外泌体提取及保存技术研究进展

韩杰 葛安 马晓霞 王佳月 杨彩婷 董丽*

(山西大学生物医学研究院, 太原 030006)

摘要 外泌体是多种活细胞经过“内吞–融合–外排”等一系列过程主动向胞外分泌的纳米级双层膜结构小囊泡, 广泛存在于血液和尿液等生物体液中。因其携带着多种蛋白质、核酸和脂质等生物活性分子, 所以外泌体不仅在细胞间物质交换和信息传递中发挥重要作用, 而且对疾病诊断、预后预测和治疗管理等均具有提示意义。外泌体的高效提取、分离和完整保存是研究其在机体内生物学作用和功能的重要前提, 也是制约基于外泌体的临床检测技术和治疗载体技术的关键。该综述将针对目前国内外外泌体提取和保存领域的最新研究进展加以综述, 并对其特点进行对比和分析, 以促进外泌体研究方法的标准化, 以及相关技术的研发和应用拓展。

关键词 外泌体; 提取; 保存

Research Progress on the Isolation and Preservation Techniques of Exosomes

HAN Jie, GE An, MA Xiaoxia, WANG Jiayue, YANG Caiting, DONG Li*

(*Institutes of Biomedical Sciences Shanxi University, Taiyuan 030006, China*)

Abstract Exosomes, nanometer-sized extracellular vesicles enveloped in a lipid bilayer membrane, are released by almost living cells through a series of regulatory processes such as “endocytosis-fusion-efflux”. Exosomes are present in numerous biological fluids such as blood and urine. Exosomes are carriers of several molecules including proteins, nucleic acids and lipids, which not only play vital roles in substance exchange and signal communication from one cell to others, but also have some implications in disease diagnosis, prognosis prediction and treatment management. The efficient extraction, isolation and intact preservation of exosomes are important prerequisites for the subsequent biological mechanism-related studies and for the development of exosomes-based testing and relevant therapeutic technique. This article reviews the latest research progress made domestically and internationally on the isolation and preservation techniques of exosomes, as well as analyzes the technical characteristics of each technique. This article aims to provide informative summarization for the methodological standardization of isolation and preservation of exosomes, as well as promote the development of related techniques.

Keywords exosome; isolation; preservation

外泌体(exosome)是多种活细胞经过“内吞–融合–外排”等一系列过程主动向胞外分泌的直径为30~150 nm的双层膜结构细胞外囊泡(extracellular vesicles, EVs)[1], 广泛存在于血液、尿液和唾液等生物体液中, 并通常作为细胞间胞质蛋白、核酸和脂质的转移载体发挥运输调控作用, 被认为是细胞间通讯的核心部分[2-4]。外泌体最早于1983年在HARDING等[5]和PAN等[6]研究羊网织红细胞分化的

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*通讯作者。Tel: 13603584627, E-mail: dongli@sxu.edu.cn

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*Corresponding author. Tel: +86-13603584627, E-mail: dongli@sxu.edu.cn

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过程中被发现, 并于1996年由RAPOSO等[7]证实能够作为一种细胞间通讯结构在免疫系统中发挥作用后得到了广泛关注。近年来, 随着外泌体在肿瘤、心血管和感染性疾病等多种疾病形成中的生物学作用和功能被逐渐揭示, 尤其是2015年MELO等[8]发现, 外泌体Glypican-1蛋白可有效区分慢性胰腺炎与胰腺癌以来, 外泌体作为疾病诊断、预后预测标志物以及药物靶向治疗载体的转化医学应用也得到了迅速的推进[9-10]。

外泌体基础医学和临床应用的精准探索和深入研究对如何准确及高纯度提取外泌体, 并完整保存提出了更高的技术要求。外泌体所在的血液等体液成分复杂, 其他细胞外囊泡亚群虽理化性质与之相近, 功能却不同[11-12], 且不同体液中外泌体含量也存在很大差别, 因此高效去除背景混杂影响, 保证外泌体浓度和质量, 不仅是研究外泌体生物学作用的重要前提, 也是制约基于外泌体临床检测技术和治疗载体技术的关键。目前, 基于外泌体粒径和密度等理化性质特点, 研究者已建立了超高速离心和免疫亲和等多种分离技术, 且保存流程和关键参数也得到了不断更新。本研究将针对近年来国内外外泌体提取分离和保存技术方面的最新研究进展加以综述, 以加速外泌体研究方法的国际标准化, 并推动相关技术的研发和转化应用。

1 外泌体的生物学特性和功能

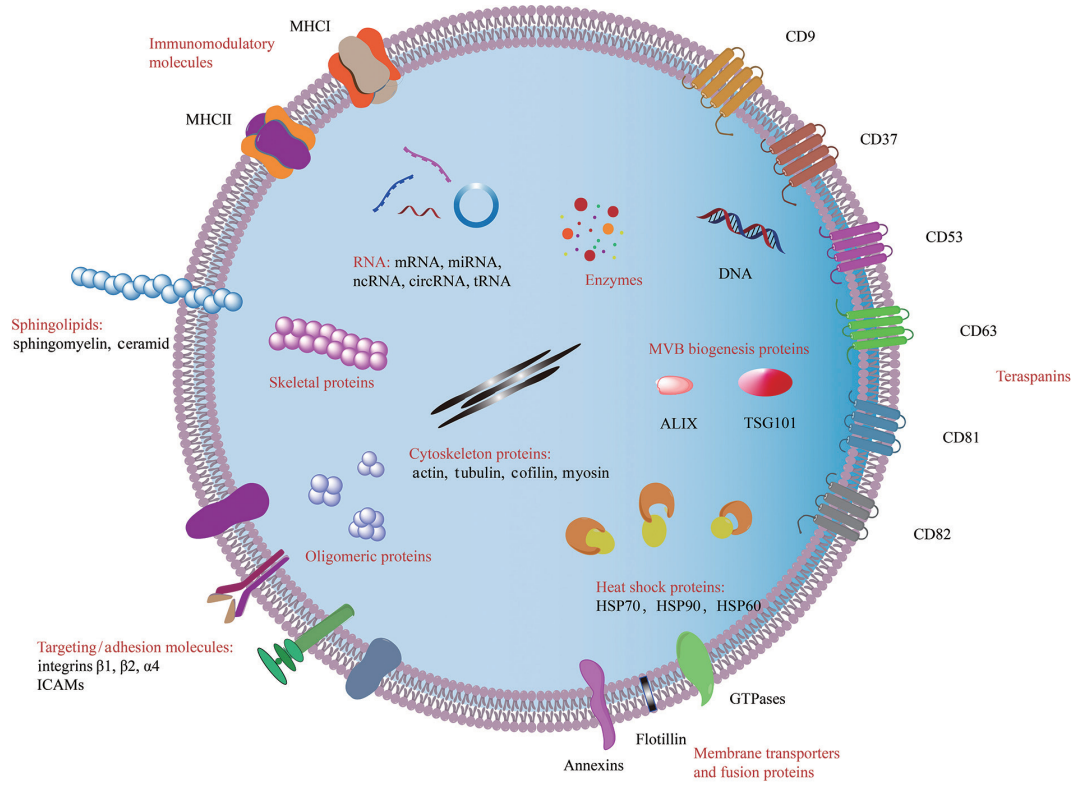
细胞分泌外泌体的过程一般分为三个阶段: (1) 质膜内陷, 形成早期内吞小泡; (2) 内吞小泡向内萌发成熟, 形成多囊泡体; (3) 多囊泡体与质膜融合, 并释放出囊泡内容物, 形成外泌体[13]。外泌体具有独特的理化性质, 其密度为1.13~1.19 g/mL[14], 由平均厚度小于5 nm的双层脂膜所包裹, 具有典型杯状形态, 呈现为扁平球形[15-16]。外泌体表面富含多糖链, 其质膜主要由溶血磷脂酸、磷脂酰丝氨酸、胆固醇、神经酰胺和鞘磷脂构成, 另有与细胞来源相关的部分特殊脂类[14,17]。外泌体可携带蛋白质、核酸和脂质等生物活性大分子, 其中主要为蛋白质, 且大部分蛋白为所有来源外泌体所共有, 只有小部分与其来源有关, 为其分泌细胞的特有蛋白, 能够反映分泌细胞的类型和生理病理状态[18-19]。除此之外, 外泌体可携带大量mRNA和miRNA等核酸, 并将其运输到靶细胞, 发挥相应的生物学功能[20]。外泌体结构及分子组成见图1。

外泌体是细胞间信息传递的重要“信使”[21], 可通过三种方式介导细胞通讯: (1) 外泌体以旁分泌的方式与靶细胞相互作用, 通过受体–配体作用黏附到靶细胞表面, 随后被内吞入靶细胞, 或直接将内容物释放到靶细胞内, 从而激活靶细胞; (2) 外泌体的胞外膜蛋白可以被蛋白酶切割, 产生的片段可以与靶细胞的细胞表面受体结合, 从而激活靶细胞; (3) 外泌体还可以与靶细胞直接接触发生膜融合, 导致外泌体中的蛋白和核酸非选择性转移到目标细胞, 从而引起靶细胞的响应[19,22]。通过介导细胞间的通讯, 外泌体不仅能够参与多种生理过程, 比如消除胞内陈旧分子[23]、呈递抗原、分化调节性T淋巴细胞或髓样细胞以抑制免疫反应[24], 而且还可以参与疾病形成的病理过程, 如通过与受体细胞的相互作用传播病原物质[25]、促进肿瘤转移过程中的血管生长和肿瘤细胞迁移[26]。

外泌体携带的与疾病进展紧密关联的分子标志物可用于疾病进展的监视、检测和诊断, 是一种潜在的非侵入性生物标志物[27]。由于外泌体本身可以作为抗原提呈小泡, 且有较长的循环半衰期, 可用于免疫学相关研究, 同时, 源自癌细胞的外泌体可以被工程化, 发挥免疫刺激作用, 可作为肿瘤疫苗应用于癌症的免疫治疗[28]。此外, 外泌体稳定而广泛地分布在器官和组织中, 具有低免疫原性、高耐受性等特点, 且良好的膜渗透性可使其通过血脑屏障, 因此可用作组织和器官特异性药物输送系统, 兼有高输送效率和低副作用[28-29]。因此, 外泌体适合用作药物载体递送治疗剂(如阿霉素或姜黄素), 治疗性miRNA、siRNA等核酸和蛋白质, 运送至靶位点后实现靶向治疗[30]。然而外泌体的传递方式会影响其在治疗方面的应用, 目前研究发现, 静脉注射的外泌体会迅速从血液循环中消失, 优先富集在肝、肺、脾、胃肠道和骨髓中。与静脉内注射相比, 腹膜内注射时胰腺和胃肠道中有更高的外泌体积累, 而皮下注射时所有器官中外泌体均为低富集[31]。

2 外泌体提取纯化技术

外泌体可以由体内或体外培养的几乎所有类型的细胞分泌, 并广泛存在于血浆、胆汁、尿液、母乳、唾液、胸腔积液、淋巴、胃酸、支气管肺泡灌洗液、脑脊液、眼泪、精液、滑膜液、羊水、腹水、鼻分泌物和子宫抽吸物液体等体液中[27,32-34]。目前, 基于



MHC: 主要组织相容性复合体; ICAM: 细胞间黏附分子。

MHC: major histocompatibility complex; ICAM: intercellular cell adhesion molecule.

图1 外泌体结构及分子组成示意图

Fig.1 Schematic diagram of molecular composition of exosomes

不同的分离原理, 研究者建立了五种主要的提取纯化技术, 分别为基于质量密度的超速离心技术、基于粒径大小的分离技术、基于溶解性质的聚合物沉淀技术、基于免疫亲和原理的分离技术和基于流体性质的微流控技术，提取纯化的技术路线总结于图2。

2.1 基于质量密度的超速离心技术

外泌体可通过差速超速离心在不同离心力下沉淀样品中不同的杂质组分, 并在100 000 ×*g*~200 000 ×*g*的转速下获取较纯的外泌体[35]。差速超速离心技术被认为是外泌体分离的“金标准”[36], 也是目前最常用的外泌体分离和浓缩方法。该方法可通过结合0.22 μm或0.45 μm孔径滤膜进行超滤来提高产物纯度减少外泌体的聚集, 其分离效率容易受到加速度、转子类型、旋转半径、沉降路径长度以及样品黏度等多种因素影响[37]。

密度梯度离心是基于差速超高速离心的改良技术。该方法需预先利用常用的梯度液介质如蔗糖、碘克沙醇和氯化铯等[38-40], 在离心管中构筑从底部到顶部密度逐渐降低的密度梯度带。根据密度梯度构建和沉降方式的不同, 又可以分为速率区带离心法和等密度梯度离心法, 前者主要根据颗粒的沉降速率分离, 介质密度均小于外泌体密度, 离心时样品在向超速离心管底部移动时, 会通过密度不断增加的密度梯度区带, 密度大的颗粒更容易穿过密度更高的梯度层, 更快地到达管底, 因此控制离心的时间很重要; 等密度梯度离心法中的密度梯度区带, 则会根据样品液中各种溶质成分来进行组合, 离心过程中, 无论离心时间多久, 不同密度颗粒仅会富集到具有相同密度的梯度区带, 而不会沉淀到底部[27]。

2.2 基于粒径大小的分离技术

外泌体作为细胞外囊泡的一个亚群, 直径集中于30~150 nm。目前, 基于粒径大小分离外泌体的方法有超滤法、尺寸排除色谱法、静水过滤透析法和非对称场流分离法等[41-44]。

超滤法利用不同孔径的滤膜, 对样品进行选择性分离以获得外泌体[41], 一般分为压力超滤和离心超滤。其中离心超滤效果更好, 不仅能减轻力对外泌体的破坏, 而且可通过增大离心力和延长离心时间提高外泌体产物浓度。但离心力过大或离心时间过长均有可能导致超滤膜或外泌体破裂[45]。此外,

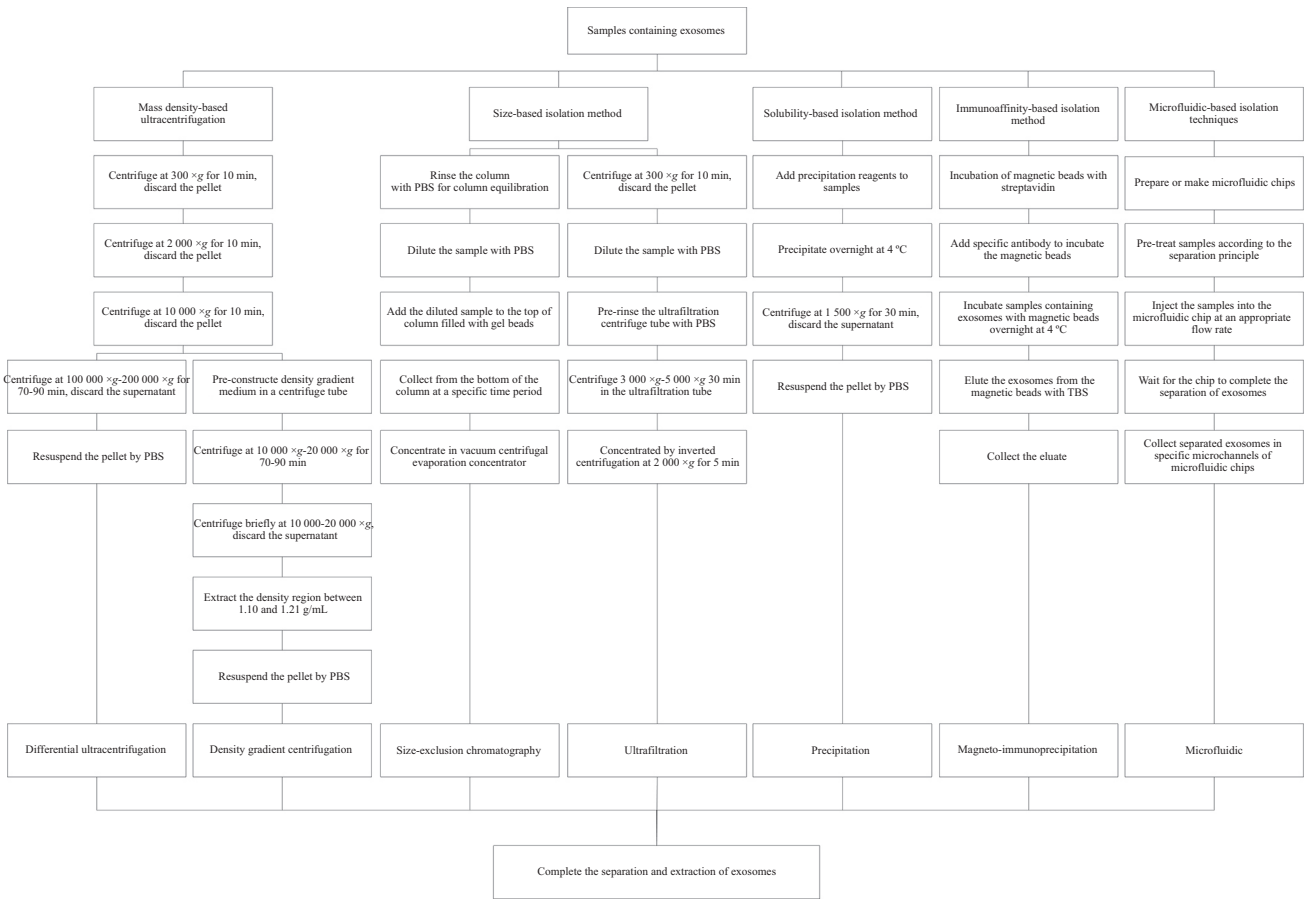


图2　外泌体常见分离方法的流程图

Fig.2　Flow chart of common isolation methods of exosomes

超滤法可以和切向流过滤法[46]、微控流法[47]、超速离心法或凝胶过滤色谱法等方法结合进一步提高分离效率[29]。

尺寸排除色谱法是使用聚合物凝胶或类似的固定相柱进行外泌体分离的技术[42], 样品以重力滴落的方式收集。流体力学半径较小的样品组分进入凝胶孔隙后, 需耗费相对较长的时间通过凝胶柱, 导致延迟洗脱, 实现不同粒径大小颗粒分离。尺寸排除色谱法可以与差速离心法结合而不会明显损失外泌体, 保证产量的同时可有效去除杂质蛋白, 更适合目标蛋白质组学和miRNA的分析[48-49]。

静水过滤透析法主要利用静水压力迫使样品中不同特定大小分子先后通过透析管, 其中溶剂和小溶质很容易通过透析管, 而较大的颗粒, 如外泌体和其他囊泡, 仍留在透析管中而被收集。静水压力可使均质流体均匀作用于一个物体表面上, 在施加较大力的同时保证目标结构的完整性。在静水过滤透析法分离后使用超速离心方法可进一步将外泌体与滞留在透析管中的其他颗粒分离[43]。

非对称场流分离技术是一种相对较新的技术, 它应用不同方向上的力场作用如离心力、引力场、温差、电场等作用力使得通道内不同体积大小的组分以不同的速度通过, 最后通过评估检测器检测到的样品组分通过顺序以及颗粒在通道上的存留分布对组分进行分析分离[50-51]。该技术具有在大尺寸范围内高分辨分离纳米颗粒的优势, 可用于分离不同的细胞外囊泡亚群[51]。

2.3　基于溶解性质的聚合物沉淀技术

通过改变外泌体的溶解性或者分散性, 可以将它们从体液或细胞培养液中沉淀出来。常见的方法是使用聚乙二醇或凝集素来沉淀样品中的外泌体。聚乙二醇是一种水溶性非离子化合物, 不含水的聚乙二醇可以通过“劫持”水分子增加疏水性蛋白和脂质分子的相互结合力, 从而迫使其脱离溶液, 进而在低速离心条件下发生沉降[52]。凝集素是一种具有高度特异性的碳水化合物结合蛋白, 可通过与外泌体质膜

糖蛋白上的糖链结合来改变外泌体的溶解性[53]。此外, 还可使用鱼精蛋白、醋酸钠、有机溶剂沉淀等方法进行沉淀分离[29]。目前, 几种商业化外泌体提取试剂盒如Exo-spin™、ExoQuick™、Invitrogen™等产品均应用聚合物沉淀法来分离外泌体。有研究显示, 蛋白酶K处理血浆可显著提高市售试剂盒分离获得的外泌体的纯度, 并且血浆样品酸化后再进行蛋白酶处理, 可增加外泌体标记物的水平[54]。

2.4 基于免疫亲和原理的分离技术

外泌体中存在着某些特定的蛋白质、脂质和多糖, 基于抗原–抗体特异性识别和结合作用原理, 可将外泌体从其他组分中分离出来。四次跨膜蛋白家族[55]、脂膜[56]、膜联蛋白[57]、上皮细胞黏附分子[58]或肝素[59]等都可以作为抗原, 而捕获外泌体的抗体可以附着在平板、磁珠、二氧化硅、树脂、膜亲和过滤器、纤维素滤膜、聚酰氨基胺树状聚合物表面和微流控器件上[29,60]。常用方法有酶联免疫吸附法和磁珠法等。

酶联免疫吸附法使用聚苯乙烯微孔板作为抗体附着介质, 其结果用吸光度值表示, 该方法可以快速分析已知表面生物标志物的表达, 也可以瞬时读出外泌体的产量和特异性。磁珠法多使用共价包覆链霉亲和素的磁珠[42], 与样品一起孵育后可通过磁泳将被结合的外泌体从样品组分中分离出来。鉴于微米级磁珠可赋予更大的接触面积, 该方法不仅具有高度特异性, 还具有比超速离心更高的外泌体产率[61]。

2.5 基于流体性质的微流控技术

微流控芯片是一种可兼容多种外泌体分离方法的新兴检测平台, 这些方法包括免疫亲和分离、膜过滤、纳米线捕获、声纳米过滤和确定性侧向位移分选等[62-63]。微流控装置是由几十到几百微米的不同直径微通道网络组成的紧凑单元, 能够处理皮升到微升范围内的黏性介质样品; 且根据特定的功能, 微通道可以相互连接, 使用额外的特定装置来微调流体运动。微流控技术能够以极高的准确性和特异性在微尺度上重现众多实验室过程, 取代昂贵的设备[64], 基于微流控技术的电化学外泌体检测芯片已经受到广泛关注。

2.6 外泌体分离技术特点的比较

国际细胞外囊泡协会(International Society for Extracellular Vesicles, ISEV)在2018年发布的“细胞外囊泡研究共识”中指出[65], 外泌体没有绝对的最佳分离方法, 其分离效果与下游应用和科学问题密切相关, 但高回收率和高特异度是两个公认的基本要求。此外, 尚需考虑操作程序的复杂程度、提取成本、生物活性以及通量等问题。由于分离原理的局限性, 目前外泌体分离技术存在其他成分污染和外泌体聚集等瓶颈问题, 如超速离心引起的蛋白质污染和外泌体聚集, 沉淀法引起的外泌体与其他杂质共沉淀及化学制剂和蛋白污染, 可选用其他方法如密度梯度离心法和尺寸排阻法避免污染和外泌体聚集[29,66]。不同分离技术具有其自身的优势和局限, 现总结如表1。

3 外泌体保存

外泌体是具有双层脂膜的囊泡结构, 其稳定性较好, 可保护内部生物分子免受体液中各种酶的影响, 从而保持其完整性和生物活性。但提取后的外泌体的完整性和生物活性也可能受到保存介质、保存温度和时间等因素的影响。

外泌体被提取后一般悬浮于磷酸盐缓冲液[67]。目前, 最常用的储存方法为冷冻保存, 但是冷冻保存可能会导致外泌体形状与物理性质的改变, 也可能导致多层囊泡的形成和聚集, 反复冻融会导致外泌体表面分子的生物特性、含量和标志物组成发生变化[68]。血清中包含外泌体在内的细胞外囊泡DNA在不同储存环境可保持稳定[69], 血浆存放于4 ℃时其RNA会显著降解, 在–20 ℃下长期保存也会导致血浆中外泌体总RNA降解, 但miRNA却十分稳定[70], 这也提示了外泌体miRNA作为生物标志物的潜力。不过也有研究表明, 血清中的miR-122和miR-145非常不稳定, 将血清在4 °C短期保存期间即发生降解[71], 这可能是由外泌体的异质性所导致的[72]。–80 °C保存被公认是保存各种生物标本如精液、尿、牛奶、血液和支气管肺泡灌洗液最适宜的保存环境[73], 虽然这样保存血浆可能产生含有大量“污染物”的外泌体[74]。4 °C保存虽然容易导致外泌体中蛋白和核酸的损失, 但却能够避免冻融过程造成的囊泡破坏[68]。

外泌体虽然建议保存于−80 °C环境下, 但在处理或运输过程中有时很难维持这种低温条件。CHAROENVIRIYAKUL等[75]提出一种冻干法以保存外泌体, 在冷冻过程中使用海藻糖作为保护剂, 海藻糖可以提供生物保护作用, 如稳定蛋白质、细胞

表1 外泌体分离技术的特点

Table 1 Characteristics of isolation techniques of exosomes

分离技术 Isolation technique	回收率 Recovery rate	特异性 Specificity	优势 Advantages	局限 Limitations
Mass density-based ultracentrifugation				
Differential ultracentrifugation	High	Low	Simplified operation, simplified sample pretreatment, high throughput, low protein fouling, no chemical reagent pollution	Requires costly instruments, time-consuming, labor-intensive, low portability, isolated exosomes tend to aggregate into clumps, low RNA yield and mix with other kind of EVs like impurity proteins, apoptotic bodies and exfoliated microvesicles[29,78-79], high centrifugal velocity may reduce exosomes yield and quality, low efficiency under highly viscous media[80-81], interference follow-up analysis
Density gradient centrifugation	Low	High	Higher purity, better biological structure and properties compared with differential ultracentrifugation[72]	Cumbersome preliminary work, complicated operation, time-consuming, treatment capacity is limited by the load zone, hard to remove high-density chemicals, subcellular water loss caused by hypertonic reagents[82-83]
Size-based isolation methods				
Ultrafiltration	Middle	Middle	Time efficient, high-portability, requires no complicated instruments, no chemical reagent pollution, the isolated exosomes RNA can be extracted directly	Hard to remove soluble proteins, the purity, the shape and charge of samples affect isolation, the filter membrane is prone to clogging, poor sustainability, the external force may damage biological activity of exosome[84]
Size-exclusion chromatography	Middle	Middle	High purity, high sensitivity, integrity and biological activity, not affected by the high viscosity of the sample, no chemical reagent pollution, prevents exosomes aggregation, scalability, easy to distinguish from high-density lipoprotein[29,66]	Time-consuming, low throughput[85], special instruments are required, low yield, costly, the purification column has strict limitations on the sample volume, subsequent concentration may be required[86]
Asymmetric flow field-flow fractionation	Low	High	Time efficient, mild conditions, full-automatic detection, reproducibility, compatible with many buffers that mimic physiological conditions	Small sample capacity, isolation according to particle size only, low throughput[50,87]
Hydrostatic filtration dialysis	Middle	Middle	Mild conditions, high integrity and biological activity, low cost, no chemical reagent pollution, exosomes can be isolated from highly diluted solutions, high throughput	Decreased efficiency for large volume of samples[43], need additional bacteria removal[29]
Solubility-based isolation methods				
Precipitation	High	Low	No special instruments or techniques are required, simplified operation, high throughput, high recovery, high integrity[88]	Tedious sample preparation, lack of proper selective isolation mechanism, specificity, RNA complexes and lipoproteins are difficult to remove, isolated exosomes particle size is not uniform, chemical reagent may damage exosomes, co-sedimentation of exosomes with contaminants[61]
Immunoaffinity-based isolation methods				
ELISA	Low	High	High specificity, suitable for separating specific exosomes, purity, intact isolated exosomes	Costly antibody and equipment, low throughput, low isolation efficiency, suitable for cell-free samples only, nonspecific adsorption of non-target vesicles on the solid phase hinders immune recognition[45]
Magneto-immunoprecipitation	Low	High	Simplified operation, good reproducibility, larger contact areas, higher capture efficiency compared with ELISA	Costly, low throughput, hard to detach and eluate exosomes from beans which may reduce the biological activity[89]
Microfluidic-based isolation technique				
Microfluidic	Low	High	Easy to automate and integrate, high portability and purity, low reagents and samples consumption, exosomes extraction and analysis can be combined[90]	Lack of standardization method, costly, complex equipment, middle-low sample capacity, low yield, the channel is prone to clogging by samples[91]

膜和脂质体; 减少冷冻过程中冰的形成; 防止蛋白质以及外泌体的聚集; 减少分离和保存过程中细胞外囊泡的损失等[76]。然而冻干法保存外泌体的期限以及于不同类型的外泌体保存的适用性尚不清楚。

值得注意的是, 不同来源的外泌体因其异质性在相同环境下保存, 其稳定性可能会大有不同[72]。WELCH等[77]研究发现, 精液经过长达30年的–80 °C冷冻保存后, 其外泌体的形状、物理性质、核酸蛋白含量和种类都没有显著变化。相反, MAROTO等[68]发现, 支气管肺泡灌洗液中外泌体冷冻保存样品与新鲜样本相比, 体积变大且有一半以上种类蛋白质的丰度发生变化甚至消失。因此, 不同种类来源的外泌体最佳的保存条件仍需探讨。

4 结论与展望

外泌体作为一种新发现的细胞间通讯载体, 与疾病发生的关系和机制正逐渐被揭示, 同时其应用研发的进程也正被快速推进。外泌体分离和保存技术虽取得一定程度的快速发展, 如商用提取试剂盒的出现, 但其应用仍存在很多挑战。目前尚缺乏能够同时实现高通量、高纯度、高回收率和高性价比的外泌体分离方法, 且现有的技术也需要在大规模研究中进行全面评估, 以确定其检测稳定性和可重复性; 如何完善外泌体保存过程中的条件参数, 确保外泌体的高生物稳定性、高生物活性、低损伤和低干扰, 不影响到后期分析也是一个难点; 此外不同来源外泌体存在异质性, 如何标准化外泌体保存方法也是目前外泌体基础研究和应用的瓶颈问题。但标准统一的保存技术是该领域持续发展的必然趋势, 仍需要更多随机分组平行试验数据, 尤其是长时间观察实验的证据予以探讨和验证。

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