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Development and Evaluation of Extracellular Vesicles Based Novel Delivery System for Enhanced Anticancer Effects

조선대학교 대학원

약 학 과

아싸두자만 엠디

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항암효과 강화를 위한 세포외소포체 기반 신규 약물전달체의 개발 및 평가

2020 년 2 월 25 일

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Contents

List of Figures

List of Table

국문초록

항암효과 강화를 위한 세포외소포체 기반 신규 약물전달체의 개발 및 평가

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세포외소포체는 세포 유래 소포로 진보된 약물 전달 시스템에 사용될 수 있다. 세포외소포체는 전구체 세포의 세포막이 지닌 핵심 특성을 보유할 수 있다. 이 특성은 표적 세포에 의한 효율적인 세포 흡수를 가능하게 한다. 또한, 대부분의 암세포로부터 유래한 세포외소포체는 망상내피계 (RES) 제거율을 낮출 수 있는 인테그린-관련 막 관통 단백질 CD47 을 함유한다. 그러나, 세포외소포체는 약물 봉입 효율이 낮다는 한계가 있다. 대조적으로, 리포솜은 약물 전달 시스템으로서 몇 가지 장점이 있지만, 암세포 내에서 세포 흡수가 제한될 뿐 아니라, RES 제거율 증가의 문제도 발생한다. 따라서, 세포외소포체와 리포솜이 융합된 새로운 약물 전달 시스템을 개발하는 연구를 수행했다. Adenocarcinomic human alveolar basal epithelial cell (A549)에서 분리된 세포외소포체의 CD47 특성을 이용, RES 제거율을 낮춘 새로운 약물 전달 시스템은 세포외소포체와 리포솜이 가진 단점을 상호 보완해 강화된 항암효과를 기대할 수 있다.

2. Introduction

All cells except specialized cells secrete several kinds of membrane vesicles. Such vesicles are known as vesicles extracellular (EVs) (1). Initially, EV secretion was considered an elimination process of unnecessary compounds from the cells (2). Nowadays, EVs are no longer considered carriers of only waste. They can exchange the components of nucleic acids, lipids and proteins within cells. EVs also act as signaling vehicles in the homeostatic processes of normal cells or under pathological changes (3, 4). Although all secreted membrane vesicles are known by the generic term 'EV', they are, in fact, vastly heterogeneous. Transmission electron microscopy, immune electron microscopy, and biochemical assays have provided insights into the biogenesis of secreted vesicles. Currently, membrane vesicles are broadly divided into two main categories: microvesicles and exosomes.

At first, the vesicles released by cultured cells were termed exosomes which later became known as reticulocytes released membranes and vesicles during the differentiation period (2). Exosomes 30–150 nm-sized cup-shaped vesicles originating from fusion between the multivesicular body (MVB) and plasma membrane, releasing exosomes to the extracellular space (5, 6). At present, it is assumed that various types of cells secrete exosomes; however, in the mid-90s, it was reported that only dendritic cells (7) and B lymphocytes (8) secrete exosomes. Microvesicles were once touted as 'platelet dust' because they are secreted by platelets to normal plasma and serum (9). In the past, microvesicles were considered mainly for their role in blood coagulation; however, at present, they are thought to play a role the intercellular communication of several cell types. Usually, microvesicles range from 50 to 1,000 nm in diameter. The basic composition is now evident to define the fate and role of each type of EVs.

Direct loading of isolated exosomes has also been reported by incubating them with the selected cargo (10, 11). Hydrophilic miRNAs as well as comparatively hydrophobic molecules such as polyphenols (e.g. curcumin), steroids (e.g. cucurbitacin) and anticancer

agents (e.g. doxorubicin) have been incorporated into exosomes by simply combining the load with the exosomes. Transfection using cationic lipids (12) and electroporation (13–16), which applies an electrical field to a suspension containing the exosomes and the intended cargo. Cargoes, especially siRNAs, have also been loaded into exosomes. However, such techniques can adversely affect exosomes and cargoes, for example by promoting aggregation (17).

The exosomal interface may need to be modified and adapted for other applications of exosomal carriers in drug delivery systems (i.e., lipid bilayer membrane). Here, we opted to a novel and simple exosome surface modification strategy using direct membrane fusion between synthetic liposomes and exosomes after parent cell secretion. This approach allows us to optimize the characteristics of the exosome surface to reduce its immunogenicity and increase its colloidal stability, and to improve the half-life of blood exosomes. Nevertheless, lipid molecules' hydrophobic properties prohibit direct loading of lip ids into exosomes. Genetic alteration of the lipid membrane of the exosomes is also difficult because lipid biosynthesis involves a large number of proteins and there is no clear understanding of the process through which lipids are sorted from the host cell to the exosome. In this context, we proposed a new strategy for developing an exosomes and liposomes fusion system using the freeze-thaw process (Figure 1) (18).

Most of the cancer cells contain the surfactant protein CD47. The binding of CD47 to SIRPα initiated signal transduction events. The molecular signaling cascade ultimately results in the inhibition of phagocytosis of CD47-expressing targets by SIRPα-expressing macrophages. The inhibitory effect of CD47-SIRPα signaling on phagocytosis is largely species specific, and researchers have coined the terms “don’t eat me” when describing CD47 (19-24). There are several examples of Cd47-mediated biological inhibition of macrophage clearance. In mice, red blood cells (RBCs) lacking CD47 cells are rapidly cleared by splenic macrophages (23). Hematopoietic stem cells upregulate their surface expression of CD47 to avoid clearance by macrophages (25-26). These suggest the immunoprotective capacity of CD47 across of biological processes. Here, we also venture

to exploit the presence of CD47 in our exosomes and fusion systems to utilize this properties of CD47 to give an enhanced anticancer effect.

3. Materials and Methods

3-1. Materials

SPC was obtained from Lipoid GmbH (Ludwigshafen, Germany). NBD-DSPE, Rhodamine were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Anti CD-9, anti CD-63 & anti-CD47 were purchased from Abcam (USA). Goat anti-Mouse IgM secondary antibody, FITC and Goat anti-Rabbit IgG secondary antibody were purchased from Invitrogen (Rockford, IL, USA). ExoQuick TC–Ultra was purchased from System Biosciences (Palo Alto, CA, USA). HPLC grade acetonitrile (ACN) and methanol purchased from Avantor Performance Materials, Inc (Center valley, PA, USA) were used. All other used reagents were laboratory grade.

3-2. Cell culture

Adenocarcinomic human alveolar basal epithelial cells A549 were procured from Korea Cell Line Bank (KCLB, Seoul). A549 cells were cultured in RPMI-1640 medium supplemented with 10% Exosome Free Fetal Bovine Serum (Exo-FBS) and 1% Penicillin-Streptomycin solution. Cells were cultured in a 5% CO_2 incubator at 37°C.

3-3. Isolation and purification of exosomes

Exosomes were isolated from supernatant of A549 cell culture. Cells were grown for around 3 days / to 80% confluency. Then the exosomes were pelleted using the Exoquick-TC Ultra (System Biosciences, USA) following the manufacturer's protocol. Briefly, 10 ml media was collected into a 15 ml falcon tube and centrifuged for 15 minutes at 3,000 × g to remove cellular debris. Transferred to another tube and added 2 ml of ExoQuick-TC solution to the media. Mixed well by inverting /flicking the tube, and incubated on ice for overnight at 4°C. Centrifuged the ExoQuick-TC/cell media mixture at 3,000 × g for 10 minutes at 4°C. Upon centrifugation, the EVs appeared on the tube's bottom as a beige

or white pellet. Carefully aspirating off the supernatant. Residual ExoQuick solution was spinned down, taking great care not to disturb the precipitated EVs in the pellet. Resuspended the pellet in 200 µl of Buffer B (Figure 2). Measured and recorded sample protein concentration.

200 µl of Buffer A was added to resuspend EVs. Centrifuged at 1,000 x g for 30 seconds to remove the storage buffer. The flow-through was removed and the column was put back into the collection tube. Column was washed by removing the cap and 500 µl of Buffer B was applied on top of the resin and centrifuged at 1,000 x g for 30 seconds. Discarded the flow through. Repeated the column washing one more time. Plugged the bottom of the column with the bottom closure. 100 µl of Buffer B was added on top of the resin to prep it for sample loading. The entire content from step 1 (or up to volume equivalent of 4 mg of total protein) was added to the resin. Placed the screw cap on the top of the column. Mixed at room temperature (RT) on a rotating shaker for 5 minutes. Loosen the screw cap and the bottom closure was removed, and immediately transferred to 2 ml Eppendorf tube. Centrifuged at 1,000 x g for 30 seconds to obtain purified EVs. Exosomes were collected in solution in 2 ml Eppendorf tube and used for downstream application or keep in the refrigerator at -80°C for further use.

3-4. Preparation of liposomes

Liposomes were prepared by thin film hydration method (27). Briefly, 60 mg of Soy Phosphatidylcholine (SPC100) was dissolved in 2 ml chloroform within a 25 ml round flask. 1 ml of drug containing solution (3 mg/ ml of Docetaxel in methanol) was added to the mixture. Organic solvent was evaporated using rotary evaporator for 30 minutes at 100 RPM and 45°C temperature in the water bath. After evaporation a thin film was produced. Hydrated the film with 3 ml of PBS conditioned about 75°C to produce suspension of multilamellar vesicles. Sonicated the liposome suspension for 30 minutes. Extruded the liposome suspension 11 times through Avanti Lipid Extruder maintaining the temperature about 50°C. Filtered the collected small unilamellar liposome suspension with 0.45µm syringe filter to eliminate free drug and stored at 4°C for future use.

3-5. Exosomes and liposomes fusion system

An established freeze-thaw method was used to fuse the exosomes and liposomes preparations. Exosomes from 10 ml supernatant were mixed with NBD-DSPE & Rhodamine containing liposomes preparation (1:1 by volume). Mixtures of exosomes and liposomes were frozen in liquid nitrogen and thawed at room temperature for 30 minutes. 10 cycles of freeze-thaw was carried out. The efficiency in fusion capacity was measured using established FRET assays. Briefly, the fluorescence of the mixture containing fluorescence coupled NBD-DSPE and Rhodamine were measured using an F-7000 Fluorescence Spectrophotometer (HITACHI, Tokyo, Japan). Excitation of NBD-DSPE at 460 nm induces fluorescence emission at 526 and 594 nm, corresponding to the emissions from NBD-DSPE and Rhodamine, respectively. The fluorescence intensity increases at 526 nm and decreases at 594 nm due to dilution of Rhodamine owing to membrane fusion with NBD-DSPE. The exosome-liposome mixture's FRET dissolution efficiency has been described as EFD = F526/ (F526 + F594), where F526 and F594 represent the fluorescent intensities at 526 and 5948 nm, respectively.

3-6. Physical Characterization

The size distribution, Polydispersity index (PDI) and zeta potential of exosomes and liposomes and their fusion system were analyzed using a zetasizer instrument Otsuka Electronics Ltd, Japan) equipped with zetasizer software version 2.3. The particle suspensions were diluted to 100 times with PBS.

3-7. Protein marker characterization by western blotting

Exosomes were suspended in RIPA buffer and protease inhibitor was added. Then added the sample loading buffer and heated for 5 min at 95 °C. Exosomes were run on 12% SDS–polyacrylamide gels, transferred to membranes of nitrocellulose, and blocked with skimmed milk. The blots were incubated with primary antibodies to CD9, CD47 and CD63 at 4°C overnight. After washing, the membranes were probed with respective

secondary antibodies at room temperature for 1 hour. Signals were detected using ECL western blotting detection reagent.

3-8. Protein marker characterization by flow cytometry

Solution of fusion systems were incubated with aldehyde/sulfate-latex beads (Invitrogen, CA) for 15 minutes at room temperature. Then BCB (PBS supplemented with 0.1% BSA; Sigma, and 0.01% $NaN_{3;}$ Daejung) was added and the sample incubated overnight on rotation. Bead-coupled exosomes were pelleted by centrifugation, washed with BCB and centrifuged again. The pellet was resuspended with BCB and stained with CD9, CD47 and CD63 primary antibodies. Washed with BCB solution once. Then the samples were stained with FITC-conjugated secondary antibodies. Washed with BCB solution twice. Data was acquired by MACSQuant (Miltenyi Biotec, Germany).

3-9. Docetaxel loading system

To calculate EE and LC, concentrations of Docetaxel encapsulated in fusion system were analyzed. In brief, 500 μl of solution of fusion system was mixed with 500 μl ACN and sonicated for 30 min using bath sonicator. The solution obtained was centrifuged at 4 ° C for 15 min at 1 2,000 g. Using high-performance liquid chromatographic method (Azura, Germany), docetaxel in the supernatant was analyzed. For HPLC analysis, C18 column (4.6 mm × 150 mm, 5 μm; Phenomenex, CA, USA) was used and maintained at 40°C. The mobile phase consisted of a 25:75 v/v mixture of water and Methanol. The rate of flow was set to 1.0 ml / min and the volume of injection was 20 μl. The detection wavelength was fixed at 230 nm. ClarityChrom® software (Version 6.1.0.130, Knauer, Germany) provided with the system was used to evaluate the chromatograms.

EE and LC were addressed according to the equations, respectively.

$$\mathrm{EE}(\%) = \frac{\text{Encapsulated drug (μg/ml)}}{\text{Total drug (μg/ml)}} \times 100$$

$$\mathrm{LC}(\%) = \frac{\text{Encapsulated drug } (\mu g/ml)}{\text{Total lipid } (\mu g/ml)} \times 100$$

4. Results and discussion

4-1. Fusion of exosomes and liposomes

In this study, we developed a fusion system of exosomes and liposomes using the freeze–thaw method. The freeze-thaw method (28-30) was selected to prepare the hybrid exosomes by membrane fusion as a relatively simple physicochemical process. The freeze-thaw method has been used to incorporate water-soluble molecules in the internal liposome water phase, suggesting that this method interferes with plasma membranes by temporarily forming ice crystals (31–33). The removal of water molecules from the hydrophilic surface of the lipid bilayer membrane by freezing therefore seems to cause significant structural and functional changes, including hydration-dependent phase changes, lateral phase separation of membrane components, and membrane fusion; the latter may occur as a result of membrane reconstitution (34-35). Such results led us to use the freeze-thaw approach to cause fusion or mixing of membranes of exosomes and liposomes. This physical procedure, in addition to being simple, prevents contaminating the membranes with, for example, unnecessary chemicals such as calcium or PEG that are used in other chemical fusion methods. To optimize the number of cycles that needed for fusion of exosomes and liposomes we applied zero to fifteen cycles of freeze-thawing. However, after 10 cycles the fusion efficiency does not changes much. Considering the time with unchanged characteristics of fusion properties 10 cycles of freeze-thawing was selected for further experiment (Figure 3).

4-2. Physical characterization

Physical characterization is one of the key characteristics for exosomes and liposomes. Dynamic light scattering (DLS) was employed to determine the physical characteristics of exosomes, liposomes and their fusion system. Also referred to as photon correlation spectroscopy (PCS), DLS is a technique used to assess vesicle size distribution.

This is done by analyzing laser light fluctuations in temporal intensity, dispersed by a dispersion of these freely spreading EVs. The mean particle diameter, PDI and zeta potential of exosomes, liposomes and their fusion system were evaluated (Table 1; Figure 4). The particle diameter of liposomes and exosomes were 141.93 ± 0.51 and 184.87 ± 1.04 nm, respectively. Whereas fusion system showed a larger diameter of 241.37 ± 2.46 nm. From the obtained data, it was suggested that the larger diameter of fusion system is due to the fusion of liposomes and exosomes.

4-3. Protein marker characterization

4-3-1. Characterization by western blotting

The aims of the study were to establish a western blot method for the analysis of exosomes samples. To characterize the exosomes by Western blotting, exosomes samples were tested by optimizing the antibodies used. Western blot is regularly used in exosome studies to confirm the presence of vesicles in the sample (36). Therefore, it's always important to establishment the Western blot method for the conduction of further studies. Several antibody dilutions and different blocking and the incubation conditions for each antibody was needed to optimize. Every exosomes has some common surface protein marker. To claim the presence of EVs/exosomes in preparations, one need to show the presence of more than one proteins. Since one absolute marker protein for exosomes does not exist, the investigation of several proteins is necessitated. It is also important for reliability to verify more than one protein that is expected to be enriched in exosomes. Tetraspanins are transmembrane proteins involved in functions of the plasma membrane. They consist of four transmembrane domains, two extracellular regions and three intracellular regions (37). Some tetraspanins, especially CD9, CD63 and CD81, are commonly used as EV marker proteins, for they have been shown to be selectively enriched in EVs (8, 38, 39). We tried to prove the presence of CD9, and CD63 in exosomes' containing sample. We also tried to prove the presence of CD47 for its specific don't eat me characteristics that

are going to be utilized in our hypothesis. Here, got the presence of CD9 and CD63. Besides, the presence of CD47 was also confirmed (Figure 5).

4-3-2. Characterization by flow cytometry

We were also interested at investigating whether the protein markers on the exosomes surface were preserved in the fusion system. Characteristic protein markers of exosomes, such as the CD9, CD47 & CD63 were assayed by adsorbing exosomes and fusion system onto latex beads and detecting via flow cytometry with addition of a secondary antibody conjugated with a fluorescence tag. Exosomes alone were used as positive control. Liposomes were used as negative control. Fusion system assayed with the selected protein markers exhibited a similar type of marker presence, compared to the positive control. However, the intensities were too low (Figure 6). This hints the presence of exosome markers in the fusion system, but need a more optimization to get a more prominent conclusive results.

4-4. Docetaxel loading system

Docetaxel was loaded into the liposomes and fusion system with thin film hydration method. The percentage encapsulation efficiency of docetaxel in liposomes and fusion system were 92.99 ± 2.39 and 88.49 ± 3.73 respectively. Whereas loading capacity of docetaxel in liposomes was 15.65 ± 0.29 (Figure 7). So, we found that the encapsulation efficiency of fusion system is very much comparable. It is suggested that similar encapsulation efficiencies were measured when docetaxel was loaded into liposomes and fusion systems produced from liposomes & exosomes fusion. This suggests that the fusion process does not negatively impact the loading potential of fusion system.

5. Conclusion

We have established a novel hybrid drug delivery system involving the fusion of exosomes and liposomes. We successfully optimized the fusion condition and the drug loading in our fusion system. In case of drug loading the result is very much comparable with liposome alone. This system can be used as a drug delivery system for targeted delivery of chemotherapeutics. However, a better optimization is needed to get a conclusive result to show the presence of exosomes markers in our fusion system. Besides, further studies are needed to carry out the *in vitro* cellular uptake, cytotoxicity tests as well as *in vivo* studies to use this system as a drug delivery for enhanced anticancer effect.

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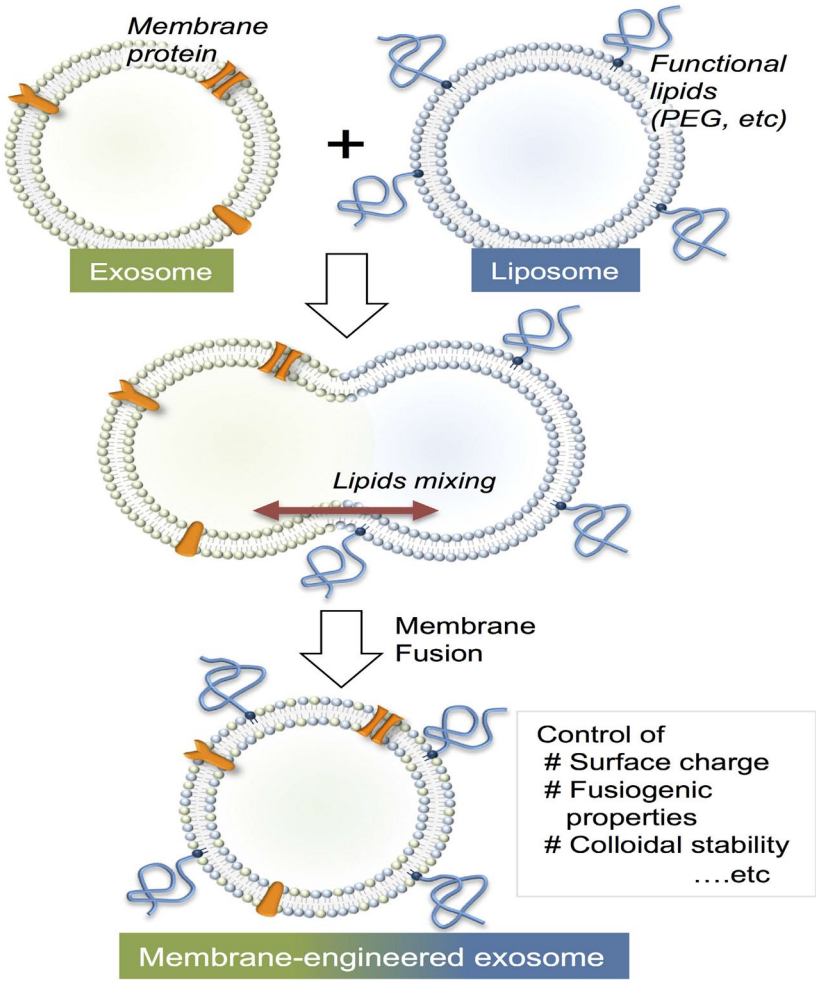


Figure 1. Schematic of method used to prepare the exosome-liposome fusion system.

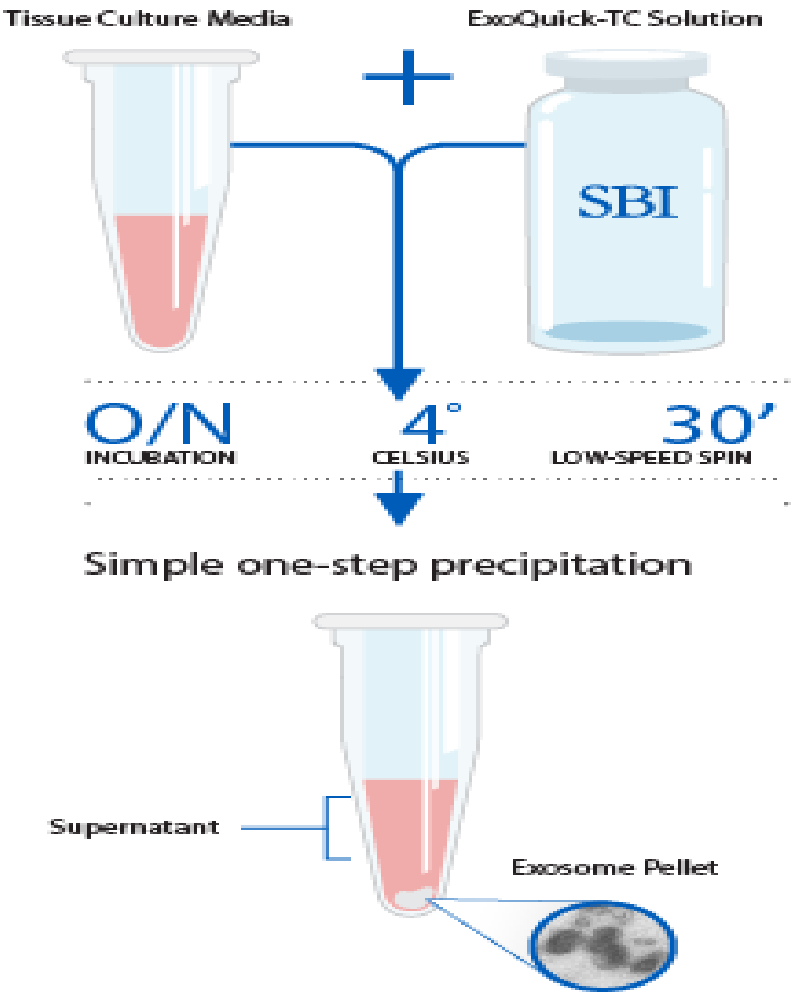
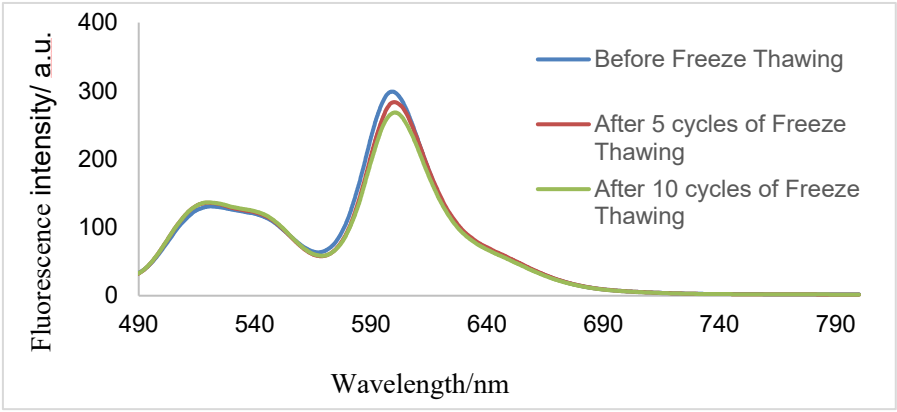


Figure 2: Exosome isolation with ExoQuick-TC solution

A



B

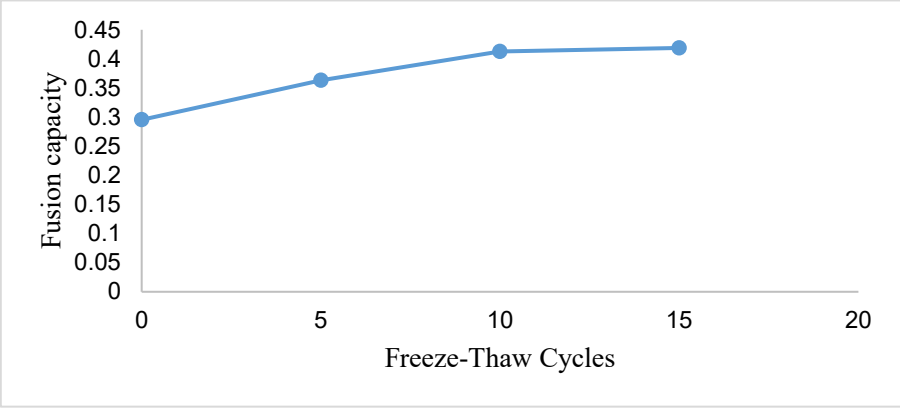


Figure 3: A: Fluorescence spectra with excitation at 460 nm. B: Fusion capacity with respect to freeze-thaw cycles

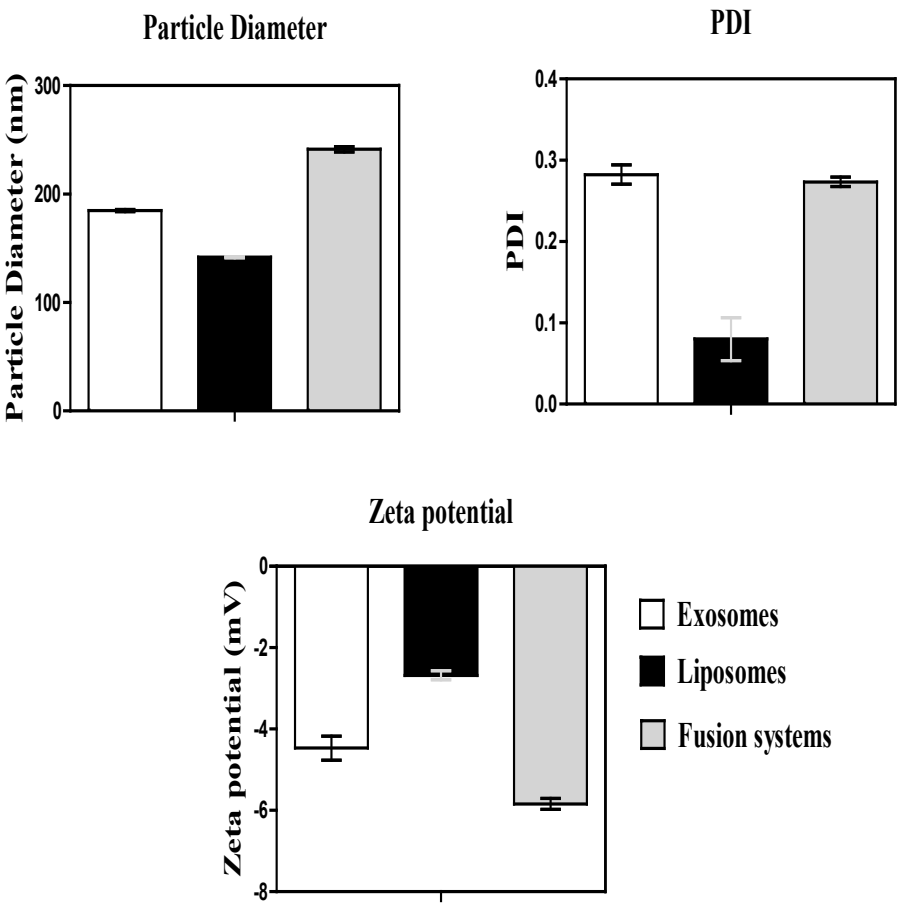


Figure 4: Particle size, PDI and zeta potential value

Data are presented as the mean ± SD (n=3)

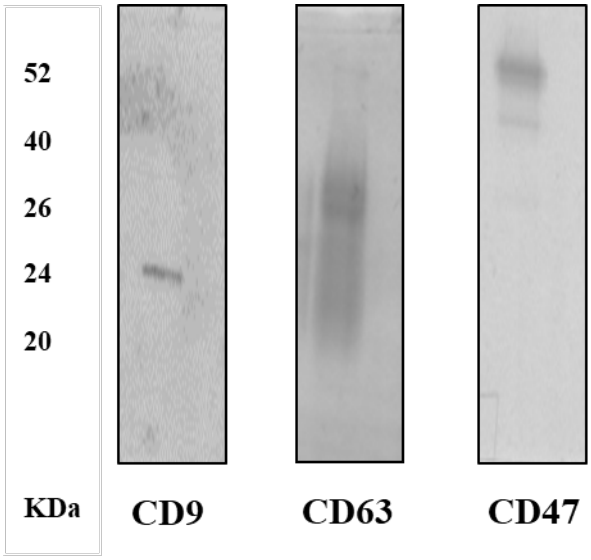


Figure 5: Western blotting showed the expression of specific proteins CD9, CD63 and CD47 in A549 derived exosomes.

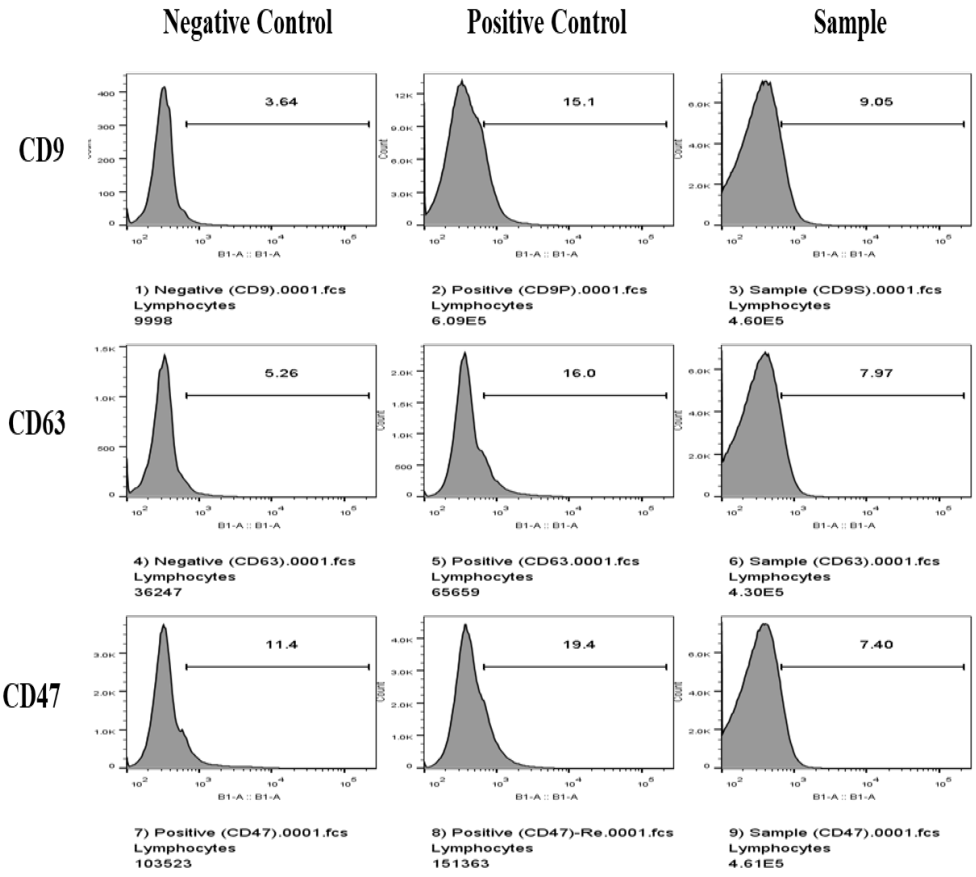


Figure 6: Surface protein characterization of fusion system

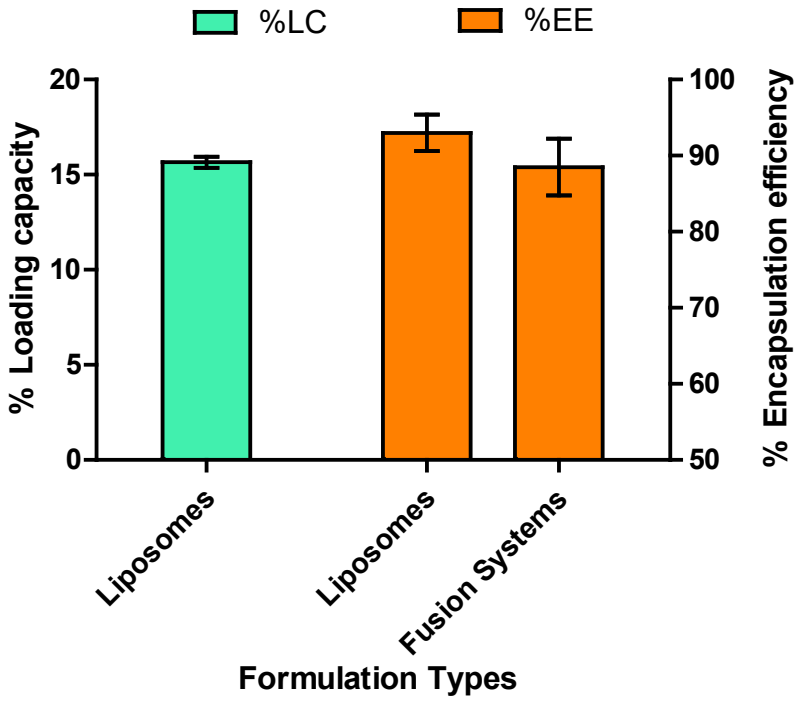


Figure 7: Docetaxel Loading in Liposomes and fusion systems

Data are presented as the mean ± SD (n=3)

Table 1: Physical Characterization

Data are presented as the mean ± SD (n=3)

	Liposomes	**Exosomes**	**Fusion System**
Particle diameter (nm)	141.93±0.51	184.87±1.04	241.37±2.46
PDI	0.08±0.03	0.28±0.01	0.27±0.01
Zeta potential (mV)	-5.84±0.14	-2.68±0.11	-4.47±0.30

ABSTRACT

Development and Evaluation of Extracellular Vesicles Based Novel Delivery System for Enhanced Anticancer Effects

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Extracellular Vesicles are cells derived vesicles that can be used for advanced drug delivery system. Extracellular Vesicles (EVs) can preserve key features from the cell membrane of their precursor cells. This property is responsible for an efficient cellular uptake by target cells. Again, EVs from most of the cancer cells contain integrin-associated transmembrane protein CD47 which can lower reticuloendothelial system (RES) clearance. However, EVs have limitations of low drug loading efficiency. In contrast, liposomes have several advantages as delivery system, but they have limited cellular uptake within cancer cells and also suffer from increased RES clearance. Thus, we are trying to develop a novel delivery system by fusing EVs and liposomes as well as exploit the CD47 properties of EVs isolated from adenocarcinomic human alveolar basal epithelial cells, A549 to escape from RES clearance to give enhanced anticancer effects.