JOURNAL OF CLINICAL MEDICINE OF KAZAKHSTAN (E-ISSN 2313-1519)
Review Article DOI: https://doi.org/10.23950/jcmk/17533

Extracellular Vesicles and Lyophilization: Getting Functionally Stable Cell-free Biomedical Products

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Received: 2025-11-27.
Accepted: 2025-12-11.

J Clin Med Kaz 2025; 22(6): 117–125

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Abstract

During the last decade, clinical application of extracellular vesicles (EVs) is of growing interest. Despite the progress in exploring the therapeutic potential of EVs, e.g. as disease markers or the carriers for therapeutic substances, it is important to identify proper storage conditions – this issue is indeed challenging. A subtype of EVs known as exosomes is of great importance in the therapeutic applications because they participate in the regulation of intercellular communication. Currently, exosomes are considered as a promising tool in the regenerative medicine. The therapeutic potential of exosomes and other subtypes of EVs, especially for their using in immunomodulation and drug delivery, dictates great attention to the methods for their storage, in particular for a long periods of time. Lyophilization is one of the best such methods designed to preserve cell-free EV-based products. In our mini-review, we discuss the main methods developed for stabilizing cell-free products and getting stable solid forms of EVs that are capable for long-term storage. We also point out that the methods need to be following both the ease of transportation and the retaining the functionally important properties for in vivo applications. The development of optimal protocols for storing the EVs are therefore crucially important for warranting that structural and functional integrity of EVs, exosomes in particular, are maintained intact or at least modified as less as possible. For comprehensiveness of our review, we refer to original studies which investigated how the storage temperature and freezing methods may affect stability of the final EV product. We summarize advances in the area of freeze-drying EVs (exosomes), the selection of optimal process parameters and lyoprotectants, the interplay between lyophilization parameters and specific functional properties of exosomes, and the preservation of their biological activity after reconstitution before application in vivo.

Keywords: extracellular vesicles, exosomes, lyophilization, cryopreservation, biomedical cell-free products

1. Introduction

Cells are constantly engaged in the exchange of molecular signals, and one of the most efficient ways they communicate is through the release of extracellular vesicles (EVs). This form of intercellular interaction relies on a multistep intracellular machinery that governs the formation, packaging, and secretion of vesicular structures [1]. At the final stages of their maturation, EVs appear as lipid-bounded particles that transport a broad spectrum of biologically active components. It includes proteins, diverse RNA species and their fragments, nucleic acids, metabolites, and lipids synthesized inside the parent cell [2]. The complexity and heterogeneity of the cargo is considered

a key feature of EVs because it extends their functional versatility and allows them to regulate a wide array of biological processes. Indeed, they are important mediators in the cell-to-cell inter-talk because they transport various ready-to-use proteins (including receptors and ion channels). In turn, it makes possible to perform different functions by the same carriage. For example, EVs contain certain types of RNA (mRNA, miRNA, siRNA, etc.) and can also contain DNA fragments that is critical in inter-cellular signaling (paracrine regulation). In addition, they can affect function of distant cells at the systemic level via circulatory system. Under physiological conditions, EVs contribute to tissue homeostasis and support normal regulatory pathways. However, their involvement becomes even more pronounced during pathological events, when cells release vesicles enriched with stress-associated or damage-related molecules. In this scenario, the function of recipient cells can be regulated both directly (via fusion to the cell membrane and releasing the content inside the cell) and indirectly (via activation of extracellular signaling molecules).

Virtually all cell types are capable of releasing EVs, making this mechanism one of the most energy-efficient routes of cell-to-cell signaling aside from direct electrical communication. Nevertheless, EVs generated by terminally differentiated cells tend to carry limited regenerative potential. In contrast, vesicles secreted by stem cells have been shown to reproduce many of the therapeutic functions attributed to mesenchymal stem cells (MSCs) themselves [2, 3]. Current classifications describe three main categories of EVs – microvesicles, exosomes, and apoptotic bodies – which differ in their origin, release pathways, morphology, cargo composition, and functional features. The main characteristics and differences between the different types of EVs, including their functional role in intercellular signaling, are summarized in Table 1.

Among different EV types, exosomes represent the most mobile and biologically active population. They are widely detectable in blood and can also be isolated from various body fluids such as urine, breast milk, saliva, bile, lymph, and cerebrospinal fluid. The molecular profile of exosomes includes numerous signaling factors that demonstrate selective tropism toward particular cell types, with their composition strongly influenced by the microenvironment of the secreting cells [1, 2, 3]. The simultaneous circulation of diverse exosome populations in extracellular fluid allows fine modulation of multiple recipient cells (and diverse types of cells) at once. When cells experience stress or damage, they release exosomes enriched with pathological molecular signatures, including proteins and miRNAs characteristic of disease states. This explains their pivotal contribution to tumor progression and metastasis but has also been proven as a key player in numerous non-cancerous disorders [4, 5]. Consequently, exosomes have emerged as promising diagnostic and prognostic biomarkers, with high relevance for personalized therapeutic decision-making.

Table 1 Main characteristics of intercellular vesicles of various types involved in intercellular signaling

Properties	Exosomes	Microvesicular bodies	Apoptotic cells
Size	30-150 nm	100-1000 nm	500-2000 nm
Origin	Endosomal pathway (exocytosis of microvesicular bodies)	Detachment from plasmatic membrane	Cell fragmentation in apoptosis
Composition	Proteins, lipids, RNA, microRNA	Membrane-bound proteins, lipids, RNA	Cytoplasmic fragments, organelles, DNA
Functions	Intercellular communication, immune regulation	Transfer cell signaling	Removal of cell debris
Protein markers	CD9, CD63, CD81, Alix, TSG101, Flotillin 1,2	Matrix metalloproteases, ARF6, CD40	Phosphati-dylserine, calreticulin, CD45, Caspase 3, C1q
The mechanism of release	Exocytosis	Budding from the cell	Cell damage and destruction
Nature	Endogenic	Endogenic	Endogenic

Exosomes also interact with immune cells by presenting antigen structures on their surface and can influence recipient cells directly after fusion with their plasma membranes. These properties open possibilities for engineering therapeutic exosomes loaded with tailored protein or nucleic-acid cargo. The exosomes preloaded by biologically active molecules can be targeted to specific tissues to support their protection and repair [1, 2]. Their involvement in neural regeneration – such as myelin formation, neuronal outgrowth, and the recovery of injured glial and neuronal cells – has been demonstrated in several studies with in vitro models [6, 7]. Recent studies suggest their active contribution to the regenerative capacity of mesenchymal stem cells. For example, the recovery of myocardial tissue through the reparation of damaged cardiomyocytes or triggering differentiation of immature cells to cardiomyocytes [8] or the recovery of retina in patients with diabetes mellitus [9] can be mediated by exosomal transport.

The high potential for using EVs for diagnostic and clinical purposes, along with their direct involvement into the intercellular communication, signaling, and molecular transfer, opens the possibility of using them in biomedicine [10]. EVs are currently gaining increasing attention as cell-free platforms capable of the delivery of drugs, cellular therapeutics, and clinical biomarkers [1, 2, 11]. Extracellular vesicles are very stable in vivo and they are typically not rejected when crossing biological barriers. The loading capacity of the exosomes allows for delivery of genetic information between cells, which enables sharing of epigenetic changes and affecting to recipient cells by genetic reprogramming [7, 11]. However, there are some limiting factors that should be considered in the context of therapeutic applications of EVs. To be used in clinical settings, EVs must be produced in sufficient quantities while maintaining their viability intact or modified in minor extent. Large-scale production of EVs while maintaining structural and functional integrity remains challenging. One of the important problems is that their long-term preservation requires specific storage techniques to prevent degradation of the carrier itself or cargo loss in time [4].

Published studies indicate that EVs behave similarly to living cells regarding sensitivity of their membrane structure (protein- and lipid-based content, morphology, etc.) to environmental conditions. Variations in storage temperatures have been shown to affect both vesicle integrity, functional recovery after thawing, fusion properties and other key features of the EVs [3, 10, 11]. Once the EVs are produced, the storage temperature significantly affects their recovery and integrity, showing that different temperature ranges promote different alterations in the EVs [5, 12]. Temperature-dependent structural changes highlight the need for proper preservation methods, among which two strategies dominate in current practice. First are deep-freezing

techniques such as cryopreservation, the second are dehydration techniques such as freeze-drying (lyophilization). In our mini-review, both approaches are examined in detail, with emphasis on their advantages, constraints, and practical implications for the long-term storage of extracellular vesicles.

2. Cryopreservation

Cryopreservation has become one of the most widely applied strategies for maintaining biological materials in a functional state over extended periods. Its routine use in laboratory and clinical practice allows long-term storage of diverse biospecimens while retaining their viability and physiological activity. The availability of cryopreserved samples on demand – including cells, tissues, and complex preparations accumulated in biobanks – significantly simplifies research workflow and ensures constant access to standardized material for analytical and transplantation purposes. This eliminates the necessity for frequent procurement of freshly isolated samples and enables thorough quality assessment prior to their clinical use. Stem cells, for instance, can be stored for prolonged periods without a measurable reduction in their regenerative or therapeutic properties, which is crucial for regenerative medicine and anticancer applications. Likewise, cryogenic storage of embryos and oocytes has long been an essential component of reproductive technologies [1, 4, 13, 14]. In addition, the proven feasibility of long-term storage of microbial cultures, established cell lines, and tissue samples provides a stable resource for research activity and supports continuous advancement in biomedicine. Preservation of genetic material of rare microbial strains plays an equally important role, as it ensures lineage continuity and creates opportunities for further genetic engineering applicable in corresponding research fields [2, 11, 15].

Cryopreservation can theoretically be applied to nearly any biological substrate — from cell-free and single-cell preparations to tissues, organs, and bacterial cultures. However, because biological material consists predominantly of water, exposure to subzero temperatures inevitably triggers ice crystallization. The formation of intracellular and extracellular crystals is one of the major factors contributing to irreversible damage, compromising membrane integrity and internal organization of cells and vesicles. Repeated freeze-thaw cycles further exacerbate this effect by reducing the concentration of bioactive molecules. In addition, these cycles induce degradation of RNA and proteins, and promote aggregation of the cellular content hindering its functional capacity. These forms of cryo-injury highlight the necessity of using cryoprotective compounds capable of mitigating structural and biochemical damage during freezing [6, 11, 16].

Cryoprotectants allow for avoiding physical effects (crystallization), osmotic damage, chemical changes like variation in pH and cytoplasmic ion content. They also maintain protein molecular stability and the spatial organization of intracellular membranes (if any cell organelles are present in a product) and outer membrane [1, 3, 17]. Two types of cryoprotectants are used now: membrane-permeable and membrane-impermeable. Membrane-permeable cryoprotectants, such as dimethyl sulfoxide (DMSO), are substances with relatively low molecular weight. Accordingly, this property facilitates their permeation through double-layer membranes without involvement of passive or active transmembrane transport. The cryoprotectants of this type stabilize the vesicular membrane from the inside. In contrast, non-permeant cryoprotectants, such as sucrose and trehalose, can not cross the membrane due to their high molecular weight. They therefore reside outside the EV membranes, mostly being incorporated into the glycocalix and affecting the structural properties of the cell membrane [4, 5, 18].

Despite the advantages of cryogenic preservation, several limitations persist. Reports repeatedly describe cytotoxicity and impairment of signaling pathways associated with the use of permeable cryoprotectants, particularly at higher concentrations or prolonged exposure [13, 14, 19]. Technical and logistical issues may also compromise sample integrity and stability. Interruptions in power supply, failure of refrigeration systems, or delays in refilling liquid nitrogen tanks may lead to unexpected sample loss, operational delays, and substantial financial burden [7, 20]. Furthermore, the requirement for uninterrupted storage at ultralow temperatures (for example, at -80 degrees Celsius or below) makes cryopreservation an inherently resource-dependent and costly approach.

3. Lyophilization (Freeze-drying)

Compared with cryopreservation, which is better suited for storing bulk cell preparations and tissues, lyophilization has emerged as one of the most suitable approaches for stabilizing cell-derived bioproducts such as peptides, proteins, extracellular vesicles, and vaccines [3, 21]. A major advantage of this method is that the biomaterial can be converted into a dry, solid form that remains its functional features for extended periods. Numerous studies show that when freeze-drying is performed

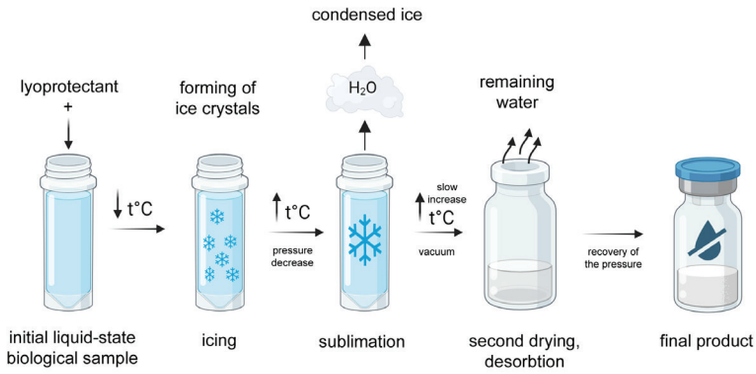

Figure 1 – The main stages of the cell lyophilization process

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in the presence of suitable cryoprotective agents, EVs preserve their morphology, size distribution, protein cargo, and overall biological activity. Importantly, lyophilized EVs maintain comparable rates of uptake and exhibit functional properties similar to freshly isolated vesicles [1, 11, 22, 23]. The principal stages of the process are illustrated in Figure 1.

First, freeze-drying is based on freezing water and then removing it away from the sample. Sublimation is the key step in the whole freeze-and-dry process, whereby water almost instantly transforms from solid state to the vapor, bypassing the liquid phase. This occurs under high pressure and at temperatures below 0°C. The process consists of three stages, which are schematically represented in Figure 1, each with different effect to the final quality of the product (see below) and different duration. During the first stage, which is the freezing, the sample solidifies with formation of ice crystals. Next, the second stage starts by the placement of the frozen sample to the vacuum allowing the ice to transform into a vapor (gas) without entering the liquid phase. The sublimation is accelerated in a heated condition. A low temperature condenser converts the evaporated water released from the vacuum chamber into a solid state [11, 21, 24, 25].

It should be noted that before freezing the biosamples must undergo specific pre-treatment. This includes adjusting the product concentration, introducing of components to enhance product stability, reducing the amount of solvent with high vapor pressure, and other technological measures. During the freezing stage, pure water ice crystals start to form and concentrate in the sample by solidification – they may present in amorphous state or in crystals or in combination of the two states [5, 10, 13, 23]. The sample temperature is lowered below freezing to achieve solidification. When temperature goes down the freeze-point of the sample's medium, the thermodynamically favorable ice clusters have higher probability to form. These clusters both act as the nuclei for initiation of medium crystallization and stimulate the crystallization process [1, 4, 5, 26]. After nucleation, ice crystals grow by increasing their volume; in a sample there are many nucleation sites appearing simultaneously, which provides nearly homogeneous freezing of the sample.

The freezing stage has direct influence on subsequent stages of the whole cycle of lyophilization. For example, higher super-cooling (lower crystal-nucleation temperature) promotes faster nucleation, resulting in appearance of larger amount of small nuclei, comprising in total much larger surface area. This prolongs primary drying time but increases the rate of desorption of unfrozen water during the next stage of lyophilization (second drying) [12, 19, 27-30]. In contrast, lower super-cooling with high crystal-nucleation temperature facilitates occurrence of much smaller amount of crystals but each crystal is larger. Altogether, it reduces the time needed for primary drying and prolongs the duration of secondary drying. However, depending on the design and capacity of the freezing apparatus, different vials of biosamples may cool under non-equal conditions. Subsequently, it may lead to variations in the rate of freezing or quality of final product between vials and batches [5, 13, 27, 31].

Of note, the cell debris, remnants of disrupted cells, and organelles should be discarded prior to the process because they do not bear functional load, need longer recovery time, and have reduced stability [14, 32]. The progression of ice formation leads to decrease in free water content. Because most of chemicals are not soluble in solid phase of the water, this in turn makes all the solutes dissolved in the liquid phase of sample's medium to be increasingly concentrated. The elevated concentrations of dissolved solutes in freezing sample contribute to the increasing in viscosity of the liquid medium until solid ice can no longer form. Because freezing conditions strongly contribute to the structure of the frozen matrix and the quality of the final product, the entire process requires permanent control, especially its first stage as most critical one [11, 21, 28, 33].

The primary drying is the main step of the whole drying process. It typically takes a longer time compared to other phases of the freeze-drying process, making it another critical step in the developing and optimizing the lyophilization process [4, 5, 22, 29]. Since primary drying step is in progress, it is needed to reduce pressure and provide sufficient heat to the sample to sublimate the ice [1, 24, 25]. During the initial drying stage, approximately 95% of the water in the material is subliming. This process can take a significant amount of time, as too much heat can alter the structure of the material.

Subsequently, while the primary drying step is occurring, the ice is continuously subliming into the vacuum. Vacuum accelerates sublimation, making it useful for targeted drying. The driving force for sublimation is the actual difference between the temperatures of ice and the surface of condenser [11, 35-38]. The heating is required for effective sublimation while the physical way it is transferred into the sample (e.g. radiation or convection) does not play a role. The heat supplied should be high enough to promote ice sublimation, but the sample temperature should remain lower enough to prevent the precipitate from collapsing after freeze-drying [3, 15, 16, 20]. The cold chamber of the condenser and its plates all represent a surface to re-solidification of water vapor. The primary role of condenser is to prevent entering the vapor to the vacuum pump, otherwise the performance of the system may be affected [18, 24, 25, 34, 39]. The chamber must be pressurized fairly below the level of saturated vapors throughout the process to promote ice sublimation. The pressure is controlled using a partial vacuum. If the pressure is lowered, it elevates the actual difference in vapor pressure between ice and condenser, but it also significantly reduces the convective heat flow, which slows down sublimation [6, 11, 19, 40-42].

The next step is secondary drying. During this stage, water is gradually evaporated by heating and vacuum [15]. However, the amount of unfrozen water may still be significant (20-50%) during this process [5, 11]. The final moisture content of the lyophilisate is a critical parameter, as it determines the product's on-shelf stability. During this stage, the temperature must be set at much higher levels compared to the settings during primary drying. This is needed for disrupting the physicochemical interactions between water molecules still adhered to the frozen sample. Because the ice is removed in primary drying, this reduces both risks of melting and sample decomposing to minimal levels [3, 24, 43, 44]. During this stage, the pressure is also typically reduced to stimulate desorption. However, the temperature increase is preferably carried out slowly (≤1 °C/min) to avoid decomposition.

One or more drying phases may be carried out at any suitable temperature. The pressure at which the drying phase is carried out may also vary because it depends on the design of the chamber, vacuumizing rate and intensity, etc. As a result, the final product is completely dry, ensuring greater stability and a longer shelf life.

The parameters of secondary drying determine the long-term stability of the final lyophilized product. The optimal residual moisture content is commonly expected to be between 0.5% and 3% [12], although certain biological materials demonstrate

better functional recovery when slightly higher moisture levels are retained [16, 22, 38]. Thus, proper optimization of secondary drying is essential for preserving structural integrity, ensuring biological activity, and achieving efficient rehydration upon use. As well, optimal residual water content is crucial for the successful recovery of lyophilized biomaterials and cells.

4. Lyoprotectants

Lyoprotectants represent a broad group of chemical compounds that safeguard biological preparations during freezing and drying by altering the physical properties of the surrounding medium. A key mechanism of their action is the ability to markedly increase viscosity, which slows or even suppresses the formation of crystalline ice. Under these conditions, water solidifies predominantly in an amorphous, non-crystalline state, forming a glass-like matrix (in contrast to typical lattice-structured ice under normal conditions). This is essential because crystal growth can deform and rupture lipid membranes, and therefore damage vesicle structure at the very initial stage of production. During sublimation, when bulk water is removed, the phospholipid bilayer tends to lose structural cohesion. Sugar-based lyoprotectants compensate for this by replacing water molecules and stabilizing the interface between the inner and outer leaflets of the membrane [4, 16, 25]. In this way, lyoprotectants prevent collapse or shrinkage of vesicles and sustain their native architecture.

A large variety of lyoprotectant formulations exists, and their composition is selected depending on the biological material, its molecular cargo, and the anticipated route of application. For some EV-based preparations, a single protective compound may suffice, whereas other formulations require mixtures that perform several roles simultaneously. These mixtures may include buffering agents, osmotic regulators, fillers, or additional stabilizers [5, 18, 28]. Proper buffering is especially important: the pH of the medium must remain stable during freezing and drying. Certain common buffers, such as phosphate-based ones, experience significant and/or non-linear pH shifts in response to temperature changes and therefore can compromise vesicle integrity. Replacing them with temperature-stable buffers helps avoid undesirable osmotic shrinkage or swelling during the critical phases of freezing [17, 19-21]. Besides sugars, biopolymers such as alginate can serve as auxiliary stabilizers. For example, alginate enhances structural durability by interacting with protein surfaces and lipid membranes. It also reduces formation of nucleation sites and supports the stability of exosomes during the freeze-drying cycle.

Among all lyoprotective agents, disaccharides are considered the most versatile. They substitute the hydration shell around vesicles by forming extensive hydrogen-bond networks with phospholipid headgroups. As a result, amorphous sugar matrix is produced which prevents aggregation, fusion, or denaturation of proteins within EVs [8, 11, 22, 45]. Trehalose is widely recognized as the most effective of these disaccharides for preserving extracellular vesicles and other delicate biological systems during lyophilization [6, 13, 17, 21]. Found naturally in yeast, fungi, bacteria, and many plants, trehalose enables these organisms to withstand severe dehydration or freezing. During lyophilization of EVs, trehalose acts as a molecular scaffold that maintains vesicle conformation as the water content decreases. Its efficacy has been demonstrated across many biological preparations, including cell-free products and multiple cell types [22, 24, 40].

Trehalose is frequently combined with membrane-permeant cryoprotectants such as 10% DMSO. This dual approach often improves post-thaw recovery, enhances proliferative capacity of thawed cells, and preserves membrane integrity and intercellular contacts when multicellular constructs are being cryopreserved. Experimental findings point to an optimal working range of 100-400 mM trehalose. Higher concentrations of trehalose can compromise viability through osmotic stress. Interestingly, introducing trehalose enables the use of lower DMSO concentrations, which is particularly beneficial considering the cytotoxic potential of high DMSO levels. The natural origin, affordability, and broad compatibility of trehalose make it a preferred choice for both cellular and acellular products.

Evidence accumulated over recent years clearly shows that trehalose-based formulations maintain the structure and biological activity of EVs over long periods of storage [18, 24, 25, 34]. Because lyophilization inevitably exposes samples to physical, mechanical, and chemical stresses, the strategic use of lyoprotectants – whether individually or in combination – is essential for minimizing molecular degradation, preserving vesicle morphology, and maintaining functional characteristics during all stages of the freeze-dry process [11, 13, 46, 47].

5. Discussion

Contemporary experimental evidence highlights the broad potential of exosomes and EVs in regenerative medicine, dermatological applications, oncological therapy, and other biomedical areas. It becomes a valuable tool for anti-tumor and regenerative therapy among other well-documented approaches including application of engineered tissues and genetic modification of target cells [49]. Their functional capacities and therapeutic roles are summarized in Figure 2 (see the next page).

In the context of EVs production and preservation, the advantages of lyophilization include minimal chemical deterioration during storage, the formation of a dry, stable formulation, improved sterility management, and the option for transport and storage under non-refrigerated conditions. On the other hand, successful freeze-drying requires continuous monitoring of several critical factors. Foremost among them the shelf temperature during processing, the pressure profile within the sublimation chamber, and the duration and sequence of each phase of the freeze-dry cycle. Additional factors and actions, such as maintaining an optimal pH range, incorporating thermal stabilizers, and selecting appropriate formulation additives, are also necessary to maintain the functional integrity of sensitive EV components. It is particularly important to ensure that the final lyophilized product contains a controlled level of residual moisture, as this parameter directly affects long-term stability, physical robustness, and resistance to degradation, which is crucial for membrane-bound vesicles. A properly designed freeze-drying procedure allows EVs to remain in a dry, solid state without losing their biological properties, even when kept at ambient temperature. Studies demonstrate that exosomes preserved with trehalose maintain key activities, including anti-inflammatory and anti-fibrotic effects, at levels comparable to freshly isolated preparations [48]. Nonetheless, several challenges remain unresolved. For example, there is still no universally accepted standard for evaluating the quality of lyophilized EVs, nor is there consensus on how freeze-drying

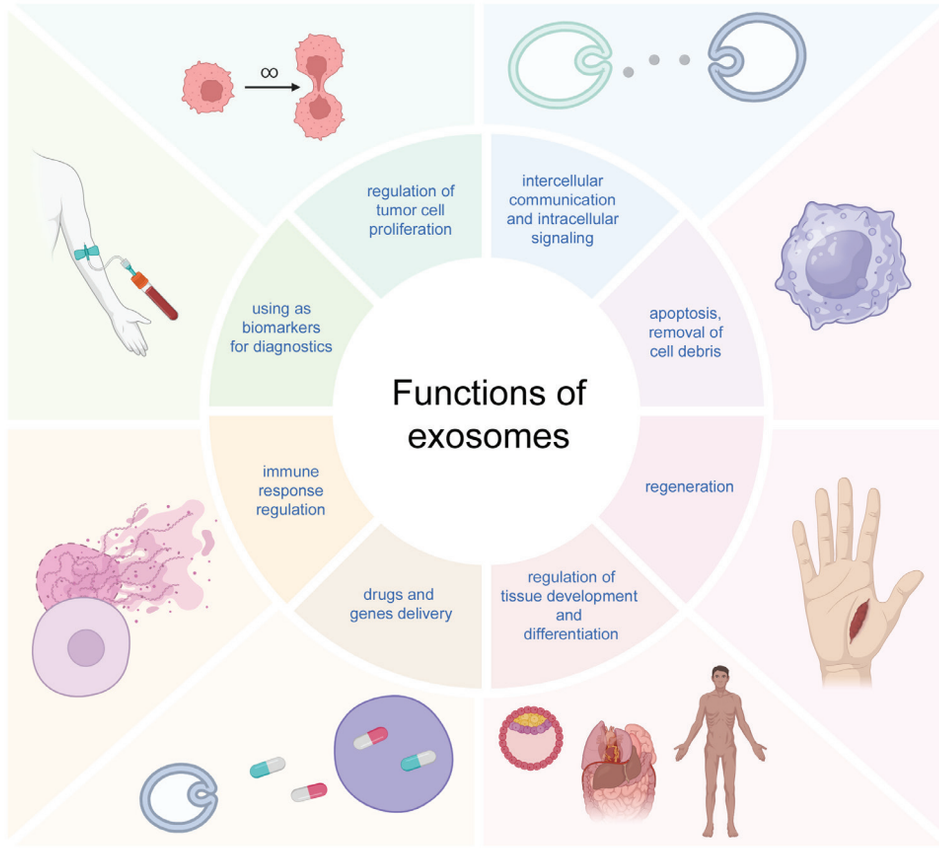

Figure 2 – Key functional features of exosomes in cell therapy. Image was created in Biorender.com

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may influence the specificity of their biological action or alter mechanisms underlying their therapeutic effects.

Current research aims to address these issues and improve the suitability of freeze-dried products for pharmaceutical and clinical applications, an effort that remains crucial for the advancement of EV-based therapies [50]. In this context, one of the most promising approaches is the implementation of AI-based algorithms and tools for various aspects related to the production, storage, and infusion of therapeutic EVs, similar to the AI-based approaches that are already in action in other fields of medicine [51]. Currently, AI is considered a powerful tool to design EVs with enhanced specificity to the target cells, e.g. due to the attaining highly precise delivery to the tissues [52]. Specifically, AI-based tools are used to characterize exosomes for their utilization in anti-cancer treatments as well as in other diseases [53]. It may also include the development of AI-assisted models for simulation of thermal changes in structural properties of the EVs during cryopreservation and dry-freeze, physicochemical stability of their cargo composition, and how the membrane-fusion ability of exosomes can be affected by the environmental changes like pH, temperature, or concentration of medium components. These AI-assisted approaches may facilitate designing and optimizing robust protocols of production and storage of EVs taking into account the type of original cells, culture medium composition, and the needed quantity of final cell-free product.

Conclusion

Lyophilization is increasingly recognized as a promising and technically mature approach for stabilizing cell-free biological therapeutics, particularly exosomes and other extracellular vesicles. By this approach, it is possible to create product that can be stored and transported for extended periods without reliance on deep-freeze conditions and, importantly, without losing its functional quality. When the process is supplemented with carefully selected cryo- and lyophiloprotectants and finely tuned operational parameters, the essential structural attributes and biological functions of these nanoscale vesicles – including membrane architecture, molecular cargo, and bioactivity – can be reliably preserved. At the same time, specific limitations persist, such as the length of the lyophilization cycle, the necessity of sterile reconstitution before use, and the dependence on lyoprotectants to maintain EVs activity at levels comparable to native vesicles. Furthermore, the regulatory classification and approval of lyophilized EV-based products as medicinal agents remain in early stages and require clearer legislative frameworks. For these reasons, progress in this field depends on coordinated interdisciplinary efforts. Unified actions by basic and applied researchers, engineering solutions for reproducible manufacturing, and implementation of validated quality-assurance systems constitute the potential for advancements. Such developments are essential for establishing a new class of stable, clinically applicable cell-free biopharmaceuticals.

Author Contributions: A.B., E.C., A.Zh., A.A., and A.G. – conception of the work; A.Zh., A.A. – reviewing literature, writing the draft; A.Zh. – visualization; A.B., E.C., A.A., A.Zh., and A.G. – revision of final version. All authors reviewed and approved the final manuscript for submission.

Disclosures: There is no conflict of interest for all authors.

Acknowledgements: None.

Funding: This work was supported by the Ministry of Education and Science of the Republic of Kazakhstan (Grant No. BR28713159).

Data availability statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

AI Usage Statement: Not applicable.

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