DNA Loading Using Extracellular Vesicles

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Gene therapy is a therapeutic strategy of delivering foreign genetic material (encoding for an important protein) into a patient's target cell to replace a defective gene. Nucleic acids are embedded within the adeno-associated virus (AAVs) vectors; however, preexisting *immunity* to *AAVs remains a significant* concern that impairs *their clinical application*. Extracellular vesicles (EVs) hold great *potential* for therapeutic applications as vectors of nucleic acids due to their endogenous intercellular communication functions through their cargo delivery, including lipids and proteins. So far, small RNAs (siRNA and micro (mi)RNA) have been mainly loaded into EVs to treat several diseases, but the potential use of EVs to load and deliver exogenous plasmid DNA has not been thoroughly described.

Keywords: exosomes, extracellular vesicles, gene therapy, × ; DNA loading, plasmid DNA ×

1. Introduction

In recent years, the functions of extracellular vesicles (EVs) have sparked interest in a new model of introducing foreign *genetic materials* [1]. EVs are endogenous delivery systems with a diameter range of ~50 nm to 1µm [2]. EVs are formed in endosomal compartments and secreted after fusion with the plasma membrane by most cell types [3][4]. They can mediate and transmit a variety of intercellular signaling molecules packaging biological cargo, including nucleic acids, *small RNAs* (sRNAs), proteins, and lipids altering the gene expression, proliferation, and differentiation of recipient cells during physiological and pathological conditions [5]. Although the classification of EVs is continuously evolving, they generally are classified on their biogenesis and release pathways, such as exosomes (Exo) (~40 to 160-nm in diameter) [6][7]; ectosomes [8]; or shedding microvesicles (SMVs), apoptotic blebs (ABs) (1 to 5-mm in diameter) [9], and other EVs subsets [8], generating a heterogeneous group of components able to redistribute their biological cargo into the entire organism.

2. Nucleic Acid-Loaded Extracellular Vesicles: Current Methods

2.1. Transfection-Mediated DNA Loading

Transfection is the procedure of a non-viral-mediated delivery of foreign genetic material into host cells [10]. Depending on the aim of the experimental research, it is essential to distinguish between transient and stable transfections. Through transient transfections, the transfected cells express the foreign gene, not integrating it into their genome. Therefore, the new gene will not perform DNA replication. These cells express the transiently transfected gene for a limited period—usually several days—after which, the foreign gene is lost through cell division or other factors [10]. Stably transfected cells begin with transient transfection, followed by an infrequent but essential process of serendipity. In a small proportion of transfected cells, the foreign gene has integrated into the genome to become part and, afterward, to be duplicated. The transfection-based technique has been conducted with exosomes derived from different cellular sources. The successful loading of exogenous genetic material in exosomes derived from murine dendritic cells has been performed by Seow et al. [11], while exosomes derived from HEK-293 cells were loaded with a plasmid containing GE11 peptide DNA [12]. As mentioned above, DNA loading through transfection strategies results in it being more efficient with plasmid DNA compared to linear DNA. However, some drawbacks have been raised in this technique; in particular, the issue has shifted to the remainders of the transfection reagents (like calcium phosphate and diethyl aminoethyl (DEAE)-dextran) that might cause incorrect or inefficient incorporations, leading to an undesirable *downregulation of gene expressions* in recipient cells. Similar importance is the localization of the transfection reagents [13]. The transfection reagents might remain partly hooked to the transfected acid nuclei to be (co)secreted in the culture medium. This may significantly affect the uptake behavior of the released transfected acid nuclei and its localization in either the exosome or protein complexes. Thoughtfulness should also be given to the complexity that characterizes the transfection process, from the initial DNA attachment to the plasma membrane and internalization via endocytosis, its release from the endosome followed by the dissociation of the vector from the DNA until its transfer into the nucleus, suggesting that an interplay of several essential

parameters needs to be considered to achieve an efficient DNA delivery. Furthermore, due to their coprecipitation with exosomes at high centrifugal forces, complexes of transfection reagents and plasmid DNA might be mistakenly deducted as exosome-encapsulated acid nuclei. Following transfection, the risk of confounding the outcomes by analyzing the presence of foreign nucleic acid into exosomes is high; therefore, protocols that rigorously characterize the purification methods are still necessary.

2.2. Electroporation Procedure-Mediated DNA Loading into Extracellular Vesicles

The electroporation approach seems to guarantee a more reliable encapsulation of foreign genetic material, maintaining EV integrity and functionality, and seems to be a viable alternative for cell types that are not responsive to the transfection method. An electrical pulse at an optimized voltage (1° C/pulse) has been applied to not damage the EVs membrane and overcome the cell membrane capacitance, disturbing the phospholipid bilayer of the membrane and creating transient membrane pores through which charged molecules like DNA cross[14]. Lamichhane and colleagues [15] reported exogenous linear foreign genetic material loaded into EVs using this technique in quantities sufficient for plasmid DNA. Besides, the authors established that the loading efficiency and capacity of linear DNA in EVs are dependent on the DNA size. Indeed, an increase of linear dsDNA fragments from 250 to 4000 bp in lengths was packaged into EVs through electroporation. Plasmid DNA fell within the range 750–1000 bp exhibiting low incorporation into EVs. One of the main advantages of electroporation is that it has a minimal effect on exosomal components such as ligands and receptors present on its membrane surface. However, as has been reported by Hood et al. [16] and Johnsen et al., [17] electroporation may trigger the aggregation of EVs and change their morphological characteristics. Thus, careful *deductions need to be* given while interpreting the loading using the electroporation method. The electroporation procedure can also induce cell toxicity; this issue might be minimized through *experimental optimization methods* and may be counterbalanced by increased transfection efficiencies. Another limitation of the electroporation technique is linked to the types of equipment that can vary among laboratories influencing the electroporation outcome and the electrical proprieties of the plasma membrane. Finally, cells are well-known to have a plasma membrane that consists of a phospholipid bilayer, which forms a stable barrier between two aqueous compartments and is a good electrical insulator [18]. This electrical property characterization of cells raises a question: According to the principle of electroporation that applies to all cells, how can its efficiency depend on the electrical circuit of the plasma membrane? The difference of conductivity in a cell represents an important matter to reflect during an electroporation approach [19].

2.3. Additional Methods Used for Loading in EVs: The Sonication and Saponin Methods

There is another method for DNA loading in EVs, such as sonication [20][21]. Sonication is the “cleanest” method, with a high loading efficiency, because it does not use enzymes or chemicals that might be carried throughout the sequencing workflow, negatively affecting the read quality. However, this method is restricted to the loading of smaller DNA molecules. Besides, the loading of DNA in EVs induces a prolonged release of catalase, as measured by retained the catalase activity over time [22]. Noteworthy, EVs loaded using the sonication approach do not show sound therapeutic effects in vivo, likely due to disruption of the exosomes’ integrity, making them more vulnerable to degradation via the reticuloendothelial system. Besides, after the loading process, the EV integrity and the loss of intrinsic contents and biological properties deserve further attention. Although it has never been used to load plasmid DNA into EVs, another loading method consists of making permeable the EV membranes through saponin use. Saponin is a detergent-like molecule able to interact with and remove cholesterol from EV membranes, forming pores without leading to morphological and functional alterations of vesicles [23]. Indeed, even though the sonication and permeabilization method share similar loading efficiencies and sustained releases of EV-encapsulated cargos, as mentioned above, EVs loaded by sonication appear to have undergone size and morphological alterations. The saponin permeabilization was utilized in a study assessing the use of protein catalase-loaded exosomes derived from macrophages as a drug delivery system for PD treatment [24]. An important aspect that emerged from this study, EVs loaded using saponin showed a prolonged release of their encapsulated cargos. However, although saponin permeabilization represents a straightforward loading method, it has never been wholly deepened in other preclinical and clinical studies. Moreover, an important saponin permeabilization-associated drawback consists of removing the residual trace of saponin after use, because, like many detergents, it can remain hooked to EVs, affecting their morphology.

3. DNA Loading Varies Across Extracellular Vesicles Subsets

The discrepancies emerged concerning the content of different part-genomic DNA (gDNA) [25] and carrying and deliver nucleic acids to recipient cells depending on the EV subsets urge studies aimed at understanding whether the plasmid DNA loading could vary in different subsets of EVs. Kanada et al. [26] isolated exosomes and ectosomes from the same cell source (HEK293T), reporting a differential loading of nucleic acids between exosomes and MVs. Furthermore, they

observed that ectosomes loaded with DNA were more efficient than exosomes delivering functional nucleic acids to target cells. These results are consistent with those reported by Lamichhane et al. [15], where, by electroporation-mediated DNA loading, they observed MVs exhibit an expanded capacity for effective linear DNA sizes (4–6 kb) than exosomes. Whereas exosome-like EVs originate from MVBs inside cells [27], MVs are plasma membrane-derived vesicles; therefore, it might be possible that both EV subsets have a different lipid composition. Ectosomes and exosomes are endowed of the same membrane topology of donor cells [28], and consolidated evidence indicates that the composition of the lipid bilayer in exosomes differs from the lipid composition of MVs [29]. Besides, changes in the membrane lipid composition may alter the membrane fluidity and their curvature. This variability of the lipid compositions into exosomes and MVs might affect their permeability to electroporation-mediated DNA loading.

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