Application Note

AN2501

Multi-parameter quality assessment of exosomes by using specific tetraspanin staining combined with zeta potential analysis

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Abstract

When extracellular vesicles (EVs) are purified for research, produced as standards for biomedicine, or commercialized as safe and effective therapeutics from specific cells, several quality attributes must be ensured. Nanoparticle tracking analysis (NTA) is very well suited for EV quality control because it works on the single particle level. However, due to the lack of specificity in scatter-based NTA, different fractions of particles, also called subpopulations, that differ in the composition of their surface molecules cannot be detected within the total number of particles. In this study, we therefore characterized a sample of CD63 e-GFP overexpressing HEK293 EVs from HansaBiomed with fluorescence-based NTA (F-NTA) to specifically determine the CD63 e-GFP positive fraction within the total number of particles. Since we were able to detect only slightly more than 50% of CD63 e-GFP positive EVs, we further increased the specificity of the analyses by using the Particle Metrix F-NTA EV Tetraspanin Detection Kit 520, which is based on antibodies directed against the exosome markers CD9, CD63 and CD81. In this kit, all three surface markers were conjugated with the same fluorophore (PAN staining) in order to specifically detect typical exosomes. Thus, we benefit from the detection of a second particle fraction and were able to distinguish the subpopulation of CD63 e-GFP-positive particles from the PAN-stained exosomes using F-NTA.

In detail, we performed in-depth EV characterization to analyze **size distribution, concentration, colocalization** and **zeta potential** of individually fluorescence labelled EV subpopulations by using one single measurement protocol in the ZetaView® instrument.

PAN staining resulted in a very efficient detection of exosomes. PAN- and e-GFP- labelled particles exhibited a smaller diameter (105.8nm & 92.1nm) compared to the total particle population (122.1nm) assessed by scatter based NTA and colocalization measurement revealed 45% particles positive for CD63 e-GFP and PAN staining. In addition, exosomes detected in both fluorescence channels revealed different zeta potential (-19.5mV) compared to the total particle count (-24.2mV). Using the Particle Metrix F-NTA EV Tetraspanin Detection Kit 520, we could clearly distinguish fluorescently labelled exosomes from the CD63 e-GFP positive EV fraction. For zeta potential analyses, PAN exosome tetraspanin staining turned out to be necessary to provide an additional differentiating quality marker that proved colloidal stability of the characterized EV standard.

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Introduction

Extracellular vesicles (EVs) are a heterogeneous group of membrane-bound nanoparticles that include endosomes, exosomes and cell membrane-derived microvesicles [1]. Due to their importance in intercellular communication, they are attracting increasing interest in biomedical research as they have been proven to act as key players in various diseases. However, they are also considered as drug carriers and are therefore intensively studied for their therapeutic potential [2-6]. The focus is mainly on a subfraction of EVs, namely exsomes, which, in contrast to microvesicles and apoptotic bodies, belong to the small EVs with a size range between 30-150nm [1, 2]. If exosomes are used for research or as a medical vehicle during therapy, quality and quantity assessment of the purified particles is essential. Therefore, comprehensive quantification of the biophysical properties, such as size and concentration are indispensable factors for both, characterization and subsequent use of EVs [7]. In the last decade, nanoparticle tracking analysis (NTA) has become one of the most common methods for size and concentration measurement of exosomes and extracellular vesicles [8, 9]. Although well established, NTA was primarily used in the scatter mode in the past, which meant that no distinction could be made between EVs and other nanoparticles, such as protein aggregates or cell debris within their size range [10]. Advanced NTA devices such as the ZetaView® models PMX-130, PMX-230 and PMX-430 from Particle Metrix (Germany) extend EV analysis on single particle level with fluorescence capability (F-NTA) [11] to characterize individual EV subpopulations stained with antibodies within a sample fraction in terms of size and concentration. The number of detections representing EVs after staining with specific fluorescently labelled antibodies can be measured using individual fluorescence channels. In addition, colocalization NTA (C-NTA) provides far more bio-specific information by rapid detection of different fluorescence signals from phenotypically distinct EVs within the sample in only one single measurement [12].

One additional physical property of EVs that can be determined by means of a further expansion stage of the NTA method is the surface charge, which is described by the zeta potential (ZP). ZP has gained increasing interest as an additional factor for assessment of the stability and integrity of EVs in suspension [13-17]. The zeta potential of EVs is determined by their surface chemistry and therefore mainly influenced by their lipid and protein composition. A charge of at least ± 30mV indicates complete colloidal stability [18, 19]. The zeta potential is therefore an excellent tool to study colloidal stability and represents a promising method to investigate quality of EVs in biological processes.

We performed a comprehensive characterization of the CD63 e-GFP overexpressing HEK293 EV standard from HansaBiomed by determining their size, concentration and zeta potential after labelling all particles with the Particle Metrix F-NTA EV Tetraspanin Detection Kit 520. Using F-NTA, subpopulations of the particles could be clearly distinguished from each other in individual fluorescence channels and from the scatter signal. Similar results were observable in zeta potential analyses. Colocalization measurements showed that 45% of the EVs were positive for CD63 e-GFP and at least one other surface marker from the PAN exosome staining. With these results, we were able to analyze the composition of the sample in

much more detail than with classical flow cytometry, which neither allows accurate measurement of size and size distribution nor does it provide insight into the colloidal stability by analysis of surface charge at single particle level.

Methods

Extracellular vesicles used:

For this study, we used lyophilized fluorescent EVs from HEK293 cells (CD63 e-GFP) provided by HansaBiomed (#HBM-HEK-EGFP63). 100µg of the EVs were reconstituted with 100µl particle free distilled water and stored according to the manufacturer's recommendations until further use.

Fluorescence labelling:

For fluorescence labelling, the Particle Metrix F-NTA EV Tetraspanin Detection Kit 520 was used. 1µl of the dissolved EVs were stained according to the instructions provided in the labelling kit. For more accurate zeta potential measurements however, 10% PBS was used instead of 100% PBS.

Detergent controls were prepared in the same way as tetraspanin PAN staining. However, after incubation, 10µl aliquots of the labelled EVs were supplemented with 10µl of 1% NP-40 solution resulting in a final concentration of 0.5% NP-40. After incubation for 30 minutes at room temperature in the dark, each lysed cocktail was supplemented with 980µl 10% PBS resulting in a final measurement volume of 1000µl.

NTA, colocalization and zeta potential measurements:

NTA measurements of size and concentration, colocalization and zeta potential were carried out using a Particle Metrix ZetaView® PMX-430 QUATT instrument equipped with the Particle Metrix Software Suite (PMSS) 1.4 including ZetaNavigator software version 1.4.7.6. and Particle Explorer software version 4.3.4.4.

Additional controls and the used measurement script which consists of 7 measurement specifications can be found in the supplementary data (Link: https://lmy.de/UPLaF).

Results

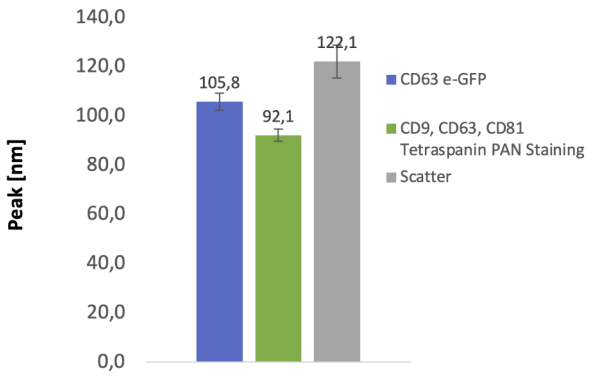
Characterization of EVs via size and concentration measurements

NTA measurements of the HEK293 CD63 e-GFP EVs in scatter mode resulted in a peak diameter of 122.1nm and a total concentration of $5.4x10^{10}$ particles per ml (see figure 1A+B). To specifically characterize EV phenotypes in terms of size and concentration, we analyzed the sample via F-NTA. The CD63 e-GFP positive fraction, detected in the 488F500 channel, revealed a peak diameter of 105.8nm (figure 1A) and a concentration of $3x10^{10}$ particles per ml (figure 1B). This is in good agreement with the concentration given by the manufacturer and corresponds to a proportion of 54.2% of e-GFP positive particles in relation to the total particle count in the scatter mode (figure 1C). Again, this finding fits pretty well with 40-60% yield of fluorescent particles stated by HansaBiomed.

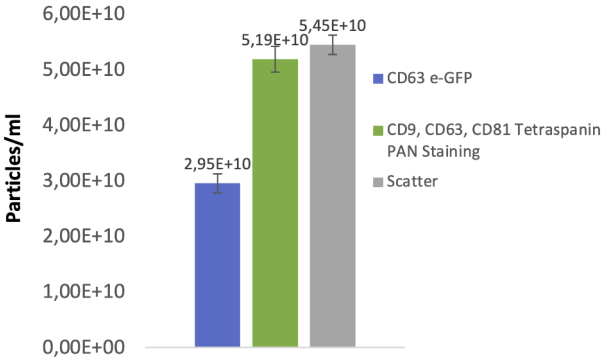
Tetraspanin PAN staining using the EV-typical surface markers CD9, CD63 and CD81 was analyzed in the 520F550 fluorescence channel

and showed a peak diameter of 92.1nm (figure 1A) and resulted in 5.2x10[10] particles per ml (figure 1B). This is a significant higher particle count compared to the e-GFP positive particles and corresponds to 95.2% labelled EVs relative to the scatter signal (figure 1C) proving an excellent purity of the exosome standard. In general, the fluorescent populations were smaller in size than the total particle population detected in scatter mode, indicating the presence of larger particles which are presumably non-EVs.

A



B



C

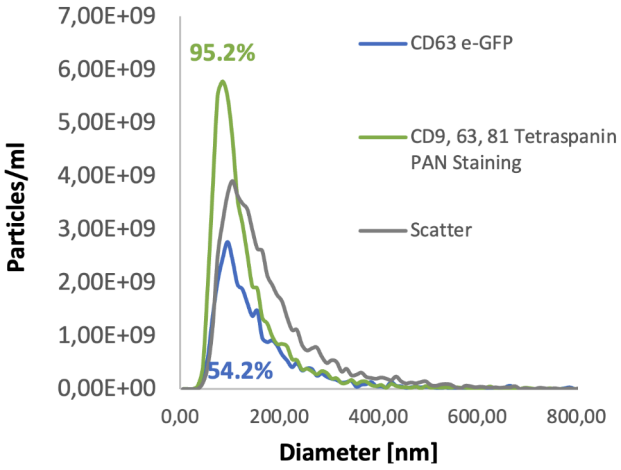


Figure 1: ***A:*** *Peak diameter of e-GFP EVs and EVs subjected to fluorescence tetraspanin PAN staining. Total particle count in scatter mode reveals slightly larger particles than in F-NTA.* ***B:*** *Particle concentration of the e-GFP positive EV fraction and EVs positive for fluorescence tetraspanin PAN staining.* ***C:*** *Size distribution of e-GFP- and tetraspanin PAN stained EVs. e-GFP and PAN stained EVs show a fraction of 54.2% and 95.2% relative to the scatter signal.*

Colocalization studies via C-NTA

For more detailed insights into the particle fraction exhibiting CD63 e-GFP and PAN stained EVs, we performed an additional approach for EV characterization via colocalization-NTA (C-NTA) which represents a further development of F-NTA [12]. Our studies refer to the detection of the co-presence of CD63 e-GFP on the one hand and CD9, CD63 and CD81 stained with the F-NTA EV Tetraspanin Detection Kit 520 on the other hand.

Based on a total count of 828 particles, C-NTA revealed 45% of the fluorescent EVs to express CD63 e-GFP as well as at least CD9, CD63 or CD81, indicated by the positive PAN staining

signal. Looking at the two fluorescence channels separately, 46% and 9% were found to be single positive for CD63 e-GFP and tetraspanin PAN staining, respectively (figure 2).

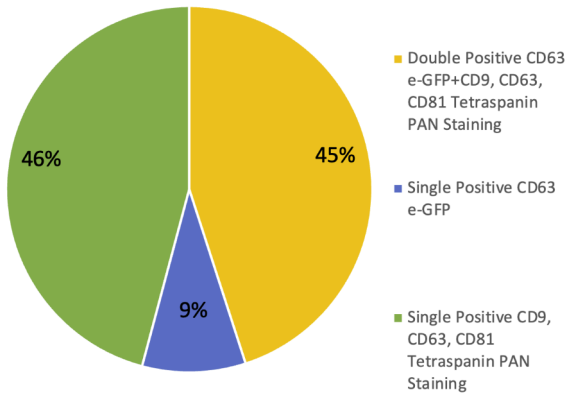


Figure 2: *Pie chart showing the result of the colocalization analysis of the tetraspanin labelled HEK293 CD63 e-GFP EVs. 45% of fluorescent particles are considered double positive for e-GFP and PAN staining whereas 46% or 9% appear single positive for PAN staining or e-GFP, respectively.*

Characterization of EVs by zeta potential analysis

To further examine the quality of the isolated EV fraction, we performed a zeta potential measurement. In a dispersed system, such as a colloidal dispersion of EVs, the net surface charge of the nanoparticles, indicated by the zeta potential, is an important parameter in determining the stability which is a crucial factor for downstream EV applications, including therapeutic approaches. By determining the electrophoretic mobility of fluorescently labelled exosomes, zeta potential was found to be -19.5mV in both subpopulations detected in the individual fluorescence channels (figure 3). In contrast, total particle population in the scatter mode revealed significant lower zeta potential of - 24.2mV. This result shows that F-NTA, in combination with zeta potential determination of the exosomes in the individual fluorescence channels can clearly distinguish CD63 e-GFP-positive EVs and tetraspanin PAN stained particles positive for either CD9, CD63 or CD81 from the total particle concentration detected in scatter mode.

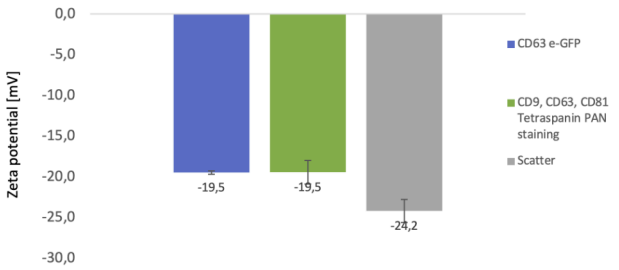


Figure 3: *Zeta potential of e-GFP and PAN staining positive EV subpopulations detected in both fluorescence channels (488F500 & 520F550) as well as overall particles of the sample analyzed in the scatter mode. The subfraction of the genuine EVs in fluorescence shows significant higher zeta potential than the total particle population detected in the scatter mode.*

Summary and Conclusion:

Size and concentration

Nanoparticle tracking analysis using the ZetaView® models from Particle Metrix has made a significant leap forward with the implementation of fluorescence and colocalization analyses, particularly in biomedical research. Since this technological advancement, the limitation of mere scattered light measurement, which only allows an estimate of the total number of particles, no

longer exists. Individual subpopulations of EVs can now be specifically analyzed after fluorescence staining and by using multiple fluorescence channels. In combination with the capability to determine the zeta potential of nanoparticles via analysis of the electrophoretic mobility, current nanoparticle analysis with the ZetaView® is perfectly suited for comprehensive characterization of EVs via size, concentration, screening of surface markers and colloidal stability.

For this study, we used a commercially available EV standard from HansaBiomed to perform in-depth characterization of exosomes at single particle level using NTA. The EVs already expressed e-GFP on CD63, so that they were clearly visible as a subpopulation in the 488F500 fluorescence channel as a fraction of 54.2%. Counterstaining with the F-NTA EV Tetraspanin Detection Kit 520 (PAN staining) increased the scope of characterization of the sample and further specified the phenotype of the particles with regard to the typical tetraspanin markers CD9, CD63 and CD81. The fraction of 95.2% of the particles positive for PAN staining relative to the scatter signal can be considered as genuine exosomes, as they were detectable as a second subpopulation in the 520F550 fluorescence channel. Thus, the F-NTA EV Tetraspanin Staining Kit 520 demonstrated that a very high percentage of the EV standard consists of exosomes that could not be detected by the intrinsic CD63 e-GFP signal alone. In addition to CD9 and CD81-positive membrane particles, labelling with the F-NTA EV Tetraspanin Detection Kit 520 most likely also included staining of CD63-positive exosomes, which are presumably free of e-GFP and therefore could not be detected in the fluorescence channel 488F500. Thus, PAN counterstaining with the F-NTA EV Tetraspanin Detection Kit 520 is justified in order to also detect CD63 surface molecules that are not e-GFP positive. In general, these results not only demonstrate that the percentage of CD63 e-GFP positive particles measured via F-NTA is consistent with the data provided by HansaBiomed (40%-60% CD63 e-GFP positive), but the CD9, CD63, CD81 PAN staining also provides evidence that the sample used is an EV standard with very high purity.

Colocalization

A relatively new achievement in NTA technology is the colocalization measurement (C-NTA). Basically, C-NTA represents the ability to prove the co-existence of two markers on one particle, analyzed in one run. A colocalization event occurs when a particle carrying two different fluorescent labels elicits detections in both channels within a certain proximity range [12]. The colocalization analyses performed in this study extend the scope of characterization of the EV standard from HansaBiomed in terms of how many CD63 e-GFP positive EVs carry at least one additional tetraspanin-typical marker that can be detected by the fluorescence signal of the PAN staining. These experiments provide information on the molecular composition of the membrane surface and thus enable further assignments as to which subpopulation is present in terms of co-existence of surface markers. Of all fluorescently labelled EVs, we were able to detect 45% that were double positive for CD63 e-GFP as well as for CD9, CD63 and CD81 PAN staining (see figure 2). Since the three typical EV markers were conjugated to the same fluorophore, more precise insights into which EV subpopulation carries an individual marker cannot be carried out, as the F-NTA EV Tetraspanin Detection Kit is designed to specifically detect and screen for exosomes in

the sample and to distinguish them from other non-EV particles. However, we estimate that most colocalization events occur on those particles carrying the CD63 antibody from the tetraspanin EV PAN staining, since around 45% of the exosome population is already CD63 e-GFP positive (see figure 2).

Zeta potential

The membrane of extracellular vesicles is a highly dynamic and interactive surface responsible for the interaction of EVs with the extracellular environment [20]. Therefore, depending on the cellular origin, the zeta potential of EVs varies widely and is determined by both the content and distribution of lipids, proteins and carbohydrates. It is assumed that the mostly negative charge detected on EVs is associated with the side chains of various ligands and receptors such as aspartate, lysine, arginine and histidine [21-23].

With a zeta potential of -19.5mV in the 488F500 and 520F550 fluorescence channels, we were able to characterize genuine extracellular vesicles in our experimental approach not only in their surface charge, but also clearly distinguish them from the total particle population detected in scatter mode (- 24.2 mV) (figure 3). Some studies have also reported zeta potential effects of storage conditions, such as the pH, particle concentration, EV isolation and overall colloidal stability [24, 25]. These parameters must also be taken into account during production purification and storage of EV-based medical products. Typically, zeta potential of ± 15mV is considered as the threshold for agglomeration, whereas ± 30mV is considered as the limit above which the colloidal system becomes thoroughly stable. In between 15 and 30mV, the system may be either agglomerated or dispersed [18, 19]. The results of our zeta potential measurements therefore indicate a partially stable solution of the EV standard with the clear tendency being dispersed. Furthermore, measurement of the change in zeta potential in the biomedical field can be used as a physiological and molecular biological marker for cellular reactions and malignant degeneration of cells in various diseases [26, 27].

Experimentally, the zeta potential is strongly dependent on pH, salt content and the ionic strength of the solvent used [26, 28]. Associated with this is the conductivity of the dispersing medium because it can influence the stability of the double layer of the membrane by altering the repulsive forces between the EVs, which in turn augments the interparticular interactions due to forces acting at a molecular scale, such as the Van der Waals force [28]; thus, interfering with the stability of the colloidal system of EVs. For reasons of comparability and reproducibility, knowledge of parameters such as pH, ionic strength and salt concentration of the solvent used is just as essential as the fact that the same buffer is always used in order to be able to assess colloidal stability of EVs in the subpopulations.

In summary, in this study we have presented a comprehensive characterization of a commercially available EV standard via NTA at the single particle level. The Particle Metrix F-NTA EV Tetraspanin Detection Kit 520 clearly distinguished PAN stained exosomes from the CD63 e-GFP positive particle fraction via F-NTA by investigating size and concentration of the respective fluorescently labelled particle fractions.

In addition, we have shown that the use of the F-NTA EV Tetraspanin Detection Kit 520 can

serve as a counterstaining tool which helps to provide more detailed information on the molecular composition of the membrane surface in colocalization NTA and thus enable further assignments as to which subpopulation is present in terms of co-existence of surface markers. PAN tetraspanin staining can also be used as a tool necessary for zeta potential analyses to obtain an additional differentiating quality marker for colloidal stability assessment and characterization of EVs. Immunolabelling with specific EV markers can therefore provide excellent quality analysis of EVs in terms of size, distribution, concentration, colocalization and zeta potential using a single NTA measurement script.

Key words: F-NTA EV Tetraspanin Detection Kit 520, colocalization, Nanoparticle Tracking Analysis, extracellular vesicles, PAN staining, CD9, CD63, CD81, e-GFP, antibody, fluorescence, colloidal stability, quality assessment.

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