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Purification and analysis of exosomes
Purificació i anàlisi d'exosomes.

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«Res en la vida és per ser temut, és només per a ser comprès. Ara és el moment d'entendre més, de manera que puguem témer menys».

Marie Curie

M'agradaria primer de tot donar les gràcies al Dr. Fernando Benavente per la seva paciència, ajuda i coneixement. Gràcies als companys de Bioanàlisi: a la Montse pels riures en les petites estones que coincidíem, al Roger per ajudar-me amb tots els dubtes que m'han anat sorgint al llarg del meu treball, i sobretot a l'Hiba per ajudar-me durant tota la meva estància al laboratori.

REPORT

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1. SUMMARY

Exosomes are 30-150 nm extracellular nanovesicles that can be found in biological fluids such as blood, urine, sweat, tears, etc. They are secreted by all types of cells through exocytosis processes. Exosomes contain a wide variety of biological relevant molecules such as lipids, proteins, mRNAs, and microRNAs. They are thought to function as messengers between cells due to their ability to endocytosis and membrane fusion. Therefore, they are involved in many physiological and pathological processes, including tumor initiation and immune response.

In this study, as an alternative to the techniques traditionally used to isolate exosomes, such as ultracentrifugation, it was explored polyethylene glycol (PEG) precipitation, which allows a facile, low-cost and effective isolation of exosomes from blood serum. Different variables of the method were studied, and the size of the isolated exosomes was measured by dynamic light scattering (DLS).

In addition, a novel capillary electrophoresis (CE) with ultraviolet (UV) detection method was developed to complement these particle size measurements. Different concentrations of hydroxypropyl cellulose (HPC, 0.2, 0.5, 0.8% v/v) were added in the background electrolyte (BGE, 0.1 M tris + 0.25 M boric acid pH= 7.9) to reduce the adsorption of the exosomes to the inner wall of the separation capillary. The best results were obtained with a 0.5% v/v of HPC in the BGE. In order to homogenize the charge of the exosomes and make the separation only size dependent 0.1% v/v of sodium dodecyl sulfate (SDS) was also added to the BGE. Under these optimized conditions, a characteristic electrophoretic profile of the isolated exosomes was obtained, and separation showed the highest reproducibility and shortest analysis times.

Keywords: Capillary electrophoresis (CE), coating, dynamic light scattering (DLS), electroosmotic flow (EOF), exosomes, extracellular vesicles (EVs).

2. RESUM

Els exosomes són nanovesícules extracel·lulars de 30 a 150 nm que es poden trobar en fluids biològics com la sang, l'orina, la suor, les llàgrimes, etc. Són secretades per tot tipus de cèl·lules mitjançant processos d'exocitosi. Els exosomes contenen una gran varietat de molècules biològiques rellevants com els lípids, les proteïnes, els ARNm i els microARN. Es creu que funcionen com a missatgers entre cèl·lules gràcies a la seva capacitat d'endocitosi i fusió de membrana. Per tant, estan involucrats en molts processos fisiològics i patològics, incloent l'inici de tumors i la resposta immune.

En aquest estudi, com a alternativa a les tècniques tradicionalment utilitzades per aïllar els exosomes, com la ultracentrifugació, es va explorar la precipitació mitjançant el polietilenglicol (PEG), que permet un aïllament fàcil, de baix cost i efectiu dels exosomes a partir del sèrum sanguini. Es van estudiar diferents variables del mètode i es va mesurar la mida dels exosomes aïllats mitjançant dispersió dinàmica de llum (DLS).

A més, es va desenvolupar un nou mètode d'electroforesi capil·lar (CE) amb detecció d'ultraviolada (UV) per complementar les mesures de mida de partícula. Es van afegir diferents concentracions d'hidroxipropil cel·lulosa (HPC, 0.2, 0.5, 0.8% v/v) a l'electròlit de separació (BGE, 0.1 M tris + 0.25 M àcid bòric pH= 7.9) per reduir l'adsorció dels exosomes a la paret interior del capil·lar de separació. Els millors resultats es van obtenir amb 0.5% v/v d'HPC al BGE. Per homogeneïtzar la càrrega dels exosomes i fer que la separació només depengués de la mida, es va afegir 0.1% v/v de dodecilsulfat sòdic (SDS) al BGE. En aquestes condicions optimitzades, es va obtenir un perfil electroforètic característic dels exosomes aïllats i la separació va mostrar la reproductibilitat més alta i els temps d'anàlisi més curts.

Paraules clau: Dispersió dinàmica de llum (DLS), electroforesi capil·lar (CE), exosomes, flux electroosmòtic (EOF), recobriment, vesícules extracel·lulars (EVs).

3. INTRODUCTION

3.1. EXOSOMES

In 1983 C. Harding et al[1] and B. T: Pan et al[2], independently, decided to study the pathway of transferrin receptors from the plasma membranes into reticulocytes. To do it they cultured immature red blood cells with labeled transferrin receptors. They observed that the labeled receptors go into reticulocytes forming vesicles of about 50 nm and these vesicles are secreted to the extracellular space. In 1989, R. Johnstone et al[3] called these extracellular vesicles (EVs) exosomes.

Exosomes are a type of EVs of about 30-150 nm size that are secreted to the extracellular space by most of cells. These nanoparticles are composed of a lipidic bilayer of about 5 nm of thickness. This bilayer can also contain different proteins that characterize the exosomes and can be used as targets for their isolation and quantification. Some of these proteins are common among all kind of exosomes regardless of the cell source that they come from. Another minority of proteins are cell-specific, depending on the type and conditions of the secreting cells. Furthermore, exosomes may contain inside other specific biomarkers, including other proteins, deoxyribonucleic acid (DNA) or ribonucleic acids (RNA), such as messenger RNA (mRNA) or microRNA[4,5,6,7].

Exosomes have received a huge attention in the last years due to their importance as messengers between cells and biological fingerprints of the cells from where they come from. Today, it is widely accepted that they are involved in different physiological and pathological processes, including cancer[4,5,6,7].

3.2. EXOSOMES ISOLATION

There are many techniques to isolate exosomes. In this section we will present some of the most important ones[4].

3.2.1. ULTRACENTRIFUGATION

Ultracentrifugation (UC) methods basically involve a serial centrifugation of the sample with different centrifugal forces and durations to isolate the exosomes from the rest of particles based on the density and size differences (Figure 1). The centrifugal forces range from 300 to $12 \cdot 10^4$ x g.

In general, there is a clean-up step (e.g. filtration) before the isolation to get rid of the large particles of the biological samples. As UC is a long procedure, protease inhibitors are typically added to prevent the degradation of exosomes proteins.

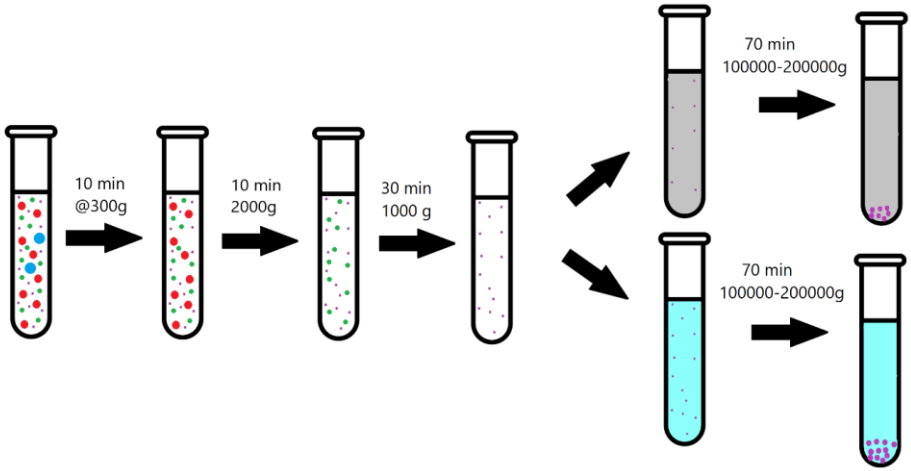

Figure 1. UC schema

Between each centrifugation step the supernatant is discarded, and the pellet is resuspended in an appropriate buffer. The centrifugal force is increased gradually.

UC is the most widely applied technique for the isolation of exosomes, but it has also disadvantages due to the required expensive specialized equipment, the heterogeneity of the exosomes, the long processing times and the extensive sample handling, which can cause contamination or loss. As an example, UC was studied by T. Baranyai et al[8] to isolate exosomes and by C. Lässer et al[9] to analyze RNA from exosomes isolated by UC.

3.2.2. SIZE BASED TECHNIQUES

Exosomes can be also isolated by sequential ultrafiltration (UF), using membrane filters with a defined molecular weight cut-off (MWCO) (i.e size exclusion pore size) (Figure 2)[10]. In general, before UF, cell debris and large particles are removed after a normal prefiltration step. This procedure was used by S. T. Peter et al[11] to isolate urinary exosomes.

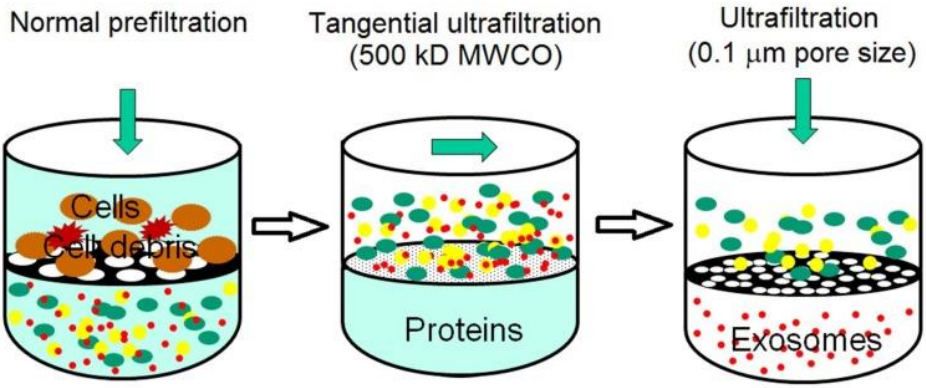

Figure 2. UF schema[10].

Size-exclusion chromatography (SEC) can be also used to isolate exosomes. In SEC macromolecules and particles are separated using a stationary phase with an appropriate pore size. Particles with sizes within the working range of the column are separated according to their size, while the smaller and larger particles are excluded[8].

3.2.3. PRECIPITATION WITH POLYETHYLENE GLYCOL

This isolation method is based on the precipitation of the exosomes with polyethylene glycol (PEG). The procedure consists in mixing a PEG solution with a sample containing the exosomes, followed by incubation at 4°C. Then the mixture is centrifuged at low speed to separate the pellet containing the exosomes from the supernatant.

The structure of PEG is commonly expressed as $H-(O-CH_2-CH_2)_n-OH$. It is a hydrophilic polymer with a variety of applications in medicine, chemistry, biology, industry, etc. It is widely used to precipitate EVs from biofluids. PEG wrap water molecules and force the less soluble particles to precipitate[4]. Several studies have demonstrated isolation of exosomes using PEG of different average molecular weights (MWs) and concentrations[5,6,7]. PEG allows a straightforward, low-cost, reproducible and effective isolation of exosomes from small volumes of sample. Furthermore, it does not require specialized equipment.

3.3. EXOSOMES ANALYSIS

3.3.1. NANOPARTICLE TRACKING ANALYSIS

Nanoparticle Tracking Analysis (NTA) uses the properties of both light scattering and Brownian motion to obtain particle size distribution in a liquid suspension. The mobility is related to the viscosity and temperature of the solvent; and it is not influenced by particle density or refractive index[12].

NTA applies a laser beam through the sample chamber. When the beam collides with the suspended particles the light scatters. The scattered light can be easily detected through a 20x magnification microscope with a video camera. It can be captured the Brownian movement of the light scattering points, which is to say “the random movement of particles in a liquid due to the bombardment by the molecules that surround them”[13]. The movement of the particles is analyzed individually. Later, applying the Stokes-Einstein equation, it can be calculated their hydrodynamic diameters[12].

NTA has been used to study nanoparticle toxicology, drug delivery, microvesicles[14], bacterial membrane vesicles, exosomes and other small biological particles[15,16]. It is the technique of choice to measure nanoparticle size but requires expensive specialized equipment.

3.3.2. ELECTRON MICROSCOPY

Electron microscopy (EM) can be used to visualize and characterize particles present in biological samples. It applies a beam of electrons creating an image of the particles studied. The electron beam goes through the sample generating a secondary electron beam that is collected and magnified by special lenses. EM is used for EVs visualization, and the diameters are measured from the collected images. EM demonstrates the presence of microvesicles and exosomes in biological fluids, but it is not quantitative and requires extensive sample preparation.

There are two main types of EM, transmission electron microscopy (TEM) and cryoelectron microscopy (cryoEM). The sample analyzed by TEM should be fixed and dehydrated before the measurement. Additionally, the image acquisition is carried out under vacuum conditions and the electron beam may also cause damage to biological samples. To avoid these problems cryoEM can be applied. This EM variant introduces a different protocol for sample preparation where the sample is kept and studied on vitreous ice at the temperature of liquid nitrogen[12,14].

EM has been applied to study all kinds of microvesicles[14], such as malignant ascites-derived exosomes[17] (Figure 3).

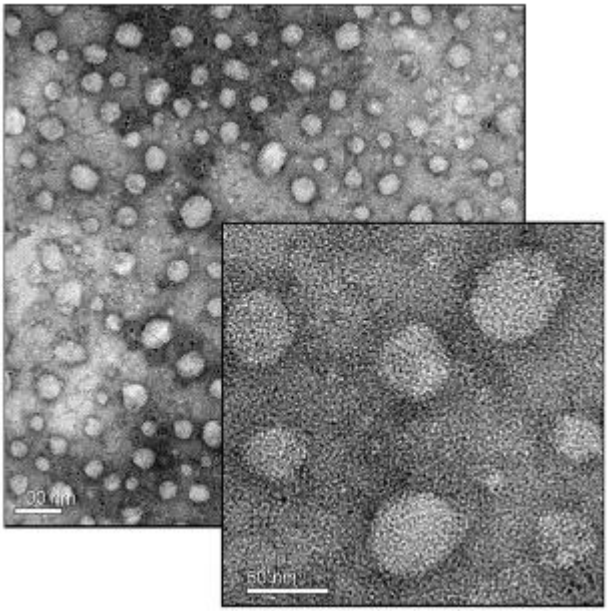

Figure 3. TEM image of exosomes from malignant ascites. Exosomes were defined as round shaped membrane vesicles in size 30-100 nm [17].

3.3.3. ATOMIC FORCE MICROSCOPY

Atomic force microscopy (AFM) detects and records the interactions between a probing tip and the surface of the sample. The surface is probed by a flat spring (cantilever) with a sharp tip mounted at its free end. When the tip is close to the sample the interaction of forces leads to a change on the cantilever course that is recorded by a photodiode detector.

One of the advantages of AFM includes the ability to measure the samples in their native conditions. We can obtain a real and high resolution 3-D surface topography of the samples by attaching the vesicles into flat surfaces as mica[12]. As EM, AFM also requires expensive specialized equipment. This technique has been applied to characterize nanovesicles from blood[18, 19].

3.3.4. DYNAMIC LIGHT SCATTERING (DLS)

Dynamic light scattering (DLS) is a very powerful and simple tool to study the diffusion behavior of macromolecules and dispersed particles in solution, which depends on their size and shape.

DLS has demonstrated to be able to assess the size distribution of EVs. It can provide the diameter range of the analyzed EVs but it is unable to give any information about their origin. DLS was applied by M. Wei et al[17] to analyze malignant ascites-derived exosomes and control culture media exosomes of by S. K. Wiedmer et al[20] to establish the size distribution of liposomes.

In DLS, which is also called photon correlation spectroscopy or quasi-elastic light scattering, a monochromatic laser beam goes through a suspension of particles. If the beam collides with a particle, the light is dispersed or scattered in all directions (Figure 4).

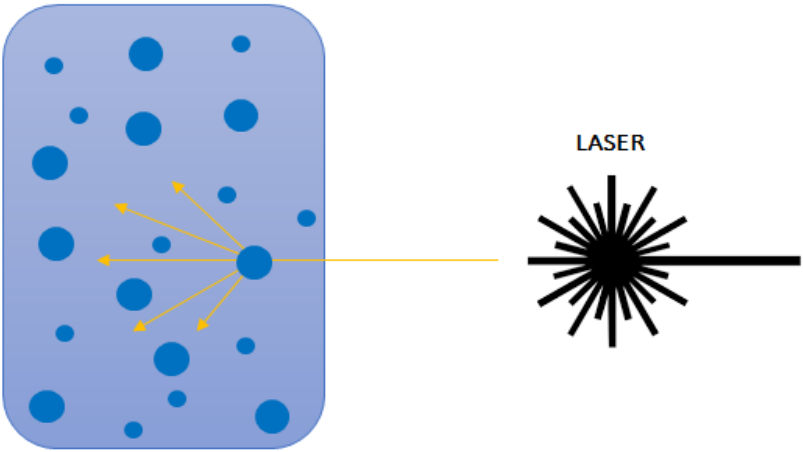

Figure 4. Representation of the scattered light

The fluctuations of the intensity can be shown as a result of the Brownian motion of the suspended particles. Small particles move faster than the large ones, and this is used to measure the particle size, taking into account very well-established theories.

During Brownian fluctuations, the distance between scattered light beams depends on the time. The light scattered by the different particles interfere between them, and it is showed in the recorded spectrum as minima or maxima (i.e. destructive and constructive interferences, respectively (Figure 5))[12,21,13].

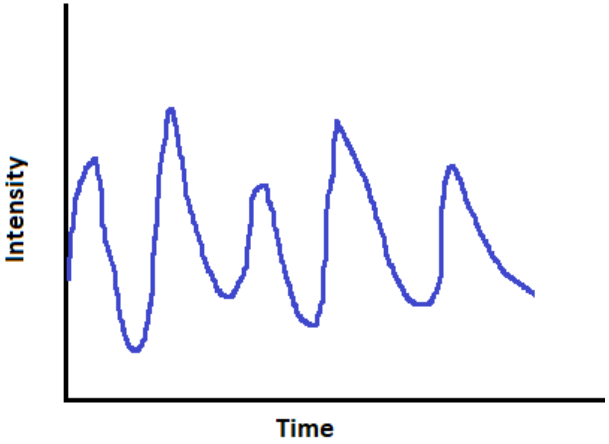

Figure 5. Fluctuation of the scattered light intensity during DLS due to constructive and destructive interferences.

The analysis is relatively easy when suspended particles do not interfere with each other through collisions or electrostatic forces. The size distribution of the particles is obtained from the autocorrelation function of the intensity that is generated[21].

In DLS we measure $G_2(\tau)$, an intensity correlation function that describes the particle movement and can be expressed as an integral over the product of intensities at time t and the delayed time $(t + \tau)$:

$$G_2(\tau) = < I(t) \cdot I(t + \tau) > \quad \text{(Eq. 1)}$$

Where τ is the lag time between two points, $G_2(\tau)$ can be normalized as:

$$g_2(\tau) = \frac{< I(t)\, I(t + \tau) >}{< I(t) >^2} \quad \text{(Eq. 2)}$$

We cannot know with precision how particles move in solution, but we can know the relative movement between each other by a correlated function of the electric field (E), $G_1(\tau)$.

$$G_1(\tau) = < E(t) + E(t + \tau) > \quad \text{(Eq. 3)}$$

$G_1(T)$ can be normalized as:

$$g_1(T) = \frac{< E(t)\, E(t+T) >}{< E(t) >^2} \quad \text{(Eq. 4)}$$

Combining the intensity and electric field correlations functions and considering that the scattering is homodyne (i.e. that the dispersion has a single frequency) and that the number of photons is a random Gaussian process we obtain:

$$g_2(T) = B + \beta |g_1(T)|^2 \quad \text{(Eq. 5)}$$

Where B is the baseline (~ 1) and β is the coherence factor depending on the area, optical alignment and scattering properties of the particles. Considering that the particles are monodisperse we have:

$$g_1(T) = e^{\Gamma T} \quad \text{(Eq. 6)}$$

Where Γ is the decay constant and it is directly related to the diffusion behavior (D_T):

$$\Gamma = -D_T q^2 \quad \text{(Eq. 7)}$$

Where q is the Bragg wave vector which is proportional to the refractive index of the solvent, n:

$$q = \frac{4\pi n}{\lambda} \sin\left(\frac{\theta}{2}\right) \quad \text{(Eq. 8)}$$

Where λ is the wavelength of the incident light and the θ is the angle at which detector is placed.

Therefore, equation 5 can be expressed as:

$$g_2(T) = 1 + \beta e^{-2D_T q^2 T} \quad \text{(Eq. 9)}$$

Equation 9 relates the particles motion with the measured fluctuations.

DLS instrument used in this study (Zetasizer Nano Malvern Instrument) employs a backscatter detection system at 173°. At this high scattering angle, the D_T can be directly obtained because the rotational diffusion contribution can be ignored. This translational diffusion coefficient depends on the concentration, it should be measured at different concentrations and extrapoled to infinite dilution. D_T is used to calculate the hydrodynamic radius (R_h):

$$D_T = \frac{K_B T}{6\pi\eta R_h} \quad \text{(Eq. 10)}$$

Equation 10 is known as Stokes-Einstein equation. Where K_B is the Boltzmann coefficient, T is the absolute temperature and η is the viscosity of the medium. Therefore, the light scattering relies on the viscosity and refractive index of the medium.

As we mentioned before, the particle size is related to the Brownian motion of particles through the Stokes-Einstein equation. Therefore, the correlation function $G_2(\tau)$ is also related to the particle size. The decay rate (eq. 7) is much faster for small particles than for large ones. (Figure 6).

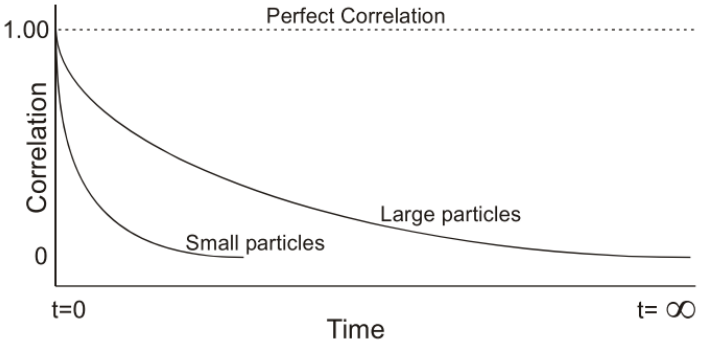

Figure 6. Representation of the correlation function $G_2(\tau)$ [13].

Once the correlation function $G_2(\tau)$ is measured, the information obtained is used to extract the decay rates for the different size classes by algorithms, and by this way the size distribution is obtained. The size distribution can be expressed in three different ways by intensity, volume and number. The fundamental size distribution obtained by DLS is the intensity distribution, and hence the R_h is deduced on these measurements. Volume and number distribution can be obtained applying Mie theory, which describes the scattering of light by a homogeneous sphere, to the size distribution by intensity. However these additional distributions must be used only as supporting information[13,22]. It is also worth remembering that the scattered Intensity depends on the size of the molecule. The scattered intensity by large particles is bigger compared to the little ones (Eq.9 and 10). This is because the intensity depends on the 6^{th} power of the particle size. Therefore, intensity is proportional to d^6 (where d is the particle diameter).

Another important parameter is the count rate (CR) that indicates the number of photons detected per second. To compare the signal strength of two different samples the derived count rate (DCR) can be calculated. The derived count rate is the measured count rate divided by the attenuation factor, which is automatically adjusted and changes between samples[23,24,25]: The larger the DCR value the higher the particle concentration.

3.3.4.1. SAMPLE PREPARATION

Sample preparation for size measurements by DLS involves specific preparation methods[13].

- Low concentration may cause light scattered not to be enough to make a measurement.
- High concentration may cause multiple scattering (i.e. the light scattered by a particle is scattered itself by another).

The sample with the dispersed particles should be slightly turbid, but the maximum concentration may allow free diffusion without particle interactions. The most appropriate concentration of the sample depends on the particle size. For exosomes (30-150 nm) Table 1 shows the minimum and maximum recommended concentrations, considering a sample density of 1 g/cm^3, and no difference between the refractive index of exosomes and water (i.e. 1.37 and 1.33)[26].

Minimum concentration	Maximum concentration
0.01 mg/ml (10^{-3} % m/m)	1% m/m

Table 1. Minimum and maximum concentrations for exosomes analysis by DLS.

Within this concentration range undesired particle interactions are avoided. Other item to keep in mind is that all the solvents for sample dilution should be filtered to avoid contaminations. Aqueous dispersants are generally filtered down to 0.2 μm. If possible, the samples are not filtered to avoid particle size distribution alterations. Similarly, ultrasonication should be only used to remove air bubbles or to breakup agglomerates if it is strictly necessary.

3.4. CAPILLARY ELECTROPHORESIS (CE)

Separation in capillary electrophoresis (CE) is based on the different migration of charged molecules (or particles) under the application of an electric field. Separation is typically carried out in 50-75 μm inner diameter fused silica capillaries. The use of these capillaries allows high efficient separations while reducing the effects of Joule heating thanks to the high electrical resistance and the large area-volume ratio of the capillary that guarantee an efficient heat dissipation. CE is a simple high-performance microscale separation technique that requires

simple instrumentation, small volume of reagents and sample and does not generate organic waste.

The operation is based on placing at each end of the narrow capillary a vial with background electrolyte (BGE), which is a buffer in most cases. In these reservoirs we have the electrodes too. After conditioning the capillary, the inlet BGE vial is replaced by the sample, which is injected into the capillary by the application of an electric field or pressure (a few nL). After placing again, the inlet BGE vial, the separation is performed by applying an appropriate voltage (between 10 and 30 kV). A schematic representation of a CE instrumental set-up is shown in Figure 7.

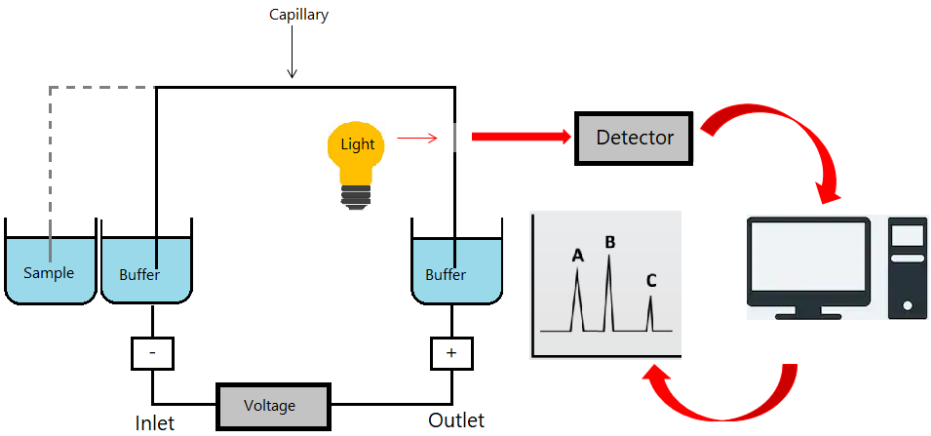

Figure 7. Schematic representation of a CE instrumental set-up.

In CE there are different modes of operation, including capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF), and capillary isotachophoresis (CITP).

Capillary zone electrophoresis method (CZE) is one of the most used because of his simplicity and versatility. This is the reason why it is widely named as CE. The separation in CE is due to migration of the ionic analytes in discrete zones at different velocities when the electric field is applied. This performance is expressed by:

$$v = \mu_e \times E \qquad \text{(Eq. 12)}$$

Where v is the ion velocity, μ_e is the electrophoretic mobility and E the electric field (V/cm). The μ_e is a characteristic of each ion and it is expressed as:

$$\mu_e \cdot \alpha = \frac{Fe}{Ff} \qquad \text{(Eq. 13)}$$

Where F_e is the electric force and F_{fis} the frictional drag force that the ions experience through the medium.

The ions move toward the electrode of the opposite charge according to their charge-to-radius (q/r) ratio, hence small and highly charged ions have large μ_e values. Furthermore, μ_e will depend on the composition and pH of the BGE.

There is another important factor which affects the apparent mobility ($\mu_{app)}$ of the different species in solution which is called electroosmotic flow (EOF).

The EOF is the flow of liquid through the capillary that it is generated in bare fused silica capillaries because of the negative charge of the ionized silanol groups (SiO^-) on the surface of the inner wall. After applying the electric field, a double layer is formed by the cations of the BGE, which are dragged to the cathode end. The movement of the solvated cations drag the solution towards the cathode generating the EOF. Therefore, the cationic analytes will be detected first because the μ_e towards the cathode and the EOF are in the same direction, the neutral analytes will be detected with the EOF, and the anionic analytes will be detected after the EOF, but only if the EOF is larger than their μ_e towards the anode end, which is in the inlet (Figure 8).

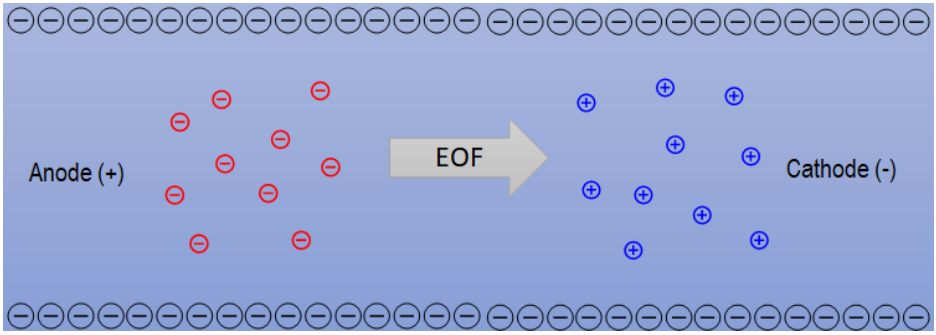

Figure 8. Analyte migration order under cathodic EOF.

The EOF varies with pH of the BGE. At high pH silanol groups are deprotonated (SiO-) and therefore the EOF is greater than at low pH where silanol groups are not ionized. The ionic strength of the BGE has also a great influence on the EOF because the double layer is compressed with high ionic strength and as consequence the EOF decreases.

The separation selectivity in CE can be also altered by changing the pH of the BGE, or by adding certain compounds. These additives are also useful to modify the EOF by dynamically coating the capillary wall. Additives can increase, decrease, suppress or reverse the charge of the inner wall and therefore the EOF. Below we can see some of the properties of some of the additives typically used.

ADDITIVE	EFFECT
Surfactants (Sodium dodecyl sulfate (SDS), Cetyl trimethyl ammonium bromide (CTAB), etc)	EOF modification; Solubilization of hydrophobic solutes; Ion pairing; Over CMC leads to MEKC.
Linear hydrophilic polymer (polyvinyl alcohol (PVA), polyethylene glycol (PEG), hydroxypropyl cellulose (HPC), etc)	EOF modification; minimization of sample adsorption; At high concentrations leads to CGE.

Table 2. Some additives typically used in CE[27].

Several authors have shown that CE can be used for the separation and characterization of different types of nanoparticles. Capillary gel electrophoresis (CGE) with fluorescence detection has been employed for the analysis of quantum dots nanoparticles using a polymer as sieving media. The BGE used was tris-boric acid at pH 8.8[28]. Capillary electrophoresis with ultraviolet detection (CE-UV) has been also used to analyze liposomes, using a mixture of dibasic sodium phosphate (Na_2HPO_4) and monobasic potassium phosphate (KH_2PO_4) at pH 7.4[29] and 3 mM phosphatidylcholine/polystyrene (POPC/PS) 80/20 mol/mol in 50 mM of N-(1,1-Dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid (AMPSO) at pH 8.3[20]. Viruses were also analyzed by CE with fluorescence detection using borax at pH 9.2[30,31]. However, to the best of our knowledge, the analysis of exosomes by CE has not been reported yet.

4. OBJECTIVES

The main objective of this study is to optimize the best conditions to isolate exosomes from human serum with PEG precipitation and to characterize the isolated exosomes.

The specific objectives of the study are:

- Evaluation of different concentrations of PEG for the isolation.
- Measurement of the size distribution of the isolated exosomes by DLS.
- Development of a novel CE-UV method for exosome analysis.

5. EXPERIMENTAL SECTION

5.1. INSTRUMENTATION

CE-UV experiments were performed with a 7100 CE instrument (Agilent technologies, Waldbronn, Germany) equipped with a photodiode array detector (DAD). Results were subsequently processed with OpenLab CDS C.01.07 SR3 ChemStation edition software (Agilent technologies). The DLS measurements were done with a Zetasizer Nano ZS apparatus ZEN2112 (Malvern Instruments, Malvern, UK). The centrifugation was performed with a Mikro 220R Hettich Zentrifugen. For measurements of pH a Micro pH 2002 potentiometer (Crison Instruments, Barcelona, Spain) equipped with a ROSS electrode 8102 (Orion Research, Boston, MA, USA) was used.

5.2. CHEMICAL AND REAGENTS

Water was purified by a Milli-Q gradient system from Millipore (Bedford, MA, USA) was used for all the experiments. All chemicals and reagents were analytical grade or better. As a precipitant we used PEG with an average MW of 8000 Da (PEG8000, 50% v/v, Fluka Biochemika). The following reagents were also used: sodium chloride (NaCl, Sigma Aldrich), potassium chloride (KCl, Sigma), disodium phosphate (Na_2HPO_4, Merck), potassium dihydrogen phosphate (KH_2PO_4, Sigma Aldrich), sodium hydroxide (NaOH), tris (J.T. Baker), boric acid (Merck), hydroxypropyl cellulose with an average MW of 80000 Da (HPC, Aldrich) and SDS (Promega).

5.3. PROCEDURES

5.3.1. SERUM PREPARATION

Blood from a healthy donor (44-year-old male) was collected in an 8.5 mL dry tube with clot activator (BD Vacutainer SST II advance REF366468). The blood samples were kept at room

temperature for 2 h, and then at 4°C overnight to improve the clot retraction. To separate the supernatant from the clot, the tubes were centrifuged at 1200 x g for 5 minutes at 4°C.

The serum obtained was collected with a micropipette (1 mL) into a 1.5 mL Eppendorf tube. Then, it was centrifuged at 1200 g for 20 min at 4°C. The pellet which formed in the bottom of the tube was discarded and the supernatant was collected. The serum was fractionated in 500 and 100 µL aliquots that were stored in the freezer at -20°C until their use.

5.3.2. BUFFER AND BACKGROUND ELECTROLYTE PREPARATION

The composition of the phosphate buffer saline (PBS) solution (pH 7.3) is described in Table 3.

Reagent	*Concentration (mM)*
NaCl	137
KCl	2.7
Na_2HPO_4	10
KH_2PO_4	2

Table 3. Composition of the PBS solution[35].

The BGE (pH 7.9) contained 0.1 M of tris and 0.25 M of boric acid[27].

5.3.3. EXOSOMES ISOLATION

Three 500µL human serum aliquots were thawed. PEG was added in different concentrations (5, 7.5 and 10% v/v) to each aliquot. Then, the samples were kept at 4°C for 2h followed by centrifugation at 3000 x g for 10 min at 4°C. The supernatant was discarded, and the pellet was washed three times with 50 µL of PBS. In each wash, the sample was centrifuged at 3000 x g for 3 min at 4°C to separate the pellet. The final pellet was resuspended in 125 µL of PBS [5,6,7]. Exosomes samples were diluted and analyzed immediately or were kept in the refrigerator at 4ºC for no longer than 48 h.

5.3.4. DLS

For DLS measurements a 1:10 v/v dilution in PBS was prepared for each sample (50 µL of the purified exosomes were diluted in 450 µL of PBS in a 1.5 mL Eppendorf). Diluted samples were analyzed as obtained, as well as after filtration (0.2µm nylon filters) or centrifugation (1250 x g for 3 min at 25 ºC).

A quartz cuvette cell Z2112 was used for all the experiments and 75 µL of sample were required for the analysis. Before adding the sample, the cuvette was washed 3 times with acetone and water and then dried. The backscatter employed was with a detection system at 173°. The equilibration time was 5s and the scan duration was 70s. Measurements were performed in triplicate at 25 ºC, setting the refraction index at 1.37 for exosomes and water as a dispersant.

5.3.5. CE

Fused-silica capillaries (Polymicro Technologies) of 75 µm internal diameter (i.d.) and 55 cm total length (LT) were used for all the experiments. The detection window was placed at 46.5 cm from the inlet of the capillary. All the solvents and solutions were filtered with 0.22 µm nylon filters.

Capillary activation:

New capillaries were activated by flushing (930 mbar) with the following solutions:

1. 20 min H_2O
2. 20 min NaOH 1M
3. 20 min H_2O
4. 20 min BGE

Preconditioning:

Between workdays capillaries were flushed with:

1. 5 min NaOH 1M
2. 5 min H_2O
3. 5 min BGE

Between runs the capillaries were flushed with:

1. 2 min NaOH 1M

2. 2 min H_2O
3. 2 min BGE

Injection and separation

Samples were analyzed at least in triplicate.

- Injection: Hydrodynamic injection, 50 mbar 10s.
- Temperature: 25 ºC of cassette and sample tray.
- Voltage: 25 kV (positive polarity).
- Analysis time: 60 min.
- The electropherograms were recorded at 210.4, 254.3, 280.4 and 585.4 nm. The UV-spectrum was stored between 200 and 600 nm at 4 nm intervals.

BGE for separation

1. BGE 1: 0.25 M tris + 0.1 M boric acid. (pH= 7.9)
2. BGE 2: (0.25 M tris + 0.1 M boric acid) + (0.2, 0.5, and 0.8 % v/v) HPC. (pH= 7.9)
3. BGE 3: (0.25 M tris + 0.1 M boric acid) + 0.5 % v/v HPC + 0.1 % v/v SDS. (pH= 7.9)

Postconditioning:

- Flush with water for 2 min.

6. RESULTS AND DISCUSSION

6.1. ISOLATION RESULTS

PEG precipitation was used to isolate the exosomes because of the simplicity and low-cost. Several authors have demonstrated isolation of exosomes using PEG of different MWs and concentrations[5,6,7]. In this study, a PEG with an average MW of 8000 Da at different concentrations (5, 7.5, and 10% v/v) was investigated. After incubation of the serum samples with PEG at 4°C for 2h, the expected pellet of exosomes was observed in all cases (Figure 9). From Figure 9, it can be seen that the precipitated exosomes slightly increased with PEG concentration until the highest amount was apparently obtained with 10% v/v of PEG.

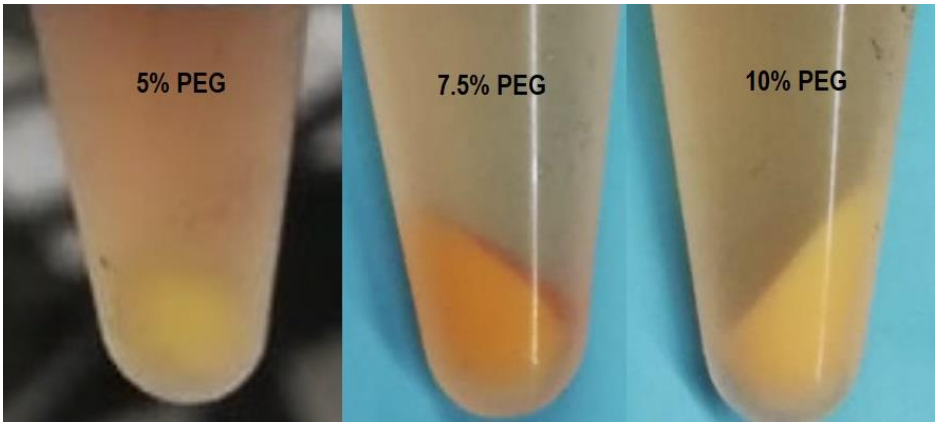

Figure 9. Isolation of exosomes from serum with different PEG concentrations.

6.2. DLS

DLS was used to measure the size distribution of exosomes that were isolated using different concentrations of PEG (5, 7.5 and 10% v/v). Since the solutions were very concentrated and turbid and not suitable for DLS measurements, the samples were diluted 1:10 v/v with PBS.

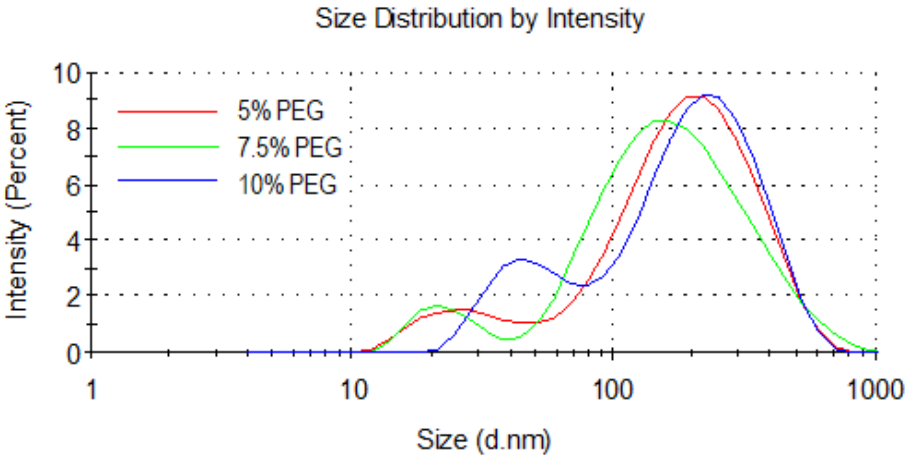

Figure 10. Size distribution by intensity of the exosomes by DLS. Sample dilution 1:10 v/v in PBS. Different concentrations of PEG.

As can be seen in Figure 10, nanoparticles were detected in the isolated exosomes samples with all the PEG concentrations. The results obtained were very similar and differences were within the experimental error of DLS. In the graph two distinct peaks could be observed. Considering the sample with 10% v/v PEG, the most intense peak corresponded to particles with sizes ranging from approximately 127 to 355 nm, and an average size of 240 nm. Meanwhile, the less intense peak corresponds to particles ranging approximately from 34 to 64 nm, with an average size of 49 nm. The measured sizes were slightly different than the typically reported sizes for exosomes (i.e. 30-150 nm). The differences must be due to the use of PEG, which may induce clumping or agglomeration of the largest exosomes (i.e. >64 nm). However, the existence of other large EVs in the samples could not be definitively discarded.

At this point, it is worth mentioning, as it was already explained in the introduction, that large particles scatter lighter because of the proportionality of the intensity with particle size. Intensity

is directly proportional to the 6th power of the particle diameter, hence, the intensity graph does not indicate the number of particles of a certain size, but the light intensity scattered by them.

The graph of size distribution by volume (Figure 11) shows the relative volume of particles in the samples. The results obtained were again very similar with the different concentrations of PEG, but now only a single intense peak could be clearly observed. As expected, considering the sample with 10% v/v PEG, this peak corresponded to the particles with sizes ranging from approximately 34 to 64 nm, and an average size of 49 nm, which were the most abundant in the samples. A similar conclusion can be drawn from Figure 12 that shows the relative number of particles in the samples.

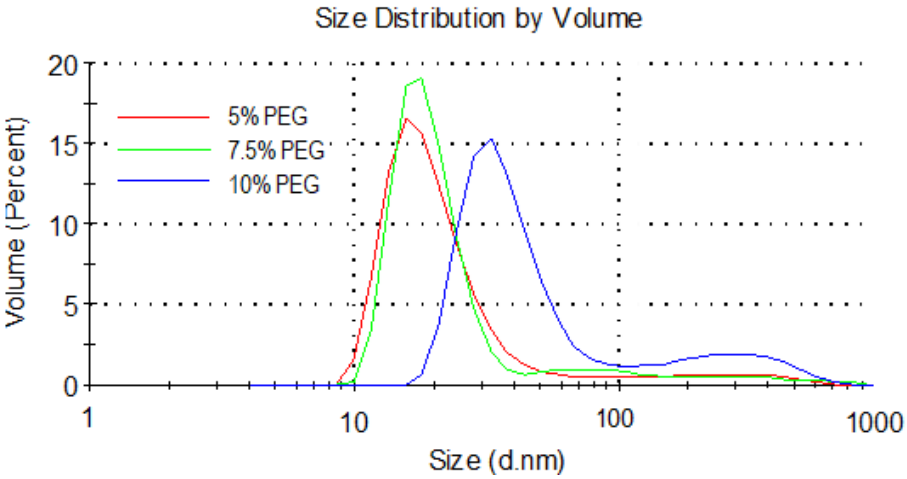

Figure 11. Size distribution by volume of the exosomes using DLS. Sample dilution 1:10 v/v in PBS. Three different concentrations of PEG.

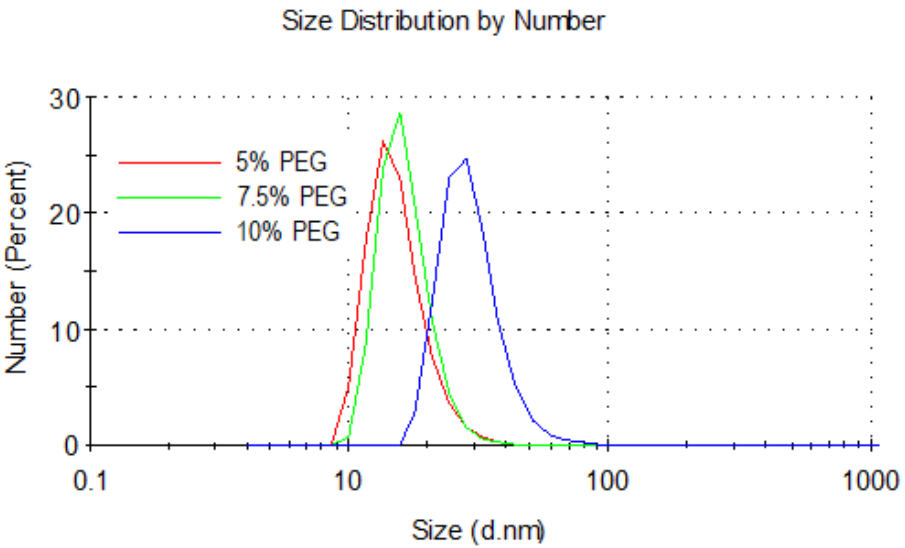

Figure 12. Size distribution by number of the exosomes using DLS. Sample dilution 1:10 v/v in PBS. Three different concentrations of PEG.

The effect of filtration and centrifugation of the samples was also investigated. Figure 13 shows the graph of size distribution by intensity for the raw samples with a 10% v/v PEG without any sample pretreatment, after filtration and after centrifugation.

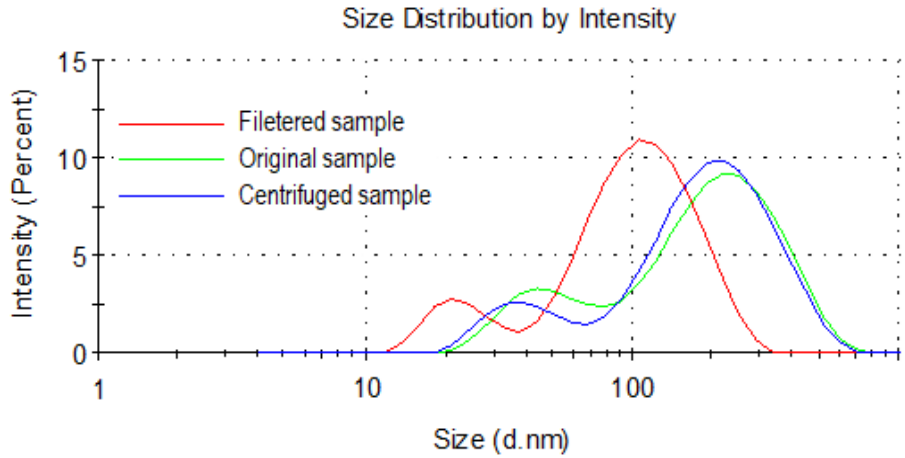

Figure 13. Size distribution by intensity of the exosomes using DLS. Sample dilution 1:10 v/v in PBS. Raw sample with 10% v/v PEG with different pretreatments.

As can be observed in Figure 13, similar results were obtained for the raw and centrifuged samples. In contrast, the particle size distribution was shifted to lower values for the filtered samples, suggesting that large particles were eliminated to a certain extent during the filtration step. The sizes measured for the particles of the filtered samples were ranging from approximately 67 to 171 nm, and an average size of 119 nm, and from 17 nm to 30 and an average size 24 nm. These sizes were within the size limits expected for exosomes (i.e. 30-150 nm) and would indicate the necessity of filtering the samples to get purer exosomes. Although after PEG precipitation the exosomes pellet was washed with PBS, the silky PEG film would keep wrapping the exosomes, forming aggregates (Figure 14). With the filtration process the exosomes aggregates would be eliminated and as a consequence the sample analyzed would contain mostly PEG-free exosomes.

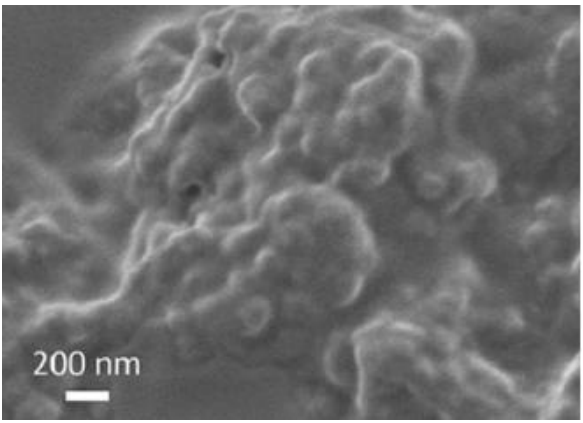

Figure 14. EM image. Multiple exosomes isolated from fetal bovine serum covered with silk-like PEG films[6].

In order to compare the particle concentration in the isolated exosomes samples with the different PEG concentrations and pretreatment procedures, the derived count rate (DCR) parameter was calculated. In general, the larger the DCR value the higher the particle concentration[23,24]. As can be observed in Table 4, the largest DCR parameter values were obtained for the raw and centrifuged samples with the different PEG concentrations, confirming that centrifugation was not significantly altering the original sample. The use of a 5% v/v of PEG

promoted a great decrease on particle concentration, compared to 7.5 and 10% v/v of PEG, confirming that exosome recovery was the lowest at the lowest PEG concentration (Figure 9). As expected, filtration promoted in all cases a great decrease on particle concentration, due to elimination of the large particles during the pretreatment step. Therefore, the elimination of the large exosomes aggregates with PEG is at a cost of decreasing the total exosomes recoveries. As the exosomes recovery was the highest with a 10% v/v of PEG without any sample pretreatment, these samples were used to develop a novel CE-UV method for exosome analysis.

Sample condition		DCR (kcps)
5% v/v	filtered	415
	centrifuged	7083
	normal	6908
7.5% v/v	filtered	8161
	centrifuged	47674
	normal	50580
10% v/v	filtered	3654
	centrifuged	50649
	normal	53414

Table 4. DCR parameter values for different PEG concentrations and sample pretreatments.

6.3. CE

Before analyzing the exosome samples by CE-UV, the Ohms' Law (Eq. 14) was studied with the BGE (0.1 M tris and 0.25 M boric acid, pH 7.9)

$$I = \frac{V}{R} \qquad \text{(Eq. 14)}$$

As can be observed in Figure 15, the Ohm's law was fulfilled within the studied voltage range, guaranteeing that the Joule heating was appropriately dissipated, as well as good separation efficiency and reproducibility.

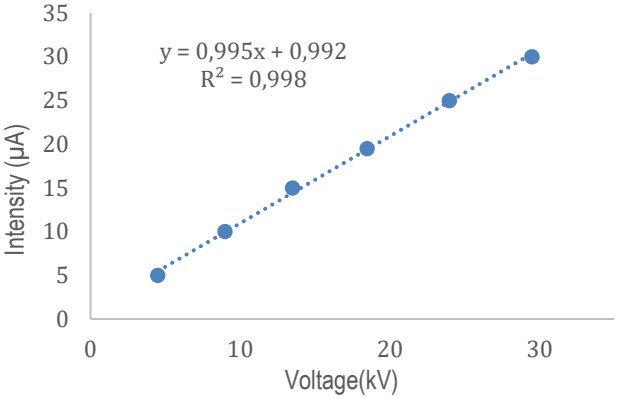

Figure 15. Representation of the Ohm's law for the CE system.

Three different separation conditions were investigated for the analysis of the exosome samples with 10% v/v PEG by CE.

The first results were obtained using the BGE of 0.1 M tris and 0.25 M boric acid (pH 7.9). As blank samples, PBS and PBS with 10% v/v PEG were analyzed. As it can be seen from the electropherogram of Figure 16, the results obtained under these conditions were not reproducible. Some peaks were detected in the electropherograms of the exosome samples that were not detected in the blanks, but results differed between the different replicates. Furthermore, the electrophoretic profiles did not correspond to the expected profiles for a mixture of nanoparticles with a relatively broad size distribution. These poor results may be due to the adsorption of the exosomes on the inner capillary wall of the bare fused silica capillary.

It is widely accepted that at physiological pH, which is close to the pH of the BGE used in this experiments, exosomes have a total negative charge[32]. Therefore, they are expected to migrate after the EOF. However, exosomes are zwitterions that present basic proteins on the surface. These proteins would be positively charged at the separation pH and they would interact

with the negatively charged silanol groups of the inner capillary wall. As a result, exosomes would be adsorbed and could not be detected.

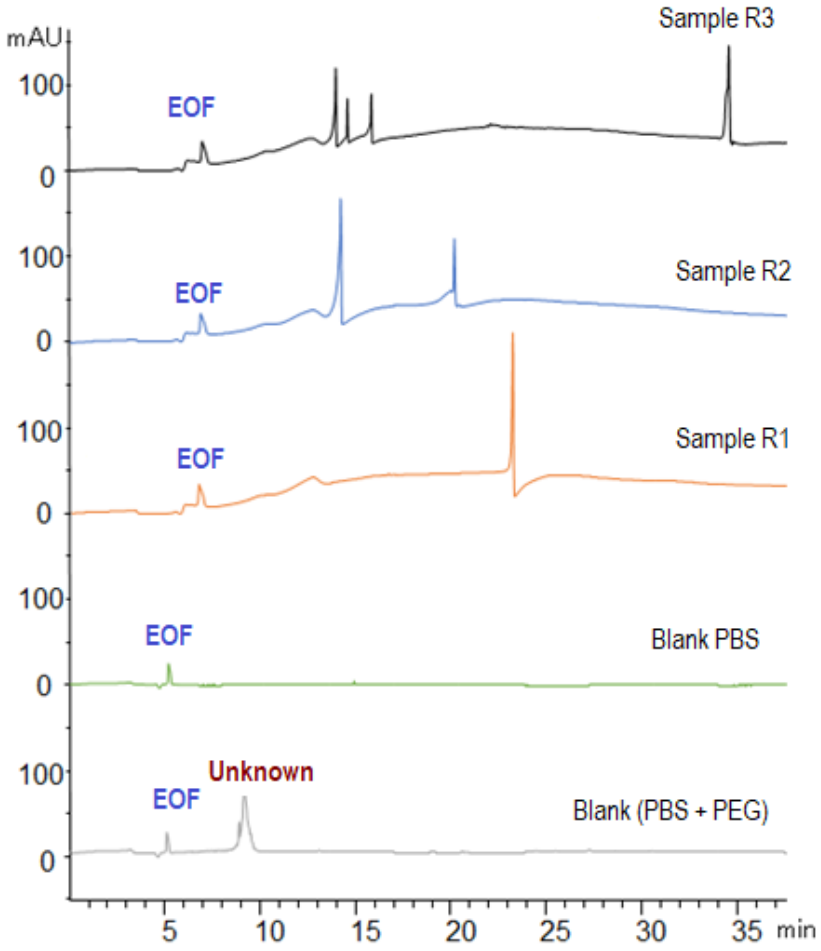

Figure 16. Electropherograms for three replicates of the exosome samples isolated with 10% v/v PEG and for the blank samples. BGE (0.1 M tris + 0.25 M boric acid, pH 7.9). Detection wavelength was 210.4 nm. A neutral marker solution (5% acetone) was analyzed to identify the EOF peak.

In order to reduce the adsorption of the exosomes to the ionized silanol groups, a dynamic capillary coating was investigated. This coating was done by adding different concentrations of

the neutral polymer HPC (0.2, 0.5 and 0.8% v/v) to the BGE. The results are shown in the Figure 17.

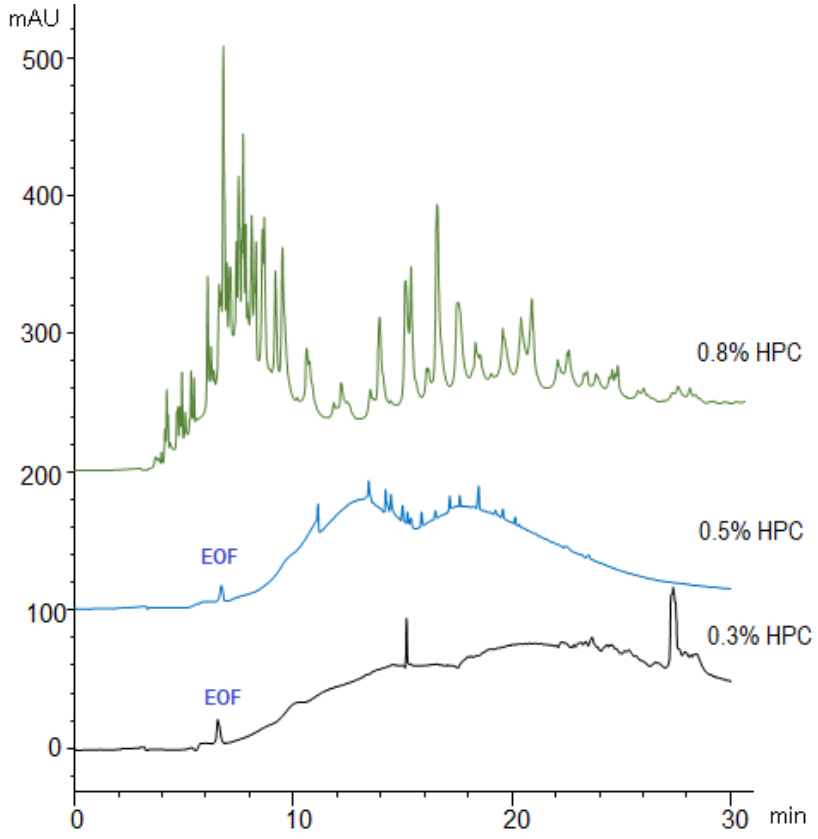

Figure 17. Electropherograms for the exosome samples isolated with 10% v/v PEG for the three HPC concentrations in the BGE (0.1 M tris + 0.25 M boric acid, pH 7.9). Detection wavelength was 210.4 nm.

As can be seen in Figure 17, as the concentration of HPC increased the total analysis time decreased. The results obtained were very different between the different concentrations of HPC. A very wide band was observed for the concentration of 0.3% v/v HPC. By increasing the concentration to 0.5% v/v results improved and two bands with a smaller width were clearly visible. These bands were dotted with very narrow peaks, which were randomly distributed along the bands when replicate analyses were done (Figure 18). Therefore, they were probably due to air

bubbles trapped in the heterogeneous mixture of exosomes with different sizes and total negative charges migrating after the EOF. The two bands were less clearly visible with a 0.8% v/v of HPC because the number of narrow peaks greatly increased, as a consequence of the increase of the viscosity of the BGE. As the best results in terms of reproducibility were obtained with a concentration of 0.5% v/v of HPC, this concentration was used for the rest of experiments.

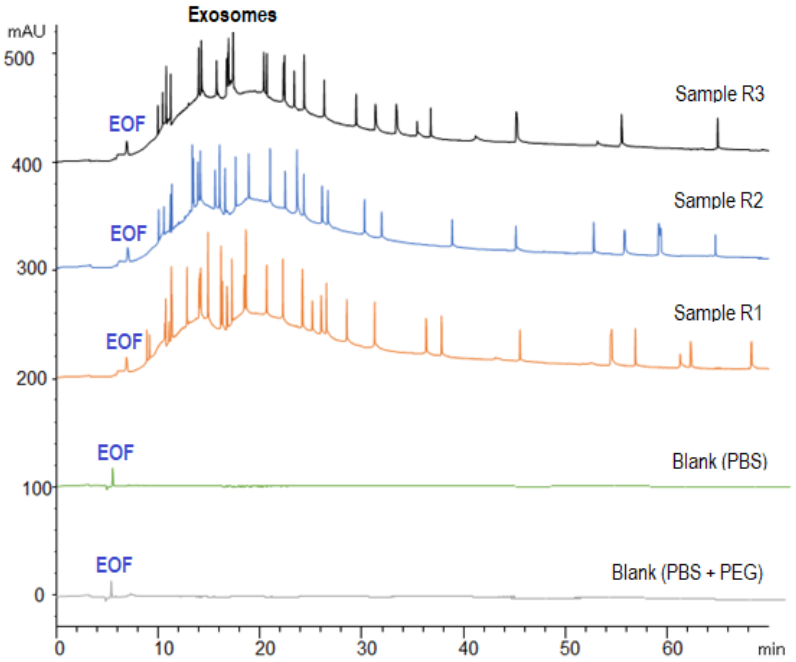

Figure 18. Electropherograms for three replicates of the exosome samples isolated with 10% v/v PEG and for the blank samples. BGE: (0.1 M tris + 0.25 M boric acid, pH 7.9) + 0.5% v/v HPC. Detection wavelength was 210.4 nm.

At this point, it is worth highlighting, that at the slightly basic pH value of the BGE (7.9), the small amount of HPC added in the BGE was not enough to significantly reduce the EOF (compare the EOF migration time in Figures 16 and 17)[33,34]. Therefore, while preventing the adsorption on the capillary, the EOF remained high enough for the negatively charged exosomes to migrate to the detector.

To further improve reproducibility, 0.1% v/v of SDS was added to the BGE with 0.5% v/v of HPC. The role of SDS was to homogenize the total negative charge of the exosomes as it is an anionic detergent. In this case the separation would be carried out depending on the exosome size.

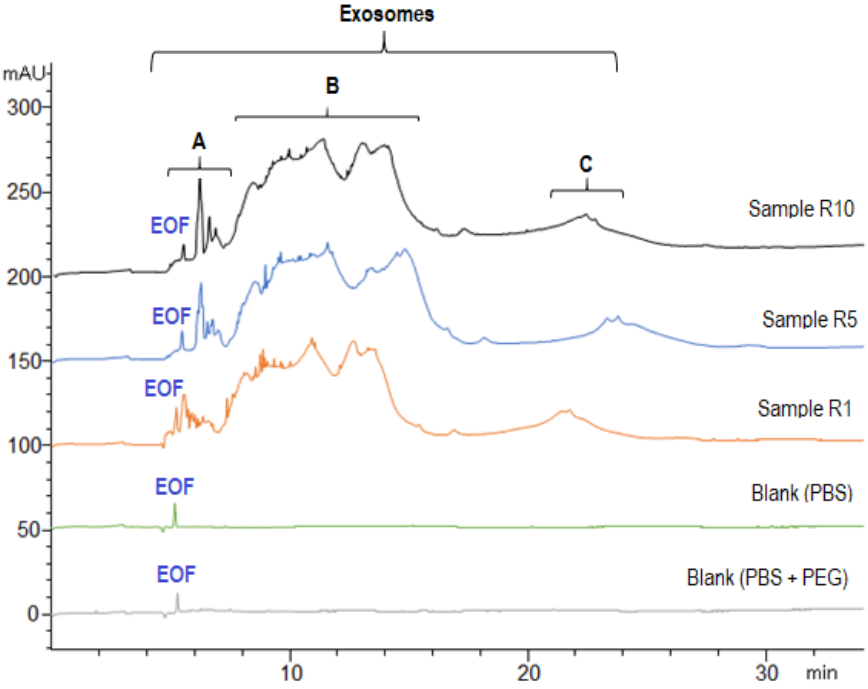

Figure 19. Electropherograms for the replicates 1, 5 and 10 of the exosome samples isolated with 10% v/v PEG and for the blank samples. BGE: (0.1M tris + 0.25M boric acid, pH 7.9) + 0.5% v/v HPC + 0.1% v/v SDS. Detection wavelength was 210.4 nm.

Figure 19 shows the electropherograms for ten replicate analyses of an exosome sample (replicates 1, 5 and 10) and for the blank samples under these conditions. As can be observed, exosomes were detected now as peaks and bands after the EOF and total analysis time was reduced to less than 30 min, air bubbles were not detected, and the electrophoretic profile was reproducible. The reduction of the detection time window, the elimination of air bubble artifacts and the enhanced reproducibility could refer to the fact that the total negative charge of the exosomes was more homogeneous after the addition of SDS, but it cannot be also discarded a positive effect on dispersing the exosomes or preventing adsorption on the inner capillary wall. Figure 20 shows the characteristic UV spectrum for the groups of peaks and bands labelled as A,

B and C in Figure 19. As can be observed, some differences could be observed between 210 and 250 nm, suggesting that different types of exosomes were detected. We are currently planning further experiments with commercial standards of exosomes from human serum and filtered PEG precipitated samples to improve the understanding of these findings.

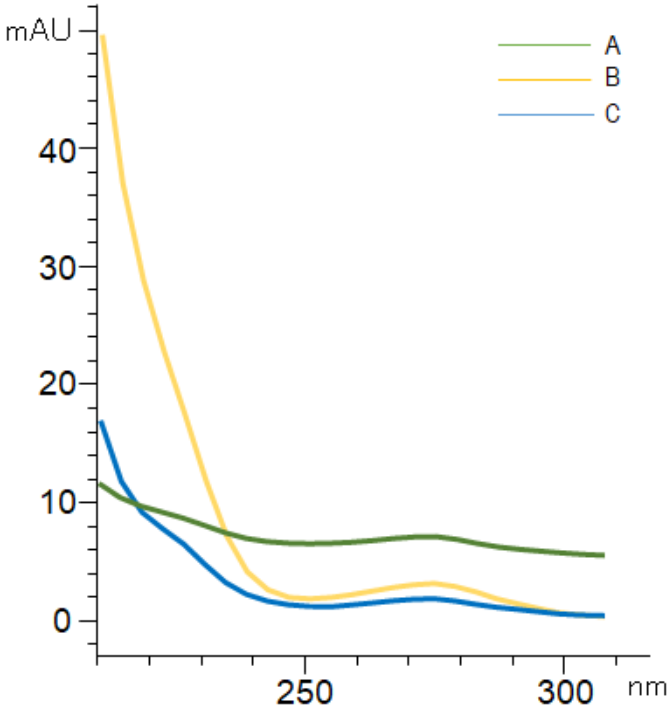

Figure 20. UV-Spectra for the groups of peaks and bands labelled as A, B and C in Figure 19.

Regarding the reproducibility of the results with different samples, they were similar for two exosome samples purified in two different days. CE analyses were also repeated after storing the samples in the refrigerator (4°C) for four days and the electrophoretic profiles changed. Therefore, as the lifetime of the samples seems to be quite short, analysis of the exosomes by DLS and CE-UV within the first 48 h after the isolation is recommended.

7. CONCLUSIONS

Precipitation with PEG was successfully applied for the isolation of exosomes from human serum samples. Different concentrations of PEG (0.5, 7.5 and 10% v/v) were used. The concentration of the isolated vesicles increased with the concentration of the PEG.

The size distribution of the isolated exosomes was measured by DLS, after diluting the samples 1:10 v/v with PBS. The most abundant particles in samples with 10% v/v of PEG showed an average size 49 nm, but 240 nm average size particles were also detected. Filtration allowed the elimination of the large particles, and the average sizes of the detected particles were shifted to 119 nm and 24 nm for the most abundant and less abundant particles, respectively. These sizes were within the size limits expected for exosomes (i.e. 30-150 nm) and would indicate the necessity of filtering the samples to get purer exosomes, but at a cost of decreasing the recoveries.

A novel CE-UV method for exosome analysis was developed using as a model the exosomes isolated with a 10% v/v of PEG without any sample pretreatment. It was observed that exosomes were very easily adsorbed on the inner capillary wall of the bare fused silica separation capillaries using a BGE of 0.1M tris and 0.25 M boric acid at pH 7.9. Addition of HPC and SDS to the BGE was investigated to reduce the adsorption. The most reproducible and shortest separations were obtained adding 0.5% v/v of HPC and 0.1% v/v of SDS. Under these optimized conditions, a characteristic electrophoretic profile of the isolated exosomes was obtained. Results were also reproducible for samples prepared in different days, but analysis within the first 48 h after the isolation is recommended. Different experiments are being planned with commercial standards of exosomes from human serum and filtered PEG precipitated samples to further the understanding about these electrophoretic fingerprints.

8. REFERENCES

1. Harding C, Heuser J, Stahl P. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J Cell Biol*. 1983;97(2):329-339. doi:10.1083/jcb.97.2.329
2. Pan BT, Johnstone RM. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. Cell 1983;33:967–978.
3. Johnstone RM, Bianchini A, Teng K. Reticulocyte maturation and exosome release: Transferrin receptor containing exosomes shows multiple plasma membrane functions. *Blood*. 1989;74(5):1844-1851. doi:10.1182/blood.v74.5.1844.bloodjournal7451844
4. Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in exosome isolation techniques. *Theranostics*. 2017;7(3):789-804. doi:10.7150/thno.18133
5. Vlassov A, Li M, Zeringer E, Conrad R. Methods and compositions for exosome isolation. 2014.
6. Weng Y, Sui Z, Shan Y, et al. Effective isolation of exosomes with polyethylene glycol from cell culture supernatant for in-depth proteome profiling. *Analyst*. 2016;141(15):4640-4646. doi:10.1039/c6an00892e
7. Antes TJ, Kwei K, Wu F. Methods for microvesicle isolation and selective removal. 2015.
8. Baranyai T, Herczeg K, Onódi Z, et al. Isolation of exosomes from blood plasma: Qualitative and quantitative comparison of ultracentrifugation and size exclusion chromatography methods. *PLoS One*. 2015;10(12):1-13. doi:10.1371/journal.pone.0145686
9. Lässer C, Eldh M, Lötvall J. Isolation and characterization of RNA-containing exosomes. *J Vis Exp*. 2012;(59):1-6. doi:10.3791/3037
10. Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in exosome isolation techniques. *Theranostics*. 2017;7(3):789-804. doi:10.7150/thno.18133
11. Peter S.T. Yuen, and Robert A. Star. Anita Cheruvanky1, Hua Zhou, Trairak Pisitkun, Jeffery B. Kopp MAK. *Rapid Isolation of Urinary Exosomal Biomarkers Using a Nanomembrane Ultrafiltration Concentrator*. Vol 292.; 2007. doi:10.1038/jid.2014.371
12. Szatanek R, Baj-Krzyworzeka M, Zimoch J, Lekka M, Siedlar M, Baran J. The methods of choice for extracellular vesicles (EVs) characterization. *Int J Mol Sci*. 2017;18(6). doi:10.3390/ijms18061153
13. Instruments M. Manual: Zetasizer Nano user manual (English) MAN0485. 2012.

14. Dragovic RA, Gardiner C, Brooks AS, et al. Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis. *Nanomedicine Nanotechnology, Biol Med*. 2011;7(6):780-788. doi:10.1016/j.nano.2011.04.003

15. Sokolova V, Ludwig AK, Hornung S, et al. Characterisation of exosomes derived from human cells by nanoparticle tracking analysis and scanning electron microscopy. *Colloids Surfaces B Biointerfaces*. 2011;87(1):146-150. doi:10.1016/j.colsurfb.2011.05.013

16. Gardiner C, Ferreira YJ, Dragovic RA, Redman CWG, Sargent IL. Extracellular vesicle sizing and enumeration by nanoparticle tracking analysis. *J Extracell Vesicles*. 2013;2(1):1-11. doi:10.3402/jev.v2i0.19671

17. Wei M, Yang T, Chen X, et al. Malignant ascites-derived exosomes promote proliferation and induce carcinoma-associated fibroblasts transition in peritoneal mesothelial cells. *Oncotarget*. 2017;8(26):42262-42271. doi:10.18632/oncotarget.15040

18. Yuana Y, Oosterkamp TH, Bahatyrova S, et al. Atomic force microscopy: A novel approach to the detection of nanosized blood microparticles. *J Thromb Haemost*. 2010;8(2):315-323. doi:10.1111/j.1538-7836.2009.03654.x

19. Ashcroft BA, De Sonneville J, Yuana Y, et al. Determination of the size distribution of blood microparticles directly in plasma using atomic force microscopy and microfluidics. *Biomed Microdevices*. 2012;14(4):641-649. doi:10.1007/s10544-012-9642-y

20. Wiedmer SK, Hautala J, Holopainen JM, Kinnunen PKJ, Riekkola ML. Study on liposomes by capillary electrophoresis. *Electrophoresis*. 2001;22(7):1305-1313. doi:10.1002/1522-2683(200105)22:7<1305::AID-ELPS1305>3.0.CO;2-Q

21. Stetefeld J, McKenna SA, Patel TR. Dynamic light scattering: a practical guide and applications in biomedical sciences. *Biophys Rev*. 2016;8(4):409-427. doi:10.1007/s12551-016-0218-6

22. Bhattacharjee S. DLS and zeta potential - What they are and what they are not? *J Control Release*. 2016;235:337-351. doi:10.1016/j.jconrel.2016.06.017

23. Nano Z, Apd T, Nano Z, Nano Z, Nano Z. What is the Derived Count Rate ?

24. Nobbmann U. Derived count rate – what is it? *Malvern Mater Talks*. 2015:9-12. http://www.materials-talks.com/blog/2015/06/11/derived-count-rate-what-is-it/.

25. Instruments M. Inform White Paper Dynamic Light Scattering. *Malvern Guid*. 2011:1-6.

26. Nobbmann U. *Refractive index-gold nanoparticles. Do refractive index and absorption matter for nanoparticles? faq: how important are refractive index absorption for nanoparticles?* 2014.

27. Heiger D. High-performance capillary electrophoresis. In: Agilent Technologies, ed. *High Performance Capillary Electrophoresis. An Introduction*. First ed. Germany: Agilent Technologies; 2000:84-85. doi:10.1039/9781847550521-00208

28. Song X, Li L, Qian H, Fang N, Ren J. Highly efficient size separation of CdTe quantum dots by capillary gel electrophoresis using polymer solution as sieving medium.

Electrophoresis. 2006;27(7):1341-1346. doi:10.1002/elps.200500428

29. Roberts MA, Locascio-Brown L, MacCrehan WA, Durst RA. Liposome behavior in capillary electrophoresis. *Anal Chem*. 1996;68(19):3434-3440. doi:10.1021/ac9603284
30. Mironov GG, Chechik A V., Ozer R, Bell JC, Berezovski M V. Viral quantitative capillary electrophoresis for counting intact viruses. *Anal Chem*. 2011;83(13):5431-5435. doi:10.1021/ac201006u
31. Azizi A, Mironov GG, Muharemagic D, Wehbe M, Bell JC, Berezovski M V. Viral quantitative capillary electrophoresis for counting and quality control of RNA viruses. *Anal Chem*. 2012;84(21):9585-9591. doi:10.1021/ac302525y
32. Deregibus MC, Figliolini F, D'Antico S, et al. Charge-based precipitation of extracellular vesicles. *Int J Mol Med*. 2016;38(5):1359-1366. doi:10.3892/ijmm.2016.2759
33. Tamisier-Karolak SL, Pagliarusco S, Herrenknecht C, et al. Electrophoretic behavior of a highly water-soluble dendro[60]fullerene. *Electrophoresis*. 2001;22(20):4341-4346. doi:10.1002/1522-2683(200112)22:20<4341::AID-ELPS4341>3.0.CO;2-I
34. Mitnik L, Salomé L, Viovy JL, Heller C. Systematic study of field and concentration effects in capillary electrophoresis of DNA in polymer solutions. *J Chromatogr A*. 1995;710(2):309-321. doi:10.1016/0021-9673(95)00500-5
35. Burkova EE, Grigor'eva AE, Bulgakov D V., et al. Extra Purified Exosomes from Human Placenta Contain An Unpredictable Small Number of Different Major Proteins. *Int J Mol Sci*. 2019;20(10):1-16. doi:10.3390/ijms20102434

12. ACRONYMS

AFM: Atomic force microscopy

AMPSO: N-(1,1-Dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid

BGE: Background electrolyte

CE-UV: Capillary electrophoresis with ultraviolet-visible detection

CGE: Capillary gel electrophoresis

CIEF: Capillary isoelectric focusing

CITP: Capillary isotachophoresis

CMC: Critical micelle concentration

Cryo-EM: Cryo-electronic microscopy

CTAB: Cetyl trimethyl ammonium bromide

CZE: Capillary zone electrophoresis

DCR: Derived count rate

DLS: Dynamic Light Scattering

DNA: Deoxyribonucleic acid

EM: Electronic microscopy

EOF: Electroosmotic flow

EVs: Extra-vesicles

HPC: Hydroxyl propyl cellulose

i.d.: Internal diameter

LT: Total length

MEKC: Micellar electro-kinetic chromatography

MWCO: Molecular weight cut-off

NTA: Nanoparticle tracking analysis

PBS: Phosphate-buffered saline

POPC: Phosphatidylcholine Persistent organic pollution

PS: Polystyrene

PVA: Polyvinyl alcohol

q/r: Charge-to-ionic radius ratio

RNA: Ribonucleic acid

SDS: Sodium dodecyl sulfate

SEC: Size exclusion chromatography

TEM: Transmission electronic microscopy

Tris: Tris(hydroxymethyl)aminomethane