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Effects of Hydrogels on Mesenchymal Stem/Stromal Cells Paracrine Activity and Extracellular Vesicles Production

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ABSTRACT

Mesenchymal stem/stromal cells (MSCs) are a valuable source of paracrine factors, as they have a remarkable secretory capacity, and there is a sizeable knowledge base to develop industrial and clinical production protocols. Promising cell-free approaches for tissue regeneration and immunomodulation are driving research towards secretome applications, among which extracellular vesicles (EVs) are steadily gaining attention. However, the manufacturing and application of EVs is limited by insufficient yields, knowledge gaps, and low standardization. Facing these limitations, hydrogels represent a versatile three-dimensional (3D) culture platform that can incorporate extracellular matrix (ECM) components to mimic the natural stem cell environment in vitro; via these niche-mimicking properties, hydrogels can regulate MSCs' morphology, adhesion, proliferation, differentiation and secretion capacities. However, the impact of the hydrogel's architectural, biochemical and biomechanical properties on the production of EVs remains poorly understood, as the field is still in its infancy and the interdependency of culture parameters compromises the comparability of the studies. Therefore, this review summarizes and discusses the reported effects of hydrogel encapsulation and culture on the secretion of MSC-EVs. Considering the effects of cell-material interactions on the overall paracrine activity of MSCs, we identify persistent challenges from low standardization and process control, and outline future paths of research, such as the synergic use of hydrogels and bioreactors to enhance MSC-EV generation.

1 | Introduction

Mesenchymal stem cells (MSCs) are known for their capacity to differentiate and transdifferentiate into clinically relevant cell types. However, as their secretory potential has become a key determinant of their biological function (Gnecchi et al. 2016), they are now a promising tool to produce clinically-relevant amounts of anti-inflammatory, anti-apoptotic, immune-modulatory, pro-regenerative and angiogenic bioactive factors (Yeo et al. 2013). This remarkable paracrine role, besides their strong homing and migration capacities (Fu et al. 2019; Sordi 2009), has earned them the nicknames of 'medicinal signaling cells,' site-regulated 'drugstores' and the 'paramedics' of the human body (Caplan 2017; Caplan and Correa 2011), encouraging research into cell-

free applications with a safer profile than conventional cell-based approaches (Kumar L. et al. 2019; Foster et al. 2017; Toupet et al. 2013).

While the paradigm in the field of MSC therapies shifted from cell-based towards secretome-based focus, there has been a parallel change in the understanding of extracellular vesicles' (EVs) paracrine function. From being considered as a cellular byproduct (Feng et al. 2022), EVs have emerged as an orchestrator of immune responses and cellular communication (Rashed et al. 2017; Gowen et al. 2020) that are also endowed with high biological permeability (Liang et al. 2021) and resistance to cryo-storage (Kou et al. 2022; Keshtkar et al. 2018). Therefore, they are also considered optimal delivery systems for biological factors targeting cells and tissues. Besides that, while MSCs are the leading source for stem cell therapy, due to their lower risk of tumorigenesis and fewer ethical constraints (Moghadasi et al. 2021; Johnson et al. 2021; Gemayel et al. 2023), stem cell-derived EVs should offer even lower immunogenicity, non-infusion toxicity, easier preservation and further insurance from ethical issues (Tan et al. 2024). On this basis, a number of preclinical and clinical studies have evaluated mesenchymal stem cell-derived EVs (MSC-EVs) efficacy as biopharmaceutical vehicles, therapeutics, and diagnostics (Casajuana Ester and Day 2023; Luo et al. 2021; Ciferri et al. 2021); MSCs are the most common cell type to derive EVs for clinical studies (49 studies, 67% of the total), with 3094 participants expected to receive MSC-derived EVs as of 2022 (Duong et al. 2023); MSC-EVs are being clinically tested as a therapeutic agent against different diseases, including acute respiratory distress syndrome (ARDS), kidney diseases, graft-versus-host disease (GvHD), osteoarthritis, stroke, Alzheimer's disease, and type 1 diabetes (Lotfy et al. 2023), while there were more than 200 preclinical studies on MSC-EVs on a number of different animal models (Tieu et al. 2023; Elahi et al. 2020). Moreover, MSC-EVs are currently investigated for osteochondral regeneration, autoimmune disorders, spinal cord injury, skeletal muscle injury, skin regeneration, treatment of lung fibrosis and neuro- (functional recovery and neurovascular plasticity), hepato- (improvement of liver fibrosis), reno- (improvement renal injury in the acute and chronic stages), and cardio-protection (neoangiogenesis and reduction of cardiac apoptosis and fibrosis) (Kou et al. 2022; Keshtkar et al. 2018; Varderidou-Minasian and Lorenowicz 2020; Kouroupis et al. 2022; Leñero et al. 2022; Kouroupis et al. 2023; Liebmann et al. 2024). Recent clinical trials are also evaluating the safety and efficacy of MSC-EVs in patients with a variety of diseases based on their immunomodulatory, analgesic and anabolic therapeutic effects (Kou et al. 2022; Keshtkar et al. 2018; Varderidou-Minasian and Lorenowicz 2020; Jones et al. 2024; Rizzo et al. 2023).

Unfortunately, current MSC-EVs manufacturing methods are inefficient and rely on space-demanding culturing platforms (Gowen et al. 2020), mostly in stiff plastic materials that provide only non-physiological 2D conditions, producing batches with inconsistent quality across cell sources and expansion protocols (Kou et al. 2022). This is where three-dimensional (3D) culturing systems, particularly hydrogels, present a promising solution, as they increase the surface-to-volume ratio and support the expansion of a larger biomass in a more space- and cost-efficient process, potentially improving EV manufacture and yield (Bordanaba-Florit et al. 2021). Hydrogels are capable of providing an in vitro environment that closely mimics the natural extracellular matrix (ECM) (Madl and Heilshorn 2018), offering a homogeneous, flexible and highly permeable hydrated matrix (Leach and Whitehead 2018) while potentially influencing the production and characteristics of EVs by modulating cell behavior through various biochemical and biomechanical cues.

The basic structure of a hydrogel involves a crosslinked polymer with available hydrophilic groups, such as hydroxyl, carboxyl, amine and sulphate. Therefore, they are able to hold large amounts of fluids, while the interlinked polymer chains prevent the hydrogel from dissolving (Mathew et al. 2018). Numerous allow the modification of mechanical properties, and can be classified based on several characteristics, like their origin (natural vs. synthetic), crosslinking method (physical vs. chemical), composition (homopolymeric vs. copolymeric), charge, structure or responsiveness (Dodda et al. 2023). The properties of the polymeric network also regulate the interaction of the encapsulated MSCs with external biological entities, provide protection from physical forces (Mazzitelli et al. 2013), and influence the diffusion of particles from and into the matrix. However, as the various biophysical cues are often interdependent, it is challenging to study the effect of specific hydrogel properties on the paracrine activity of MSCs. Designing advanced materials for cell therapy requires a precise understanding of how these signals interact with the cells (Li, Liu, et al. 2021), including the impact from biological and culturing variability. The adaptability of hydrogel systems allows the modulation of biochemical and biomechanical cues, favouring enhanced EV secretion or altering EV composition. Through the modification of polymer properties, hydrogels can create a controlled microenvironment that impacts the cellular processes involved in EV release, offering a potentially tool for scaling up the production of MSC-EVs and improving their therapeutic efficacy (Lenzini et al. 2020; Zavala et al. 2020; Khan et al. 2022).

Therefore, this review identifies key parameters in hydrogel design for MSC-EVs production, accounting for MSC-EVs' benefits and disadvantages towards clinical application. We compared the studies on cell-material interactions of hydrogel-encapsulated MSCs in the context EV secretion and overall paracrine function, while discussing the state-of-the-art of MSC-EV engineering and the general outlines of MSC-hydrogel interactions.

2 | Challenges and Efforts to Improve MSC-EVs Generation for Clinical Application

EVs are non-replicable lipid-based structures that are released via exocytosis from different cell types (Varderidou-Minasian and Lorenowicz 2020; Kouroupis et al. 2022), from which MSCs are remarkable producers (Yeo et al. 2013). EVs are generally classified as exosomes (30–150 nm) and microvesicles (100–1000 nm) (Di Vizio et al. 2009); however, these ranges overlap (Kou et al. 2022; Jeppesen et al. 2023) and are not rigorously used in the literature, despite their distinctive biological characteristics (Park et al. 2019). Exosomes have a density of 1.11–1.19 g/mL and a flat-spherical shape (Kou et al. 2022; Colombo et al. 2014); they mature in multivesicular bodies through a cargo-sorting process, and are released to the extracellular space by fusion of the multivesicular body into the cell membrane (Phan et al. 2018;

Urbanelli et al. 2013). Microvesicles, on the contrary, are released from the outward budding of the plasma membrane without a cargo-sorting process and have a lower density than exosomes (1.04 to 1.07 g/mL) (Johnson et al. 2021). It is important to note that, similar to microvesicles, apoptotic bodies cleave from the membrane without a maturation process, but they contain a more indiscriminate mix of fragments of nuclei, proteins, DNA, miRNA and other molecules (Madl and Heilshorn 2018).

The membrane of MSC-EVs is rich in cholesterol, sphingomyelin, ceramide and lipid raft proteins (Harrell, Jovicic, et al. 2019; Gazdic et al. 2015). It also exhibits common EV-specific surface markers (CD9, CD63 and CD81), MSC-specific surface markers (CD44, CD73, and CD90) (Kou et al. 2022), and adhesion molecules (e.g., CD29, CD59 and CD166) that enable their homing to injured tissues, among other functions (Harrell, Jovicic, et al. 2019). MSC-EVs contain messenger RNAs (mRNA), microRNAs (miRNAs), enzymes, cytokines and growth factors that regulate target cell's phenotype, function, and homing (Harrell, Jovicic, et al. 2019; Qiu et al. 2019; Harrell, Fellabaum, et al. 2019). However, while micro-vesicles are loaded with a similar composition to the cytosol of their parental cells, exosomes are selectively enriched in proteins and nucleic acids to exert specific functions (Park et al. 2019; Riazifar et al. 2017; Baek et al. 2019). The encapsulated mRNA and miRNA is the molecular basis for MSC-EVs functions (Kou et al. 2022; Qiu et al. 2018); however, miRNAs better correlate to downstream functionality than mRNAs. According to previous studies, specific miRNAs in MSC-EVs are linked to cardiovascular diseases, wound healing, neural damage, protection against hepatic and renal injuries, and both cancer-induction and cancer-suppression (Park et al. 2019).

Protein cargo does not vary significantly among different MSC sources, as almost half of the loaded proteins are similar across MSC-EVs from different tissues (van Balkom et al. 2019). Some of these proteins have defined biological functionality (Qiu et al. 2019), for example, they are related to immunomodulation (MOES, LG3BP, PTX3, S10A6, Cox2, PD-L1, galectin-1, TGF-β), neuroprotection (neprilysin and catalase), wound healing (type VII collagen and Wnt4), and angiogenesis (PDGF-D, Jagged1, EMMPRIN, c-kit, SCF, Angiopoietins 1 and 2). Although the correlation of EVs' cargo and function is nowadays better understood, MSC-EVs biogenesis and pharmacokinetics still have extensive knowledge gaps that prevent a reliable clinical translation (Keshtkar et al. 2018; Huldani et al. 2022). Current data also show differences in the activity of MSC-EVs subpopulations, hindering the identification of therapeutically beneficial EV subsets (Varderidou-Minasian and Lorenowicz 2020), and therefore requiring a better classification of EVs' subtypes and their functionalities (Tsiapalis and O'Driscoll 2020). Similarly, the parental MSC's tissue source affects MSC-EVs function. While MSC-EVs from various tissues promote angiogenesis, EVs from bone marrow (BMSC-EVs) and umbilical cord (ucMSC-EVs) specifically enhance tissue repair. BMSC-EVs also have lower immune-suppressive effects (Cai et al. 2020), exhibiting the greatest ability to remodel the ECM with the lowest amounts of proinflammatory cytokines (Chen et al. 2019). Besides that, while no difference in the yield and protein concentration of EVs from BM-MSCs and UC-MSCs has been reported (Naskou et al. 2024), proteomics analysis has revealed that the tissue origin contributes to distinct protein profiles among EVs from adipose, bone marrow, and synovium tissue, affecting their cartilage and bone regenerative capacities (Li, Yu, et al. 2021). Similarly, tissue source and passage number impact the angiogenic capacity of rat MSC-EVs derived from bone marrow and adipose tissue (Liu et al. 2024). To address this complexity, a recent study mapped the heterogeneity landscape of different MSC-EVs for precise selection in translational medicine, including EVs produced by MSCs from human umbilical cords, bone marrows, adipose tissues, dermal tissues and dental pulps (Li, Chen, Liu, et al. 2023).

While it is certain that EVs have lower immunogenicity and a better safety profile than cell-based therapies (Zhou et al. 2021), their biodistribution is not fully understood and strongly depends on the administration route (Sun et al. 2021). The therapeutic potential of EVs changes with treatment duration and within the pathological micro-environment (Gimona et al. 2021), thus increasing the risk to affect unwanted targets. Because of that, even though MSC-EVs therapies are theoretically cheaper than MSC-based treatments (Varderidou-Minasian and Lorenowicz 2020), the definition of the therapeutical doses is still unclear, especially in the context of biological variability. The limited proliferation of MSCs and their variable physiological state (Börger et al. 2017) are also frequent concerns for large-scale EV manufacturing (Kou et al. 2022). Differences in donor pathology and immunogenicity are responsible for functional alterations (Park et al. 2019), metabolic dysfunction (Capasso et al. 2015) and loss of mesenchymal plasticity and anti-inflammatory effects (Galipeau 2013).

To counter MSC-EV biodistribution and sourcing challenges, several attempts have been made to harness the EV's tropism (Rosenkrans et al. 2024). Membrane proteins can be fused with viral proteins to target specific cells (Di Rocco et al. 2016). For example, MSC-EVs modified with the rabies virus glycoprotein accumulate in tumours and concentrate in acetylcholine-receptor-rich organs (Wiklander et al. 2015), while POxylation and PEGylation stabilize MSC-EVs in plasma and preserve their native properties, increasing EV half-life by 6-fold at 6 h post-injection (Simon et al. 2025). Besides membrane modification, some aspects of the natural biodistribution of MSC-EVs are now better understood and can be used to differentially target injured tissues. MSC-EVs exhibit tropism towards the lungs in acute lung injury models in mice (Tieu et al. 2023), and can dose-dependently target radiation-injured intestinal tissues (He et al. 2024). MSC-EVs also penetrate tumours better than the EVs from other sources, such as the A431 squamous cell carcinoma line (Cohen et al. 2021). There are also efforts to better understand the impact of the administration route on MSC-EVs biodistribution (Wiklander et al. 2015; Tolomeo et al. 2023) and of the use of different labelling dyes in the interpretation of biodistribution assays (Rosenkrans et al. 2024; Chen et al. 2023). Significant work has also been done on clarifying the MSC-EV dosage regimes (Williams et al. 2023), upon which two clinical trials have already progressed to dose optimization in osteoarthritis treatments (Jones et al. 2024). Unfortunately, there is still no consensus on the units with which MSC-EVs dosages are calculated, as sometimes they are presented in micrograms, some by the number of particles, while others simply state the number of MSCs used to generate EVs, further hindering the comparison of doses among different studies (Lotfy et al. 2023).

The cargo of MSC-EVs is also sensitive to culture conditions (e.g., oxygen and growth factor availability) and can be modified through genetic engineering to improve antigenic, anti-inflammatory, immunosuppressive, analgesic, or reparative properties (Varderidou-Minasian and Lorenowicz 2020; Liebmann et al. 2024; Alonso-Alonso et al. 2022). However, stimulating the secretion of paracrine factors can lead to overshooting, calling for timely control strategies beyond current technological capabilities (Lagneau et al. 2023). The diversity of expansion platforms and the variety of culturing configurations also lead to inconsistent and contradictory findings (Elahi et al. 2020). Additionally, while 3D niches can improve culture screening and throughput (Madl and Heilshorn 2018), they are also more complex than 2D systems, preventing the translation of 2D findings into 3D models and between different 3D platforms (Zonderland and Moroni 2021). It is challenging to decouple the impact of a biophysical cue on cellular proliferation from the impact on other functions (Leach and Whitehead 2018), much more to account for synergic effects from the material's biochemical cues (Wechsler et al. 2021). The manufacturing of EVs also requires precise methods for purification and characterization and standardized procedures for harvest and enrichment (Kou et al. 2022). However, precise isolation and storage guidelines should be developed with considerations for the variability in MSC sources, culture systems and enrichment processes (Muralikumar et al. 2021). Given the inefficiency of the current purification methods and the unclear dosage regimes, treatment comparisons require a careful estimation of the total cost of goods for an equivalent clinical outcome (Elahi et al. 2020), raising the actual expenses of the therapy.

Due to the complexity and heterogeneity of EV preparations, quality control is a crucial element in the production of clinical-grade vesicle formulations (Nagelkerke et al. 2021). Beyond cell-intrinsic factors, cell culture conditions and downstream processing also affect the batch reproducibility of the MSC-EV product, but as such parameters are present in any manufacturing process of biologics, they can be controlled by appropriate process controls, following the good manufacturing practice (GMP) for biologics and cell therapy industries (Gimona et al. 2021). Unfortunately, many of the currently available methods and technologies for purification, quantification and characterization of EVs are inconsistent, and GMP standards need to be developed towards a reproducible, practical and scalable clinical GMP-grade EV application (Johnson et al. 2021). As a result, differences in batch-to-batch consistency based on the EV-isolation techniques have been reported multiple times, highlighting the multiplicity of factors impacting EV quantity and quality, and the need for better methods to ensure EV reproducibility (Wiest and Zubair 2020).

To compensate for these challenges, there is growing interest in the use of bioreactors and chemically defined medium, besides the preconditioning of parental MSCs to enhance their therapeutic potential (Courageux et al. 2022). As examples, MSC-EV therapy for myocardial infarction has used advanced bioreactor systems that led to consistent clinical outcomes (Shimizu et al. 2024), MSC-EV therapy for neurodegenerative diseases integrated quality by design (QbD) principles from early development stages to ensure process fidelity and product quality standards (Burns 2023), while, in the context of diabetic wound healing, MSC-EV therapy integrated automated and closed-system manufacturing procedures to minimize human intervention and optimize production efficiency (Shimizu et al. 2024; Fernández-Santos et al. 2022). Similar efforts are made to provide standardized EV culturing parameters for conventional static 2D systems (Barekzai et al. 2024), which are frequently used as controls for more complex 3D systems and bioreactors. Additionally, harmonized standards such as the minimal information for studies of extracellular vesicles (MISEV) from the International Society for Extracellular Vesicles (ISEV) (Witwer et al. 2021), the ISSCR Guidelines for Stem Cell Research and Clinical Translation (Lovell-Badge et al. 2021), and the set of guidelines on human mesenchymal stem cell-derived small EVs from China (Q. Li, B. Li, Ye, et al. 2023) enable comparability, cooperability and standardization within studies on MSC-EVs. Initiatives such as EV-TRACK also play a crucial role in promoting collaborative endeavours to enhance best practices (Shimizu et al. 2024; EV-TRACK Consortium et al. 2017). Extensive research has also been made to compare different isolation methods, such as ultracentrifugation versus size exclusion chromatography (Balbi et al. 2024), ion exchange chromatography versus ultrafiltration (Malvicini et al. 2024), and ultracentrifugation versus high-speed centrifugation and sucrose cushion ultracentrifugation (Abyadeh et al. 2024).

It may be too soon to answer whether MSCs' paracrine function can be precisely tuned with current technological capacities; meanwhile, MSC-EVs-based therapies could be partially considered a 'therapeutic black box' (Lagneau et al. 2023). Every step of the manufacturing process faces variability and complexity. MSCs are isolated from different tissues and donors, then expanded in multiple and unstandardized culturing configurations. After culturing, the supernatants are harvested and enriched under diverse protocols that generate EV batches with heterogeneous sub-populations. There is also high interdependency of culturing parameters, knowledge gaps in the understanding of EVs' biogenesis, processing inefficiencies, and a clinical context of uncertain biodistribution, pharmacokinetics and cost. In these challenging conditions (Figure 1), customizable substrates, such as hydrogels, support the development of studies to interrogate specific biophysical and biochemical cues and clarify their incidence on cell behaviour (Caliari and Burdick 2016), advancing in the establishment of integrated control strategies. At the same time, their injectability, outstanding biocompatibility, and biodegradability make them suitable tissue-engineered biomaterials (Deng et al. 2020) and provide a valuable tool to compensate for donor and host variability.

3 | Using Hydrogels' Properties to Guide MSC Functionality and Paracrine Action

Hydrogels are water-rich biomaterials that can encapsulate cells in a network and provide them with complex physicochemical interactions. These materials can mimic salient elements of native ECM (Caliari and Burdick 2016) and influence the physiological (e.g., cell adhesion, spreading, proliferation, migration, differentiation) and pathological (e.g., apoptosis, fibrosis, immunological rejection) processes of the cell (Cao et al. 2021). Hydrogels' manufacture depends on the polymer's nature, although it generally involves 3 key components: a backbone

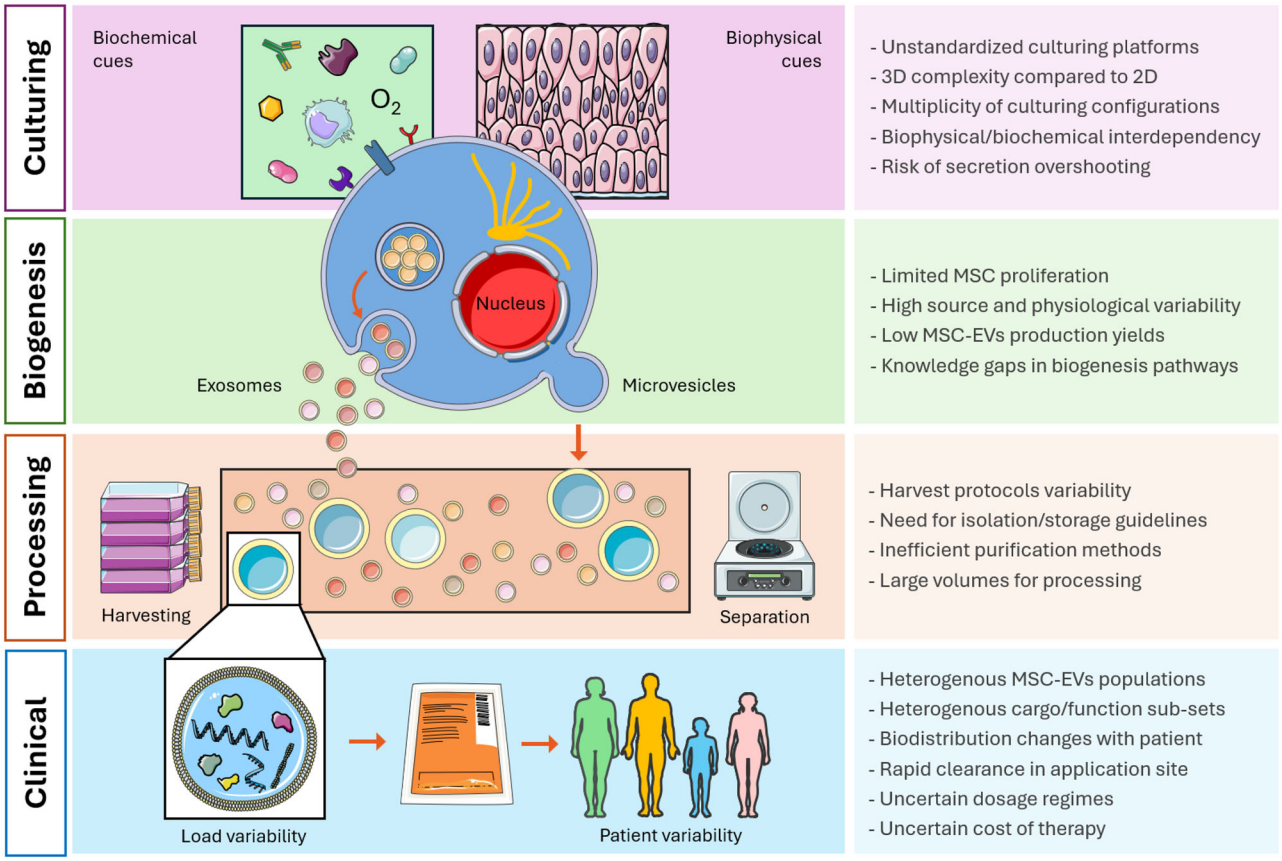

FIGURE 1 | Challenges in MSC-EVs manufacture in the context of 3D culturing towards clinical translation. MSC-EVs, mesenchymal stem cell-derived extracellular vesicles.

polymer (or polymers), a crosslinker, and, if necessary, an initiator for crosslinking (Ahmed 2015). There are several physical or chemical crosslinking processes according to the desired type of bonds and the crosslinking mechanism. Physically crosslinked hydrogels, frequently called 'reversible hydrogels,' are linked by hydrophobic interactions, electrostatic interactions or hydrogen bonding (Parhi 2017). These bonds are generally triggered by environmental parameters like temperature, pH, or charge density (Ermis et al. 2018). Chemically crosslinked hydrogels usually involve a crosslinker that interconnects separated polymer chains. These hydrogels tend to be mechanically stronger than physically crosslinked hydrogels (Jiang et al. 2016), as they involve covalent bonds formed through radical polymerization, high-energy irradiation, enzymes or photoinitiation (Ermis et al. 2018). In terms of cytotoxicity, physically crosslinked hydrogels are more advantageous, as they are free of organic solvents and expose the cells to milder processing conditions. In contrast, chemically crosslinked hydrogels demand harsher conditions for the cells during crosslinking (Akhtar et al. 2016).

Through the combination and modification of the hydrogel components, biophysical and biochemical cues can be incorporated to guide MSC functionality (Wobma et al. 2018). Biophysical cues are primary elements in biomaterial design and have a longer lifetime than biochemical cues. These include stiffness, porosity, degradability and viscoelasticity (Li, Liu, et al. 2021). When framing these properties in the context of MSC culture, the biophysical cues can be further grouped into two categories: mechanical and architectural properties (Figure 2). The mechanical properties define the material's stability and influence cell function by presenting instructive physical cues. The architectural features are mainly related to the diffusion of particles from and into the matrices, and the space availability to support cell growth (Lenzini et al. 2020; Paniushkina et al. 2020). Beyond these two categories, practical parameters such as hydrogel precursor sterilizability and injectability should be considered towards clinical applications, along with other fundamental parameters including hydrogel cytocompatibility and biodegradability (Lagneau et al. 2023). It should also be noted that MSCs from diverse tissue sources might react differently to hydrogel encapsulation, requiring attention to the material's biocompatibility and mechanical properties (Wehrle et al. 2019) to assure cell functionality. For example, the adipogenic and neurogenic differentiation potential of adipose and bone marrow-derived MSCs can be influenced by controlling matrix stiffness, while the gel's adhesion properties (Lee et al. 2020) and the optimal range of mechanical parameters to suppress MSCs' differentiation bias are tight for cells from identical donors and vary among cell populations from different donors (Miyoshi et al. 2023).

3.1 | Mechanical Properties: Stiffness and Viscoelasticity

The influence of matrix stiffness on MSCs morphology, proliferation and differentiation is better understood than its influence

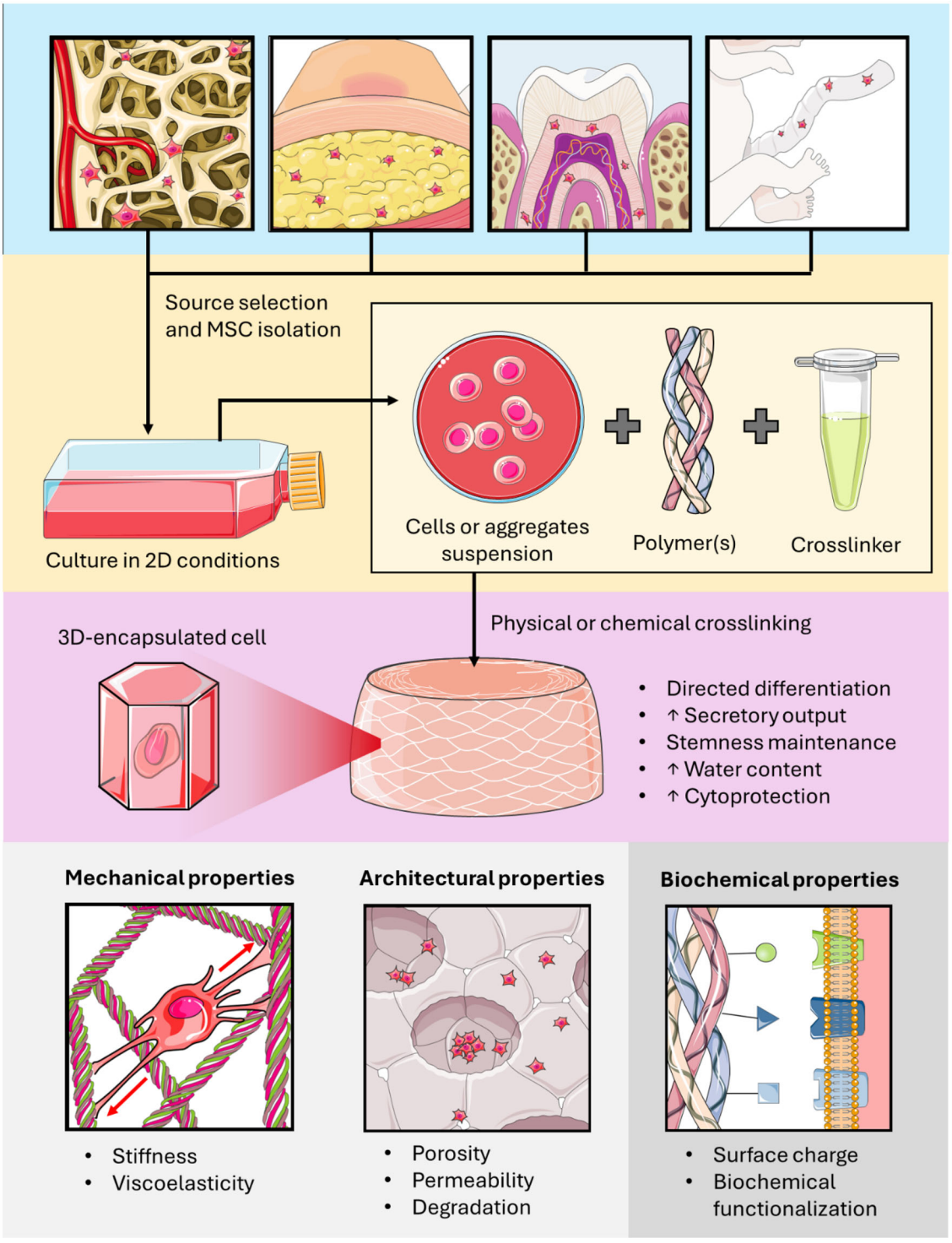

FIGURE 2 | Transitioning from 2D to 3D culture and main hydrogel properties for MSCs culture. MSCs, mesenchymal stem/stromal cells.

on paracrine action (Wechsler et al. 2021). Stiffer materials seem to direct the cells towards spindle-like morphology (Whitehead et al. 2018), promote mechano-transduction (Steward et al. 2013) and enhance osteogenic differentiation (Wu et al. 2018), while softer materials enhance proliferation (Whitehead et al. 2018), adipogenesis (Huebsch et al. 2010; Zhao et al. 2014) and chondrogenesis (Lagneau et al. 2023; Lee et al. 2020; Toh et al. 2012). Stiffness, however, is not enough to trigger certain MSC functionalities which demand additional priming to be started (Kouroupis et al. 2019); for example, the regulation of chondrogenic differentiation is restricted when solely biophysical cues are used without stimulant soluble factors (Guo et al. 2021; Bian et al. 2013). Prolonged exposure of MSCs to soft or stiff matrices can result in a strong commitment to the lineages specified by the matrix elasticity, even when the cells are exposed to contradictory soluble differentiation cues. This phenomenon is called 'mechanical memory' (Wechsler et al. 2021), and it biases MSC towards fibrogenesis and osteogenesis when cultured in

supra-physiologically stiff materials, such as 2D tissue culturing polystyrene (Yang et al. 2014). Specifically, miR-21 is a long-term memory keeper of the fibrogenic program in MSCs and remains elevated for 2 weeks after the mechanical stimulus. Soft matrix priming can erase such 'mechanical memory,' attenuating MSCs' fibrogenesis differentiation and increasing MSCs' therapeutic effects (Li et al. 2017).

Substrate stiffness might be the predominant factor to guide certain secretory profiles, as MSCs cultured on 30 kPa gels produce more immunomodulatory factors than MSCs cultured on 100 kPa gels (Ogle et al. 2020). It is also stiffness, instead of cytokine stimulation, that dominates IDO1 expression, as well as the up-regulation of NFkB-p65 and COX-2. Thus, the mechanical properties could act as a switch for processing of independent molecular inputs (Darnell et al. 2018). Prolonged culture of MSCs on 2D also decreases their mechano-sensing abilities and reduces the secretion of cytokines such as TGF-β1, VEGF, GDNF and EGF, but if the MSCs are transferred to soft hydrogels, it rescues the secretion of key proliferation and growth factors (Rao et al. 2018). Unfortunately, the effects of mechanical memory are still widely unknown for EVs production.

Soft hydrogels may enhance MSC secretion through an increased maintenance of stemness, which implies an association between differentiation and secretory activity (Lagneau et al. 2023). Similarly, pathways regulating MSCs immunomodulatory functionality seem to be related to differentiation pathways (Gao et al. 2023). However, the hydrogels that influence viability and proliferation may simultaneously impact the magnitude of the secretory activity, thus hindering any isolated conclusions on the effects of the material's stiffness on secretion, if a proper normalization is not performed (Lagneau et al. 2023; Cai et al. 2016). Overall, MSC cultured in 3D soft materials ($\leq$ 5 kPa) produce significantly higher levels of immunomodulatory factors when compared to stiff materials in 2D conditions (Ji et al. 2019). However, higher matrix stiffness also increases cytoskeletal tension, potentially leading to a more open nucleus, and thus enhancing the translocation of key transcriptional factors for downstream gene expression towards secretory upregulation (Wechsler et al. 2021). MSCs within stiff hydrogels secrete preferentially VEGF, while PGE2 secretion is predominantly upregulated in soft hydrogels. VEGF secretion is so sensitive to the mechanical properties of the material that it can be tuned with different levels of stiffness (Murphy et al. 2017). Nevertheless, a direct correlation of substrate stiffness and MSC's immunomodulatory function is still ambiguous. Contradictory results were found in different studies, as some show that soft matrices enhance inflammatory activation of MSCs (Wong et al. 2020), whereas others show that they induce MSCs to attenuate inflammatory response (Zhuang et al. 2022). This discrepancy could be attributed to differences in study design, material configurations and culture parameters (Gao et al. 2023). An upregulation of immunomodulation-related genes has been observed with soft hydrogels primed with TNF-α (Wong et al. 2020). Similarly, an enhanced capacity to promote the polarization of macrophages to the M2 phenotype has also been described, though it still depends on the introduction of an inflammatory stimulus (Sridharan et al. 2021). Interestingly, the paracrine function of MSCs on dynamic stiffening materials seems to be higher than on constant stiffness materials (Chen and Lv 2022).

The impact of viscoelasticity on cell behaviour is less understood than the impact of stiffness, even though it is a relevant physiological and pathological parameter (Lagneau et al. 2023). In order to produce viscoelastic materials, polymers are usually crosslinked by non-covalent or dynamic covalent bonds that allow for network reorganization upon deformation (Lagneau et al. 2023; Teng et al. 2019; Zhao et al. 2010); the speed of stress relaxation can be then increased keeping a similar initial elastic modulus (Chaudhuri et al. 2015). Faster stress relaxation enhances both spreading and proliferation of MSCs compared to low relaxation hydrogels (Chaudhuri et al. 2016). Additionally, it increases osteogenesis and mineralization capacities, but only in matrices with an appropriate stiffness (Chaudhuri et al. 2016; Darnell et al. 2017). Substrates can also be tuned to increase differentiation into myogenic lineages (Cameron et al. 2011) through an increased activation of Rac1 GTPase (Cameron et al. 2014). Regarding its effect on paracrine action, conditioned medium retrieved from MSCs cultured in viscoelastic hydrogels contains more proangiogenic factors than that from MSCs from purely elastic hydrogels (Hung et al. 2020). MSCs cultured on low stiffness substrates also change osteopontin expression depending on the viscoelastic properties of the material (Liu et al. 2017). Similarly, MSCs cultured on compliant substrates showed upregulated expression of IL-8, MCP-1, IL-21, brain-derived neurotrophic factor (BNDF), and stromal cell-derived factor (SDF)-1a genes (Wechsler et al. 2021). Other studies reported that a fast-relaxing hydrogel leads to the upregulation of COX-2 and TSG-6 genes in combination with a higher secretion of PGE2 by encapsulated MSCs (Lagneau et al. 2023; Vining et al. 2019).

3.2 | Hydrogel Architecture: Diffusion, Porosity and Degradation

While biomechanical properties directly instruct cell functionality, hydrogel architecture is better related to the way cells can access and interact with their environment (medium and surfaces). In this way, the architectural properties mainly affect the diffusion from and into the cell niche, the availability of adhesion sites and surface geometry, the ease of matrix remodelling and the space available for cell growth. Above all, modifying the material's physicochemical properties affects molecular diffusion, thereby impacting secretion measurements (Lagneau et al. 2023). This is also relevant from a permeation perspective, as increasing porosity improves nutrient diffusion and removal of cellular waste, leading to better proliferation rate and osteogenesis of MSCs (Zhao et al. 2021; Bakhshandeh et al. 2023). Pore size can also influence the penetration of TNF-α and IL-17, hindering the reception of biochemical signals from the medium. To circumvent these diffusion limitations, paracrine signals and drugs can be encapsulated within the material to further regulate the local microenvironment, raising questions on whether the MSC-secreted paracrine factors could efficiently leave those materials for proper harvest (Wobma et al. 2018; Moshaverinia et al. 2015). The mechanical and architectural properties are interdependent; whenever a crosslink density affects hydrogel elasticity (especially in covalently crosslinked hydrogels), it simultaneously impacts porosity, anchoring points proximity and material permeability (Trappmann et al. 2012). Notably, most studies do not assess such complex interactions, calling for caution on conclusions that consider the mechanical

properties as the sole direct modulator of cell secretory functions (Lagneau et al. 2023).

Cells embedded in hydrogels with a mean pore size of ~5 nm secrete lower levels of cytokines and re-generative factors (e.g., HGF, FGF-2 and IGF) than in alginate scaffolds with an average pore size of 120 μm. However, the effect was not attributed to diffusion differences but to cell–cell interactions during culture, which are prevented in tightly encapsulated matrices (Qazi et al. 2017). Alginate hydrogels with smaller pores and higher elasticity suppress the infiltration of proinflammatory cytokines and T-lymphocytes and the activation of NF-kB signalling (apoptosis and pro-inflammatory regulation pathway) (Chen et al. 2019; Ansari et al. 2017). Similarly, MSCs encapsulated in hydrogel-type collagen scaffolds induce lower MHC-II expression and lymphocyte proliferation compared to MSCs encapsulated in sponge- and membrane-type collagen scaffolds (Yuan et al. 2011). Some of these findings can also be explained as too large pores might limit cell adhesion (Kress et al. 2012); although porous biomaterials are typically used for 3D culture, an excessive pore size could also result in cells adhering to the inner walls of the scaffold, effectively creating a 2D culture system (Chen et al. 2019).

Besides the direct effect of pore size on the diffusion of paracrine factors, other architectural elements are related to cell growth and differentiation, such as matrix remodelling and space availability. Ionically cross-linked hydrogels have relatively weak bonds that can be ruptured by MSCs, while covalent bonds can withstand it. For ionically crosslinked hydrogels, intermediate stiffness instead of elevated stiffness induces the highest osteogenesis and the lowest adipogenesis (Guo et al. 2021). At the same time, an optimal intermediate stiffness for osteogenesis has been reported in alginate, agarose and PEG hydrogels (Huebsch et al. 2010). On the contrary, there is no upper stiffness differentiation limit in 2D, as demonstrated by efficient osteogenic differentiation on extremely stiff polystyrene and glass. This upper limit in 3D likely arises from a reduced ability for cells to remodel the matrix and gather adhesive ligands. Only adipogenesis and no osteogenesis occur in covalently crosslinked hyaluronic hydrogels, irrespective of hydrogel stiffness (Zonderland and Moroni 2021; Khetan et al. 2013).

Similarly, the inhibition of certain enzymes has enabled the evaluation of the effect of matrix remodelling on cell function. Hyaluronidase inhibition in hyaluronic acid hydrogels avoids osteogenic differentiation (Ferreira et al. 2018), while inhibiting membrane-bound MT1-MMP (a collagen-degrading enzyme) in MSCs encapsulated in type-I collagen gels hinders osteogenic differentiation (Lu et al. 2010; Tang et al. 2013) and enhances adipogenic differentiation (Zonderland and Moroni 2021). Unfortunately, the effect of matrix remodelling on paracrine action has not been explored as much as on differentiation and growth. However, it is known that remodelling and degradation are related to the mechanical properties of the hydrogel; matrix degradation likely promotes ligand clustering and tension generation, in agreement with studies of mechanosensitive MSC differentiation in alginate gels (Madl and Heilshorn 2018; Huebsch et al. 2010; Chaudhuri et al. 2016; Vincent and Engler 2013). Besides that, the pores within hydrogels are the channels for the flow of cells; when the pores become larger, cell mobility is enhanced, and the contact between cells becomes more frequent (Hu et al. 2022), which may affect the immunomodulatory capacity of MSCs (Gao et al. 2023).

3.3 | Hydrogel Composition and Inclusion of Biochemical Cues

Isolating the effect of the material's biochemistry from its biophysics is complicated; most studies evaluate the same material with different mechanical and architectural properties, but it is difficult to study different materials while keeping constant their biophysical properties. The surface biochemistry of a biomaterial can have a greater influence than its mechanical characteristics on certain cell functions, and potentially on MSCs paracrine activity (Chen et al. 2019). Hydrogels composed entirely of zwitterionic poly(carboxybetaine) monomers, can contribute to the maintenance of MSC multipotency and phenotype independent of differentiation-promoting media, cytoskeletal-manipulation agents, and the stiffness of the hydrogel matrix (Bai et al. 2014). Also, MSCs encapsulated within collagen-based hydrogels show a better secretory profile compared to MSCs cultured in gelatin-based hydrogels, which are stiffer, demonstrating that higher bulk stiffness alone does not augment the paracrine activity of MSCs. The functionalization of the polymer backbone with thiol-modified glycoproteins also leads to further changes in the paracrine profile of MSCs (Drzeniek et al. 2021).

Some polymers might modulate MSCs' paracrine action by themselves. For example, alginate encapsulation (with or without attached RGD sequences) seems to enhance the immunomodulatory abilities of MSCs, preventing PBMC proliferation (Follin et al. 2015). Additionally, MSCs encapsulated in alginate may produce more PGE-2, which enhances their ability to modulate experimental inflammation regardless of external stimuli (Stucky et al. 2015). Nonencapsulated MSCs show an indiscriminate increase in cytokine secretion in response to IL-1β, while alginate-encapsulated MSCs show a targeted secretory response with increased expression of pro-inflammatory (IL-1β, IL-6, IL-7, IL-8), anti-inflammatory (IL-1RA) and chemotactic (G-CSF, MDC, IP10) cytokines (McKinney et al. 2022). It would be ideal to conclude whether the polymer itself has a predominant role in these outcomes, but without proper 3D controls, the changes in paracrine function could be just a consequence of 3D encapsulation itself (Wobma et al. 2018).

There is plentiful evidence on how the inclusion of biochemical cues can bolster the efficacy of MSCs (Wobma et al. 2018) by improving survival (e.g., tenascin-C [Yates et al. 2017] and RGD peptide [Dhillon et al. 2019]), homing (e.g., SDF-1α [Kim and Tabata 2016]), and paracrine factors secretion (GFs and immunomodulatory factors [Pumberger et al. 2016]). Some matrix components are also known to alter MSCs differentiation. The collagen-mimetic ligands DGEA and GFOGER increase osteogenic and chondrogenic differentiation, while the laminin-derived IKVAV motif enhances osteogenic and adipogenic differentiation. Additionally, the incorporation of chondroitin sulphate, hyaluronic acid, and heparan sulphate alters chondrogenic differentiation (Madl and Heilshorn 2018). There are also many examples of the impact of including adhesion motifs (e.g., RGD, HAVDI, GRGDS and GFOGER) on MSC's paracrine action.

Specifically, the inclusion of the RGD peptide enhances VEGF secretion (Ho et al. 2016) and improves the therapeutic effect of BMSC on induced acute lung injury in rats (Ding et al. 2022), the HAVDI-peptide (an N-cadherin epitope) increases the production of growth factors (e.g., IL-10, TGF-β1, GDNF, VEGF) (Lagneau et al. 2023; Caldwell et al. 2020; Qazi et al. 2020), the GRGDS, an ECM derived peptide, enhances MSC's proliferation, secretion, cell morphology, metabolic activity and potential to induce neuronal proliferation (Silva et al. 2013), and GFOGER improves secretion of inflammatory factors IL-8 and IL-6, and chemotactic factor monocyte chemoattractant protein-1 (MCP-1) (Wechsler et al. 2021; Clark et al. 2020). Cytokines can also be tethered to the hydrogel. For example, tethering IFN-γ preserves its biological activity and causes significant differences in MSCs cytokine secretion and ability to halt activated T-cell proliferation and monocyte-derived dendritic cell differentiation (García et al. 2019).

4 | Using Hydrogels Properties to Guide MSC-EVs Production and Release

The understanding of the cell-material interactions that lead to MSC-EVs production is less developed than with other paracrine factors (such as VEGF, GNDF, TGF-β1, PGE-2, HGF, FGF-2, IGF, IL-1β, IL-6, IL-7, IL-8, IL-10 and others). Several hydrogel formats have been used to produce MSC-EVs, which have been tested in various animal models, but the field is relatively new (Table 1). From an initial interest in chitosan and alginate, research has moved towards collagen, gelatin and PEG-based hydrogels, including several biochemical cues that complicate the comparability of the results. Additionally, only a few studies have performed mechanical evaluations of the substrates or measurements of porosity, stiffness and other architectural parameters. EV secretion has been confirmed in both soft (3.7 kPa) (Hodge, Decker, et al. 2023) and stiff hydrogels (500 kPa) (Han et al. 2022), replicating the mechanical properties of adipose and spinal cord tissues. However, MSCs are reported to secrete twice as many EVs in soft hydrogels compared to stiff hydrogels, with similar expression of late endosomal markers CD63 and CD9 (Lenzini et al. 2021). Besides low stiffness, the use of fast stress-relaxing hydrogels increases the yield of EVs and their cargo (Choi et al. 2022), but other studies have failed to confirm this effect (Wong et al. 2020).

The effect of the hydrogel's architectural properties on the production of MSC-EVs is not as well studied as the effects on EV diffusion, which is extensively discussed for MSC-EVs modified release applications (Ma et al. 2023; Zhao et al. 2024; Zhang et al. 2023; Garcia-Motta et al. 2024; Ju et al. 2023; Pulido-Escribano et al. 2023; van de Looij et al. 2023; Brennan et al. 2020; Khayambashi et al. 2021; Simon et al. 2023; Yang, Li, Zhao, and Shang 2024; Yang, Li, Zhao, Wang, et al. 2024). Hydrogels can absorb large amounts of fluids and cellular secretions (Li and Mooney 2016), and, in comparison to metals and ceramics, their controllable structural porosity allows for easier personalized release mechanisms (Zhang et al. 2023). Such characteristics are useful to induce in situ angiogenic/osteogenic differentiation, immunomodulation and analgesia signalling (Mantha et al. 2019), while also helping to mitigate undesired EV biodistribution and avoid rapid clearance by the immune system (Ma et al. 2023). However, the requirements for a controlled release application are different from the ones for EV production. Moreover, a controlled release hydrogel is designed to keep and deliver EVs in a timely manner, while EVs production demands a straightforward release to facilitate harvest in the culturing medium. Otherwise, the EVs would be released only when the hydrogel is fully degraded (Ma et al. 2023), either by progressively degrading the material, or by accumulating EVs within the hydrogel and harvesting by final total polymer degradation. Therefore, hydrogel's porosity, swelling rate and surface charge should be controlled to favour EV release kinetics in culturing protocols (Ju et al. 2023; Li and Mooney 2016).

EV diffusion is mainly driven by the polymer network's mesh size, which has some degree of heterogeneity. In principle, when EVs are smaller than the mesh size, they can diffuse freely out of the matrix (Ma et al. 2023). Since EVs size generally lies between 50 and 250 nm, nanoporous (mesh size below 100 nm) and microporous (between 100 and 1000 nm) hydrogels are optimal for controlled release applications (van de Looij et al. 2023; Yáñez-Mó et al. 2015; Elbert 2011), while micro- and macroporous (above 1 μm) hydrogels are better for MSC-EVs production. Increased crosslinking density or polymer concentration may result in high porosity but small pore size, effectively prolonging the retention of EVs (Ma et al. 2023). Very small mesh size (15–34 nm) could also make EVs release dependent on the hydrogel's swelling (Ju et al. 2023; Mardpour et al. 2019). Additionally, if the internal cavities in a 3D culturing system have a size below 5 μm, they can hinder cell proliferation and migration (Doolin and Stroka 2019), which in turn might impact EV secretion.

The amide, amine, hydroxy and carboxylic groups on the proteins and peptidoglycans found on the EV's membrane can interact with the hydrogel through hydrogen bonds or van der Waals forces (Chen et al. 2022). Besides that, since EVs exhibit a negative charge due to phosphatidylserine (Seo et al. 2022), positively charged polymers are widely studied for EVs controlled release (Ma et al. 2023). For instance, chitosan-containing hydrogels, having a relatively weak cationic charge, can provide a prolonged release of EVs for over 6 days (Tao et al. 2017), and the presence of the cationic polymer poly-L-lysine can retain EVs into a scaffold for a sustained release over 35 days (Tao et al. 2022). EVs adhesion molecules, such as α integrins, allow their adhesion to ECM matrix components (type I collagen, fibronectin, and other derivative adhesion peptides), which also affects the release of EVs from the hydrogels (Ju et al. 2023; Huang et al. 2021). Despite its relevance, the effect of hydrogel's porosity, swelling rate and surface charge is generally not discussed in the development of MSC-EVs production protocols.

Several biochemical factors enhance the therapeutic potential of MSC-EVs (Park et al. 2019) and could be included in hydrogels as biochemical cues. For example, lipopolysaccharides increase the secretion of EVs from UC-MSCs (Ti et al. 2015), IL-1β induces greater immunomodulatory effects in MSC-EVs (Song et al. 2017), TNFa, interferon gamma, or TGF-β induce MSC-EVs able to decrease cytokine expression in splenocytes and increase regulatory T cell differentiation (Zhang et al. 2018), PDGF enhances angiogenic potential of MSC-EVs (Lopatina et al. 2014), and erythropoietin enhances the MSC-EVs' protective effects on renal injury (Wang et al. 2015). However, just one study has actually

TABLE 1 | Polymers and modifications that have been studied for the generation of MSC-EVs, their basic mechanical and architectural properties, and the animal models where they have been tested.

Reference	Polymer	Conc. (% w/v)	Modification	Pore size	Young modulus (kPa)	Application	Animal model	Study
Han (2023) (Han et al. 2023)	GELMA	5	None	ND	ND	Cerebral ischemia repair	Mouse	Middle cerebral artery occlusion
Han (2022) (Han et al. 2022)	GELMA	5	None	ND	500	Spinal cord injury	Rat	Spinal cord injury model
Wang (2023) (Wang, Wei, et al. 2023)	Gelatin	NR	NR	ND	ND	Spinal cord injury	Rat	Spinal cord injury model
Hodge (2023) (Hodge, Robinson, et al. 2023)	PEG based	NR	Collagen/fibrin or fibronectin coating	ND	ND	ND	ND	ND
Hodge (2023) (Hodge, Decker, et al. 2023)	PEG based	NR	Fibronectin coated	300 μm	3.7	ND	ND	ND
Yan (2023) (Yan et al. 2023)	Acellular cartilage	5	NR	ND	ND	Osteochondral repair	Rat	Osteochondral defect model
Ying (2022) (Liu et al. 2022)	β-chitin	0.3	Nanofibers	200 μm	ND	Wound healing	Rat	Circular full-thickness cutaneous wounds
Choi (2022) (Choi et al. 2022)	Gellan gum	2	Various	180 μm; adjustable	Various; shear-thinning behaviour	ND	ND	ND
Cao (2022) (Cao et al. 2022)	Matrigel	5	None	Variable	Various; shear-thinning behaviour	Wound healing	Mouse	60 % volume of Gluteus maximus removal or infliction of excisional wounds
Yu (2022) (Yu et al. 2022)	Collagen	0.3	None	250–750 nm	9.32	Bone regeneration	Rat	Infliction of alveolar bone defect
Yu (2021) (Yu et al. 2021)	Collagen	0.3	Added Fe3O4 nanoparti-cles	300 nm to 1 μm	1.4 & magnetic stretching	Bone regeneration	Rat	Infliction of alveolar bone defect
Khan (2021) (Khan et al. 2022)	Chitosan	2.9	Added Hyaluronic acid	ND	ND	Myocardial infarction	Rat	Ligation of left anterior descending artery
Lenzini (2021) (Lenzini et al. 2021)	Alginate	2	RGD modification	ND	3 and 20	Acute lung injury	Mouse	LPS injection to induce acute lung injury
Zavala (2020) (Zavala et al. 2020)	Cellulose	1.8	Sulphate group	ND	ND	ND	ND	ND
Boido (2019) (Boido et al. 2019)	Chitosan	2.5	None	122 μm	ND	Spinal cord injury	Mouse	Spinal cord transection to interrupt ascend-ing/descending tracts

assessed the effect of a hydrogel-bound biochemical cue (integrin ligand density) on the generation of MSC-EVs, finding that lower ligand densities cause a higher EV production (Lenzini et al. 2020). Some ions have also been studied as biochemical cues in scaffolds and could be explored in hydrogels. Akermanite bioceramics containing Mg ions enhance exosomal miR-196a-5p cargo, with positive effects on osteogenesis (Qi et al. 2024), and lithium-substituted bioglass ceramics direct BMSCs to generate chondrogenesis-promoting exosomes, thanks to the upregulation of exosomal miR-455-3p transfer (Liu et al. 2023). It should be noted that EVs themselves can serve as biochemical cues; however, more research into the role of other cell-derived EVs on the potency of MSCs in terms of EV secretion will be needed in order to obtain optimal therapeutic outcomes (Park et al. 2019).

5 | Influence of Hydrogel Formats on the Production and Performance of MSC-EVs

Several MSC sources have been used to produce MSC-EVs in hydrogels (Table 2). These include bone marrow, periodontal ligament, amniotic membrane, menstrual blood, umbilical cord, and adipose tissue, generally from human donors. Due to senescence and genetic variability, heterogeneity of donors, tissue source, and initial passage, it is difficult to perform a straightforward comparison among the results from the available studies. Furthermore, differences in study design, such as MSC isolation methods, seeding density, culturing duration, media formulations, and MSC-EVs collection strategies and isolation methods, beside the impact from the hydrogel's production method (casting, moulding, jet-spray and 3D printing), also influence the generation of the MSC-EVs. Only a few studies have been conducted in physioxic conditions, and although aMEM and DMEM are the most used media, other branded media have been used with diverse levels of FBS and glutamine supplementation, providing different levels of stimulation to the cells.

There are broadly two types of hydrogel formats, which mainly differ in the exposure of the cells to the crosslinking process and the resultant hydrogel network's architecture (Figure 3). In the first method, called bulk encapsulation, the cells are mixed with the polymer and then exposed to the crosslinking process. This creates a tight hydrogel where individual cells or aggregates are encapsulated within a bulk that entirely encloses and fixes them in place. In the second method, the polymer is crosslinked without the presence of the cells, and it is later re-hydrated to create a porous matrix; the cells are then added to the scaffold-like structure and allowed to migrate through the channels in the material. These two methods, namely bulk and scaffold-like encapsulation, greatly influence the expansion patterns of the cells, the permeability of the hydrogel, and the cell–cell interactions; hence, they need to be investigated separately.

Among the studies on MSC-EV production from hydrogels, the seeding density varies from 2 to 10 million cells/mL in bulk encapsulation and from 15 to 50 cells/mm^2 in scaffold-like hydrogels. These differences in cell concentration produce significant changes in cell-to-cell proximity and nutrient uptake patterns, both known to affect the quality and yield of EVs (Hettich et al. 2020; Patel et al. 2017). Culture and collection times also vary in the published protocols, ranging from 3 to 14 days and 24 to 72 h, respectively. These differences also influence the cell's adaptation to 3D conditions and the total yield of EV collection. The collection strategy, independent of the medium used, is either based on serum-free medium or exosome-depleted serum-supplemented medium, which affects cell stimulation levels and consequently the characteristics of the generated EVs (Lehrich et al. 2021). Despite these significant differences, some general outlines can be discussed from the findings. However, such conclusions should be kept within the range of the tissue source and culturing strategy used in each study, for which we recommend the reader consult Table 2 and Table S1 for reference. Besides the comparison among hydrogel formats on MSC-EVs, we have included in this chapter a discussion on the potential of bioreactors to improve the performance of 3D cultures; these technologies have been extensively employed with similar culturing substrates (e.g., microcarriers and hollow fibres), and achieved positive results on MSC-EVs yield, quality and function.

5.1 | Bulk Hydrogels

Bulk hydrogels for MSC-EVs production have been generated with protein-type polymers (GelMA, Collagen and Matrigel) or polysaccharide-types (Cellulose, Gellan gum and Chitosan) (Table 3), which are fundamentally different in regard to the availability of adhesion sites. The degree to which the MSCs can attach to the encapsulating material greatly influences the cytoskeleton configuration and membrane binding to the ECM, affecting their paracrine function. Therefore, some of these polysaccharides need to be modified or combined with secondary polymers to enable cell adhesion (Zavala et al. 2020; Choi et al. 2022). The crosslinking strategy also varies depending on the need to create covalently or ionically crosslinked networks, affecting mesh architecture and matrix availability for remodelling. These crosslinking strategies expose the cells to external factors (e.g., temperature changes, pH shifts, high shear or free radical species), which may impact cellular behaviour and further complicate the comparability of the studies. Jet-spray methods, for example, may use temporary high temperatures and potential high shear rates in the spray nozzle. However, studies have shown high cell viability post-spray, implying a less damaging environment to the cells due to the short period of exposure (Dell et al. 2022). Casting and moulding methods seem to be less aggressive, as they are based on dispensing the polymer either freely over a surface or confined into a mould, creating planar structures with some degree of control on their thickness. Unfortunately, the dimensions of these cast and moulded hydrogels are seldom reported in the studies, despite the impact that both size and shape have on particle diffusion and cell migration (Ju et al. 2023).

MSCs encapsulated in bulk hydrogels are smaller, rounder, more closely connected and uniformly distributed than 2D cultured cells (Han et al. 2022, 2023; Yu et al. 2022). At the same time, they show good cytocompatibility in both casting (Han et al. 2022; Choi et al. 2022; Boido et al. 2019) and in jet spray methods (Cao et al. 2022). Cell proliferation is rarely characterized in the hydrogels, preventing the normalization of the EVs yield from different culturing strategies. However, there are some interesting findings in this regard. In vivo data suggest that MSCs

TABLE 2 | Sources, culturing, collection and isolation strategies for MSC-EVs produced from hydrogel 3D culturing.

Reference	Species	Tissue source	Passage	Seeding density	Cuture time	Colletion time	Collection medium	Isolation method
				Bulk-hydrogel culturing				
Han (2023) (Han et al. 2023)	Mouse	Bone marrow	3 to 5	2,000,000 (cells/mL of gel)	NR	48 h	Exosome-free serum	Ultracentrifugation
Han (2022) (Han et al. 2022)	Rat	Bone marrow	3 to 5	2,000,000 (cells/mL of gel)	7 days	NR	NR	Ultracentrifugation
Choi (2022) (Choi et al. 2022)	Human	Bone marrow	6	2,000,000 (cells/mL of gel)	3 days	48 h	Serum-free media	ExoQuick ultraEV isolation kit (SBI)
Yu (2022) (Yu et al. 2022)	Human	Periodontal ligament	2 to 5	5,000,000 (cells/mL of gel)	72 h	During culture	Exosome-free serum	Ultracentrifugation
Yu (2021) (Yu et al. 2021)	Human	Periodontal ligament	2 to 5	5,000,000 (cells/mL of gel)	72 h	During culture	Exosome-free serum	ExoQuick-TC (SBI, USA)
Khan (2021) (Khan et al. 2022)	Human	Amniotic membrane	4 to 7	2,000,000 (cells/mL of gel)	NR	48 h	Serum-free media	Ultracentrifugation
Zavala (2020) (Zavala et al. 2020)	Human	Menstrual blood	NR	800–1000 cells/bead	NR	24, 48 and 72 h	Serum-free media	Ultracentrifugation or total exosome isolation kit (Termo Fisher)
Cao (2022) (Cao et al. 2022)	Human	Umbilical cord	3 to 10	2,000,000 to 1,000,0000 (cells/mL of gel)	5 days	96 h	exosome-free serum	Ultracentrifugation
	Scaffold-hydrogel culturing							
Ying (2022) (Liu et al. 2022)	Mouse	Adipose tissue	NR	1,000,000 cells/scaffold	NR	NR	NR	NR
Hodge (2023) (Hodge, Decker, et al. 2023)	Human	Adipose tissue	2 to 5	50 cells/mm2	Up to 14 days	48 h	Serum-free media	ExoQuick-TC kit
Lenzini (2021) (Lenzini et al. 2021)	Human	Bone marrow	3	25 cells/mm2	NR	72 h	serum-free media	ultracentrifugation
Wang (2023) (Wang, Wei, et al. 2023)	Human	Umbilical cord	NR	100,000 cells/scaffold	14 days	48 h	Serum-free EVs secretion-promoting medium	Ultracentrifugation
Hodge (2023) (Hodge, Robinson, et al. 2023)	Human	Adipose tissue	2	15 cells/mm2	8 days	72 h	Serum-free media	ExoQuick-TC kit
Yan (2023) (Yan et al. 2023)	Human	Umbilical cord	3 to 5	NR	48 h	48 h	Exosome-free serum	Ultracentrifugation

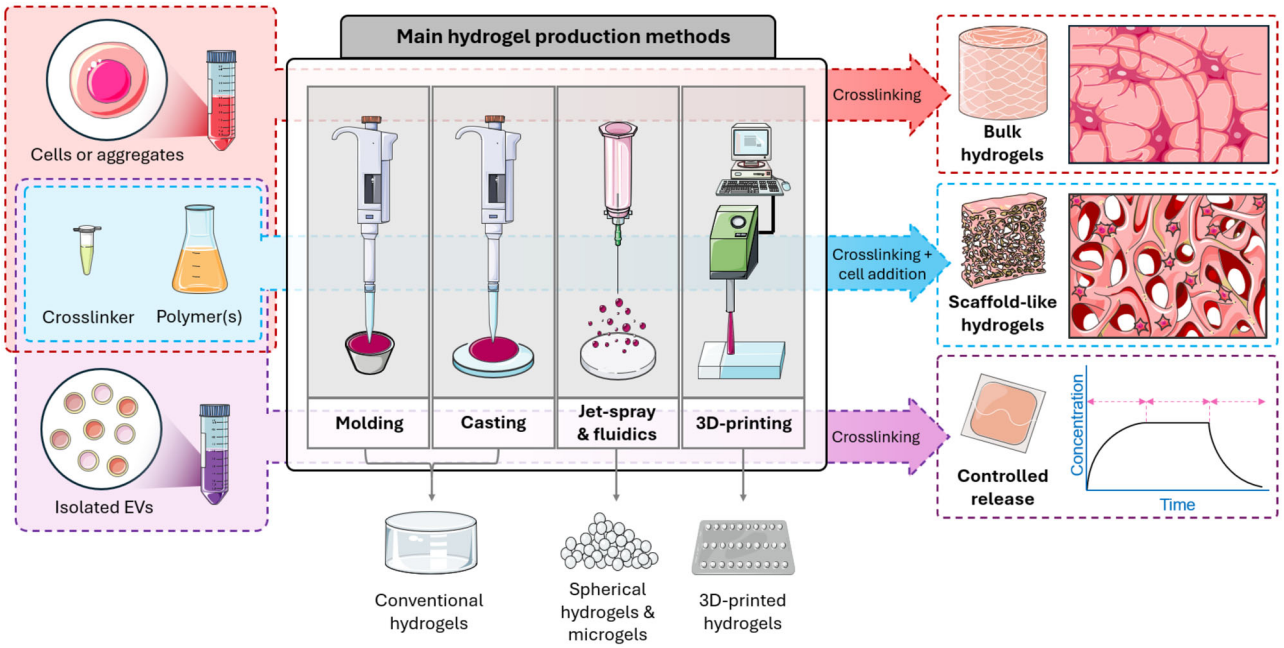

FIGURE 3 | Hydrogel formats and main production methods for MSCs and MSC-EVs encapsulation. MSCs, mesenchymal stem/stromal cells; MSC-EVs, mesenchymal stem cell-derived extracellular vesicles.

TABLE 3 | Materials, methods and shapes used for the production of EVs in bulk hydrogels.

Reference	Shape	Method	Polymer	Conc. (% w/v)	Crosslinker	Crosslinking time
Han (2023) (Han et al. 2023)	Disk	Moulding	GELMA	5	LAP	20 s
Han (2022) (Han et al. 2022)	Disk + Needle release	Moulding	GELMA	5	LAP	20 s
Choi (2022) (Choi et al. 2022)	Disk	Casting	Gellan gum	2	Temperature or CaCl2	10 m
Yu (2022) (Yu et al. 2022)	Dumbbell	Moulding	Collagen	0.3	Temperature or Ph	30 m
Yu (2021) (Yu et al. 2021)	Dumbbell	Moulding	Collagen	0.3	Temperature	30 m
Khan (2021) (Khan et al. 2022)	NR	Casting	Chitosan	2.9	Genipin	NR
Boido (2019) (Boido et al. 2019)	Drop	Casting	Chitosan	2.5	β-glycerophosphate	30 min
Zavala (2020) (Zavala et al. 2020)	Bead	Jet-spray	Cellulose	1.8	pDADMAC	NR
Cao (2022) (Cao et al. 2022)	Bead	Jet-spray	Matrigel	5	Temperature	30 m

show higher proliferation when embedded in a hydrogel than without it (Khan et al. 2022). Also, studies show that MSCs encapsulated in microgels (hydrogels whose scale is measured in microns) show higher proliferation than in cast hydrogels, especially when seeded in higher cell densities (Cao et al. 2022). On the preservation of stemness, there is some data favouring hydrogel culturing versus 2D culturing, as the markers SOX2 and Nanog are increased in hydrogel cultured MSCs compared to 2D cultured MSCs (Han et al. 2022, 2023).

The average diameter of the MSC-EVs produced in bulk hydrogels ranges between 60 (Han et al. 2022) and 123 (Zavala et al. 2020) nm, smaller than EVs produced in 2D conditions (Zavala et al. 2020; Han et al. 2022; Yu et al. 2022). However, some studies have found little difference in their dimensions (Choi et al. 2022; Yu et al. 2021). A narrower size distribution compared to 2D has also been reported, which could indicate higher EV purity (Zavala et al. 2020). All the research that measured the yield of EVs from bulk hydrogels reported a higher output than equivalent 2D

culturing conditions (Zavala et al. 2020; Choi et al. 2022; Han et al. 2023; Cao et al. 2022; Yu et al. 2022), reaching up to 2,5 fold higher yield (Cao et al. 2022, Yu et al. 2022). As previously mentioned, EV production could be increased using spherical microgels instead of cast hydrogels (Cao et al. 2022), as well as fast stress-relaxing hydrogels significantly boost EV production (Choi et al. 2022). The EVs are usually enriched in typical exosomal surface proteins (TSG101, CD81 and CD9) (Han et al. 2022, 2023; Yu et al. 2022), but in some cases EVs isolated from bulk hydrogels express lower levels of CD81 than the EVs from standard 2D culture (Zavala et al. 2020). The endocytosis signals that are found on the EVs surface are related to increased functionality in several studies (Zavala et al. 2020, Han et al. 2022, 2023; Yu et al. 2021, 2022), enabling a successful internalization of the MSC-EVs by BV2 cells (Han et al. 2022; Han et al. 2023), BMSCs (Yu et al. 2021, 2022), and astrocytes (Han et al. 2022). In some cases, the internalization rate has been higher in the EVs produced in hydrogels than in the ones produced in 2D conditions, either during early internalization stages (Yu et al. 2021) or as a whole (Han et al. 2022). Protein cargo is reported to increase by hydrogel culturing, as hydrogel-produced MSC-EVs may hold more proteins than in equivalent 2D conditions (Choi et al. 2022; Yu et al. 2022). The cargo is also enriched in proteins involved in cell-substrate adhesion and elements from the ECM (Han et al. 2023) and remodelling of the central nervous system (Han et al. 2022). Specifically, when EVs are produced from hypoxia-primed MSCs, the cargo is enriched in specific proteins involved in cell proliferation and angiogenesis: cellular communication network factor 1/2 (CCN1/2), adipocyte enhancer-binding protein 1 (AEBP1), angiopoietin-like 7 (ANGPTL7), and insulin-like growth factor-binding protein 3 (IGFBP-3).

Some results also suggest that a hypoxic environment in 3D conditions drives MSCs to secrete EVs loaded with proteins that activate Wnt. In this way, upon MSC-EV infusion, in vivo resident MSCs and other progenitors are signalled to promote cardiogenesis and cytoprotection (Khan et al. 2022). The EVs from hydrogel-cultured MSCs are reported to promote myoblast growth in a dose-dependent manner, but there is no difference between vesicles produced from spherical microgels and cast hydrogels, when identical EV doses are used for the assay. This implies that different encapsulation methods might not affect EV quality as much as they may affect the yield (Cao et al. 2022). Differences in the promotion of BMSCs growth have also been described, which show a similar trend to 2D conditions up to Day 7, when hydrogel-produced EVs exhibit a higher effect (Yu et al. 2022); another study found differences as early as Day 1 (Yu et al. 2021). Hydrogel EVs also exert a stronger promigratory effect on BMSCs when compared to 2D-produced EVs (Yu et al. 2022), while the effect of both hydrogel and 2D-produced EVs on angiogenesis is similar, indicating that the EVs potentially retain the trophic abilities of parental MSCs (Zavala et al. 2020).

EVs produced in bulk hydrogels are reported to inhibit T cell proliferation by approximately 30%; therefore, some immunomodulatory properties of MSC-EVs are kept when produced in hydrogels (Zavala et al. 2020). Additionally, EVs from spherical microgels and cast hydrogels show no significant difference in inducing anti-inflammatory-related gene expression, possessing an equivalent anti-inflammatory effect to EV products generated by conventional methods (Cao et al. 2022). When compared to 2D-produced EVs, hydrogel-generated vesicles also exert a more potent anti-inflammatory effect that attenuates glial/fibrotic scar formation in in vivo models (Han et al. 2022). There are also differences in the secretion of immuno-modulatory factors in in vivo studies. Of note, in an LPS-induced BV2 inflammation model after exposure to the EVs, the expression of TNF-α in 2D and hydrogel-produced conditions decreased by 0.57- and 0.3-fold, respectively, while the expression of IL-10 increased by 1.96- and 1.46-fold (Han et al. 2023). Another study found that IL-10 is upregulated in 2D and hydrogel conditions 2.23 and 2.8 times higher than in LPS-stimulated cells. Compared with the LPS-stimulated group, interleukin-6 (IL-6) and glial fibrillary acidic protein (GFAP) were significantly down-regulated (0.63- and 0.69-fold) in 2D conditions, while in hydrogel-produced EVs they were down-regulated (0.27- and 0.58-fold), respectively (Han et al. 2022).

The neuroprotective effect has been reported to be higher in hydrogel-derived EVs than in 2D conditions, which may be related to content differences and a more efficient penetration through the blood-brain barrier. In a middle cerebral artery occlusion model, the neurological function score regained after EV treatment increased significantly with both 2D (2.2 ± 1) and 3D (1.4 ± 0.5) produced EVs, while the cerebral infarct volume is more attenuated with 3D produced EVs than with 2D (Han et al. 2023). In another study, the effect of MSC-EVs on nerve regeneration after spinal cord injury was better than the effect of 2D-produced EVs; hydrogel-produced EVs were rich in neuroprotective-related proteins and miRNAs that regulate the transcription of cellular immune response genes (Han et al. 2022). MSC-EVs produced in hydrogels also induce a significant increase in the proliferation of neurites but with no differences when compared to 2D production (Zavala et al. 2020). On cardio protection, an improvement in cardiac function and a reduction of fibrosis were observed in a myocardial infarction model as a result of MSCs encapsulated in hydrogels. However, the regenerative properties of the hydrogel itself, combined with the higher engraftment of the stem cells in the target tissue, might promote robust improvements in cardiac function, besides the EVs-induced effect (Khan et al. 2022). Hydrogel-produced MSC-EVs upregulate the expression of COL1A1, Runx2, OPN and OCN, besides increasing matrix mineralization, proving that EVs effectively facilitate in vitro osteogenesis of BMSCs (Yu et al. 2022). Another study similarly found that BMSCs treated with MSC-EVs resulted in an increase in mRNA expression of osteogenesis-related genes (e.g., ALP, RUNX 2, OCN and COL1) and spontaneously formed the most mineralized nodules on Day 21 of osteo-induction culture. The results also verified the strongest osteogenesis in an alveolar bone defect model with the treatment of MSC-EVs and Matrigel (Yu et al. 2021).

5.2 | Scaffold-Like Hydrogels

Polysaccharides, protein-based polymers, decellularized tissue and synthetic polymers have been used to create scaffold-like hydrogels for the production of MSC-EVs (Table 4). These scaffold-like hydrogels are made by crosslinking the polymer into a specific shape, either by casting, moulding or 3D printing, followed by a hydration phase of variable duration. Some materials, like acellular tissue, also require lyophilization to form the

TABLE 4 | Materials, methods and shapes used to produce EVs in bulk hydrogels.

Reference	Shape	Method	Polymer	Concentration (% w/v)	pore size
Ying (2022) (Liu et al. 2022)	Disks	Moulding	β-chitin	0.3	200 μm
Hodge (2023) (Hodge, Decker, et al. 2023)	Block	3D printing	PEG based	NR	300 μm
Lenzini (2021) (Lenzini et al. 2021)	Drop	Casting	Alginate	2%	NR
Wang (2023) (Wang,, Wei, et al. 2023)	disks	Moulding	Gelatin	NR	NR
Hodge (2023) (Hodge, Robinson, et al. 2023)	Block	3D printing	PEG based	NR	NR
Yan (2023) (Yan et al. 2023)	NR	3D printing	Acellular cartilage	5%	NR

macro-microporous architecture necessary for cell incorporation (Yan et al. 2023). Once the structure is formed and hydrated, the cells are typically added dropwise and allowed to migrate and distribute throughout the porous microchannels of the scaffold. In this case, in contrast to bulk hydrogels, the cells are not embedded/encapsulated within the structure itself but rather form 3D networks within the porous channels (Hodge, Decker, et al. 2023). Therefore, porosity and pore size become critical parameters to understand the relationship of the cells with their microenvironment, although these parameters are rarely reported in the surveyed studies.

Cells proliferate easily and present good viability in scaffold-like hydrogels, avoiding necrosis even in the centre of pores with a diameter of 300 μm (Hodge, Decker, et al. 2023). MSCs seeded onto 3D-printed scaffolds exhibit a more characteristic spindle-shaped morphology and more prominent cell clusters than in 2D culture (Yan et al. 2023); however, they have also been reported to remain rounded on soft scaffold-like hydrogels, while showing increased cell spreading and decreased circularity as the substrate becomes rigid (Lenzini et al. 2021). The relative decline in MSC surface markers is greater in 2D attached cells than in scaffold-like cultures, while the 'stemness' genes, including FZD9, OCT4, FGF2 and ICAM1, similarly are higher expressed in scaffold-like systems, with a significant increase in senescent cells in 2D cultures (~11.5%) relative to cells within hydrogel systems (~3.6%) (Hodge, Decker, et al. 2023).

The size of the EVs from hydrogel scaffold-like cultures has been reported as just slightly bigger (Wang, Wei, et al. 2023) or smaller (Yan et al. 2023) than 2D-produced EVs, with a mean diameter ranging from 69 nm (Yan et al. 2023) to 125 nm (Wang, Wei, et al. 2023), similar to MSC-EVs produced in bulk hydrogels. All the studies that quantified the yield of MSC-EVs in scaffold-like hydrogels found an increase in EV production when compared to 2D controls (Hodge, Decker, et al. 2023; Lenzini et al. 2021; Wang, Wei, et al. 2023; Hodge, Robinson, et al. 2023; Liu et al. 2022); an increase that can be 5 times higher when the productivity is normalized per cell in culture (Wang, Wei, et al. 2023). The scaffold format has been used frequently to study the effect of mechanical properties on MSC-EV secretion. Higher secretion on soft (~20,000 particles/cell) than on stiff substrates (~10,000 particles/cell) was reported, while there is a similar level of EV secretion on faster stress-relaxing hydrogels, suggesting a marginal impact of this factor on EV production. This effect differs from the results obtained in bulk gellan-gum hydrogels (Choi et al. 2022). The combination of a soft hydrogel substrate ($E \sim 3$ kPa) and a low RGD concentration (0.16 mM) can lead to a ~10-fold increase in EV secretion per cell, relative to that of plastic 2D cultures. This combination has been discussed as evidence that integrin–ligand-mediated focal adhesions might limit EV secretion in MSCs (Lenzini et al. 2021).

MSC-EVs secreted in scaffold-like hydrogels are successfully endocytosed by fibroblasts (Hodge, Decker, et al. 2023), macrophages (Wang, Wei, et al. 2023; Yan et al. 2023), keratinocytes (Hodge, Decker, et al. 2023) and BMSCs (Yan et al. 2023), and contain higher amounts of proteins (Hodge, Decker, et al. 2023; Wang, Wei, et al. 2023; Hodge, Robinson, et al. 2023). Additionally, 116 miRNAs have been identified as significantly different between scaffold-like hydrogels and 2D produced EVs, some of them potentially associated with cartilage repair, downregulation of apoptosis, promotion of cell proliferation, promotion of stem cell chondrogenic differentiation, and promotion of macrophage M2 polarization (Yan et al. 2023). EVs from scaffold-like hydrogels also increase keratinocyte migration and proliferative activity versus 2D controls, which is a key component of epidermal regeneration (Hodge, Robinson, et al. 2023). Besides keratinocytes, scaffold-produced EVs promote the proliferation of BMSCs and chondrocytes better than 2D-produced EVs, and also promote the migration of BMSCs in vivo (Yan et al. 2023).

EVs produced from scaffold-like hydrogels and injected into spinal cord injury sites have been reported to increase the expression of the anti-inflammatory factor IL-10, and decrease the expression of the pro-inflammatory factor TNF-α, relative to 2D controls (Wang, Wei, et al. 2023). In parallel, scaffold-produced MSC-EVs promote a pro-regenerative immune microenvironment by downregulation of NF-κB p65 in macrophages and

reducing its nuclear translocation and expression of NLRP3 proteins, a key pathway for the production of inflammatory factors (Yan et al. 2023). MSC-EVs from soft hydrogel scaffolds also reduce oedema and vascular permeability in LPS-treated mice to a greater extent than MSC-EVs produced on plastic 2D cultures. Lastly, the EVs generated from different substrates have a similar level of keratinocyte growth factor (KGF) and interleukin-6 (IL-6) RNAs, mitochondrial DNAs and miRNAs (Urbanelli et al. 2013; Harrell, Jovicic, et al. 2019; Gazdic et al. 2015). Thus, soft scaffold-like hydrogels might enhance EV secretion without compromising functionality or cargo contents that can be beneficial to resolve injury (Lenzini et al. 2021).

5.3 | Bioreactors and Their Potential to Enhance Hydrogel-Based MSC-EV Generation

Bioreactors have extensively been used for the production of MSC-EVs, but in only a few instances have been paired with bulk (Nikolits et al. 2024) or scaffold-like hydrogels (Huang et al. 2023). The bioreactors used for this purpose are either perfusion or suspension-based systems. Perfusion bioreactors enable the medium to continuously refresh nutrients and remove waste metabolites while keeping the cells in place. This perfusion principle can be achieved with different configurations; however, the most common solution is the capillary-based hollow fibre bioreactor (Garcia-Aponte et al. 2021). Suspension bioreactors, on the other hand, enable the movement of medium in a vessel through the aid of impellers or other mechanical actuators. Since MSCs are adherent cells, these suspension bioreactors need to couple with some kind of particulate matrix, like hydrogels or microcarriers, to enable cell expansion.

Within the perfusion bioreactors, the hollow fibre platform has been applied in several instances for MSC-EV generation (Kink et al. 2024; Garcia et al. 2024; Jakl et al. 2023; Allen et al. 2020; Gobin et al. 2021; Bellio et al. 2022; Yan and Wu 2020; Cao et al. 2020). MSCs can grow slower in a hollow fibre system, but they achieve higher cell counts compared to 2D cell culture with no purity differences, except for a reported lower expression of HLA (Jakl et al. 2023). Remarkably, MSCs exhibit a stable and predictable metabolite and secreted factor profile during prolonged culture in hollow fibre bioreactors, and switch EVs composition after exposure to inflammatory stimuli in a reproducible way (Allen et al. 2020). The MSC-EVs yield in these bioreactors is reported to increase up to 7.5-fold (Yan and Wu 2020), 19.4-fold (Cao et al. 2020), and 38-fold (Kink et al. 2024) compared to 2D expansion, without significantly changing their identity (Kink et al. 2024; Garcia et al. 2024; Cao et al. 2020), size (Garcia et al. 2024; Gobin et al. 2021; Cao et al. 2020), protein and lipid cargo (Garcia et al. 2024), and surface glycan profile (Gobin et al. 2021). In terms of functionality, MSC-EVs derived from hollow fibre bioreactor cultivations show pro-angiogenic potential comparable to 2D-produced EVs (Garcia et al. 2024), have a consistent low immunogenicity profile and abundant immuno-regulatory and angiogenic factors (Gobin et al. 2021), can stimulate chondrocyte proliferation, migration, and matrix synthesis better than 2D-derived MSC-EVs (Yan and Wu 2020), better alleviate cisplatin-induced murine acute kidney injury than 2D-derived MSC-EVs (Cao et al. 2020), but provide no survival benefit in an acute radiation syndrome model (Kink et al. 2024). Beyond the hollow fibre bioreactor, there are alternative perfusion systems that have been tested for MSC-EV generation (Huang et al. 2023; Ferroni et al. 2023; Almeria et al. 2024; Jeske et al. 2022; Kronstadt et al. 2023; Kang et al. 2022). The VITVO system also achieves a higher yield of MSC-EVs compared to 2D systems (Ferroni et al. 2023; Almeria et al. 2024) and preserves the EV's identity (Ferroni et al. 2023), while micro-perfusion platforms can induce a 40-80-fold increase (depending on measurement method) in MSC-EVs yield compared to 2D culture (Kronstadt et al. 2023), also improving MSC-EVs' wound healing function (Huang et al. 2023; Kronstadt et al. 2023). Micro-perfusion bioreactors that include dynamic aggregation can also promote autophagy, alter metabolism toward glycolysis, and enhance MSC-EV production (Jeske et al. 2022). Simple flat-plate bioreactors can produce seven times more MSC-EVs than static culture conditions, and were used to demonstrate the link between EV biogenesis and increased calcium ion concentration under flow conditions (Kang et al. 2022). These studies support that 3D platforms (e.g., hydrogels) could be further enhanced by the continuous flow provided by a perfusion system without detrimental effects on MSC-EVs quality and function, but, on the contrary, to improve culture reproducibility and incorporate new forms of stimuli to the cells.

Suspension-based bioreactors are the most frequently used platforms for MSC-EVs generation and can be broadly divided into low shear spinning wheel reactors (SWR) and stirred bioreactors (SR). They are usually tested in combination with microcarriers (MCs), which are often catalogued as 3D culturing systems, given their similitude to scaffold-like hydrogels and the considerable expansion yield they can attain. MCs have also been extensively investigated for MSC-EVs production because of their reproducibility and straightforward transference to spinner flasks, stirred tanks, vertical wheel reactors, rotary vessels and microfluidic chips. However, not all MCs fit the criteria to be catalogued as a real 3D system or hydrogel, as most of them attach the cells only on their surface, have little porosity, and do not constitute a hydrated matrix. Non-porous plastic MCs can therefore underperform when compared to real 3D systems. The maximum cell density on an MC surface can be 5.8 times greater than on a flask-based 2D culture (Dos Santos et al. 2024), hindering EV secretion due to greater cell confluency. Higher levels of cell–cell contact decrease the available membrane surface area, which might lead to a reduction in vesicle budding (Patel et al. 2017). Direct cell–cell contact also decreases contactless communication, consequently leading to lower EV production per cell (Patel et al. 2018). Very high levels of cell and MC aggregation are frequently observed in MCs (Dos Santos et al. 2024; Jeske et al. 2023), affecting EV's proteins and miRNA cargo (Jeske et al. 2023). This phenomenon is increased when the reactor's operation is stopped (i.e., discontinuous operation), impairing cell growth and identity (Fernandes-Platzgummer et al. 2023). The formation of the aggregates typically suggests that the culture system has reached its limits, and a carrier-to-carrier passage is required to expand the culture scale (Wang, Wei, et al. 2023). This also outlines the importance of the time of harvest when developing bioprocesses to manufacture EV populations with MCs (Phelps et al. 2024). Nevertheless, high yield and purity have been consistently reported in MSC-EVs produced in plastic MCs, possibly due to the dynamic conditions enabled by bioreactors, which cause

cytoskeleton reorganization and reconfigured cell metabolism (Jeske et al. 2023).

Despite of the plausible setbacks from the use of MCs, this technology has yielded positive results, which are generally better in SWRs than on SRs. SWRs used for MSC-EVs generation (Nikolits et al. 2024; Jeske et al. 2023; Wang, Pang, et al. 2023; Larey et al. 2024; Otahal et al. 2024; de Almeida Fuzeta et al. 2020; Jalilian et al. 2022), exhibit an MSCs doubling time similar to that of a monolayer culture (Wang, Pang, et al. 2023), while their operation under hypoxic conditions further improves cell proliferation (Nikolits et al. 2024). The MSC-EVs yield per cell increases 2.5-fold (Jeske et al. 2023), 3-fold (de Almeida Fuzeta et al. 2020), and up to 100-fold (Otahal et al. 2024) versus 2D cultures, with increases of 5.7-fold (de Almeida Fuzeta et al. 2020) to 24-fold (Jalilian et al. 2022) in total EVs concentration in the medium. The SWR is also scalable, showing similar MSC-EVs secretion and cargo content in a 0.5 L versus a 0.1 L bioreactor (Jeske et al. 2023). Differences from tissue sources have already been characterized in a 60 mL SWR, where BMSCs, ADMSCs and UCMSCs yielded an average of 2.8, 3.1, and 4.1 $\times$ 10^{11} EV particles per vessel (de Almeida Fuzeta et al. 2020). Additionally, more proteins are upregulated in SWRs than in monolayer cultures (Wang, Pang, et al. 2023), as well as the expression of EV biogenesis markers, glycolysis genes and certain microRNAs and proteins in the MSC-EVs cargo (Jeske et al. 2023). A better MSC-EVs purity has also been reported in SWRs compared to static cultures (de Almeida Fuzeta et al. 2020; Jalilian et al. 2022), and SWR-produced MSC-EVs have a superior potency compared to 2D culture flasks, with regard to inhibition of inflammation, inhibition of chondrocyte hypertrophy and induction of cartilage-specific ECM production (Otahal et al. 2024).

In SRs, which offer a higher shear profile than SWRs, elevated reactive oxidative species in MSCs can be generated without increasing rates of cellular senescence (Jeske et al. 2022). On the other hand, SRs offer a more versatile platform to introduce sensors for expansion control, enabling more advanced observations towards process intensification. In SRs, a lower glucose concentration favours MSC-EVs secretion (Costa et al. 2023), but no significant differences can be seen in MSC-EVs produced in fed batch versus continuous mode operation (Fernandes-Platzgummer et al. 2023). The introduction of Raman spectroscopy sensors also contributes to streamlining the selection of optimal EV collection timepoints (Costa et al. 2023). Unfortunately, and despite the significant upregulation of EVs biogenesis genes (3–10 fold) in SRs cultures (Jeske et al. 2022), MSC-EVs yield per cell can be lower in SRs than in 2D systems (Dos Santos et al. 2024; Gaesser et al. 2024) or just slightly superior: 1.4 fold (Jeske et al. 2022) to 2.2 fold (Costa et al. 2023). In addition, the average size of MSC-EVs was found to increase at higher shear rates (Phelps et al. 2022). Despite these issues, MSC-EVs seem to keep their functionality, as they can still stimulate angiogenesis in cerebral microvascular endothelial cells (Phelps et al. 2024), keep their inflammatory suppression capability in the treatment of lung inflammation (Zhang et al. 2024), and upregulate type II collagen production by mesenchymal progenitor cells (Phelps et al. 2022). Hydrogels can offer an excellent opportunity to improve the performance of SRs, since they can shield the cells from the effect of shear stress and offer a reliable 3D culturing matrix. This could boost the implementation of QbD approaches to MSC-EVs generation.

6 | Conclusion and Future Perspectives

Despite the progress of the recent years, several uncertainties in the manufacturing of MSC-EV remain, preventing a clear clinical outline towards translation and mass-production. Harvesting and purification methods require standardization, proper dosage and pharmacokinetics concepts need to be defined, the cost of therapy needs a more integrative approach, and optimal or differential culturing conditions (e.g., physioxia, supplementation, dynamic culturing) have to be explored systematically. These knowledge gaps need to be bridged before claiming that paracrine functions of MSCs can be precisely tuned, with or without the aid of hydrogels. If hydrogels are involved for cell culture, testing different materials and modifications without characterizing their basic properties, further complicates a conclusive narrative: it is difficult to decouple the impact of a biophysical or biochemical cue on several synergic cellular functions (e.g., proliferation, stemness, metabolic activity and cellular communication), besides the interdependencies between the hydrogel's properties themselves. In the same vein, testing different seeding and culturing configurations (e.g., cell density, passage, medium, 2D vs. 3D) without proper controls may provide results, but can attribute these results to the wrong variables. Some of the studies summarized in this review suffer from these hindrances and make their conclusions applicable to a particular set of experimental configurations and therefore should be observed on a case-by-case basis.

To give a baseline for material characterization, we grouped hydrogel properties into three categories: mechanical, architectural and biochemical. The mechanical properties are well understood: high stiffness is reported to promote mechanotransduction and osteogenic phenotype, while low stiffness promotes adipogenesis and chondrogenesis; high stiffness might increase cytoskeletal tension and enhance downstream gene expression, while low stiffness overall maintains cell stemness; lastly, high stiffness reduces the secretion of immunomodulatory factors, but low stiffness recovers cytokine secretion in MSCs exposed to stiff substrates. Fast-relaxing hydrogels also improve the secretion of paracrine factors. Meanwhile, EV secretion has been confirmed in both soft and stiff hydrogels, and it is apparently higher with low-stiffness and fast-relaxing hydrogels. The effect of dynamical stiffening and the erasing of the mechanical memory in soft hydrogels (both enhancers of the overall secretion of paracrine signals) have not been explored for the secretion of EVs yet but could improve their yield and cargo. Comparatively to the effect of the mechanical properties, little is known on the effect of the architectural properties in the secretion of EVs. Beyond the impact of porosity and surface charge on vesicle diffusion, which has been extensively studied in controlled release applications, small pore size is known to decrease cytokine secretion by hindering cell–cell interactions; a similar effect is caused by substrates that are difficult to remodel or degrade. None of these phenomena have been assessed in the secretion of EVs, especially the impact of degradation kinetics, although the secretion of MSC-EVs has been confirmed both in micro-porous and macro-porous hydrogels. Finally, the biochem-

ical properties are frequently evaluated with several examples of polymer functionalization that show positive effects on the secretion of EVs.

Despite the comparability issues in studies about MSC-EVs and hydrogels, some trends can be established from their results. The size of the EVs from bulk hydrogels is similar to the size of the EVs produced in scaffold-like hydrogels, but only the EVs from bulk hydrogels are clearly smaller than the EVs produced in 2D under the same study conditions. The yield and the protein content of the EVs are increased both in bulk and scaffold-like hydrogels, if compared with 2D controls. Interestingly, spherical hydrogels seem to increase the yield even further. The internalization rate by target cells is higher in the EVs produced in bulk hydrogels than in 2D conditions, and both bulk and scaffold-like hydrogels improve the promigratory and proliferative potency of the EVs on BMSC and their neuroprotective potential. However, the effect on angiogenesis and anti-inflammatory potential is reported to be similar to 2D-produced EVs. None of the studies found detrimental effects from hydrogels culturing on the quality, yield, functionality or potency of the EVs. Taking this fact as a starting point should guide research in prioritizing the standardization of culturing and isolation conditions before further testing clinical applicability.

Questions still remain on the scalability of these technologies and the platforms required to culture the hydrogels. Just a few of these studies incorporated bioreactors to better control process parameters and culture perfusion, which has been a great advantage for alternative 2D-dynamic systems, such as microcarriers. Similarly, few studies considered the EV productivity normalized per medium or cell, which keeps hindering the estimation of the cost of therapy. This is a necessary step towards the generation of clinically relevant EV doses. Considerations upon culturing and harvesting duration, cell stimulation and hydrogel stability should also be included, as cells seem to be more resilient to senescence and external stressors when grown in hydrogels, opening the possibility to alternative culturing protocols. Overall, MSC-EVs hold great promise for biomedical applications. Nonetheless, substantial challenges persist, and it is still to be answered whether their secretion can be precisely controlled, calling for systematic approaches to standardize a field that is still in its infancy.

Author Contributions

Oscar Fabian Garcia-Aponte: conceptualization (lead), formal analysis (lead), visualization (lead), writing–original draft (lead), writing–review and editing (lead). **Simon Kahlenberg**: formal analysis (supporting), writing–original draft (supporting), writing–review and editing (supporting). **Dominik Egger**: supervision (equal), writing–original draft (supporting), writing–review and editing (supporting). **Dimitrios Kouroupis**: supervision (equal), writing–original draft (supporting), writing–review and editing (supporting). **Cornelia Kasper**: supervision (lead), writing–review and editing (lead).

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing is not applicable to this article, as no new data were created or analysed in this study.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.