermal papilla-derived EVs over a period of 7 days, resulting in improved hair growth[153].

In many cases, it is desirable to erode the polymer backbone in response to specific conditions in host tissue. In a number of diseases, such as cancer and diabetic wounds, tissue environments become acidic, presenting opportunities to release EVs in a pH sensitive manner. A previous study encapsulated EVs in a hydrogel formed by Schiff base reaction between the aldehyde group of oxidized hyaluronic acid and the primary amine group of a polypeptide, such as ε-poly-L-lysine. Since Schiff bases hydrolyze under weak acidic

conditions, this hydrogel system enables EV release in response to low pH, which was shown to be effective in treating an animal model of chronic diabetic wounds[149].

Enzyme-based degradation mechanisms can also be employed to erode the polymer backbone and release EVs. In particular, naturally-derived hydrogels or synthetic hydrogels with peptide-based crosslinkers can be used to encapsulate EVs so that they can be released when various cells in host tissue secrete MMPs in pathophysiological conditions. For instance, gelatin-methacrylate hydrogels are known to be degraded by both collagenases and gelatinases[160] and were indeed used to encapsulate and locally release EVs for treatment of myocardial infarction[151] and cartilage regeneration[142]. In addition, MMP2-cleavable self-assembling peptides were used to form hydrogels and deliver EVs in the context of renal ischemia-reperfusion injury[152].

Light-sensitive degradation of hydrogels addresses a need for noncontact-based strategies to externally trigger EV release independently of host tissue conditions. A recent study used the *ortho*-nitrobenzyl-based photocleavable linker that contains both thiol and acrylate groups. The linker molecules were first attached to EVs via disulfide bonds and then mixed with cysteine-conjugated hyaluronic acid to induce gelation via thiol-acrylate Michael addition[161]. The amount of released EVs was shown to be proportional to the number of UV-blue light irradiation, suggesting the utility of this approach in on-demand EV release.

6.2. Strategies to increase EV retention within hydrogels

Previous studies suggest that ECM-bearing EVs deposited on a cell culture surface facilitate cell migration[162–164], raising the possibility that EVs can be used as haptotactic cues to recruit cells at the vicinity of hydrogels via juxtacrine interactions. In addition, when EVs are entrapped in hydrogels, soluble factors from EVs can be released in a controlled manner[165]—some of these factors are chemotactic signals[166,167], which can recruit cells from distance. Thus, increasing the retention of EVs in hydrogels offers opportunities to recruit, program, and deploy host cells in a localized manner. Indeed, physical entrapment of EVs in nanoporous hydrogels was shown to increase EV retention *in vivo* after delivery[168–170]. However, hydrogels can be engineered to increase the retention of EVs by leveraging non-selective or selective molecular interactions (Fig. 6). The advantage of using non-selective interactions is that they can be generalized to different types of EVs regardless of their subpopulations or sources. Since the EV membrane is negatively charged, positively charged materials can be used to increase the retention of EVs via electrostatic interactions, which were shown to promote regeneration[171] and immunomodulation[172]. EVs can also be grafted to materials more permanently by covalent bonds. One study employed a photoinduced imine crosslinking hydrogel to graft EVs upon gelation and showed sustained EV retention over 2 weeks[169]. More recently, a copper-free click chemistry strategy was described, where EVs were collected from cells that were metabolically labelled with azide-containing amino acids, and encapsulated in collagen hydrogels that were modified with dibenzocyclooctyne (DBCO) to conjugate EVs, resulting in increased recruitment of macrophages and vascular growth in hydrogels[173]. On the other hand, selective molecular interactions are desirable if the goal is to elicit specific biological responses by immobilizing a subset of EVs. This has been achieved by grafting peptide sequences that bind to specific

integrins present on the EV membrane to promote EV retention and tissue regeneration, including the Arg-Gly-Asp (RGD) peptide[174,175] that binds to $\alpha_5\beta_1$ and $\alpha_v\beta_3$[176] and a laminin-derived peptide[177] that binds to $\alpha_3\beta_1$ integrin[178].

7. Material-based cell culture strategies to control EV secretion from cells

In controlling EV release and retention via engineered materials, most studies to date collected EVs from cells on 2D tissue culture plastic, followed by enrichment of EVs from conditioned media prior to interfacing with materials. However, physicochemical factors of materials used in cell culture can impact the quantity and the properties of EVs from cells (Section 3), which may subsequently influence downstream applications with EVs. Thus, it will be important to understand how materials impact EV production by cells. The insights from this understanding can be helpful not only to improve the production of EVs that will be interfaced with materials, but also to inspire material-based strategies for sustained EV release or retention via cells. Advances in biomaterial design and biomanufacturing strategies have led to tunable engineered systems that recapitulate physical, chemical and structural properties of native tissues—these systems have been leveraged to discover new insights on cellular functions, which cannot be readily studied on standard tissue culture conditions[155,179]. Recent studies have employed these advances to control and improve EV production.

One important advance is a bioreactor system where cells can be cultured and a medium can be perfused so that EVs can be collected over time. A hollow-fiber bioreactor system (e.g., Fibercell) has emerged as one of the major methods to scale up the production of EVs, since hollow fibers offer a high surface area to attach a large number of cells (over 10^9) per setup, while enabling the circulation of the medium for nutrient exchange[180–183]. In addition to concentrating EVs in a small medium volume, the system also enriches for small EV-associated proteins per protein preparation compared to plastic culture. This suggests the potential effect of hollow fiber geometry or mass transfer on increasing small EV secretion or decreasing EV reuptake. It is possible to customize a bioreactor system by replacing hollow fibers with a 3D printed scaffold from a commercial stereolithography instrument, which was shown to increase EV production from endothelial cells[184]. While these studies used rigid materials to attach cells, employing a hydrogel-based cell culture surface or a scaffold with physiological biophysical properties[26] will likely help further increase the yield of EVs from a bioreactor system.

Another emerging approach is to collect EVs from cell spheroids formed in microwells or on non-adhesive materials[185]. In one study, spheroids from gastric cancer cells were formed in an agarose microwell array and shown to increase the number of EVs per cell, while the average EV size was decreased—spheroid-derived EVs also showed an increased level of microRNAs, which subsequently downregulate proteins involved in the ADP-ribosylation factor 6 pathway that is known to mediate microvesicle shedding[186]. Thus, this study suggests that cell spheroids produce more small EVs and less large EVs. Consistently, another study showed that MSC spheroids formed by a hanging-drop method or on an anti-adhesive, poly(2-hydroxyethyl methacrylate)-coated surface increase EV number per cell compared to 2D culture[183]. In a therapeutic context, a recent study formed cell

spheroids from lung biopsy tissues on an anti-adhesive surface, followed by cell expansion and collection of EVs, which were shown to be effective in treating preclinical models of fibrotic lung injury[187]. Overall, these studies suggest the utility of forming spheroids in promoting EV production. Given the diffusion limit of spheroids for nutrient exchange, the size of spheroids will need to be controlled below 100 μm to avoid the necrotic core[188]. Combining with a bioreactor system or employing vascularization strategies will enable the use of larger spheroids with high viability to increase the yield of EVs. From a mechanistic perspective, micropatterning-based strategies to decouple cell-cell contact and cell-material interactions[189] will help dissect their relative contributions to EV production.

In principle, encapsulation in engineered materials provides cells with physiologically relevant cues in 3D microenvironments, which could be optimal for EV production compared to standard culture conditions. One study showed that the amount of EV proteins secreted per cell is increased when the medium is collected from MSCs in 3D collagen gel than cells on 2D plastic culture, and that EVs from MSCs in 3D collagen gel with pore size 1~3 μm[190] show improved efficacy in an animal model of traumatic brain injury[191]. Another study showed that encapsulating HeLa cells in a peptide nanofiber-based hydrogel with pore size ~500 nm increases cell spheroid formation compared to 2D plastic culture, resulting in a more gradual release of EVs with a unimodal size distribution and a similar miRNA expression profile as that of cervical cancer patient plasma[192]. More studies are warranted to understand how 3D environments improve EV production, since these observations can be attributed to a number of factors arising from differences in the presentation of both physical and biochemical cues by 3D collagen gel *vs.* 2D plastic culture. Unlike 2D culture where EVs are directly secreted into liquid medium, EVs can interact with a polymeric network in 3D environments, a factor that needs to be taken into consideration in evaluating EV production.

8. Outlook

Understanding EVs in the context of the ECM inspires various strategies to interface EVs with engineered hydrogels as a means to improve the therapeutic efficacy of EVs by locally controlling release or retention. Making advances in this field requires the convergence of multiple fields, including cell and matrix biology, chemistry, membrane biophysics, biomaterial design, and nanotechnology.

The presence of EVs in the ECM is reminiscent of synthetic nanocomposite hydrogels[193], materials with distinct properties due to the inclusion of nanoparticles[193], which were previously developed to achieve advanced material properties, such as rapid self-healing[194] and toughness[195]. Polymer physics teaches us that nanostructures can crosslink a polymer chain if they bind to the polymer with strong affinity and multivalency, provided that they are small enough to be bridged by the network[196]. This principle suggests the possibility that some cell-secreted nanoscale mediators may serve as primary or secondary crosslinkers of the ECM polymers, and hence influence ECM structure and ultimately function. Large EVs will likely offer greater multivalency, but small EVs may be better suited to be bridged by the network. Exomeres were shown to be smaller and more rigid than EVs[35], suggesting

the possibility that NVEPs may remain in nanoporous hydrogels after encapsulation and contribute to mechanical rigidity.

A simple negative feedback loop can be envisioned where cells initially secrete more EVs when the ECM is softer[26], but if some EVs are deposited into the ECM[20] and stiffen the network by crosslinking, this will limit the ability of cells to further produce EVs in a physiological condition. Testing this possibility will necessitate the development of materials of which properties can be dynamically tuned by incorporation of EVs from material-interfacing cells. This is also important in modeling diseases, such as cancer[197] and fibrosis[198] where the ECM stiffens in most cases, and EVs play important roles in disease progression[199,200]. The interplay of cell-secreted EVs, EV-ECM interactions, and their impact on cellular functions will help advance our understanding of pathological processes that accompany substantial structural changes in tissue microenvironments.

It has become clear that cells secrete both EVs and NVEPs with distinct properties[33–36]. Since this insight has emerged very recently, it is likely that most studies to date interfaced both EVs and NVEPs with biomaterials simultaneously. Thus, future efforts will benefit from the implementation of fractionation strategies to separate or deplete EVs and NVEPs, such as immunoaffinity-based approaches[201] prior to interfacing with biomaterials. In addition, biogenesis mechanisms and biomolecular compositions are beginning to be better understood for different types of EVs and NVEPs, offering opportunities to design biomaterials that can release or retain specific subpopulations[174,175,177]. While the field is still young and rapidly redefined, combining cell-secreted nanoscale mediators with biomaterial design offers a novel platform to advance materials science, biology, and medicine.

Acknowledgments

This work was supported by National Institutes of Health Grants R01-GM141147 and R01-HL141255, and National Science Foundation CAREER Grant 2143857. We acknowledge Dr. Stephen Badylak and Dr. George Hussey for initial discussion of the review.

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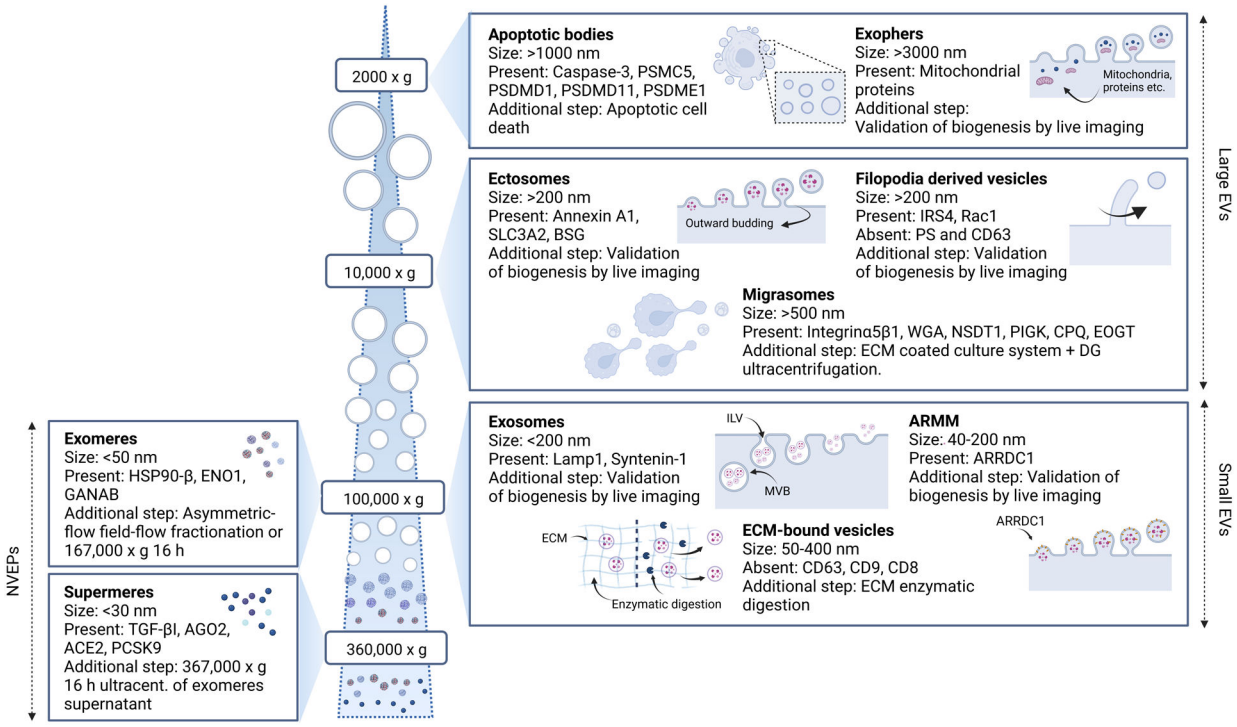

Figure 1. Cell-secreted nanoscale mediators.

Cells secrete a diverse range of nanoscale mediators with distinct physicochemical properties. In general, these mediators are classified into lipid membrane-bound extracellulr vesicles (EVs) and non-vesicular extracellular nanoparticles (NVEPs), which can generally be separated based on the size by differential ultracentrifugation. Apoptotic bodies and ectosomes (or microvesicles) are large (>200 nm) EVs and produced by membrane budding. More recently described large EVs are associated with specific biological processes, including exophers, migrasomes and filopodia derived vesicles. Exosomes belong to a subpopulation of small (<200 nm) EVs that originate from intraluminal vesicles (ILVs) in multivesicular bodies (MVBs) and are released when MVBs fuse with the plasma membrane. In addition to exosomes, small EVs consist of other subpopulations, including arrestin-domain-containing protein 1 (ARRDC1)-mediated microvesicles (ARMMs) and extracellular matrix (ECM)-bound vesicles. NVEPs, including exomeres and supermeres are generally smaller (<50 nm) than EVs, and can be isolated by additional ultracentrifugation steps.

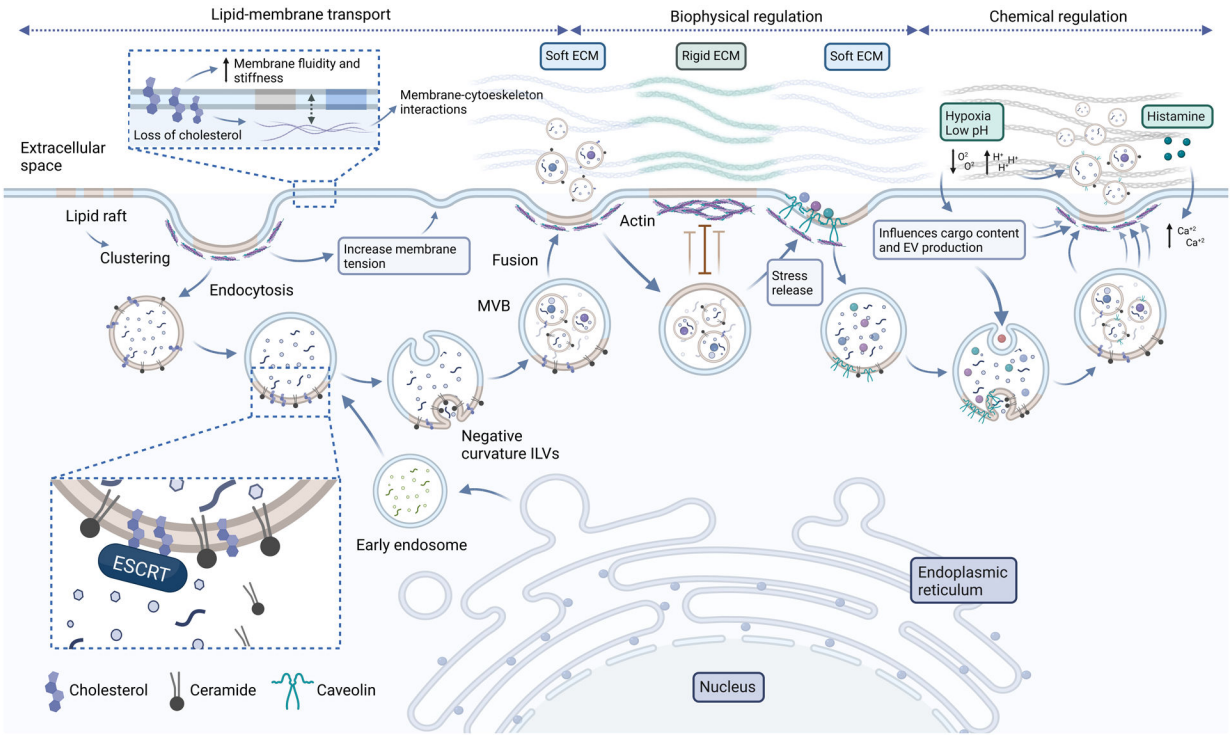

Figure 2. Biogenesis mechanisms of EVs in the ECM.

EV biogenesis is tightly linked with the lipid membrane transport process and physicochemical factors of the ECM that regulate this process. Lipid rafts serve as precursors of multivesicular bodies (MVBs) by providing lipids, including cholesterol and ceramide. Cholesterol mediates the recruitment of the endosomal sorting complexes required for transport (ESCRT) and ceramide induces negative curvature to form intraluminal vesicles (ILVs). The loss of membrane during endocytosis of lipid rafts can be counteracted by the gain of membrane during MVB fusion, thereby balancing membrane tension. When the ECM is softer, lipid rafts, including caveolae, are more readily formed because they are not used to counteract mechanical stress. In this case, lipid rafts can package some ECM molecules, which are shuttled into MVBs and released via exosomes. In addition, actin cytoskeletons are less dense in cells on a soft ECM, thereby facilitating MVB fusion and exosome release. The ECM also offers chemical cues that faciliate EV release, including oxygen tension, pH and signaling molecules that activate intracellular calcium levels.

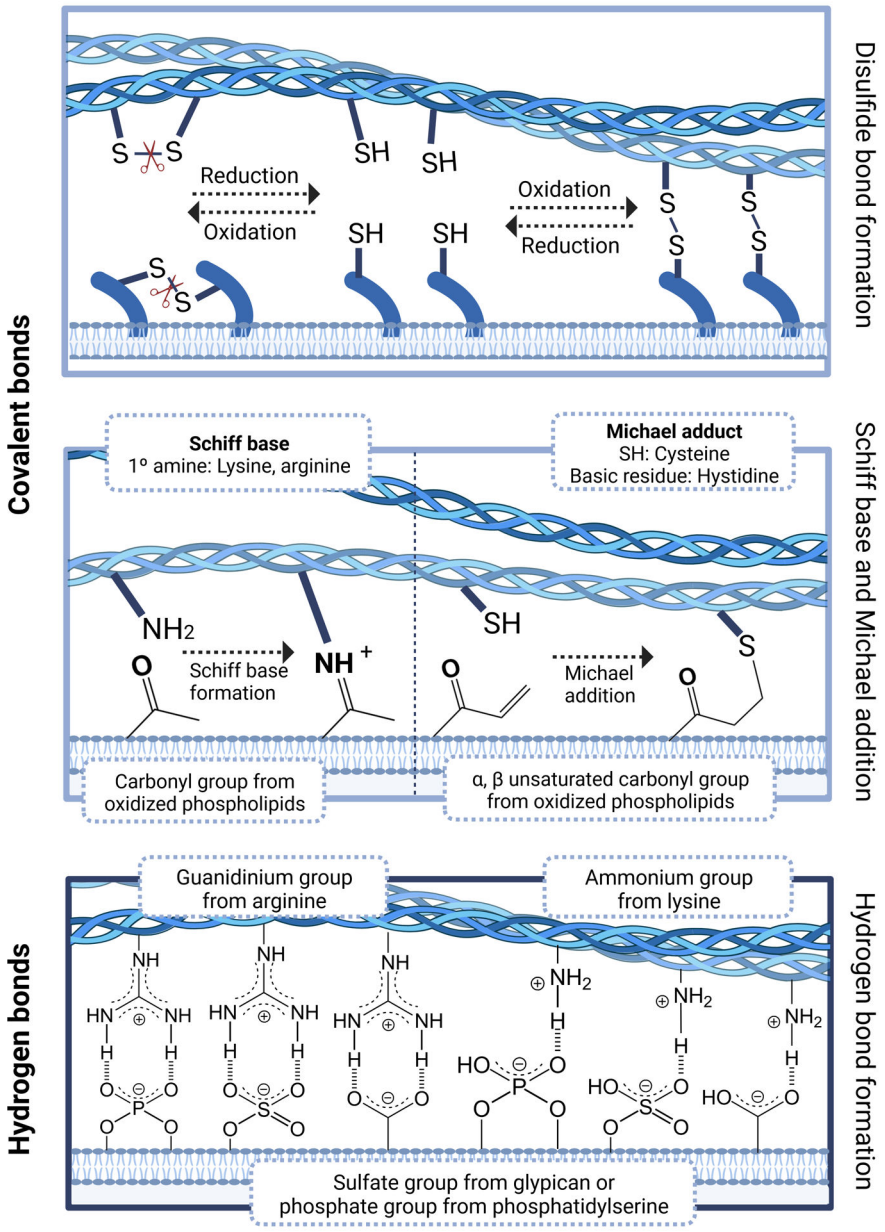

Figure 3. Biomolecular interactions between EVs and the ECM.

A number of biomolecular interactions can determine whether EVs bind to or are released from the ECM. Disulfide bonds can occur between a cysteine group of an EV membrane protein and that of an ECM protein, and are reversble depending on the redox state of the tissue environment and the availability of an extracellular enzyme that catalyzes this process. In addition, covalent bonds can be formed between a lipid molecule of the EV membrane and an ECM protein as Schiff bases or Michael adducts. EVs can also interact with the ECM via hydrogen bonds between a negatively charged heparin sulfate proteoglycan (e.g. glypican) or a phospholipid (e.g. phosphatidylserine) on the EV membrane and a positively charged amino acid (e.g. arginine or lysine from heparin binding domains) in an ECM protein.

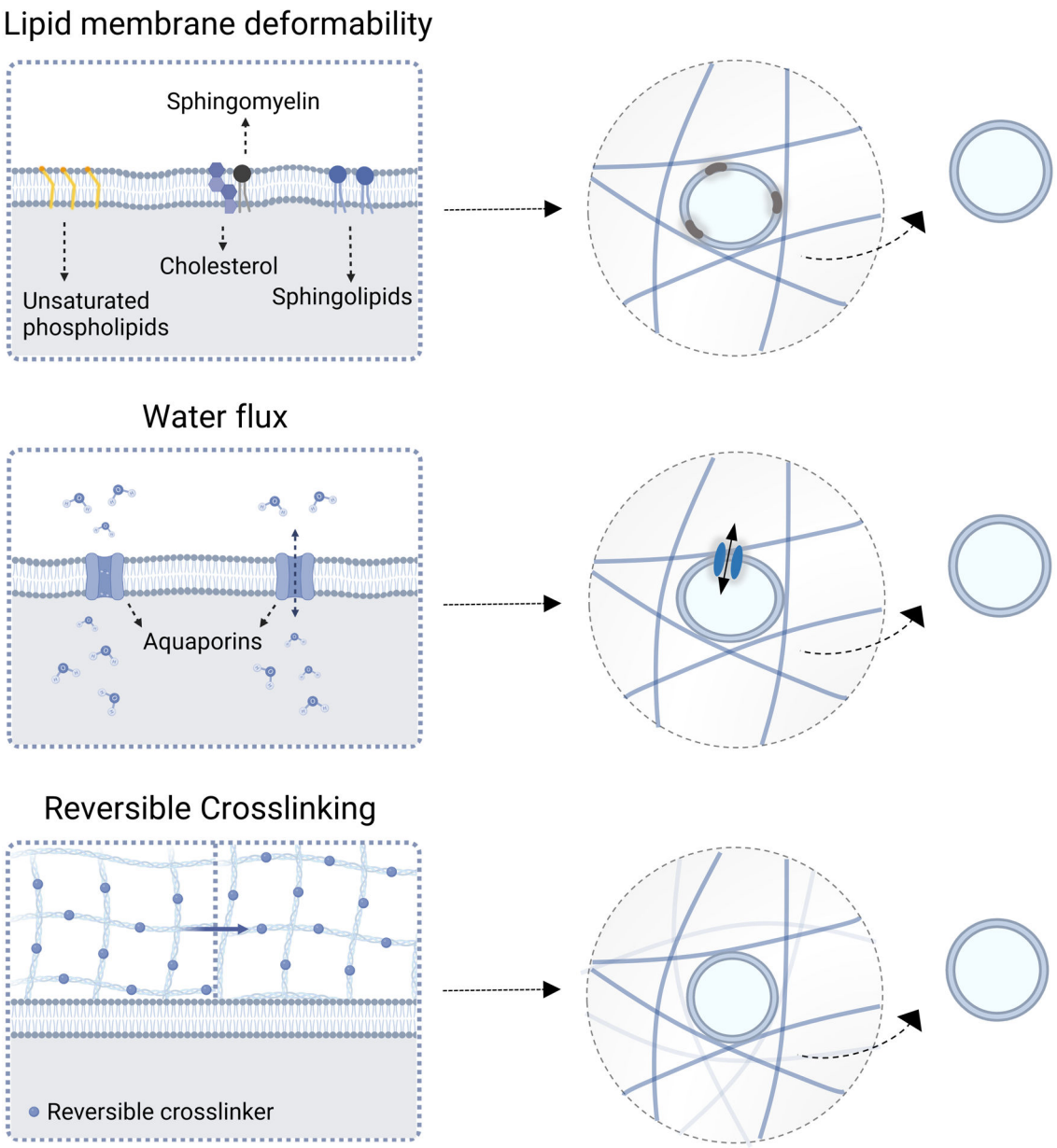

Figure 4. Biophysical mechanisms of EV transport in the ECM.

Under certain conditions, EVs can readily transport through a nanoporous network without relying on polymer degradation or convection. EVs contain a distinct set of lipids from cells or ECM-bound vesicles, including unsaturated phosphoslipids and sphingolipids, which can make EVs deformable. The ability of EVs to flux water through aquaporin enables them to deform in the network, thereby helping them resist changes in osmotic pressure. In addition to EV deformability, ECM crosslinking will likely need to be reversible, in order for EVs to bind to the crosslinks and to rearrange the network during the transport process.

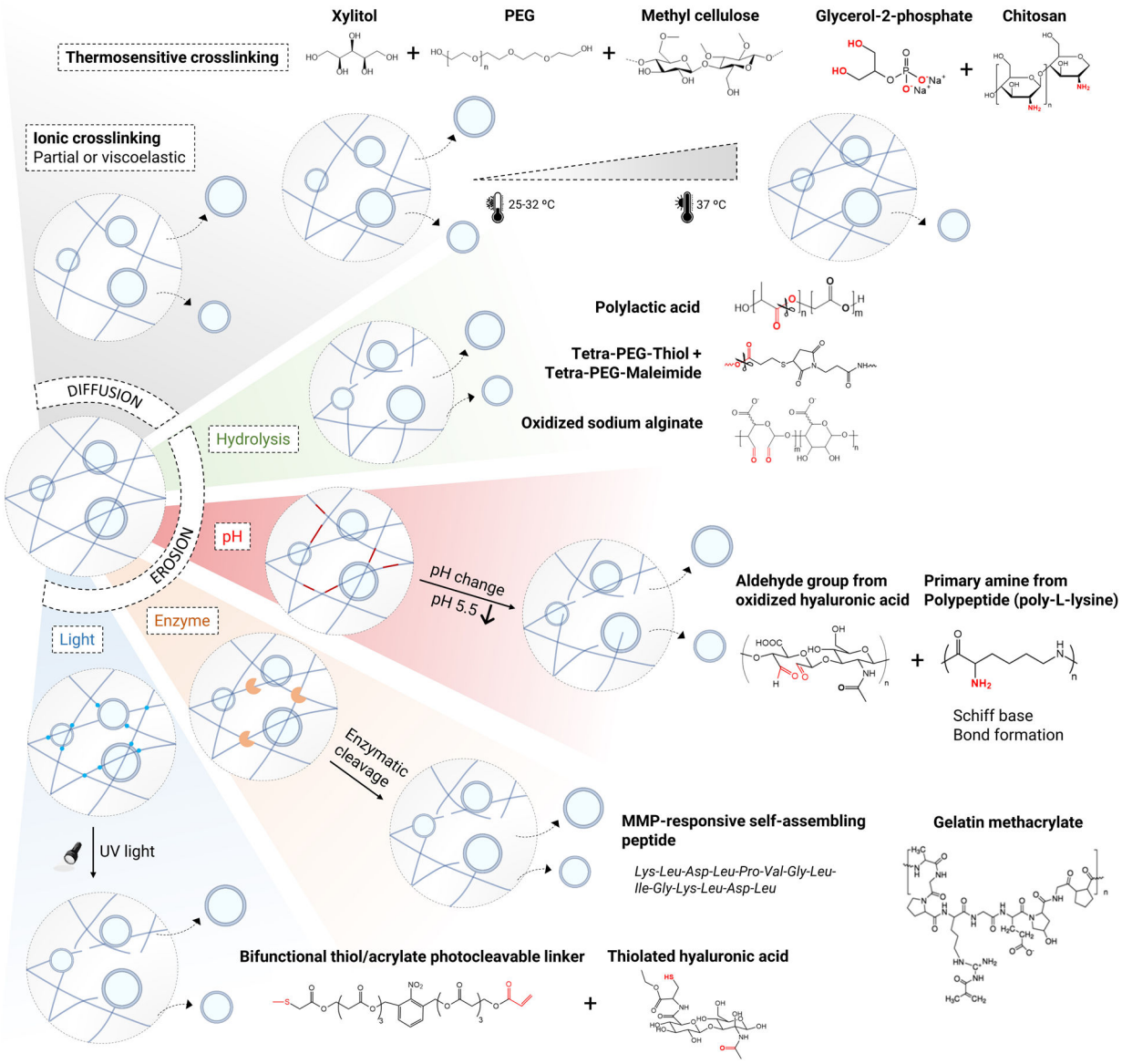

Figure 5. Biomaterial strategies to control EV release.

EV release can be controlled by either diffusion or erosion-based mechanisms. EVs can diffuse out in partially-crosslinked or viscoelastic hydrogels. Thermosensitive crosslinking can be used to tune EV diffusion from hydrogels as a function of temperature. For a more complete local release of EVs, erosion of a hydrogel network can be achieved either spontaneously through hydrolytic degradation or conditionally in response to external stimuli. The external stimuli that result in EV release by erosion of a hydrogel network can be classified into those that depend on host tissue conditions, such as pH and presence of enzymes, and those that enable on-demand release, such as light. Specific examples that were previously used to control EV release are shown for each category.

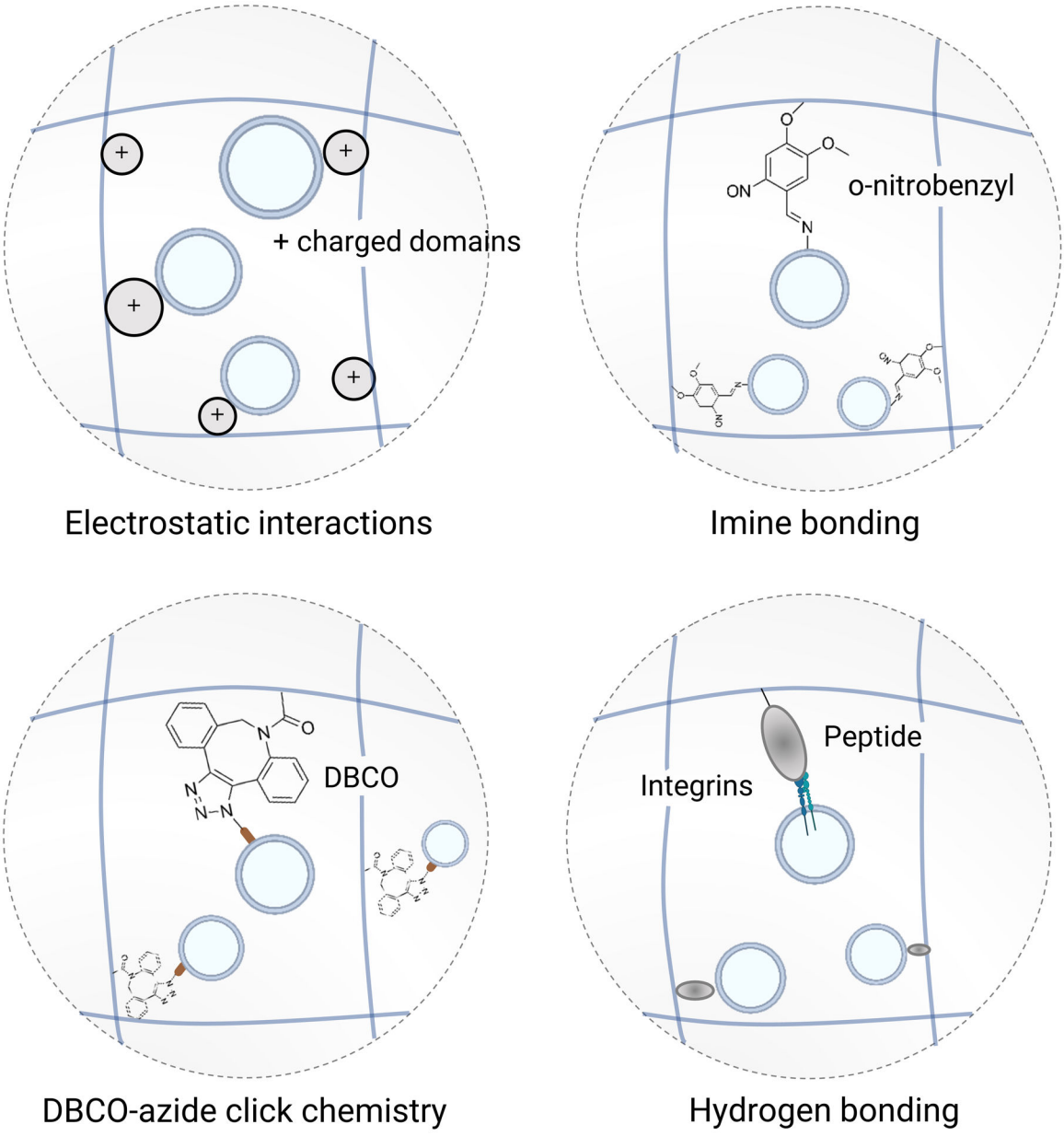

Figure 6. Biomaterial strategies to promote EV retention.

Introducing molecular interactions between EVs and a polymer network helps retain EVs within biomaterials to recruit and locally program cells. These interactions can be general, such as electrostatic interactions, imine bonding, and click chemistry (e.g. dibenzocyclooctyne (DBCO)-azide covalent bonds) of metabolically labelled EVs, in order to accomodate different types of EV subpopulations. Conversely, introducing a molecular sequence to a polymer network, such as an adhesion peptide that binds to integrins, enables the capture of a defined EV subpopulation in order to elicit a specific biological response.