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Mesenchymal Stem Cell-Derived Extracellular Vesicles: Progress and Remaining Hurdles in Developing Regulatory Compliant Quality Control Assays

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Abstract

Regenerative medicine is shaping into a new paradigm and could be the future medicine driven by the therapeutic capabilities shown by mesenchymal stem cell-derived extracellular vesicles (MSC-EVs). Despite the advantages and promises, the therapeutic effectiveness of MSC-EVs in some clinical applications is restricted due to inconsistent manufacturing process and the lack of stringent quality control (QC) measurement. In particular, QC assays which are crucial to confirm the safety, efficacy, and quality of MSC-EVs available for end use are poorly designed. Hence, in this review, characterization of MSC-EVs and quality control guidelines for biologics are presented, with special attention given to the description of technical know-how in developing QC assays for MSC-EVs adhering to regulatory guidelines. The remaining challenges surrounding the development of potency and stability of QC assays are also addressed.

Keywords

Bioprocessing · Conditioned media · Exosome · Microvesicles · Regenerative medicine · Secretomes

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Abbreviations

μm	Micrometers
AD	Adipose tissue
AFM	Atomic force microscopy
BM	Bone marrow
CB	Cord blood

CDSCO	Central Drugs Standard Control Organisation
DLS	Dynamic light scattering
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EMA	European Medicines Agency
EVs	Extracellular vesicles
FC	Flow cytometry
FDA	Food and Drug Administration
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
ISEV	International Society for Extracellular Vesicles
MFDS	Ministry of Food and Drug Safety
MISEV	Minimal information for studies of extracellular vesicles
MSC-EVs	Mesenchymal stem cell-derived extracellular vesicles
MSC-Exo	Mesenchymal stem cell-derived exosome
MSC-MVs	Mesenchymal stem cell-derived microvesicles
MSCs	Mesenchymal stem cells
MVBs	Multivesicular bodies
MVs	Microvesicles
MWCO	Molecular weight cutoff
nm	Nanometers
NTA	Nanoparticle tracking analysis
PCR	Polymerase chain reaction
PMDA	Pharmaceuticals and Medical Devices Agency
QC	Quality control
qPCR	Quantitative polymerase chain reaction
RPS	Resistive pulse sensing
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Scanning electron microscopy
sEVs	Small extracellular vesicles
TEM	Transmission electron microscopy
TRPS	Tunable resistive pulse sensing
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling
UC	Umbilical cord
WB	Western blot

1 Introductions

Mesenchymal stem cell secretome, the extracellular vehicles (MSC-EVs), has sparked attention in the last few years because of their increasing biological relevance in normal physiology and disease states (Xunian and Kalluri 2020; Hartjes et al. 2019). They have broader therapeutic effects due to its ability to carry a considerable number of functional therapeutic molecules in the form of mRNA, micro-RNAs, long-coding RNAs, DNA, and metabolites (Maumus et al. 2020). This unique characteristic allows MSC-EVs to be utilized in many forms such as the replacement of live cells in stem cell transplantation or as a vehicle in delivering specific targeted therapeutic agents (Maumus et al. 2020).

Though up to date more than 40 MSC-EV studies have been conducted broadly in preclinical animal models, however, only a few have been approved for clinical studies in which most of them are in the not yet recruiting or available phases and are confined to wound healing process and autoimmune, neurological, and cardiovascular diseases (Witwer et al. 2019; Lee et al. 2021). Inconsistent bioprocessing of EV production especially in large scale-up and lack of standardized quality control (QC) assay are the key factors downgrading the therapeutic effects of MSC-EVs products (Witwer et al. 2019; Nguyen et al. 2020). While attention has been given in addressing the issues related to the former, researchers still have difficulty in designing a proper QC assay to align with regulatory requirements.

MSC-EVs are classified as medicinal biological products by leading regulatory agencies, which means that MSC-EV-based products need to be approved by competent authorities before administration for clinical use. For the approval of MSC-EV products to be

considered, compliance to Good Manufacturing Practices (GMP) and strict regulatory practices during the in-process QC of product, finished product QC, and stability studies are crucial to ensure the safety, efficacy, and quality of the MSC-EVs available to end use. Current MSC-EV QC assays are centralized to address the characterization as there is a certain degree of overlap in terms of sizes and markers between the various subtypes (exosomes, microvesicles, apoptotic bodies). While a certain degree of attention is given to address the QC-related assays on physical properties, safety, and purity, potency assays that overall predict the biological function of the EVs are often neglected (Ludwig et al. 2019). Further, most of these QC assays were developed without proper analytical validation resulting in inconsistency in release criteria.

Hence, this review will address the progress and challenges in designing regulatory compliant QC assays that cover major key elements such as identity, purity, safety, and potency. We anticipate that a validated QC assay will lead to the better therapeutic efficacy of MSC-EVs.

2 Biological Characterization of MSC-EVs

MSC-EVs are non-replicated lipid bilayer vesicles and generally secrete into the extracellular environment (Witwer et al. 2019). Typically, EVs are divided into three subtypes – apoptotic bodies, microvesicles (MVs), and exosomes – which are generally distinguished by their sizes (Fig. 1). Exosome being the smallest of all of them with a diameter size between 40 and 120 nanometers (nm), has the main role in regulating intercellular communication carrying specific biomolecular information such as RNAs, proteins, and lipids in their intracellular compartments and transporting them to the target cells (Andaloussi et al. 2019; Colombo et al. 2014). Exosome can be easily distinguished from other subtypes by characterising marker proteins such as tetraspanins (CD9, CD63, CD81, CD82), membrane transport proteins (annexins, Rab), heat shock proteins (Hsp60, Hsp70, Hsp90), and multivesicular bodies (MVBs) formation proteins (Alix, TSG101) (Zhang et al. 2019) that are found on its membrane surface. This is followed by MVs which are in the range of size of 50 to 1000 nm and have similar function and content as exosomes (Andaloussi et al. 2019). MVs also mediate intercellular communication via the delivery of contents to recipient cells and contain cytosolic and plasma membrane-associated proteins, mRNAs, miRNAs, nucleic acids and lipids in their intracellular compartments (Andaloussi et al. 2019; Doyle and Wang 2019). The protein markers present on the membrane of MVs are

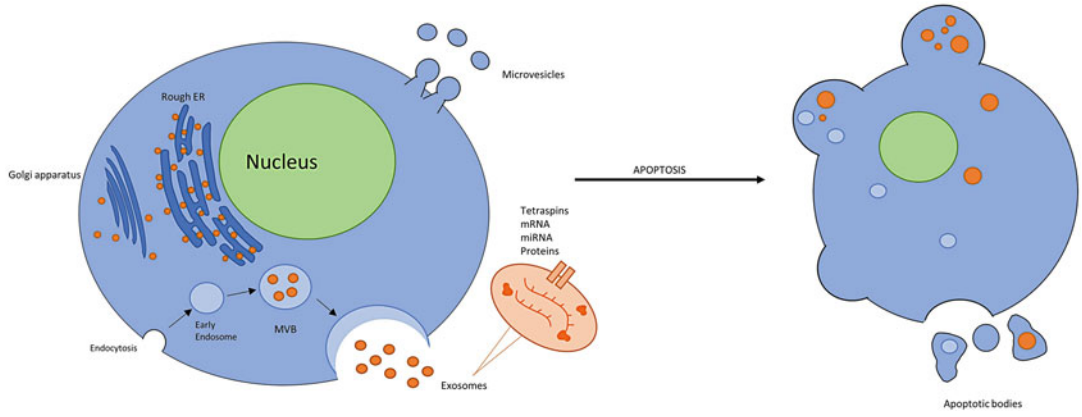

Fig. 1 The biogenesis and content of extracellular vesicles which include the subtypes of extracellular vesicles, microvesicles, and apoptotic bodies

integrins, selectins, and CD40 ligands that function as adhesion molecules (Andaloussi et al. 2019). Apoptotic bodies are larger EVs with a size between 500 and 2000 nm and formed during apoptosis. It contains a portion of a dying cell known as nuclear fractions and cell organelles and can be recognized by having large amounts of phosphatidylserine (Andaloussi et al. 2019).

Due to the overlapping of sizes, characterization markers, and functions between the subtypes of EVs, the International Society for Extracellular Vesicles (ISEV) has published minimal criteria for studies of extracellular vesicles (MISEV) in defining EVs, especially those from MSCs (Thery et al. 2018). According to guideline which is known as MISEV 2014 (later being adopted as MISEV 2018), MSC-EVs that are sized greater than 200 nanometers (nm) in diameter are termed large EVs while EVs that are smaller than 200 nm in diameter are termed small EVs (sEVs) and are named based on its contained biochemical compounds or their originating cell type (Thery et al. 2018).

ISEV further suggested that each research of EVs should be (1) outlined by quantitative measurement of EV source (e.g., total amount of conditioned medium, initial cell seeding, and final cell count); (2) characterized to the utmost practicable to determine the quantity of EVs (e.g., quantification of particles, protein, lipids, nucleic acid); (3) verified for the presence of components specific for particular EVs subtypes (e.g., characterization by protein composition or surface marker); and (4) examined for the presence of non-vesicular, co-isolated components. In other words, EVs should be characterised by the content such as protein or particle concentration, minimum three positives and one negative protein marker and two analytical measures of single extracellular vesicle. The positive protein markers of EVs include transmembrane and cytosolic protein while negative protein markers are composed of apolipoproteins A1/2 and albumin. In addition, EVs subtype can be characterized by subcellular compartments such as the nucleus, mitochondria, or extracellular protein with biological function like growth factors, cytokines or the extracellular matrix (Thery et al. 2018).

2.1 Current Scenario on MSC-EV Characterization Assays

Most of the characterization assays related to downstream activities of MSC-EVs are largely based on identifying and quantifying (Table 1). Apart from directly looking at the number of particles with the same range of sizes, the assays in this category also measure various components that are present in the EVs such as the specific protein markers and the contents of nucleic acids and proteins. Currently, nanoparticle tracking analysis (NTA) is the most commonly used method to determine the size and the number of particles followed by dynamic light scattering (DLS) and resistive pulse sensing (RPS). On the other hand, single vesicle analysis by electron microscope (EM) is more popularly used than atomic force microscopy (AFM) in most research to characterize the morphology and structure of EVs. In terms of evaluating protein marker quantification and expression, a majority of researchers used Bradford, Western blot (WB), or flow cytometry (FC) analysis. While most of the researchers are using compendial testing which is also known as pharmacopoeia standards to check on safety, only a handful of research had focused on reporting on potency testing. Further, despite many studies providing quantitative values, none of the research reported on the ratio (e.g., protein/particle, lipid/particle, or lipid/protein) to estimate EV purity which has been recommended by the ISEV.

3 Quality Control Regulation for MSC-EVs

Quality control consists of procedures done to warrant that the service or product fulfills certain requirements or achieves a certain degree of quality (Sachan et al. 2014). The Food Drug and Administration (FDA) defines biologics as products that can be used in the diagnosis,

Table 1 QC-related assays being used in MSC-EV research

Author	Cell origin	Phase	Disease	Isolation/ purification	QC assay			
					Identity/quantity	Purity	Safety	Potency
Nassar et al. (2016)	Human CB-MSC-EVs	Phase II/III	Chronic kidney disease	Differential centrifugation Density gradient ultracentrifugation	Total protein content TEM SEM FC			
Zhang et al. (2018)	Human UC-MSC-Exo	Phase I	Refractory macular holes	Differential centrifugation	Total protein content SEM WB			
Shi et al. (2021)	Human AD-MSC-EVs	Phase I	Lung injury	Differential centrifugation PEG concentration	Total protein content TEM NTA WB		Gram stain – Microscopy Sterility test – BacT/ALERT Mycoplasma – qPCR Endotoxin – Limulus assay	
Gatti et al. (2011)	Human MSC-MVs	Preclinical	Acute and chronic kidney injury	Differential centrifugation	Total protein content TEM SEM DLS		Endotoxin – Limulus assay	
Bruno et al. (2012)	MSC-MVs	Preclinical	Acute kidney injury	Differential centrifugation	Total protein content TEM SEM DLS FC		Endotoxin – Limulus assay	
Li et al. (2012)	Human UC-MSC-Exo	Preclinical	Liver fibrosis	Differential centrifugation Ultrafiltration	Total protein content TEM WB			
Arslan et al. (2013)	MSC-Exo	Preclinical	Myocardial ischemia/ reperfusion injury	HPLC Ultrafiltration Tangential flow filtration				

(continued)

Table 1 (continued)

Author	Cell origin	Phase	Disease	Isolation/ purification	QC assay			
					Identity/quantity	Purity	Safety	Potency
Bian et al. (2013)	Human BM-MSC-Evs	Preclinical	Myocardial infarction	Differential centrifugation	Total protein content TEM FC WB			Cell proliferation assay Cell migration assay Tube formation assay
Zhou et al. (2013)	Human UC-MSC-Exo	Preclinical	Cisplatin-induced nephrotoxicity	Density gradient centrifugation Ultrafiltration	TEM WB			
Zhu et al. (2013)	Human BM-MSC-MVs	Preclinical	Acute lung injury	Differential centrifugation	Total protein content SEM			
Chen et al. (2014)	Murine BM-MSC-MVs	Preclinical	Pulmonary arterial hypertension	Differential centrifugation Ultracentrifugation	Total protein content TEM NTA FC			
Zhang et al. (2014)	Human UC-MSC-MVs	Preclinical	Renal ischemia/ reperfusion injury	Differential centrifugation	Total protein content TEM FC	TUNEL assay		
Cruz et al. (2015)	Human BM-MSC-EVs	Preclinical	Allergic airway inflammation	Differential centrifugation Ultracentrifugation	Total protein content TEM NTA			
Doeppner et al. (2015)	Human BM-MSC-Exo	Preclinical	Poststroke neuroregeneration	Ultrafiltration PEG precipitation Ultracentrifugation	Total protein content NTA WB		Bacterial contamination – PCR and infectious serology	
Monsel et al. (2015)	Human BM-MSC-MVs	Preclinical	Severe pneumonia	Ultracentrifugation	Total protein content SEM WB			

Teng et al. (2015)	Murine BM-MSC-Exo	Preclinical	Myocardial infarction	Precipitation (ExoQuick-TC)	Total protein content TEM FC			Cell proliferation assay Tube formation assay
Zhang et al. (2015a)	Human UC-MSC-Exo	Preclinical	Cutaneous wound healing	Differential centrifugation Ultrafiltration	Total protein content TEM NTA WB			
Zhang et al. (2015b)	Murine BM-MSC-Exo	Preclinical	Traumatic brain injury	Precipitation (ExoQuick)	Total protein content TEM NTA WB			
Zhao et al. (2015)	Human UC-MSC-Exo	Preclinical	Acute myocardial ischemic injury	Differential centrifugation MWCO concentration Density gradient centrifugation Ultracentrifugation	Total protein content TEM NTA WB			
Lin et al. (2016)	AD-MSC-Exo	Preclinical	Renal acute ischemia/ reperfusion injury	Ultracentrifugation	EM WB			
Ophelders et al. (2016)	Human BM-MSC-EVs	Preclinical	Preterm hypoxic-ischemic brain injury	Ultrafiltration PEG precipitation Ultracentrifugation	Total protein content NTA WB		Tested for the presence of bacteria, viruses, and endotoxins	
Tamura et al. (2016)	Murine BM-MSC-Exo	Preclinical	Liver injury	Differential centrifugation Ultrafiltration Ultracentrifugation	Total protein content TEM TRPS FC			
Zhang et al. (2016)	BM-MSC-Exo	Preclinical	Myocardial repair	Differential centrifugation Precipitation (ExoQuick-TC)	Total protein content TEM FC WB			Cell proliferation assay Cell migration assay Tube formation assay

(continued)

Table 1 (continued)

Author	Cell origin	Phase	Disease	Isolation/ purification	QC assay			
					Identity/quantity	Purity	Safety	Potency
Zou et al. (2016)	Human MSC-EVs	Preclinical	Renal ischemic reperfusion injury	Ultracentrifugation	Total protein content TEM FC NTA			
Bai et al. (2017)	Human UC-MSC-Exo	Preclinical	Autoimmune uveitis	Differential centrifugation Ultrafiltration	Total protein content EM WB			
de Castro et al. (2017)	Human AD-MSC-EVs	Preclinical	Allergic asthma inflammation	Differential centrifugation Ultracentrifugation	Total protein content SEM DLS			
Drommelschmidt et al. (2017)	Human BM-MSC-EVs	Preclinical	Inflammation-induced preterm brain injury	Ultrafiltration PEG precipitation Ultracentrifugation	NTA WB		HIV, HCV, HBV – Multiplex PCR Microbiological contamination – BacTAlert bottles	
Gangadaran et al. (2017)	Murine BM-MSC-EVs	Preclinical	Hindlimb ischemia	Differential centrifugation Density gradient ultracentrifugation	NTA TEM WB			Cell migration assay Cell proliferation assay Tube formation assay
Haga et al. (2017)	BM-MSC-EVs	Preclinical	Lethal hepatic failure	Differential centrifugation Ultracentrifugation	Total protein content NTA TEM			
Mao et al. (2017)	Human UC-MSC-Exo	Preclinical	Inflammatory bowel disease	Differential centrifugation Density gradient centrifugation Ultrafiltration	Total protein content TEM NTA WB			

Song et al. (2017)	Human UC-MSC-Exo	Preclinical	Sepsis	Differential centrifugation Ultrafiltration	Total protein content TEM WB			
Stone et al. (2017)	Human UC-MSC-EVs	Preclinical	Lung ischemic/ reperfusion injury	Differential centrifugation	Total protein content NTA Imaging FC			Cell migration assay
Wang et al. (2017)	MSC-EVs	Preclinical	Myocardial infarction	Ultracentrifugation Differential centrifugation	Total protein content TEM WB			Cell proliferation assay Cell migration assay Tube formation assay
Ahn et al. (2018)	Human UC-MSC-EVs	Preclinical	Neonatal hyperoxic lung injury	Differential centrifugation Ultracentrifugation	TEM NTA			
Bandeira et al. (2018)	AD-MSC-EVs	Preclinical	Silicosis	Differential centrifugation Ultracentrifugation	Total protein content TEM/SEM NTA FC			
Cho et al. (2018)	Human AD-MSC-Exo	Preclinical	Atopic dermatitis	Differential centrifugation	TEM NTA WB FC			Cell-based assay
Jiang et al. (2018)	Human UC-MSC-Exo	Preclinical	Liver injury	Differential centrifugation Ultracentrifugation MWCO concentration Density gradient centrifugation Ultrafiltration	TEM NTA FC			

(continued)

Table 1 (continued)

Author	Cell origin	Phase	Disease	Isolation/ purification	QC assay			
					Identity/quantity	Purity	Safety	Potency
Sun et al. (2018a)	Human UC-MSC-Exo	Preclinical	Spinal cord injury	Differential centrifugation MWCO concentration Ultracentrifugation Ultrafiltration	Total protein content TEM DLS WB			
Sun et al. (2018b)	Human MSC-Exo	Preclinical	Type 2 diabetes mellitus	Differential centrifugation Ultrafiltration	Total protein content WB TEM NTA			
Wu et al. (2018)	Human UC-MSC-Exo	Preclinical	Inflammatory bowel disease	Differential centrifugation Density gradient centrifugation Ultrafiltration	Total protein content TEM NTA WB			
Hao et al. (2019)	Human BM-MSC-EVs	Preclinical	Lung injury	Differential centrifugation Ultracentrifugation	Total protein content SEM NTA FC WB			
Shi et al. (2019)	Human UC-MSC-Exo	Preclinical	Acute myocardial infarction	Differential centrifugation MWCO concentration ExoQuick-TC	Total protein content TEM NTA WB			
Shiue et al. (2019)	Human UC-MSC-Exo	Preclinical	Nerve injury-induced pain	Differential centrifugation Ultrafiltration	Total protein content TEM WB FC			
Varkouhi et al. (2019)	Human UC-MSC-EVs	Preclinical	Acute lung injury	Differential centrifugation	Total protein content TEM FC			

EM electron microscope, *TEM* transmission electron microscope, *SEM* scanning electron microscope, *NTA* nanoparticle tracking analysis, *DLS* dynamic light scattering, *TRPS* tunable resistive pulse sensing, *FC* flow cytometry, *WB* Western blot, *HPLC* high-performance liquid chromatography

prevention, and treatment of medical disorders (Code of Federal Regulations 2021). Hence, biological products, such as MSC-EVs, are subjected to several QC regulations, which cover aspects such as identity, quantity, purity, sterility, potency, and stability. Each country has its own regulatory body, governing the quality of biological products with its own set of QC guidelines (Table 2).

3.1 Establishing QC Assays for EV-Based Products

Many characterization assays are established and being used routinely in MSC-EV research work; however, the main stumbling block is that only a handful of these assays are being developed into a proper QC assay. In this regard, the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) has proposed the feasibility of developing any assays into QC assays with specific acceptance criteria to ensure the assays' reproducibility, reliability, and therapeutic value. To develop a good QC assay, ICH Q2 (R1) guideline highlights the need to conduct validation experiments based on the eight important parameters which include linearity, specificity, range, accuracy, robustness, precision, quantitation limit, and detection limit (Table 3). Here, we briefly explain

Table 2 Guideline subsections of each regulatory aspect for biological products by several regulatory bodies

	Guideline subsection by each regulatory body				
Regulatory aspects	FDA (USA) – Code of Federal Regulations Title 21, Subchapter F, Chap. 1, Part 610 (Code of Federal Regulations 2021)	EMA (Europe) – ICH Q5C – Quality of Biotechnological Products: Stability Testing of Biotechnological/ Biological Products and ICH Q6B – Specifications: Test Procedures and Acceptance Criteria for Biotechnological/ Biological Products (European Medicines Agency 1994a, 1994b)	MFDS (Korea) – Regulation on Approval and Review of Biological Products (Ministry of Food and Drug Safety 2003)	PMDA (Japan) – Guideline for the Quality, Safety, and Efficacy Assurance of Follow-On Biologics (Pharmaceuticals and Medical Devices Agency 2009)	CDSCO (India) – Biosimilar Guideline 2016 (Central Drugs Standard Control Organisation 2016)
Identity	610.14 identity	–	Article 28 (review criteria for biologics)	–	6.3.2 product characterization
Quantity	–	Q6B 2.1.5 quantity	–	6. Specifications and test procedures	6.3.1 analytical methods
Purity	610.13 purity	Q6B 2.1.4 purity, impurities, and contaminants	Article 28 (review criteria for biologics)	6. Specifications and test procedures	6.3.2 product characterization
Sterility	610.12 sterility	Q6B 2.1.4 purity, impurities, and contaminants	Article 28 (review criteria for biologics)	6. Specifications and test procedures	–
Potency	610.10 potency	Q6B 2.1.2 biological activity	Article 28 (review criteria for biologics)	6. Specifications and test procedures	6.3.2 product characterization
Stability	–	Q5C	–	4.4 stability testing	6.3.4 stability

Table 3 List of QC assays corresponding to ICHQ2 (R1) guidelines

			ICH validation criteria based on ICH Q2 (R1)								Other parameters		
Author	Assays' category	Assays	Specificity	Linearity	Range	Accuracy	Precision	Detection limit	Quantitation limit	Robustness	Acceptance range	Cost	Technicality
Adan et al. (2016); Maas et al. (2015)	Identity/ quantity	Flow cytometry (FC)	/	/	/	×	/	x	/	/	Percentage of positive or negative expression markers	↔	↓
Choudhary and Ka (2017)		Scanning electron microscopy (SEM)	/	/	/	/	/	x	/	x	The morphology and structure, either intact or not intact	↑	↑
Dragovic et al. (2011); Maas et al. (2015)		Nanoparticle tracking analysis (NTA)	/	/	/	x	x	/	/	/	The amount of particle size	↓	↓
Ghosh et al. (2014); Hartjes et al. (2019)		Western blot (WB)	/	/	/	/	x	x	/	/	Either presence or absence of the bands	↓	↓
Koritzinsky et al. (2017); Zhao et al. (2015)		Bradford assay	x	/	/	/	/	x	/	x	Detect the presence and concentration of protein in the sample of 5–50 µg/ml	↓	↓
Lu et al. (2017); Williams and Carter (1996)		Transmission electron microscopy (TEM)	/	/	/	/	/	x	x	/	The morphology and structure, either intact or not intact	↑	↑
Sharma et al. (2018)		Atomic force microscopy (AFM)	/	/	/	/	x	x	/	/	The amount of particle size	↔	↓

Doeppner et al. (2015)	Safety	Infectious serology	x	/	x	/	/	/	x	x	Positive result from this test is able to detect the presence of certain antibodies	↓	↓
Enderle et al. (2015); Garibyan and Avashia (2013); Montero-Calle et al. (2021)		Quantitative polymerase chain reaction (qPCR) for mycoplasma	/	/	/	/	/	x	/	x	Able to determine the exact CT value and also either the presence or absence of the bands for mycoplasma	↔	↓
Iwanaga (2007); Mehmood (2019)		Limulus assay	x	/	/	/	/	/	/	x	Gelation reaction once exposed to endotoxin from gram negative bacteria	↓	↓
U.S. Food and Drug Administration (2018)		BacT/ ALERT	/	/	/	/	x	/	/	x	The culture will change to yellow with the presence of microorganism contamination	↓	↓
Tripathi and Sapra (2021)		Microscopy gram stain	/	/	/	/	/	x	/	x	Detect the presence of contamination of either gram positive or gram negative microorganisms	↓	↓

Legend: ↑ = high, ↓ = low, ↔ = moderate

the role of each of these parameters and how important they are in shaping up a good QC assay.

Specificity is defined as the capacity of an assay to assess the existence of components that may be anticipated to be present within the analyte. For instance, in terms of MSC-EVs for identity or quantity purposes, specificity denotes the size of MSC-EVs and typically carries markers such as CD63, CD9, and CD81. In this regard, assays such as NTA, Bradford, WB, or FC can be used to validate the specificity of MSC-EVs. Other assays such as scanning electron microscopy (SEM), transmission electron microscopy (TEM), or AFM may be used; however, we reckon that the assays are less specific as compared to the former assays due to the limitation of the instrument itself which only visualizes the morphology and structures of MSC-EVs. In terms of safety-related assays, specificity refers to the ability to differentiate MSC-EVs from contaminants such as body fluids given that we derived the exosomes from the blood and qPCR-based assays tend to have higher specificity as compared to other assays (Ludwig et al. 2019).

This is followed by the validation of the linearity of an assay which is defined as the ability to acquire experimental results that have a directly proportional relationship to the concentration of analyte within a sample. Generally, the acceptance coefficient of determination (R-squared) is 0.99, and one who performs assays such as NTA, Bradford, total protein content, WB, and qPCR has to validate the linearity of these assays. Next, range is defined as the range between the lower and upper concentrations of analyte within a sample. With this in mind, AFM has the highest range as it can detect particle size as close as 1 nm up until 120 nm. On the other hand, accuracy is the degree of closeness between the experimental value and the value acknowledged as the conventional true value or approved reference value. For example, FC does not meet the requirement of accuracy criterion for MSC-EVs as it cannot determine the sample concentration accurately due to the swarming effect and its insensitivity toward lower size range solutes.

The precision of an assay demonstrates the proximity of agreement among a set of results obtained from several samplings of a sample under prearranged conditions. Moreover, as stated in the ICH guidelines, precision is to be tested using homogenous and authentic samples; if not possible, artificially prepared samples can be used for the investigation. To give an example for the precision criterion, Hartjes et al. and Kurian et al. stated that although AFM has a better range, it has a very low reproducibility as the technique is highly dependent on the sample size, such that it can only image a maximum height of 10–20 micrometers (μm) within a total scanning area of 150 × 150 μm (Hartjes et al. 2019; Kurian et al. 2021). The validation for precision is further divided into three subcategories, namely, repeatability, intermediate precision, and reproducibility. Precision under the identical operational conditions over a short period is expressed by repeatability and requires at least nine determinations that cover a defined range for the assay or a minimum of six determinations at 100% test concentration. Finally, intermediate precision is expressed within-laboratories variations which include different days, analysts, and equipment. Reproducibility is expressed by the prevision between laboratories which is usually as a mean for methodology standardization. In short, techniques that qualify for the categories of range, accuracy, and precision should be able to detect a range of different size EVs while maintaining optimum accuracy and precision.

Penultimately, the detection limit of a test is referred to as the least amount of analyte within a sample which could be detected. For example, techniques that qualify for this category should have the ability to detect the presence of EVs although in a low concentration. Other than that, the quantitation limit of a test is the least amount of analyte within a sample that can be precisely and accurately quantified. This parameter is mainly used for low concentrations of compounds in a sample and to determine impurities or degradation products within an analyte. For example, the low penetration of EM beam and vacuum conditions required the sample to be ultrathin

and completely dry affecting the morphology of EVs. Finally, assays that qualify for the robustness category should show reliability with a deliberate variation. For example, the low scanning speed of AFM requires a longer time to obtain an accurate image. This leads to thermal drift causing variation in image quality that can affect the analytical condition which in turn produces an invalid result. In a nutshell, to ensure the repeatability and precise result, a combination of several QC assay techniques should be implemented so that the overall result obtained will be able to fulfill the ICH validation guidelines. For example, the Bradford test, TEM, SEM, FC, and qPCR can be used in the quality control process along with NTA as they complement each other, hence producing reliable results.

4 Remaining Challenges

Hurdles persist among currently available QC assays such as challenges in developing specific potency assays and ascertaining the life span of EVs. Potency assay consists of biological (in vitro or in vivo) or nonbiological assays which test the specific biological capabilities of the product (USFDA 2011). Establishing QC-related potency assays for MSC-EVs is more challenging compared to other pharmaceutical or biological products due to several factors as listed below:

1. Differences in EV preparations may result in enrichment of different components within MSC-EVs resulting in a change of therapeutic outcomes (Gimona et al. 2021).
2. Different donors of MSC-EVs have different biological properties and components which may change the therapeutic outcome (Gimona et al. 2021).
3. The mechanism of therapeutic potential of MSC-EVs is still vague as it can be involved in more than one different pathological process making it difficult to predict the potency of MSC-EVs (Gimona et al. 2021).
4. MSC-EVs from different MSC sources can vary in therapeutic potency such that suppression of T-cell proliferation is higher in AD-MSC-EVs than BM-MSC-EVs (Adlerz et al. 2020).
5. The spatiotemporal site of action remains unknown making it challenging to determine the precise biodistribution of MSC-EVs within a cell/tissue (Gimona et al. 2021).

Once the dynamic biological activities of EVs have been identified, currently available QC assays can be customized to comply with potency assay requirements. For example, Bruno and colleagues found that combining RT-PCR, enzyme-linked immunosorbent assay (ELISA), immunosorbent assays, and antibody assays was able to measure the full potency of the MSC-EVs but only for acute kidney injury (2009). Another study on myocardial ischemia injury showed that using a combination of enzymatic assays was also able to determine the potency of the MSC-EVs (Lai et al. 2010). Within these two examples, the combination of currently available assays such as RT-PCR, ELISA, and enzymatic assays can comply with the potency assay requirements such that it is specific, accurate, precise, and quantitative. However, due to the diverse attributes of MSC-EVs, combinations of different types of potency assays can only be specific to their respective pathological processes.

Another important aspect that needs to be considered is the life span which is orchestrated by stability data collectively contributed by factors such as temperature, light, and handling procedure. Nevertheless, a poorly designed QC assay may result in contradicting outcomes. For example, a study showed that 4 days' preserved exosomes at −80 °C are not stable as compared to freshly prepared ones (Maroto et al. 2017). Surprisingly, Jeyaram and Jay reported that the optimum storage condition for exosomes was −80 °C, contradicting the former study (Jeyaram and Jay 2017). This shows the important validation of the analytical procedure for each selected QC assay to ensure consistent results.

Further, majority of the purity tests that detect only endotoxins and mycoplasma contamination often neglect potential viruses contamination. Viruses are capable of enclosing themself into

EVs due to convergence of pathways (van der Grein et al. 2018). It is also known that the size ranges of EVs and viruses are similar which could lead to EV preparation being susceptible to viral contamination (Gyorgy et al. 2011). However, implementing QC to detect the presence of viral contamination is an uphill task because most methods, such as the PCR multiplex assay, are not product-specific. To test for viral contamination, short fragments of DNA are needed to complement specific parts of transcribed viral DNA. In short, current methods are only capable of detecting specific target viruses, and development for nonspecific target assays for viral detection should be carried out.

5 Conclusion

Despite these challenges, there is still room to improve QC assays for MSC-EVs. For QC tests that involve categories such as identity, purity, and quantity, apart from complying with ICH guidelines, efforts also should be taken to establish a similar QC procedure between various laboratories around the world which leads to a standard reference. Such an initiative can also be arranged by the ISEV as part of a compliance program. The issue remains that the enrichment method for isolation of EVs still remains with challenges that are difficult to keep up with as there is a lack of consensus concerning isolation steps for EVs (Stam et al. 2021). Additionally, the preparation of MSC-EVs remains expensive and has limited scalability, and if these preparations were scaled up, QC assays for MSC-EVs would be difficult to maintain. Moreover, the key to developing a potency assay is determining and mapping the pathological processes in preclinical animal models as it will lead to a better understanding of MSC-EVs' potency. In terms of extending the life span of EVs, lyophilization of EVs can be adopted. For example, a study by Charoenviriyakul and colleagues demonstrated that EVs could be lyophilized with the use of trehalose without affecting their stability and structure which allows exosomes to be preserved at room temperature which is useful for many applications (Charoenviriyakul et al. 2018). However, immortalized MSC-derived EVs would have special considerations for QC assays as they divide infinitely and express unique gene patterns that sometimes cannot be found in regular EVs (Rohde et al. 2019). With this, it is possible to develop separate assay criteria for MSC-derived EVs and immortalized MSC-derived EVs. Furthermore, special surface markers such as tetraspanins on MSC-derived EVs should be taken into consideration when developing an identification assay as it provides specificity only to EVs. With the combination of these suggestions and improvements, precision of a standardized QC assay can be developed to not only serve as a gold standard for MSC-EVs research but to also enhance its therapeutic value.

Conflict of Interest Kong-Yong Then and Soon-Keng Cheong are directors of CryoCord Sdn Bhd and declare direct share interest in the company, whereas all other authors declare no conflict of interest.

Author Contribution Statement J.K.E.C., L.H.F., C.Y.M., H.Y.T., and X.Y.T. contributed to the writing (original and final drafting) of the manuscript. V.G. contributed to the conceptualization and writing (review and editing) of the manuscript. T.S.R., K.Y.T., A.K.D., and S.K.C. contributed to the writing (review and editing) of the manuscript.

Data Availability Statement Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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