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Review article

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# Extracellular vesicle–matrix interactions

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## Abstract

The extracellular matrix (ECM) harbours various 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 (EVs) and secrete them. Thus, EVs inherently interact with the meshwork of the ECM. In this Review, we discuss various aspects of EV–matrix interactions. Cells receive feedback from the ECM and leverage intracellular processes to control the biogenesis of EVs. Once secreted, various biomolecular and biophysical factors determine whether EVs 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 in which EV release, retention and production can be precisely controlled to elicit various biological and therapeutic outcomes.

## Sections



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## Introduction

The extracellular matrix (ECM) is a network structure consisting of various biomolecular and biophysical components essential to cellular functions and 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 constituent and indirectly by calibrating how cells generate contractile forces and tension via mechanotransduction[2,3], 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 the role 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]. Cells can secrete soluble proteins directly, but they 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], but ECM-bound vesicles were documented in other tissues only recently[13]. Studies with label-free third-harmonic generation microscopy further showed the enrichment of EVs in tissue stromal regions, which consist of dense matrix fibres[14,15]. 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 start by surveying the current knowledge of different cell-secreted nanoscale mediators. We then 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 and 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 investigation 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.

### Cell-secreted nanoscale mediators

Cell-secreted EVs are traditionally classified into apoptotic bodies, ectosomes (also called microvesicles or microparticles) and exosomes on the basis of 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]. By 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]. The issue with this classification is that validating specific cell-secreted EVs based on biogenesis pathways requires well-controlled investigations, such as using live cell imaging techniques fused with genetic approaches[26].

From a practical point of view, EVs can be classified into large (>200 nm) and small (<200 nm) EVs[27] and may include various EV subtypes in addition to apoptotic bodies, ectosomes and exosomes. Differential centrifugation can be used to separate large EVs (<10,000*g*) and small EVs (>100,000*g*). For instance, exophers are microscale large EVs that are isolated at ~1,000*g* 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 (ARRDC1)-mediated microvesicles that are formed by budding[32] and ECM-bound vesicles, which are 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 that after ultracentrifugation at 100,000*g*, the pellet contains, in addition to small EVs, non-vesicular extracellular particles (NVEPs) that do not contain a lipid bilayer. 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 100,000*g* 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 (~360,000*g*) can be done overnight on the supernatant to obtain even smaller NVEPs (<30 nm), called supermeres[36]. Some NVEPs were shown to be released via a shared pathway as exosomes[33], but the biogenesis pathway of NVEPs remains relatively unknown compared with that of EVs.

## Mechanisms of EV biogenesis in the ECM

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

### 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 to understand how EV biogenesis is regulated by the ECM. Lipid rafts are discrete, dynamic nanoscale domains in the external leaflet of the cell membrane that 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, have an essential role in the formation of MVBs: cholesterol recruits the endosomal sorting complex required for transport (ESCRT) machinery[42], and ceramide triggers the negative curvature of the MVB membrane to form ILVs in an ESCRT-independent manner[43]. 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 not only increases

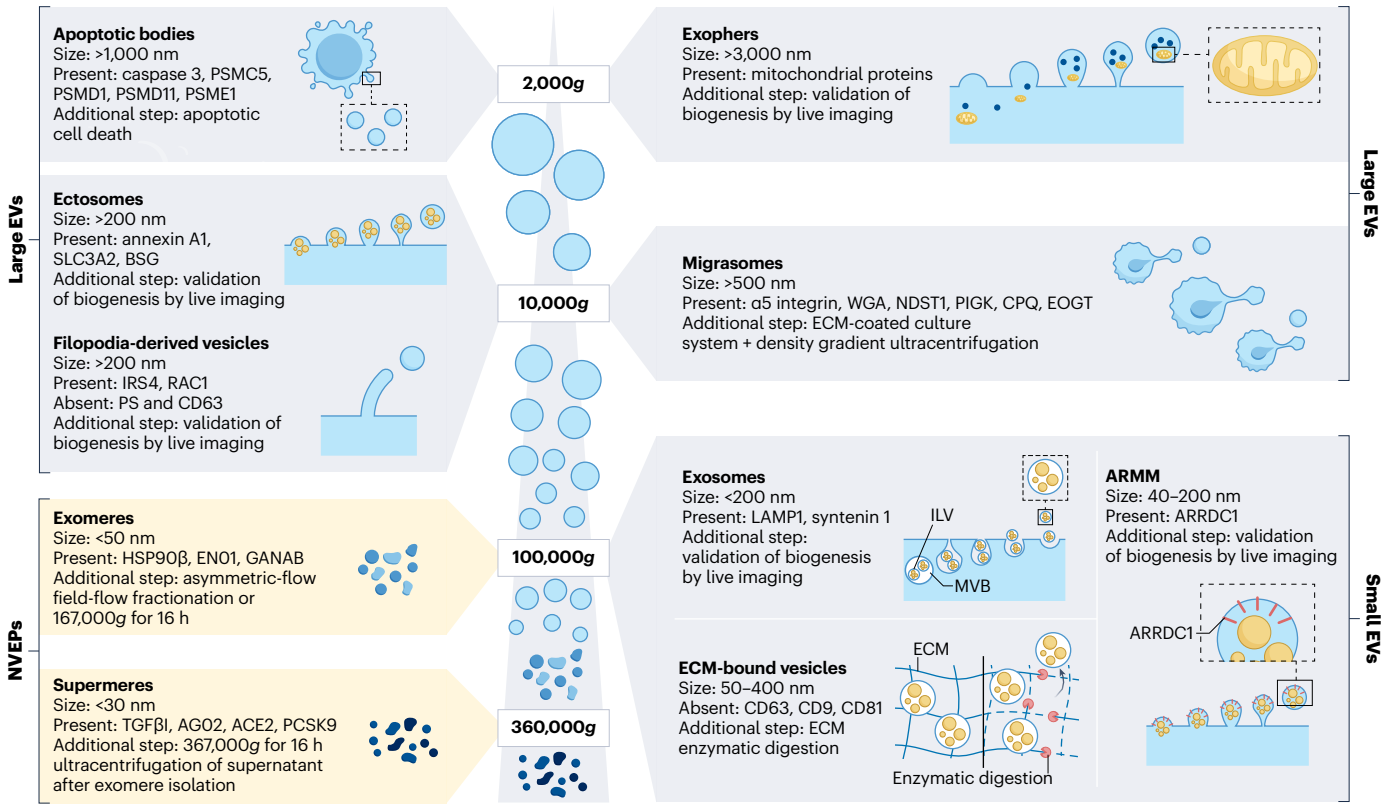



**Fig. 1 | Cell-secreted nanoscale mediators.** Cells secrete a diverse range of nanoscale mediators with distinct physicochemical properties. In general, these mediators are classified into lipid-membrane-bound extracellular vesicles (EVs) and non-vesicular extracellular nanoparticles (NVEPs), which can generally be separated on the basis of their 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 and include exophers, migrasomes and filopodia-derived vesicles. Exosomes belong to a subpopulation of small (<200 nm) EVs that originate from intraluminal vesicles 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. ILV, intraluminal vesicle; PS, phosphatidylserine.

membrane fluidity[46] but also promotes membrane–cytoskeleton interactions[47], thereby stiffening the cell membrane[48]. Thus, endocytosis of lipid rafts may result in a temporary increase in the cell membrane tension. However, this increase can be counteracted when MVBs fuse with the cell membrane to release exosomes, a 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.

## Biophysical regulation by the ECM

Because cells pull on and sense the resistive force from the ECM[2,3], the 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]. Caveolae have a role in mechanosensing, because 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]; this also happens 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. By contrast, cells on a stiffer matrix form an extensive actin network, which serves as a physical barrier for MVB transport and exosome release[26].

## 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 signalling and have 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

owing to increased ECM crosslinking[63], which by itself can impede EV production[26]. However, in these disease conditions, tissues undergo hypoxia, which decreases extracellular pH owing 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 modify EV cargo content to induce pathogenic phenotypes[67–69]. A low extracellular pH not only enhances the secretion of caveolin-containing EVs but also makes the EV membrane less fluid owing to increased incorporation of sphingomyelin, another class of sphingolipids[70].

## Biomolecular interactions between EVs and the ECM network

The molecular basis of the interactions between EVs and ECM polymers can be hypothesized on the basis of the 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). However, most of these interactions remain to be directly confirmed in the context of EV–ECM interactions.

### 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 the covalent incorporation of laminin, which is known to be

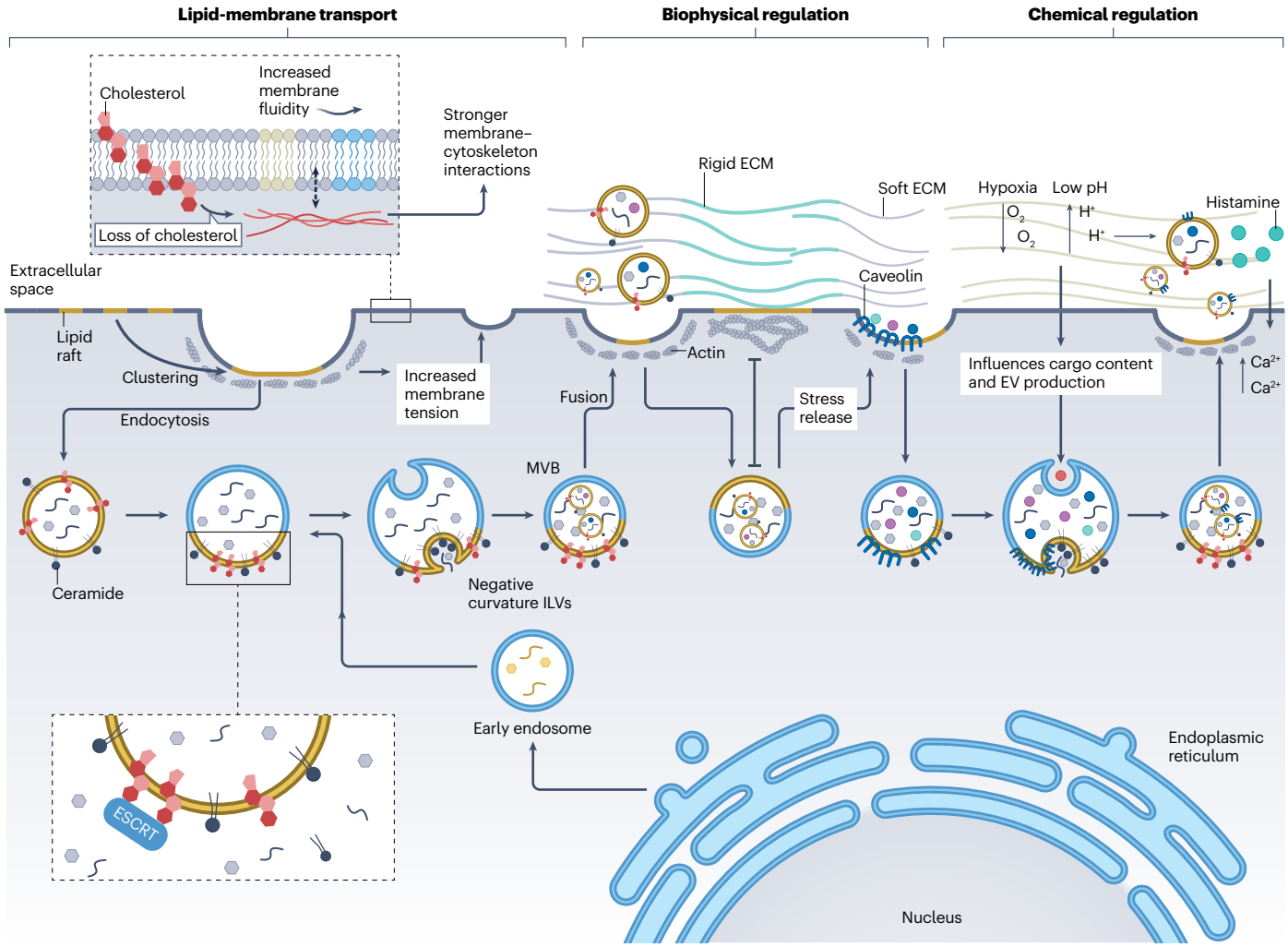



**Fig. 2 | Biogenesis mechanisms of EVs in the ECM.** Extracellular vesicle (EV) biogenesis is tightly linked with the lipid-membrane transport process and physicochemical factors in the extracellular matrix (ECM) that regulate this process. Lipid rafts serve as precursors of multivesicular bodies (MVBs) 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 facilitate EV release, including oxygen tension, pH and signalling molecules that activate intracellular calcium levels.

present in some EVs[71], into the ECM[72]. Because 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, whereas 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 are hence associated with ageing[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 in which EVs have been implicated[76,77].

### 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 bonds with negatively charged phosphate, sulfate and carboxylate groups[79]. The same principle applies to lysine, but its interaction with a negatively charged group is weaker than for arginine because lysine forms one hydrogen bond, whereas 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, the EV membrane contains a number of receptors that can bind to the ECM at places where hydrogen bonding plays important roles, including αLβ2 integrin (also known as LFA1)[83,84], α4β1 integrin[85,86] and CD44 (refs. [87,88]).

## Biophysical EV–ECM network interactions

The ECM consists of a polymer network with meshes that enable the transport of liquid and solutes. The mesh size ranges from nanometres to micrometres[89,90]. Whereas small molecules transport freely through the meshes by diffusion, EVs are often larger and more likely confined in the nanoporous ECM ($r_{mesh}/r_{EV} \leq 1$, where $r_{mesh}$ and $r_{EV}$ are the mesh size and the EV radius, respectively) owing to strong steric hinderance by the polymer. Indeed, the ECM in the interstitium is known to impede the transport of larger (>100 nm) synthetic nanoparticles and their drainage into the lymphatic system, thereby serving as a barrier for drug delivery[91]. The presence of matrix remodelling enzymes, such as matrix metalloproteinases (MMPs)[92] and lysyl oxidases[93], in EVs suggests that EVs can potentially modulate the mesh size of the ECM. However, if each EV relied on its ability to degrade the ECM 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. Indeed, the 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 the thermal motion of EVs. The hopping

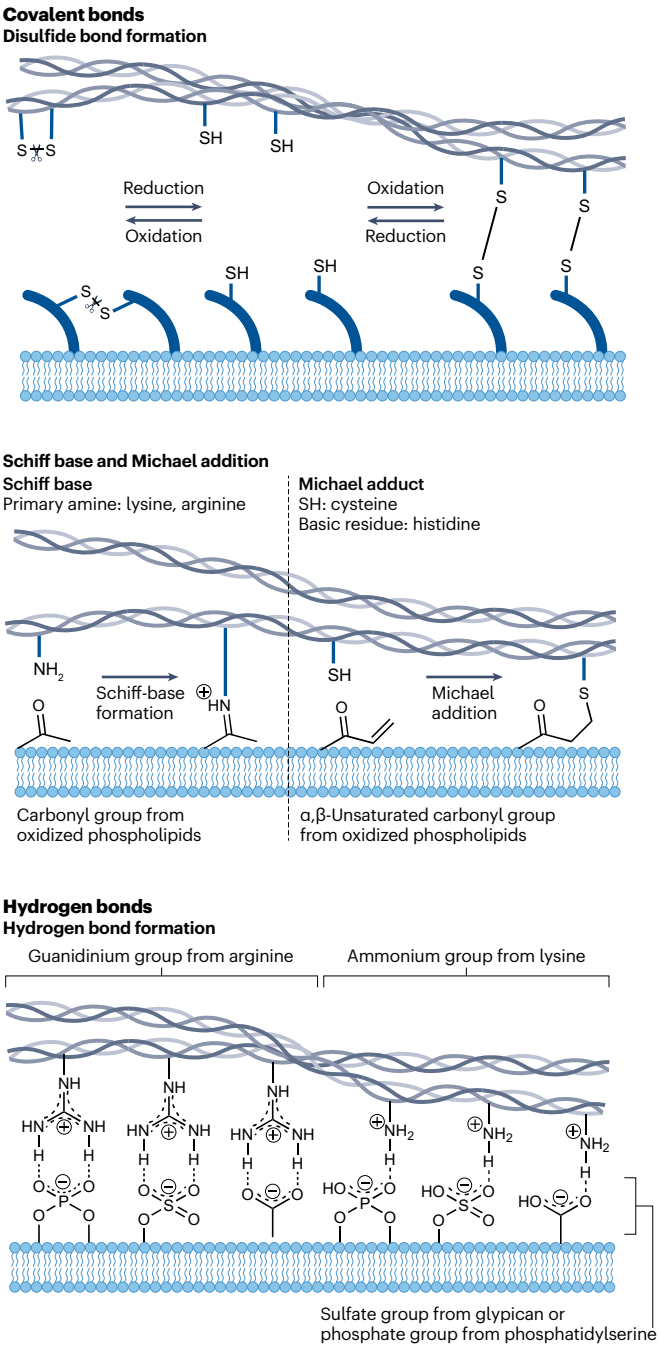



**Fig. 3 | Biomolecular interactions between EVs and the ECM.** Several biomolecular interactions can determine whether extracellular vesicles (EVs) bind to or are released from the extracellular matrix (ECM). Disulfide bonds can form between a cysteine group of an EV membrane protein and that of an ECM protein and are reversible depending on the redox state of the tissue environment and the availability of an extracellular enzyme that catalyses this process. In addition, covalent bonds can form 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 (such as glypican) or a phospholipid (such as phosphatidylserine) on the EV membrane and a positively charged amino acid (such as arginine or lysine from heparin-binding domains) in an ECM protein.

diffusion model offers a physical explanation of this concept, because it shows that trapped particles larger than the mesh size can escape at longer timescales by overcoming the free-energy barrier between the confinement cages[94]. Supporting this model, synthetic nanoparticles were observed to exhibit subdiffusive behaviours with infrequent jumps in mucus[95,96], which is made of entangled polymers without covalent crosslinking. In the context of ECM-based polymers, a number of studies over the past decades showed that the cartilage matrix allows the transport of molecules larger than its pore size (~6 nm)[90], including nanoparticles[97], a process that is facilitated under mechanical loading owing to convective flow[98,99]. Convective flow is also known to drive the transport of nanoparticles with a size of 20–50 nm in the interstitial matrix by lymphatic drainage[91]. However, 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 not only deepen our fundamental understanding of EV transport in the ECM but also inform engineering strategies to release EVs from or retain EVs in hydrogels (Fig. 4).

## EV biophysical properties

The rigidity of synthetic nanovesicles impacts their ability to transport in a confined space by deformation[100–103]. A broad range of rigidity has been reported for EVs. The majority of studies use atomic force microscopy (AFM) to characterize nanoscale vesicle rigidity in terms of Young's modulus ($E$), which is defined by the response of a material to a force applied along a 1D axis (in Pa or N m$^{-2}$). Using the Hertz model of indentation[104], $E$ for EVs has generally been reported to be in the megapascal range, variations of which depend on cell types and subpopulations. EVs from tissue preparations, including saliva[105], neuronal synapses[106] and blood plasma[107], show $E < 10$ MPa, whereas EVs secreted from cultured mammalian cells[18] and cancer cells[108,109] show $E > 20$ MPa. Within subpopulations, $E$ is lower for larger EVs than for smaller EVs and NVEPs from cancer cells[108]. Intriguingly, a study on synthetic nanovesicles showed that there exists an optimum $E$ ~ 50 MPa for which vesicles show the fastest diffusivity through mucus[102]. This value is similar to the value of $E$ of CD63$^+$ EVs from mesenchymal stromal cells (MSCs, ~100 MPa), which were shown to transport in the crosslinked, viscoelastic ECM[18]. The Hertz model is widely used because of its simplicity and independence of particle size, but it requires the assumption that EVs are purely elastic and homogeneous in composition. More recently, a modified Canham–Helfrich model was used to separately determine the bending rigidity ($\kappa$) of the EV membrane and the osmotic pressurization of the EV lumen from AFM measurements[110]. The $\kappa$ is the energy needed to deform a membrane to a different curvature from its initial one (in $k_bT$, which equals to $4.11 \times 10^{-21}$ J at room temperature)[111]. Using this model, the $\kappa$ of EVs from red blood cells was shown to be ~15 $k_bT$ (ref. [112]), whereas the $\kappa$ of EVs from breast cancer cell lines was shown to decrease from ~16 $k_bT$ to below 10 $k_bT$ with increased malignancy[113]. Systematic studies are still needed to correlate the Young's modulus and bending rigidity of EVs from different sources with their diffusivity in the ECM.

The relationship between nanoparticle rigidity and diffusivity motivates the important question of what determines the rigidity of EVs. Synthetic phosphatidylcholine-based nanovesicles exhibit an $E$ of 2–10 MPa[114,115] and a $\kappa$ of ~14 $k_bT$ (ref. [110]); the latter was also observed in microscale unilamellar vesicles[116,117]. The similarity of these values to those of EVs warrants further examination of the roles of natural lipid bilayer compositions and lumen fluid properties in determining the rigidity of EVs. Cholesterol and sphingolipids are the most abundant lipids in EVs[118–120] and hence have important roles in the structural integrity of EVs. EVs also contain different phospholipids. Early studies with microscale unilamellar vesicles showed that at a constant temperature, the presence of *cis*-double bonds (unsaturated) in the hydrocarbon tails of phospholipids introduces a structural kink that decreases molecular packing, thereby increasing membrane fluidity and decreasing $\kappa$ (refs. [121,122]). These observations were confirmed by AFM investigations of synthetic nanovesicles that showed that liposomes with a liquid-like, disordered membrane have lower κ (ref. [123]). Culturing MSCs with polyunsaturated acids increases the content of phospholipids with unsaturated fatty acyl groups in EVs[124], suggesting the possibility that the bending rigidity of EVs could be tuned ex vivo. By contrast, ECM-bound vesicles are enriched in phosphatidylglycerol[73], which increases the $\kappa$ of the membrane[125]. 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[21,126]. The rigidity of EVs from red blood cells generally decreases with increased protein-to-lipid ratios[127], although this relationship likely depends on how protein insertion impacts membrane order[115,128,129]. One important class of membrane proteins in natural vesicles is channel proteins that mediate membrane transport, because they regulate fluid content and

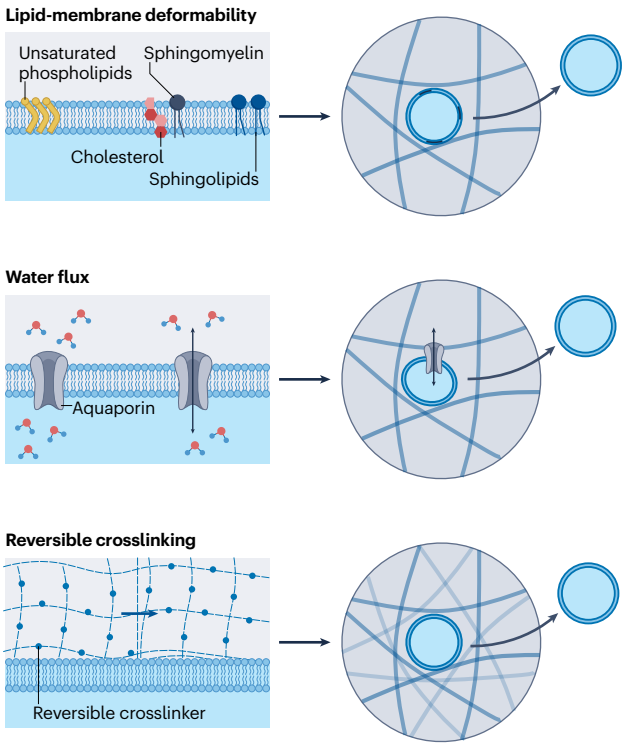



**Fig. 4 | Biophysical mechanisms of EV transport in the extracellular matrix.** Under certain conditions, extracellular vesicles (EVs) can readily transport through a nanoporous network without relying on polymer degradation or convection. EVs contain a distinct set of lipids that can make EVs deformable. The ability of EVs to flux water through aquaporins enables them to deform in the network, thereby helping them to resist changes in osmotic pressure. In addition to EV deformability, extracellular matrix crosslinking likely needs to be reversible for EVs to bind to the crosslinks and to rearrange the network during the transport process.

properties of the vesicle lumen. To date, a diverse range of ion and water channel proteins have been identified in EVs[130]. Of these, aquaporins are one of the earliest channel proteins discovered in EVs in urine[131–133] and red blood cells[134]. 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[131], whereas red blood cells secrete EVs with less aquaporin 1 under hypertonic conditions[134]. Interestingly, aquaporin-driven water flux was shown to maintain stability in plant-derived vesicles under hypertonic conditions[135], 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]. 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 likely helps to resist changes in osmotic pressure and rigidification of EVs upon deformation during the transport process.

### ECM biophysical properties

The deformability of EVs alone is likely not sufficient to overcome steric hinderance by the matrix polymer, because extreme deformation of EVs would compromise their structures. Successful EV transport also requires 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[136]. Because most tissues are viscoelastic[1], it is possible that EV transport occurs in tissues upon external load. Interestingly, a modelling study showed that in the absence of external force, a weakly crosslinked ECM polymer network can still rearrange if nanoparticles or nanovesicles in the polymer transiently bind to or interfere with the crosslinks of the polymer, thereby enabling their transport in the ECM[137]. This concept remains to be directly tested for EVs in the ECM, but a study supports this notion, because EVs but not synthetic nanoparticles can transport in ionically crosslinked hydrogels[18]. This observation raises the interesting possibility that EVs may be able to transport in viscoelastic hydrogels by influencing their crosslinks.

## Interfacing EVs with engineered materials

After systemic injection in vivo in solution form, EVs are dispersed and cleared by the liver with a half-life of hours or less[138]. Analogous to controlled drug delivery[139], material-based strategies, often based on engineered hydrogels, can be used to control either the release or the retention of EVs in a specific tissue. Implantation[140,141], injection of bulk hydrogels[142], in situ gelation[143–150] and microgels[151] have been used to deliver hydrogels containing EVs to the host. The majority of these strategies used EVs from MSCs as a means to restore damaged tissues, because they are known to contain cargo molecules with potential immunomodulatory and regenerative effects[152,153].

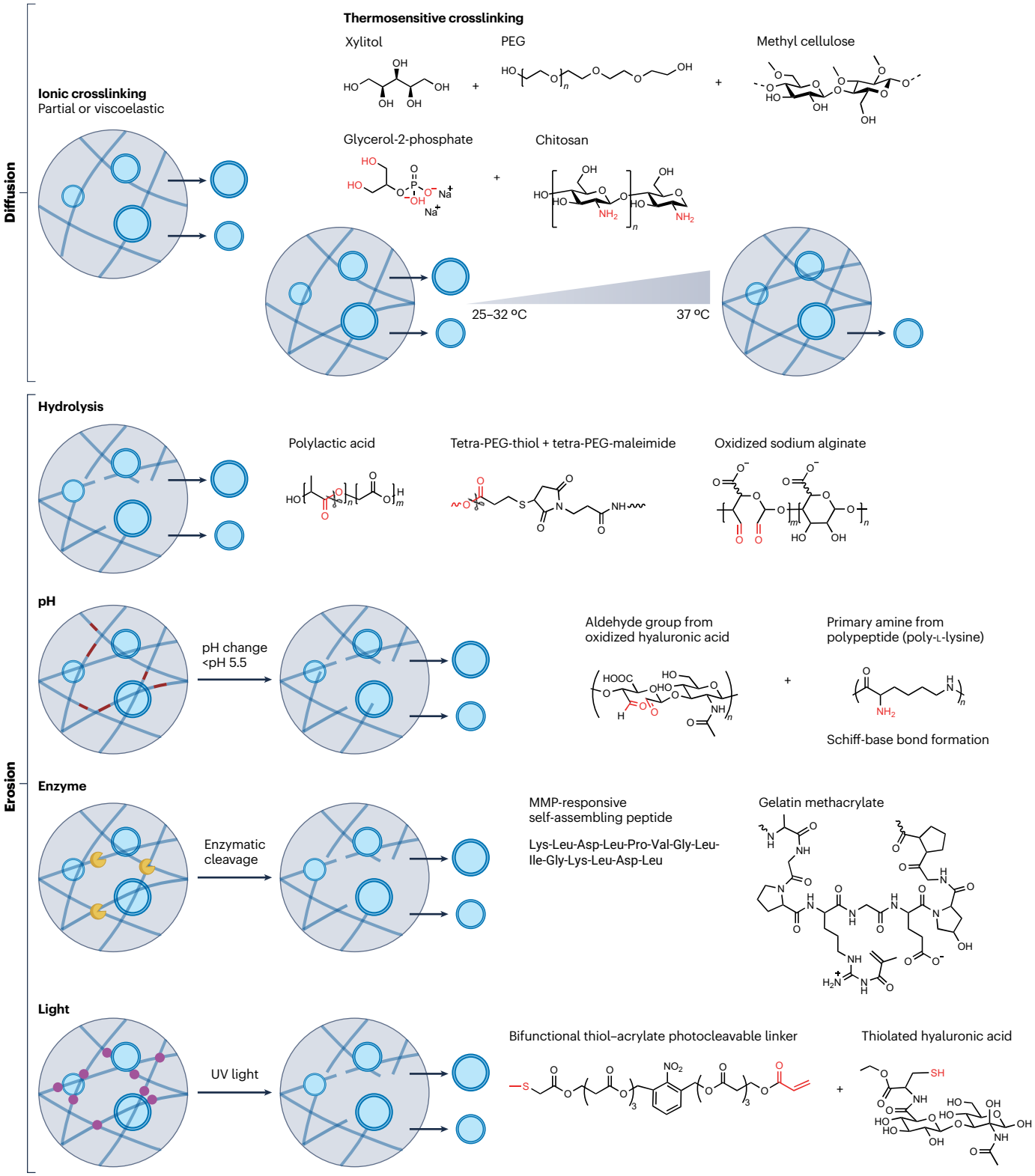
### Controlled release of EVs to the host

**Diffusion.** The ability to gradually release EVs from hydrogels will help to control the rate at which EVs become available to occupy tissue over time to achieve therapeutic effects. The first important step to achieve this goal is to crosslink hydrogels from polymer solutions in the presence of EVs, so that EVs can gradually diffuse from hydrogels over time (Fig. 5). EV transport is generally more sensitive to crosslinking than small molecule transport owing to the large particle–mesh size ratios. Thus, the choice of crosslinking strategy determines both the kinetics and the maximum amount of EV release by diffusion. A delayed release of EVs was obtained from alginate hydrogels with high molecular weight[142]. 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[154]. Viscoelastic hydrogels from purified alginate can release a substantial fraction of EVs at an optimum elasticity when crosslinked with $CaSO_4$, which offers a slower, more uniform gelation. Diffusion is partially facilitated by the ability of the EV to control deformation via water flux in confined spaces[18]. In addition to partial or reversible crosslinking of hydrogels, temperature-sensitive crosslinking of hydrogels can be effective in achieving controlled EV release, while being a useful strategy to obtain 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 gradually released and promoted corneal regeneration[145]. Another study used methylcellulose-based hydrogels with xylitol and polyethylene glycol (PEG), which undergo gelation at 37 °C via hydrogen bonds, to control the release of EVs, which is accelerated at lower temperatures. This system can potentially be useful in some disease conditions, such as critical limb ischaemia, in which the temperature of the damaged tissue is known to decrease owing to reduced blood flow[146].

**Erosion.** To ensure that EVs are as completely released as possible from hydrogels in a localized manner, several studies have used strategies to induce the erosion of the polymer backbone. These strategies can be categorized on the basis of the degradation mechanisms (Fig. 5). The simplest strategy is to engineer polymer networks so that they undergo hydrolytic degradation over time to gradually release EVs[148,151,155]. 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, which can treat bone defects[155]. Similarly, clickable PEG-based hydrogels, in which cleavage of the ester bonds in PEG-thiol derivatives leads to gradual swelling and sustained release of encapsulated EVs from MSCs over 4 weeks, were used to treat an animal model of chronic liver failure[148]. 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[151].

In many cases, it is desirable to erode the polymer backbone in response to specific conditions in the 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. For example, EVs were encapsulated 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. Because Schiff bases hydrolyse 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[147].

Enzyme-based degradation mechanisms can also be used 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 the host tissue secrete MMPs. For instance,




gelatin-methacrylate hydrogels are degraded by both collagenases and gelatinases[156] and were used to encapsulate and locally release EVs for the treatment of myocardial infarction[149] and for cartilage regeneration[140]. In addition, MMP2-cleavable self-assembling peptides were used to form hydrogels and deliver EVs in the context of renal ischaemia–reperfusion injury[150].

**Fig. 5 | Biomaterial strategies to control EV release.** Extracellular vesicle (EV) release can be controlled by either diffusion or erosion-based mechanisms. EVs can diffuse out of 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 the presence of enzymes, and those that enable on-demand release, such as light. Specific examples are shown for each category. MMP, matrix metalloproteinase; PEG, polyethylene glycol.

The light-sensitive degradation of hydrogels addresses a need for non-contact-based strategies to externally trigger EV release independently of host tissue conditions. The *ortho*-nitrobenzyl-based photocleavable linker, which contains both thiol and acrylate groups, was used for this purpose to promote wound healing[143]. 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. The amount of released EVs was shown to be proportional to the duration of UV light irradiation, suggesting the utility of this approach for on-demand EV release.

### Strategies to increase EV retention within hydrogels

Previous studies suggest that EVs deposited on a cell culture surface facilitate cell migration[157–159], raising the possibility that EVs can be used as haptotactic cues to recruit cells in 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[160]. Some of these factors are chemotactic signals[161,162], which can recruit cells from a 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[163–165].

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. Because the EV membrane is often negatively charged[166,167], positively charged materials can be used to increase the retention of EVs via electrostatic interactions, promoting regeneration[168] and immunomodulation[169]. EVs can also be grafted to materials more permanently by covalent bonds. One study used a photoinduced imine crosslinking hydrogel to graft EVs upon gelation and showed sustained EV retention over 2 weeks[164]. More recently, a copper-free click chemistry strategy was described, in which EVs were collected from cells that were metabolically labelled with azide-containing amino acids and encapsulated in collagen hydrogels that were modified with dibenzocyclooctyne to conjugate EVs. This strategy resulted in increased recruitment of macrophages and vascular growth in hydrogels[170]. By contrast, 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. Examples include the Arg-Gly-Asp (RGD) peptide[171,172] that binds to α5β1 and αVβ3 integrins[173] to promote kidney and bone repair and a laminin-derived peptide[174] that binds to α3β1 integrin[175] to treat spinal cord injury.

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

Most studies on the controlled release and retention of EVs via engineered materials collect EVs from cells on 2D tissue culture plastic and interface them with materials. However, as we discussed, physicochemical factors related to materials used in cell culture can impact the quantity and the properties of EVs, which may subsequently influence their downstream applications. Thus, it is important to understand how materials impact EV production by cells. These insights 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 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 uncover new insights on cellular functions that cannot be readily studied under standard tissue culture conditions[153,176]. Recent studies have used these advances to control and improve EV production.

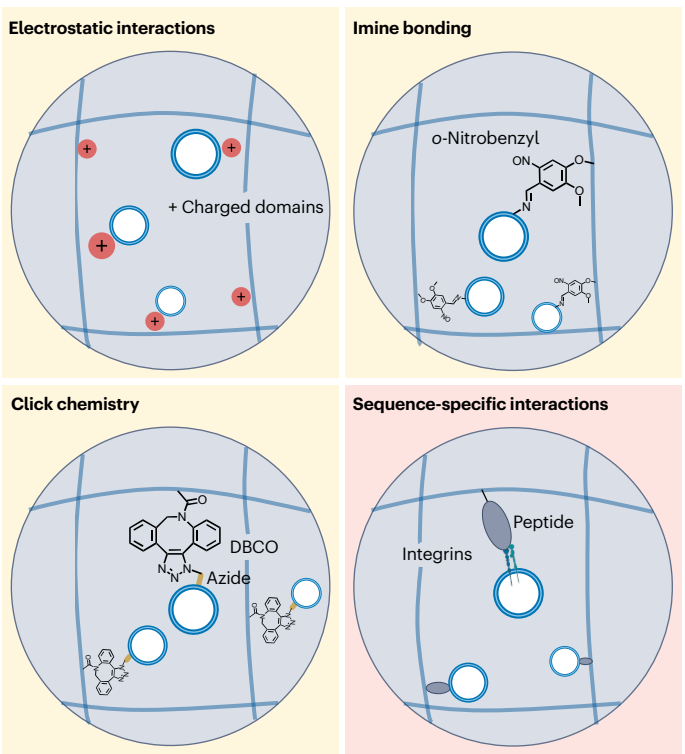



**Fig. 6 | Biomaterial strategies to promote EV retention.** Introducing molecular interactions between extracellular vesicles (EVs) and a polymer network helps to retain EVs within biomaterials to recruit and locally program cells. These interactions can be general, such as electrostatic interactions, imine bonding and click chemistry (for example, with dibenzocyclooctyne (DBCO)–azide covalent bonds) of metabolically labelled EVs, to accommodate 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 to elicit a specific biological response.

One important advance was the development of bioreactor systems in which cells can be cultured and a medium can be perfused so that EVs can be collected over time. Hollow-fibre bioreactor systems (such as Fibercell) have emerged as one of the major methods to scale up the production of EVs, because hollow fibres 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[177–180]. In addition to concentrating EVs in a small medium volume, the system also produces more proteins associated with small EVs per protein preparation compared with plastic culture. This suggests that a hollow-fibre geometry and mass transfer have potential to increase small EV secretion or to decrease EV reuptake. It is also possible to customize a bioreactor system by replacing hollow fibres with a 3D-printed scaffold from a commercial stereolithography instrument, which was shown to increase EV production from endothelial cells[181]. These studies used rigid materials to attach cells; using a hydrogel-based cell culture surface or a scaffold with physiological biophysical properties[26] will likely further increase the yield of EVs from bioreactor systems.

Another emerging approach consists in collecting EVs from cell spheroids formed in microwells or on non-adhesive materials[182]. 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, with a decreased average EV size. Spheroid-derived EVs show an increased level of microRNAs, which subsequently downregulate proteins involved in the ADP-ribosylation factor 6 pathway to inhibit large EV production, while promoting small EV production[183]. Consistently, another study showed that MSC spheroids formed by a hanging-drop method or on an anti-adhesive, a poly(2-hydroxyethyl methacrylate)-coated surface, increase the EV number per cell compared with 2D cultures[180]. In a therapeutic context, cell spheroids were formed 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[184]. 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 needs to be kept below 100 µm to avoid the necrotic core[185]. Combining this strategy with a bioreactor system or using 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[186] will help to dissect the relative contributions of these interactions 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 with standard culture conditions. One study showed that the amount of EV proteins secreted per cell increases when the medium is collected from MSCs in a 3D collagen gel compared with cells on a 2D plastic culture, and that EVs from MSCs in a 3D collagen gel with a pore size of 1–3 µm (ref. [187]) show efficacy in an animal model of traumatic brain injury[188]. Another study showed that encapsulating HeLa cells in a peptide nanofibre-based hydrogel with a pore size of ~500 nm increases cell spheroid formation compared with a 2D plastic culture, resulting in a more gradual release of EVs with a unimodal size distribution and a similar microRNA expression profile as that of plasma of patients with cervical cancer[189]. More studies are needed to understand how 3D environments improve EV production, because these observations can be attributed to several factors arising from differences in the presentation of both physical and biochemical cues by 3D hydrogels compared with 2D plastic cultures. Unlike in 2D cultures, where EVs are directly secreted into a liquid medium, in 3D environments, EVs can interact with a polymeric network, a factor that needs to be taken into consideration when evaluating EV production.

## Outlook

Understanding EVs in the context of the ECM can inspire various strategies to interface them with engineered hydrogels as a means to improve their therapeutic efficacy by locally controlling their 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[190], materials with distinct properties arising from the inclusion of nanoparticles[190], which were developed to achieve advanced material properties, such as rapid self-healing[191] and toughness[192]. Polymer physics teaches us that nanoparticles or nanovesicles 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[193]. 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 are 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 in which cells initially secrete more EVs when the ECM is softer[26], but when some EVs are deposited into the ECM[20], they stiffen the network by crosslinking and limit the ability of cells to further produce EVs in a physiological condition. Testing this possibility will necessitate the development of materials with properties that can be dynamically tuned by incorporation of EVs from material-interfacing cells. This is also important in modelling diseases, such as cancer[194] and fibrosis[195], in which the ECM stiffens and EVs play important roles in disease progression[196,197]. Understanding the interplay between cell-secreted EVs and the ECM and its impact on cellular functions will help to 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 and that they have distinct properties[33–36]. Because this insight has emerged very recently, it is likely that most studies to date interfaced both EVs and NVEPs with biomaterials simultaneously. Future efforts will benefit from the implementation of fractionation strategies to separate or deplete EVs and NVEPs, such as immunoaffinity-based approaches[198], before 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[171,172,174]. The field is still young and rapidly redefined, but it is clear that combining cell-secreted nanoscale mediators with biomaterial design offers a novel platform to advance materials science, biology and medicine.

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## Author contributions

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## Competing interests

The authors declare no competing interests.

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