Nanomed. J. 10(2): 131-137, Spring 2023

RESEARCH PAPER

Preparation and characterization of platelet-derived exosomes, as a nanostructure for bio-compound delivery

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ABSTRACT

Objective(s): Exosomes are extracellular vesicles (EV), which are released from the endosomal membrane of the cells. They are 30-120 nm in size, and carry many biological substances such as protein, lipids, and RNA. All kinds of cells release exosomes during their life. Exosomes are a small showcase of the mother cell's contents. Platelets-derived exosomes (Exo-Plt) are the most frequent nanoscale particles in the peripheral blood. Their availability and non-toxicity as an autologous carrier and a growth factor cargo made us introduce the most efficient method to isolate exosomes from platelet products and also examine their ability as a delivery system.

Materials and Methods: Two different protocols including stimulated and unstimulated were used in the present study to obtain Exo-Plt. Dynamic light scattering (DLS), BCA assay, Transmission Electron microscopy (TEM), and western blotting were performed to assess the basic characteristics (size and zeta potential, protein content, morphology, and CD markers, respectively) of the isolated particles. Consequently, the ability of Exo-plts for delivery of exogenous miRNA into the model cell line was assessed by Real-Time PCR.

Results: The stimulated method was more efficient to isolate Exo-Plt. Additionally, the applied agonist and the time of sample preparation from individuals affected the exosome yield. The Exo-Plts prosperously delivered the exogenous cargo into the cell line.

Conclusion: This research provided a standard protocol to isolate high-quality and pure Exo-Plt for bio-compound delivery and even therapeutic objectives. Exosomes provide the opportunity for cell-free therapy.

Keywords: *Drug delivery systems, Extra cellular vesicles, Platelet rich plasma, Transmission electron microscopy*

How to cite this article

Shams SF, Mohammadipour M, Deyhim MR. Preparation and characterization of platelet-derived exosomes, as a nanostructure for bio-compound delivery. Nanomed J. 2023; 10(2): 131-137. DOI: 10.22038/NMJ.2023.70974.1758

INTRODUCTION

Exosome are nanoscale particles, which arose from the endosomal system of every live cell. They carry proteins, mRNA, lipid (1, 2) , and even viral particles (1). Studies showed extracellular vesicles are divided into three categories based on size: exosomes (< 100 nm), MPs (100–1000 nm), and apoptotic bodies (≥1 μm)(3). Some mechanisms trigger exosomes generation such as stress, cell activation, apoptosis signaling and also normal function of cells (4).

The structure of two phospholipid layers and nano-size facilitate the passage of particles through the cell membrane. This feature, along with the ability to transport substances has drawn attention to the use of these particles in drug delivery systems (5). Furthermore, this bilayer structure protects the cargo from degradation and other biochemical and physical changes in the surrounding environment (6, 7). Due to the content of growth factors of platelet derived exosomes (Exo-Plt) (8), they have the potential to be used in the field of regenerative medicine (4, 9, 10), such as osteoarthritis treatment (11, 12) and regrowth of hair (13).

Considering all the points mentioned about the importance of Exo-Plts, it seems necessary to introduce a high-yield and standard protocol for achieving high-purity exosomes. So, this study was designed to compare two different methods of Exo-Plts isolation and also evaluate the cargo delivery potential of the particles.

MATERIALS AND METHODS

In this way, two different strategies were compared; they have been introduced by previous

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Note. This manuscript was submitted on January 6, 2023; approved on March 12, 2023

researches for exosome isolation via commercial kit from platelet concentrates (PC). The donor referred to the innovation center of IBTO. The whole blood product was prepared by routine phlebotomy method and the PC was obtained through routine Platelet Rich Plasma (PRP) method. Herein, we performed both protocols in two different days during the storage period (the 1st and the 5th day). The ethics committee of Iranian Blood Transfusion Organization (IBTO) confirmed the present study, and it was performed under the ethics code of IR.TMI.REC.1400.007.

Release of exosome

Cell activation pathway directly affects the quantity and the quality of the released exosomes (14). In this method, the PC bags were kept in the incubator at 22±2 °C for up to five days after the day of collection, until the extracellular vesicles (EV) released due to the natural function of platelets and accumulated in the PC bag. This protocol is called the Unstimulated method.

In the second protocol, the platelet product was stimulated by the platelet agonists to produce exosomes. Actually, 10 cc of PC was drawn, it was centrifuged 2500 g for 10 minutes in the room temperature. The supernatant was discarded, and 2 cc of Tyrod buffer (PH: 7.35) were added to the pellet; it was slowly mixed with the solution.

To stimulate and activate the cells to release exosomes, 5 µl $CaCl_2$ (1 M) as a strong platelet agonist was added to the solution. The solution was kept in incubator 37 °c for 30 minutes. In parallel, 5µl Epinephrine (Hyphen, France) (a weak platelet agonist) was also added to another tube with similar content. This protocol is called Stimulated method.

The following tests were performed to determine the characteristics of obtained exosomes. To assess the effect of storage time on the nanoparticle characteristics, both methods were applied on the specimens of the 1st and the 5th days of the same PC bag.

Exosome Isolation

Exosome isolation kit (Ana Cell Teb, Iran) was used to separate the Nano scaled EVs. In the first step, the PC was centrifuged 3000 g for 10 minutes. The pellet containing debris and platelets was discarded and the supernatant filtered through a 0.2 filter. It was mixed with reagent A in the ratio of 1/5 (Reagent/Specimen). Then, it was incubated for 12 hr in 4°C. After the end of the incubating time, the solution was centrifuged 3000rpm for 45 minutes in 4°C. In this step, the supernatant was discarded and the white pellet (exosome) was dissolved in PBS buffer (equal volume to the first volume of PC).

Dynamic light scattering (DLS)

To determine the size of the particles the nanoparticle analyzer (HORIBA scientific-SZ100, Japan) was used. This step was done in the central laboratory of the school of chemistry, university of Tehran.

BCA assay

BCA assay (DNA biotech. IRAN) was performed to determine the protein concentration of the samples. Standard curve was prepared according to the kit's instruction. In the first step, 1cc of the obtained exosomes was lysed via RIPA buffer (DNA biotech. IRAN). PMFS as the protease inhibitor was also added to the RIPA buffer (1/100 v/v) immediately before adding the exosome specimen. The procedure was performed according to the instruction. Finally, the absorbance of the solutions was read at 625 nm, and the protein content of each sample was determined according to the standard curve.

Western blotting

To verify the identity of the isolated particles western blotting was done for CD63 marker, which is one of the specific marker of exosomes. The protein content of samples was extracted according to the steps mentioned in the previous section. The samples were run on the SDS PAGE poly acryl amid gel 10% to separate different molecular weight proteins. The bands were transferred to the PVDF membrane (Roche. Swiss) via 30 vol overnight; it was blocked in the skim milk solution 5%. In the next step, the membrane was incubated with anti CD63 (Padza Padtan. IRAN) in 4 c overnight. It was washed with tween 20 three times, and then incubated with secondary antibody (anti IgG mouse) (Cytomatin Gene. IRAN) for 3 hours in a dark condition. Subsequently, the membrane was washed with tween 20. The enhanced chemiluminescence kit (ECL) (Cytomatin Gene. IRAN) was used for band detection. The ECL imaging system prepared the pictures from membrane.

Transmission electron microscopy (TEM)

Extracted nanoparticles from both methods were diluted in PBS, and subjected to the negative staining. The TEM imaging was done in Aria Rastak laboratory (IRAN. Tehran).

Exosome loading with miR-150

The cargo loading ability of the isolated exosomes were also examined. The $CaCl_2$ transfection method was applied according to the Zhang's study (15) to load the miRNAs in to the Exo-Plt. A solution consisted of 2.5 µl miR-150 (Bioneer, South Korea) (100 pmol/µl), exosome (1/22) (vol/vol) and $CaCl_2$ (1 M) was prepared. The CaCl2 concentration was adjusted to 0.1 M.

Consequently, the RNA extraction process by Trizole reagent (Roche, Swiss) was performed according to the published protocol of Rio et al. (16). The efficiency of loading process was determined using the miR-150 Real Time-PCR (RT-PCR) (Ana Teb. IRAN). cDNA synthesis process and also the RT-PCR were done according to the kits instruction. The $2^{-\Delta\Delta Ct}$ method was used to examine the results of miR-150 expression.

Cell transfection with the loaded Exo-Plts

M07-e cell line was used as a model cell to evaluate the possibility of cargo delivery of Exo-Plts. It was obtained from national cell Bank of pasture institute (Tehran. Iran). The cells were cultured in RPMI 1640 supplemented with 10 ng/ml IL-3 (Stem cell, Canada), 15% Fetal Bovine Serum (FBS) (Gibco, USA), and 1% penicillin/streptomycin (Gibco, USA). The cell Flasks were kept under the condition of 5 % CO_2 and 37°C. After the cells proliferated sufficiently, they were transferred into the six well cell culture plate (4× 105 cells for each well).

In the next step, the loaded Exo-Plts were added to one of the wells, one well also considered as control and without any additives. The third and the fourth wells were also treated with unloaded Exo-Plts and miR-150. After 24 hr, it was harvested in combination with the control groups and the RNA extraction was performed.

Evaluation of the Exo-Plt ability to transport miR-150 into the cell line

The RT-PCR miR-150 was also done to examine the level of miR-150 in the cells. miR-U6 was considered as the internal control gene in the RT-PCR. The reactions were performed in the total volume of 10 µl. The following ingredients were mix, master mix 2X (Amplicon, Denmark) (5 µl), CDNA (1 µl), forward and revers primers (1 µl for each), and DEPC water (2 µl). The RT-PCR was done by Corbett Rotor gene and the following condition (Table 4) was set.

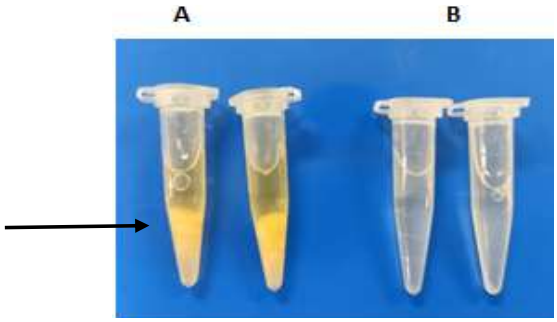

Fig. 1. Micro tubs contain exosomes after cold incubation period. A: the unstimulated method with high sediment of protein and the yellow color of plasma. B: micro tubs contains exosomes obtained via platelet activation by two mentioned agonists

Statistical analysis

Data analysis was performed using Graph Pad Prism 9 software. Alfa error of 5% was considered. Data were expressed as mean ± SD. All comparison tests were calculated as two-tailed. In this analysis, Chi-Square and two-way ANOVA tests and Tukey's post hoc test were used to analyze the exosome characteristics, and welch, One Way Repeated Measures, and ANOVA were performed for RT-PCR results comparison. All experiments were done in duplicate, and repeated three times.

RESULTS

Exosome precipitation of the applied methods using commercial kit is shown in Fig. 1.

Exosome size and its distribution

The mean diameter of exosomes is shown in Table 1. Size distribution of the isolated particles has been listed in Fig. 2 and Table 2. According to the published articles, the size range of 30-120 nm was considered as the exosome area.

Table 1. Mean diameter of the isolated particles

	Unstimulated Protocol	Stimulated Protocol	
		Epinephrine	$CaCl_2$
The 1st day	22.1±7.1	51.5±30.2	118.5±37.5
The 5th day	26.9±9.5	88.6±35.3	43.2±15.8

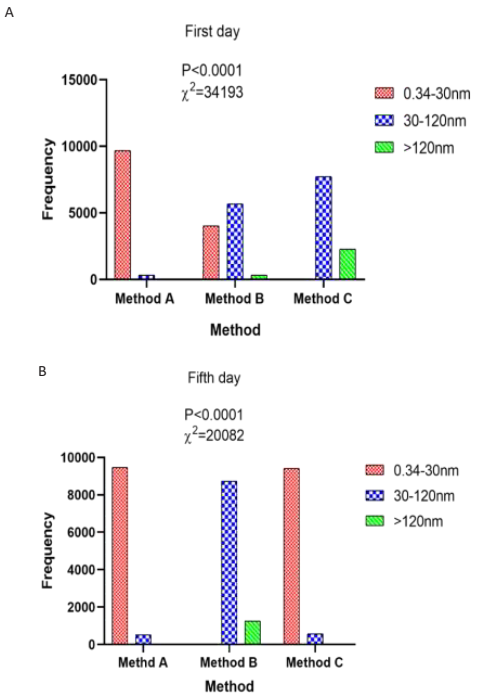

Fig. 2. Distribution of particles size in different days of study. Method A: Unstimulated, Method B: Stimulated by Epinephrine, Method C: Stimulated by $CaCl_2$

The mean size of exosomes in the unstimulated protocol did not change significantly between the two days of study (P-value> 0.05). In the simulation method (Epinephrine) the mean diameter of nanoparticles increased during the storage time, but it decreased in the stimulation via $CaCl_2$; size changes were statistically different in the stimulated method between the two days of the experiment (P-value< 0.05). Exosome size distribution of the applied methods has been cited in Table 3.

Comparison of the results of size distribution between the two agonists of $CaCl_2$ and Epinephrine (a strong and a weak, respectively) shows significant differences within and between the studied days. Stimulation with Epinephrine reduced the number of exosome-sized particles on the fifth day compared to the first day, but stimulation with $CaCl_2$ increased the relative frequency of the exosome-sized particles significantly between the two days of experiments.

Protein content

The protein concentration was statistically different between the stimulated and unstimulated methods, but it was not statistically different between the Epinephrine and $CaCl_2$ stimulation groups in the first and the fifth days. Fig.

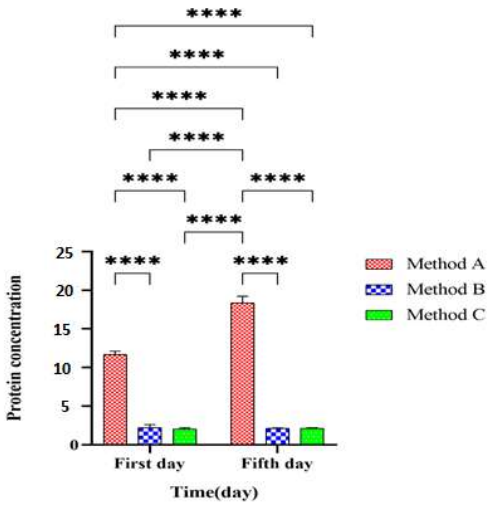

Fig. 3. Protein concentration of isolated exosomes by different methods (mgr/ml). Method A: Unstimulated, Method B: Stimulated by Epinephrine, Method C: Stimulated by $CaCl_2$

Table 2. Mean diameter of the isolated particles

	Unstimulated Protocol	Stimulated Protocol	
		Epinephrine	$CaCl_2$
The 1st day	22.1±7.1	51.5±30.2	118.5±37.5
The 5th day	26.9±9.5	88.6±35.3	43.2±15.8

Table 3. Size distribution analysis of particles by volume (%)

Method		day	Size distribution		
			<30	30-120nm	>120
Unstimulated		1st	96.66	3.23	<1
		5th	94.65	5.13	<1
Stimulated	Epinephrine	1th	0	78.20	22.03
		5th	94.16	5.44	<1
	$CaCl_2$	1st	40.13	56.72	3.14
		5th	0	87.34	12.65

Table 4. Protein concentration of the exosome specimens (mgr/ml)

Day	Unstimulated	Stimulated	
		Epinephrine	$CaCl_2$
1st	11.66± 0.44	2.24 ± 0.369	2.05± 0.15
5th	18.37± 0.84	2.12 ±0.07	2.16±0.05

and Table 4 show the protein concentration of the samples from different methods and in different time points.

Western blotting

The results of western blot showed three distinct bands of CD63 (Fig. 4A). CD63 belongs to the tetraspandin family and is one of specific marker for exosome identification (17). The results are shown in Fig. 4.

TEM imaging

The TEM images are shown in Fig. 4B.

miR-150 loading into the Exo-Plts

The results of miR-150 RT-PCR confirmed the efficient loading of exogenous miRs in to the Exo-Plts. The loaded Exo-Plts have 18.89-fold increase in miR-150 compared to the unloaded exosomes (P-value<0.05).

Successful Cell Internalization of Exo-Plts

The expression of miR-150 in the M07-e cells increased 2.28 fold during 24 hr of cell transfection onset, which was statistically significant compared to the control groups (P-value<0.05).

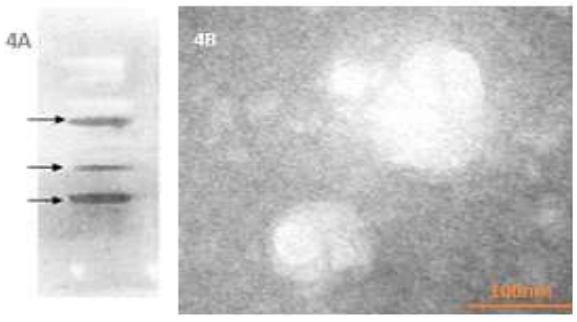

Fig. 4. Western blot results and TEM imaging of exosomes
The orange line in 4B is the scale of 100 nm
3A: Western blot results of CD63. It has three different molecular weight (32 KDa,35KDa and 50KDa), which is due to N- glycosylation process (17)
3B: TEM image of exosomes of the unstimulated method in a context of protein

DISCUSSION

Regarding the cargo-loading potential of EVs, their importance in drug and genetic material delivery has been highlighted. Besides Exo-Plts are enriched of platelet growth factors, which are effective in tissue damage repair (18); regarding the applied agonists and the method the growth factor and cytokine content were completely different (19), as well as protein concentration and size distribution of the EVs. One of the findings of the present study was introducing the most efficient protocol to isolate exosomes from PC bags to facilitate future preclinical investigations via Exo-Plts for therapeutic aims.

Herein, it was shown that the protein concentration of the specimen obtained via the unstimulated method was extremely higher compared to the stimulated method either via Epinephrine or $CaCl_2$. Actually, during exosome precipitation in the unstimulated method a high volume of white pellet was also precipitated in the micro tube (Fig. 1); it consists of protein and contains coagulation factors.

Precipitated coagulation factors are the main agent in increasing the protein concentration in the exosome samples. High protein concentration may interfere with the subsequent tests, such as RNA extraction, RT-PCR, ELISA, and every test based on turbidometery; in the present study, it also affected the quality of the TEM images (Fig. 4B). High concentration of proteins in the unstimulated method also lead to the formation of jelly state in the cell culture media. Although the coagulation factors interrupt other laboratory tests, but in terms of regenerative medicine they would be advantageous. For example, fibrinogen promote fibroblast growth and accelerate the process of wound and scar healing (20).

The western blot results of a similar study confirmed that the stimulation method besides no interference of coagulation factors has a minimal amount of contamination with cellular protein residues (19). Azevedo et al. also performed the unstimulated method and separated the accumulated Exo-Plts, despite washing the platelet pellet by Tris-Buffered Salin (TBS) high protein concentration of Exo-Plt (5.8 ± 0.96 and 16.5 ± 0.99 mg/ml) have been reported (21).

On the other hand, in the stimulated method the size distribution of the isolated particles

showed an extremely higher relative frequency of exosomes produced via both agonists ($CaCl_2$ and Epinephrine). In this regard, Saumell-Esnaola .et al provided new inisghts into the morphological and biochemical charectristics of Exo-Plt. Activation of platelets with $CaCl_2$ triggered release of more exosomes compared to the unstimulated method (19). Furthermore, Dash and colleagues announced that different isolation techniques directly affect the characteristics of isolated exosomes (22). According to the low frequency of exosome-sized particles in relation to smaller and larger ones, it seems that the unstimulated method was not enough efficient to isolate exosomes from PC bags.

$CaCl_2$ as a strong agonist induced larger population of nanoparticles on the first and the fifth days compared to Epinephrine; moreover, Epinephrine produced a significant number of exosome sized particles only on the first day. The platelet count decreased during the storage time (480 ×103/µl to 415×103/µl). As the PC approaches the expiration date the platelet viability and activation potential (23) and also the potential to produce exosomes decreases; so, Epinephrine as a weak stimulus in the first days of storage will be more effective in producing exosomes, but CaCl2 as a strong agonist successfully and significantly stimulated the remaining active platelets and also produced a higher proportion of exosomes, even on the 5th day of the storage period. In addition, an indirect relationship was found between the nanoparticle size and the frequency in the stimulated method.

In the same direction, Collier et al. applied PAR-A1 peptide as an agonist to activate the platelets; despite low protein concentration in the stimulated method, they performed washing steps by HEPES buffer to remove the extra protein of plasma. In the following, they loaded the Exo-Plts with miR-223 and successfully induced protein expression in the Monocytic cell line (24). In line with the present study findings, Exo-Plts acted as an efficient delivery system.

A summary of our findings and theirs showed that the stimulation method has the minimum concentration of proteins, which less interferes with further analysis of the study. In the current study, protein concentration decrement in the stimulation method was the result of washing PRP with Tyrod buffer. From the data explained before, we concluded the isolation method and applied agonist and even the individual characteristics of platelets could affect the particles size and also the size distribution. Among isolation methods, ultracentrifugation significantly removes the disturbance proteins of the specimens but has special limitations such as lack of ease of access (25).

The findings of the present study implied that despite easy access and economic efficiency, commercial kit leads to protein deposition in the isolated particles of the PRP sample. To fix this defect, the stimulated method is preferred due to the washing step with Tyrod buffer removing excess proteins.

CONCLUSION

Platelet exosomes are used by many studies as available and inexpensive natural carriers for the delivery of biomaterial and a valuable source for regenerative medicine. The method of obtaining high-quality exosomes may be challenging for many researchers; so, introducing the principled and effective method to isolate the nanoscale particles could improve the future trials in the field of gene and drug delivery and also regenerative medicine.

Taken together our results proposed that the stimulated method is more efficient than the unstimulated protocol, and also EVs are released with different quality and quantity in response to different agonists.

LIMITATIONS

One of the most important limitations of this study was inaccessibility to Nano tacking analyzer (NTA) instrument to count the amount of particles of each method.

ACKNOWLEDGEMENTS

The authors are thankful to the personnel of the research center of Iranian blood transfusion organization, especially Ms Sahar Afaridon for her technical supports. This article is one of the extracted articles from the Ph.D. thesis of Seyyede Fatemeh Shams from the Iranian blood transfusion organization. This work is based upon research funded by Iran National Science Foundation (INSF) under project No.4005320.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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