Study of the physicochemical properties of exosome dispersions obtained by ultrafiltration

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Abstract. The article presents the results of studying the physicochemical properties of exosome preparations obtained by ultrafiltration, which indicate a high degree of the composition and properties dependence of the obtained product on the material of the filters used. Quantitative determination of proteins and nucleic acids in exosome samples using UPN-50 filters allows us to conclude that the content of the main impurity compounds in the preparation is significantly reduced compared to dispersions obtained using filters with pore sizes of 220 and 450 nm. Analysis of flow cytometry data made it possible to demonstrate that when using the UPN-50 filter, an increase in the contribution to the dispersion of all types of fractions of non-exosomal size was observed, the appearance of which can result from fraction destruction associated with pore size or filter material properties. drying of the dispersion was observed in the studied exosome samples. Fraction sizes ranged from 40 to 450 nm (an average of about 200 nm). Exosomes from the entire variety of membrane vesicles are fractions that have the most suitable characteristics that allow them to be used as a nanoscale drug delivery vehicle while ensuring the necessary quality control of the drug at the sample preparation stage.

Introduction

From the moment of discovery until the beginning of the 21st century, biovesicles were not given much importance, but as soon as their direct participation in intercellular communication was proved, many scientists of the world showed a genuine interest in studying of exosomes.

Exosomes, as a rule, include a fraction of membrane vesicles with a diameter of 40-100 nm, which are secreted into the intercellular space by various types of tissues and organs cells. It is known that the structure of the exosomal capsule is a double lipid membrane with an integrated layer of external transmembrane proteins. Microvisicles are found in various biological fluids (bronchoalveolar lavage, serum, urine, breast milk, cerebrospinal fluid, saliva, malignant pleural effusions, etc.), both during normal functioning of the body and in the event of various diseases. It has been established that exosomes are released by B- and T-lymphocytes, platelets, dendritic cells, mast cells, as well as epithelial cells. They provide transportation of proteins, lipids and nucleic acids in organs and systems, bypassing the

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plasma membrane, which allows them to be used as containers for targeted delivery of drugs to target cells [1].

Literature data indicate that the bulk of studies of the therapeutic efficacy of exosomal drugs are directed to oncopathology, Alzheimer's and Parkinson's. Molecules of the substance (microRNA, mRNA, hormones, enzymes, antitumor compounds, antioxidants) integrated into exosomes have improved pharmacological characteristics: increased bioavailability, solubility and stability of the drug, reduced toxicity [2, 3, 4, 5, 6].

The heterogeneity of the membrane vesicle population in terms of physical, biochemical characteristics, as well as the presence of molecules or subcellular formations with similar physical or biochemical characteristics in any biological fluid makes it difficult to widely use exosomes in clinical practice due to the lack of a single standard method for isolating a "pure" product [7, 8].

One of the main promising directions in the development of pharmacology is the development of selective drugs and effective approaches to their production using nanoscale drug delivery vehicles. Providing the necessary therapeutic concentration of drugs in target cells without affecting healthy ones is not an easy task, which requires a specific drug carrier. In our opinion, the most promising is the use of an exosomal delivery system, since it does not require expensive equipment, it allows the production of nanopreparations in large volumes.

There are many ways to isolate microvesicles from blood, but all of them are based on the use of expensive equipment, reagents and commercial kits, and are also long and laborious, which makes it difficult to reproduce these techniques in wide laboratory diagnostic practice [9, 10, 11].

The aim of our study was to study the dependence of the composition and physicochemical properties of the obtained exosomal dispersions on the pore size and characteristics of the membrane materials used.

For the successful implementation of the task, the optimal conditions for the production and registration of exosomes were determined, as well as the strategy for their isolation, which allows to reduce the presence of impurities of other microvesicular structures.

Materials and methods

The source of exosomes was the blood serum of outbred males of the Therapy and Pharmacology Department barn of Stavropol State Agrarian University. The manipulations were carried out in accordance with the European Convention for the Protection of Vertebrate Animals Used for Scientific Purposes. Blood was taken from the saphenous vein of the forearm, after preliminary processing the place of sampling (cut hair along the veins, skin disinfection), into vacutainers with coagulation activator. For thorough mixing of blood with a coagulation activator, after which it was centrifuged at 3000 rpm for 10 min [11]. Aliquots of 1.8 ml of blood serum were taken into 2 ml tubes, then centrifuged using a MiniSpin microcentrifuge (Eppendorf, Germany) at 4000 x g rpm for 30 min. 1 ml of the obtained supernatant was taken with a medical syringe and filtered simultaneously through the following membranes: Millex-GV (Durapore) with a membrane diameter of 25 mm and a pore size of 0.22 μm ; LCR (hydrophilic PTFE, Millipore), with a membrane diameter of 25 mm and a pore diameter of 0.45 μm , and on a Millipore installation (USA) with a vacuum pump using UPN-50 filters (50 kDa, Vladipor, Russia).

In the course of further work, the properties of the obtained experimental series of exosomes were evaluated. Electron microscopy of exosome preparations was performed using Carl Zeiss EVO LS 10, in SE1 mode.

Determination of protein concentration was performed on a pre-calibrated Qubit 2.0 fluorimeter (Life Technologies, USA) using the recommended commercial reagent kits in

accordance with the manufacturer's instructions. A Qubit TM Protein RNA concentration solution was prepared by mixing Qubit TM Protein reagent and Qubit TM Protein buffer. Flow cytometry of dispersions was performed using an Attune cytometer (Applied Biosystems, USA),.

Statistical processing of the research results was carried out using a package of statistical programs: the Russified version of the program STATISTICA 6.0 (StatSoft - Russia, 1999), BIOSTAT (S.A. Glantz, McGrawHill, translation into Russian - "Practice, 1998). The main statistical characteristics were determined: mean, standard square deviation. The significance of differences was calculated using Student's T-test in the case of equality of variances, its modification (T-test with separate estimates of variances) in the case of variance inequality and Bonferroni correction for multiple comparisons. Differences were considered statistically significant with a probability of at least 95% (p <0.05).

Results and discussion

Exosome preparations obtained by ultrafiltration through UPN-50, 220 nm, and 450 nm membranes were studied by flow cytometry; the results are shown in Figures 1-3.

Fig. 1. Flow cytometry results of an exosome preparation obtained by ultrafiltration (UPN-50, 50 kDa)

Fig. 2. Flow cytometry results of an exosome preparation obtained by ultrafiltration (220 nm)

Fig. 3. Flow cytometry results of an exosome preparation obtained by ultrafiltration (450 nm)

Visualization and identification of the structural components of the obtained exosomal dispersions was carried out by electron microscopy (photo 1-3).

Fig. 4. Electron micrograph of an exosome sample obtained by ultrafiltration (x27420)

Fig. 5. Image from Carl Zeiss EVO LS 10

Fig. 6. Electron micrograph of exosomes sample obtained by ultrafiltration (x49000)

The measurement of the fluorescence level and recalculation of the protein concentration in the sample was carried out in accordance with the calibration line and the value of the dilution factor, the results are presented in table 1.

Table 1. The results of determining the total concentration of proteins in samples using specific fluorescence.

Sample No.	Ultrafiltration	Protein (µg / µl)	DNA (ng / µl)	RNA (ng / µl)
1.	220 nm	3,9± 0,13	3,52± 0,18	3,14±0,21
2.	450 nm	3,96± 0,20	2,84± 0,14	2,62± 0,11
3.	UPN-50	1,1± 0,08	0,324± 0,09	0,460±0,11

We previously suggested that when exosomes are obtained, the use of filters with a smaller pore diameter at the ultrafiltration stage can lead to a regular decrease in the concentration of proteins and nucleic acids. The results obtained indicate a high degree of influence on the composition of the preparations of the material properties of the filters used, in particular their hydrophilicity, i.e. the ability to hold the individual components of the dispersion, regardless of their size. The use of UPN-50 filters makes it possible to isolate the fraction of particles and other components of blood serum with a mass of less than 50 kDa. The results of the quantitative determination of proteins and nucleic acids in the exosome preparation allow us to conclude that the content of the main insoluble impurity compounds in the preparation is significantly reduced compared to dispersions obtained using filters with pore sizes of 220 and 450 nm. At the same time, it should be noted that when filtering on the UPN-50 membrane, a noticeable decrease in the concentration of exosomal particles is observed.

Multiparameter analysis of flow cytometry data indicates that the use of a PTFE filter with a pore size of 450 nm made it possible to obtain an exosomal fraction with a more compact subpopulation of particles (the presence is more than 98%), the total concentration of which in the finished product was higher than when using an LCR filter with a pore size of 220 nm. At the same time, in the exosome dispersions obtained using the LCR membrane, there was the presence of additional subpopulations of fractions, possibly associated with the formation of larger associates or the presence of fractions different from exosomes in morphology and size. The preparation filtered through a UPN-50 membrane was a dispersion of exosomes characterized by a significantly lower content of fractions forming 2 subpopulations.

An analysis of flow cytometry data made it possible to demonstrate that when using the UPN-50 filter, an increase in the contribution to the dispersion of all types of fractions of non-exosomal size was observed (see Figure 1), the appearance of which may result from fraction destruction associated with pore size or filter material properties. At the same time, a decrease in the concentration of fractions obtained by filtration on UPN-50 during the analysis of flow cytometry data can be caused by limitations of the equipment used. The literature provides information on the significant limitations of this approach when detecting fractions less than 200 nm in size. It should be emphasized that fractions whose

size is not within the detection limit of flow cytometry cannot be discriminated against from the noise of the device, which can “artificially” lower the concentration of the measured microviscules in the sample [3]. Undoubtedly, flow cytometry is a powerful method for the analysis of membrane vesicles, however, due to some limitations, the interpretation of the data on membrane vesicles should be carried out carefully.

When conducting electron microscopy using Carl Zeiss EVO LS 10, the presence of fractions, mainly of a spherical and ovoid form, forming associates upon drying of the dispersion was observed in the studied exosome samples. Particle sizes ranged from 40 to 450 nm (an average of about 200 nm).

It is known that exosomes are actively involved in many physiological processes (morphogenesis, hemostasis, immune control, etc.) from embryogenesis to aging. The main physiological role of membrane vesicles is to transport substances from cells to the extracellular space, with their delivery to cells of distant tissues and organs. The expansion of the resolution capabilities of the observation and analysis of nanosized fractions over the past twenty years has contributed to an increase in interest in the study of microvesicular intercellular transport.

The most widely used among researchers was the exosome isolation protocol, which includes ultracentrifugation in a sucrose density gradient [6]. The lack of a unified standard approach for producing exosomal dispersions is actively discussed in the literature; attempts are made to standardize protocols. An additional difficulty is the fact that neither the qualitative nor the quantitative composition of microviscules can be strictly specific.

It should be noted that the use of methods such as differential centrifugation of dispersions of microvesicles increase the risk of their fragmentation into smaller fragments [10]. To eliminate this problem, we recommend the use of ultrafiltration through membranes. In addition, in any physiological fluid and in the intercellular space, obviously, at the same time, populations of subcellular formations with a size in the range of 30-100 nm can be present simultaneously. Therefore, the size of exosomes is not an absolute criterion for their difference, since both viruses and protein complexes can be detected in this range. To study the effectiveness of secreted membrane vesicles, the study of individual populations of vesicles is of great importance. In addition to membrane vesicles, biological fluids can also contain a large number of inclusions whose sizes are close (exosomes are similar in size to viruses and lipoproteins, the size of the remaining microviscules coincides with the size of bacteria) or molecules with a tendency to form aggregates or complexes. The presence of such inclusions can not only significantly complicate the detection of membrane vesicles, but also facilitate their joint deposition with various populations of the desired fractions. Of the entire variety of membrane vesicles, exosomes are fractions that have the most suitable characteristics, which allow them to be used as a nanoscale drug delivery vehicle while ensuring the necessary quality control of the drug at the sample preparation stage.

Conclusion

Thus, the data obtained allow us to conclude that, despite the larger pore size (450 nm), the use of polytetrafluoroethylene filters is more preferable in comparison with Millex-GV filters, 220 nm (Durapore). In addition, a larger pore size promotes the preparation of a drug with a high content of exosome fractions up to 450 nm, which, in turn, can lead to a relative increase in the efficiency of inclusion of biologically active substances with the further use of dispersions.

Reference

1. L. Console, *Clinica Chimica Acta*, **488**, 165-171 (2019) doi.org/10.1016/j.cca.2018.11.009.
2. R. Shanmuganathan, *International Journal of Biological Macromolecules*, **130**, 727-736 (2019) doi.org/10.1016/j.ijbiomac.2019.02.060
3. P. Calvo, C. Remuñán-López, J. Vila-Jato, M. Alonso, *Journal of Applied Polymer Science*, **63**, 125-132 (1997)
4. K. Balagangadharan, *International Journal of Biological Macromolecules*, **104**, 1372-1382 (2017) doi.org/10.1016/j.ijbiomac.2016.12.046
5. M. Martínez-Martínez, *European Journal of Pharmaceutics and Biopharmaceutics*, **136**, 174-183 (2019) doi.org/10.1016/j.ejpb.2019.01.009
6. A. Prata, *Carbohydrate Polymers*, **116**, 292-299 (2015) doi.org/10.1016/j.carbpol.2014.03.056
7. R. Shanmuganathan, *International Journal of Biological Macromolecules*, **130**, 727-736 (2019) doi.org/10.1016/j.ijbiomac.2019.02.060
8. T. Aminabhavi, *Chitosan Based Biomaterials*, **2**, 1-29 (2017) doi.org/10.1016/B978-0-08-100228-5.00001-8
9. J. Park, *Nanomedicine: Nanotechnology, Biology and Medicine*, **13** (6), 2015-2025 (2017) doi.org/10.1016/j.nano.2017.04.012
10. V. Engkagul, *Nanomedicine: Nanotechnology, Biology and Medicine*, **13** (8), 2523-2531 (2017) doi.org/10.1016/j.nano.2017.07.001
11. L. Du, *Nanomedicine: Nanotechnology, Biology and Medicine*, **10** (7), 1411-1420 (2014) doi.org/10.1016/j.nano.2014.04.001