Exosome labeling by lipophilic dye PKH26 results in significant increase in vesicle size

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Abstract

Exosomes are membrane vesicles secreted by cells and distributed widely in all biofluids. Exosomes can modulate the biological activities of cells in a paracrine or endocrine manner, in part by transferring their content, such as miRNA, following uptake in recipient cells. Fluorescent labelling of exosomes is a commonly used technique for understanding their cellular targeting and biodistribution. Lipophilic fluorescent dyes such as those in the PKH family have been widely used for exosome labelling. One concern with the use of lipophilic dyes is an increase in the exosome size due to membrane fusion or intercalation. This size shift alone may undermine the validity of exosomes tracing studies as small changes in the size of inorganic nanoparticles are known to affect their cellular uptake and biodistribution. Here, the possibility of minimizing the size shift of PKH labelled exosomes was systematically studied by changing the labelling condition. Unfortunately, the size shift towards larger particles was observed in all the PKH labelling conditions, including those where the labelled exosomes were below the fluorescent detection limit. As opposed to lipophilic dyes, no significant shift in the size of labelled exosomes was detected with protein binding dyes. Since the size shifts identified in all the PKH labelling conditions are likely to affect the cellular uptake and biodistribution, PKH may not be a reliable technique for exosomes tracking.

Keywords: Exosomes, Extracellular Vesicles, PKH, CFSE, Size, Nanoparticle Tracking Analysis, Cellular Uptake, Biodistribution, Fluorescent Labelling, Lipophilic dye.

Introduction

Exosomes are small-membrane bound vesicles (30–150 nm in diameter) secreted by all cell types examined and can be found in almost all biofluids including blood, breast milk, urine, saliva and even in cell culture media [1,2]. Exosomes mediate cell-cell communication by exchanging proteins, DNA, RNA and lipids between donor and recipient cells and activating signaling pathways in target cells via receptor ligand interaction [3,4]. They have been shown to play a role in regulating both physiologic and pathologic processes including immune regulation and cancer [5]. Exosomes secreted by cells into the extracellular environment have been shown to be internalized through different routes and mechanisms including fusion with the plasma membrane and a range of endocytic pathways such as receptor-mediated endocytosis, phagocytosis, lipid raft-dependent endocytosis and micropinocytosis [6–9]. Exosomes target a wide range of recipient cells such as dendritic cell [10], macrophage [11], dermal fibroblast [12], endothelial and myocardial cells [13].

Studies that examine exosome uptake into target cells and *in vivo* biodistribution have utilized a range of exosome labelling and tracking approaches to follow exosome fate [14]. The most common technique for studying exosome biodistribution and target cell interaction involves labelling of exosomes with fluorescent dyes. Exosomes membranes have been stained using the fluorescent lipid membrane (lipophilic) dyes like PKH26 [15–17], PKH67 [18], DiI [19], and DiD [20]. The PKH family have been the widely used dyes in the lipophilic dyes class as they have a highly fluorescent polar head group and long aliphatic hydrocarbon tails which readily intercalates into any lipid structure leading to a long-term dye retention and stable fluorescent[15,20].

Despite the widespread use of lipophilic dyes (PKH) for labelling of exosomes, up to now, far too little attention has been paid to artifacts generated during the PKH labeling of exosomes such as the formation of numerous nanoparticles which consist exclusively of micelles/aggregates of PKH, without exosomal content [21,22]. It was further shown that in terms of size, surface area and fluorescent intensity, the PKH nanoparticles cannot be distinguished from PKH labelled exosomes and were taken up by astrocytes. This capacity for cell uptake of PKH nanoparticles may lead to false positive signals in exosomal tracking studies [21].

A second artifact of lipophilic dye labelling of exosomes is the generation of larger species which may derive from either nanoparticle fusion or dye intercalation with exosomes [18,21,23]. This larger PKH-labelled exosome species may alter uptake into target cells as previous studies have shown that cells preferentially uptake smaller exosomes [24]. However, the contribution of size versus exosomes constituents in this uptake phenomena is unclear. In order to examine the impact of particle size alone, the size-dependent uptake of nanoparticles composed of inorganic materials including those made of polystyrene [25]and silica [26] has been studied. These studies have shown that the interaction and uptake of nanoparticles with living cells are sensitive to the size of nanoparticles regardless of their composition. Lower cellular uptake of nanoparticles was consistently observed with increasing nanoparticle size, possibly due to the increased energy required to take up the larger particles [14,27–29]. Furthermore, size affects the biodistribution of nanoparticles *in vivo*, for instance, more rapid accumulation of larger nanoparticles was observed in liver and spleen [30]. Large particles also tend to exhibit shorter circulation half-life, which may be due to the activation of the complement system and quick removal of large nanoparticles from blood [31].

Since PKH family has been widely used for labelling exosomes, formation of larger species after labelling exosomes with PKH may trigger abnormalities in their tissue distribution and cellular uptake in both *in vivo* and *in vitro* studies. The objective of this study was to investigate the possibility of minimizing the formation of larger labelled exosome species in order to reduce non-physiological effect of these larger species on cellular uptake, biodistribution and circulation half time. In order to minimize the formation of larger labelled exosomes, the PKH to exosome ratio

was systematically changed and the size of particles was determined using Nanoparticle Tracking Analysis (NTA). In all labelling conditions including where the labelled exosomes were below the fluorescent detection level, exosomes size was increased.

Materials and Methods

Fluorescent staining: Lyophilized urinary exosomes (HansaBioMed, Estonia) were diluted in ultra-pure water to a protein concentration of 0.1 μg/mL, following the manufacturer's instructions. Prior to staining, 1 μM of PKH26 (Red Fluorescent Cell linker for General Cell Membrane, Sigma-Aldrich) stock was added to 300 μL of diluent C and incubated at 37° C for 15 minutes. Then, 1 μL of exosomes stock was added to PKH26 in diluent C, resulting in a sample with 0.3 μg/mL of exosomes and 4 μM of PKH26. Other concentrations of exosomes and PKH were made accordingly. CFSE (CellTrace™ CFSE Cell Proliferation Kit, Thermo Scientific Fisher) stock was made following the manufacture instructions by adding 18 μL of Dimethyl Sulfoxide (DMSO, Sigma-Aldrich) to the CFSE vial resulting in 5 mM stock. In order to stain exosomes with CFSE, 1 μL of CFSE stock was added to 300 μL of ultra-pure water prior to staining and then 1 μL of exosomes from 0.1 μg/mL exosomes stock was added and incubated for 2 hours at 37° C as it was previously described [22].

Nanoparticle tracking analysis (NTA): For each run, 300 μL of the prepared samples was injected into the sample chamber of a NS300 instrument (NanoSight, Aumesbery, UK). Seven measurements of the same sample were performed for 30 seconds. For the "Blur", "Minimum expected particle size", and "Minimal track lengths" the auto adjustment settings provided by software developer were used. The camera level and detection threshold were adjusted manually for each experiment as recommended by the manufacture. For data capturing and analysis, the NTA analytical software (NanoSight NTA 3.2) was used. Briefly, from the recorded video, the mean square displacement of each detected particle was determined. Then, using the Stokes-Einstein equation, the diffusion coefficient and sphere-equivalent hydrodynamic radius were determined by the software. Particles size distribution and concentration were displayed for each sample.

Fluorescent imaging and analysis: Fluorescent images were taken at 40X using Keyence BZ-X700 microscope (Keyence Corp. of America, MA, USA) with the same exposure time for all samples ($1/12$ s). The line scan analysis was conducted using NIH ImageJ.

Results and Discussion

Lipophilic dyes such as the PKH family have been widely used to label a range of cell types such as mesenchymal stem cells [17,32] and tumor cells [33] in proliferation and migration studies [32,34]. Since exosomes have a lipid bilayer structure similar to that of the cell's plasma membrane, PKH dye family have been adapted for exosome labelling. As noted above, Dominkus et al. [21] have recently shown that PKH labelling of exosomes generates larger species. In order to confirm these findings, exosomes were labelled with a similar PKH to exosomes molar ratio and the resulting particles' size was assessed using NTA. NTA is a technique for measuring the size and concentration of nanoparticles in suspension in real time based on tracking the light scattered from suspended particles undergoing Brownian motion [35–37]. As was seen previously [21,22], nanoparticles were present in PKH-only controls and critically, the generation of large species, greater than that found in either the exosomes or PKH-only controls, was observed following labelling of exosomes with PKH (Fig. 1). Additionally, the interaction of polystyrene (PS) nanoparticles with PKH was studied as a control experiment. In contrast to exosomes, where PKH fusion and intercalation and thus generation of larger particles can occur, PKH should not fuse with PS nanoparticles. As expected, the

formation of larger particles was not observed when 100 nm PS nanoparticles were added to the PKH sample (Figure 2).

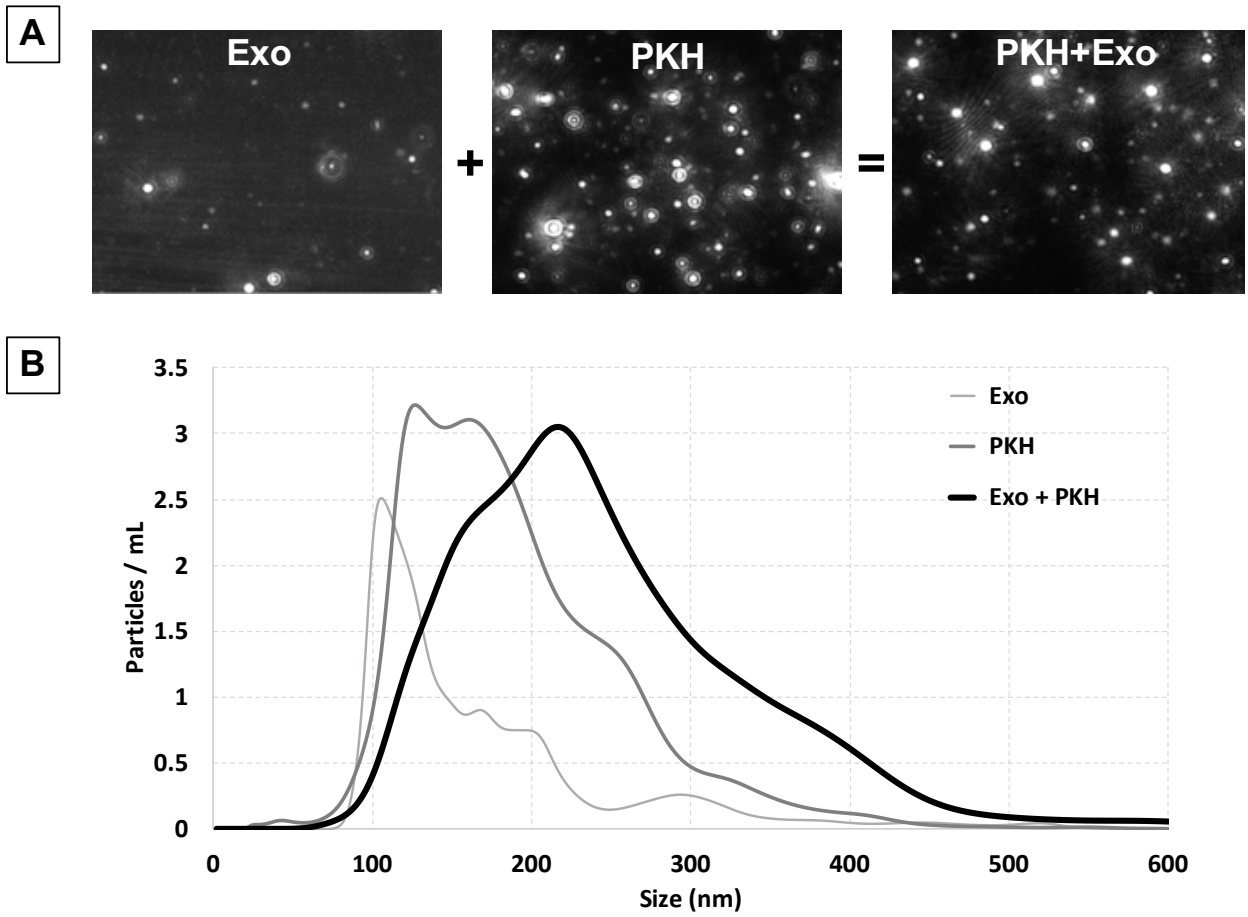

Fig. 1. Size characterization of PKH exosome labelling by Nanoparticles Tracking Analysis (NTA). A) NTA video frames of exosome-only (Exo) as control, PKH-only (PKH) as control, and PKH-labelled exosome (PKH+Exo). B) Size distribution of exosome only control (Exo), PKH-only control (PKH), and PKH-labelled exosome (PKH+Exo) samples (n=7).

Possible mechanisms causing the observed size increase are the fusion of PKH nanoparticles with exosomes or the intercalation of PKH molecules into exosomal membranes, both of which would result in the formation of larger species. This suggests the possibility of minimizing the size increase due to dye intercalation or nanoparticles fusion with exosomes by reducing the PKH input level, while maintaining detectable fluorescent signal from PKH labelled exosomes. To assess this possibility, the fluorescent detection range of PKH-labelled exosomes samples was determined. PKH/exosome ratios were adjusted by changing the concentration of PKH while holding the exosome concentration constant. The fluorescence level of these samples along with that of the PKH and exosome-only, and background-only control groups was visualized using fluorescent microscopy and quantitated by sampling the cross-sectional fluorescent intensity of captured images (the line scan).

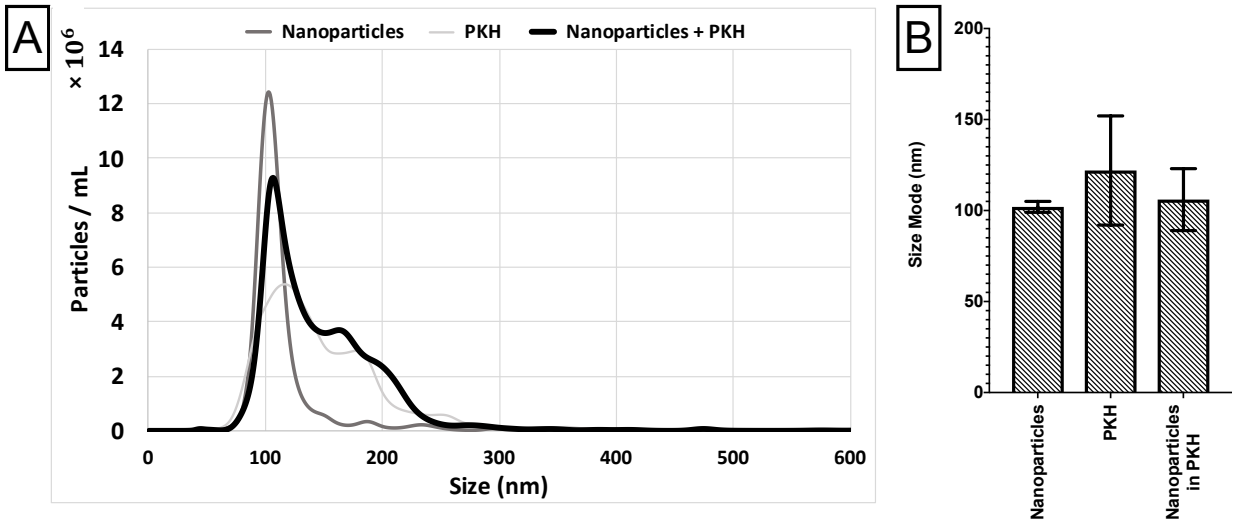

Fig. 2. Size characterization of polystyrene (PS) nanoparticles in PKH by Nanoparticles Tracking Analysis (NTA). A) Size distribution of PS nanoparticles-only control , PKH-only control, PS nanoparticles in PKH sample (n=7). B) Size mode of PS nanoparticles-only control , PKH-only control, PS nanoparticles in PKH sample (Error bars represent 95% confidence interval of the mean).

Initially, the same concentration of PKH and exosomes was used as in figure 1. Weakly fluorescent features consistent with the presence of PKH nanoparticles in the PKH-only control were observed by fluorescent imaging (Figure 3A). In comparison to the PKH-only control, several brighter features were seen which are likely the larger particles formed after PKH exosomes labelling (Fig. 3B). The line scan of PKH-labelled exosome samples showed higher fluorescent intensity compared to the background signal (Fig. 3E). Line scan analysis of the PKH-only control and PKH-labelled exosome samples revealed a reduction in the baseline signal possibly as the result of floating PKH dye interacting with exosomes (Figure 3E). Furthermore, larger spikes in the PKH-labelled exosome sample again suggesting the presence of larger labelled exosomes (Fig. 3E). In contrast, a 25 times reduction of PKH concentration led to a decrease in the fluorescent intensity to the same signal level as the background, as well as causing the loss of the fluorescently bright features observed at higher concentrations of PKH (Fig. 3C-E).

After determining the fluorescent detection range of PKH labelled exosome samples, the effect of PKH concentration on the particles size distribution was explored. The concentration of PKH was systematically varied while holding exosome concentration constant. Representative examples of particle size distribution measured by NTA for different concentration of PKH labelling of exosomes can be seen in figure 4. For all concentrations of PKH, NTA analysis of particle size distribution showed that exosome labelling with PKH caused the formation of larger species relative to exosomes and PKH alone and exosome alone control groups (Fig. 4D). Quantitative determination of NTA results was done by comparing the mode of the nanoparticle size (Figure 4E). Consistent with size distribution results, a shift in the mode towards larger particles was observed in all PKH-labelled exosome samples (PKH+Exo) compared to the exosome alone control. Furthermore, no size shift was observed when the suspension buffer (diluent C) was added to exosomes in the absence of PKH confirming that PKH is the cause of the size shift. Additionally, Characterization of the particles size mode when 100 nm PS nanoparticles were added to the PKH sample revealed no size shift in the PS nanoparticles after adding to the PKH

sample compared to the PS nanoparticles alone. This finding again suggests that PKH is causing the size shift in the labelled exosomes samples (Fig. 2B).

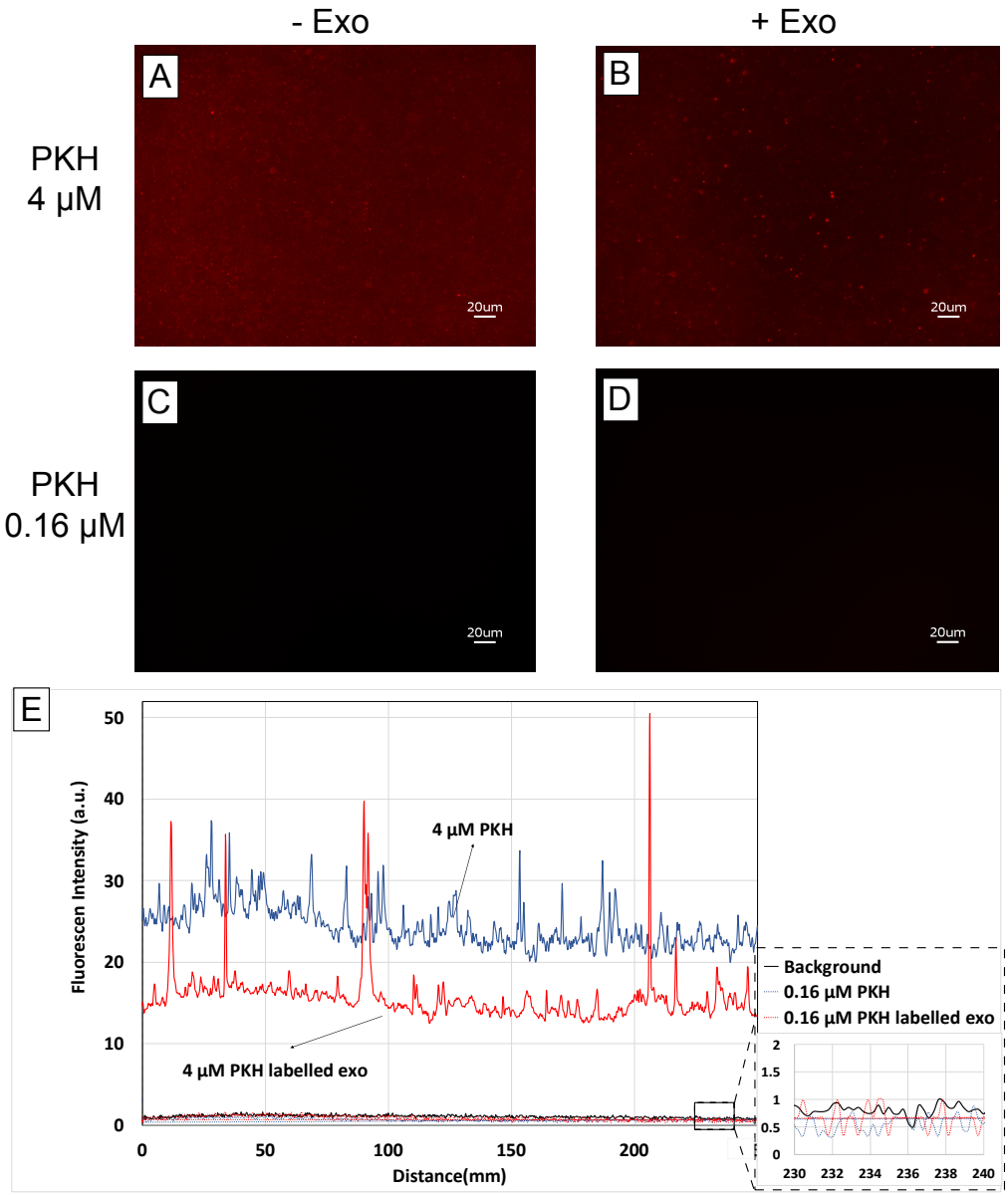

Fig. 3. Determination of the fluorescent detection range. Fluorescent images of A) 4 µM PKH-only control B) 4 µM PKH-labelled exosomes C) 0.16 µM PKH-only control D) 0.16 µM PKH-labelled exosomes. E) Representative line plot analysis of fluorescent images (A-D). Inset is showing the line plot analysis of background, 0.16 µM PKH-only control and 0.16 µM PKH-labelled exosomes.

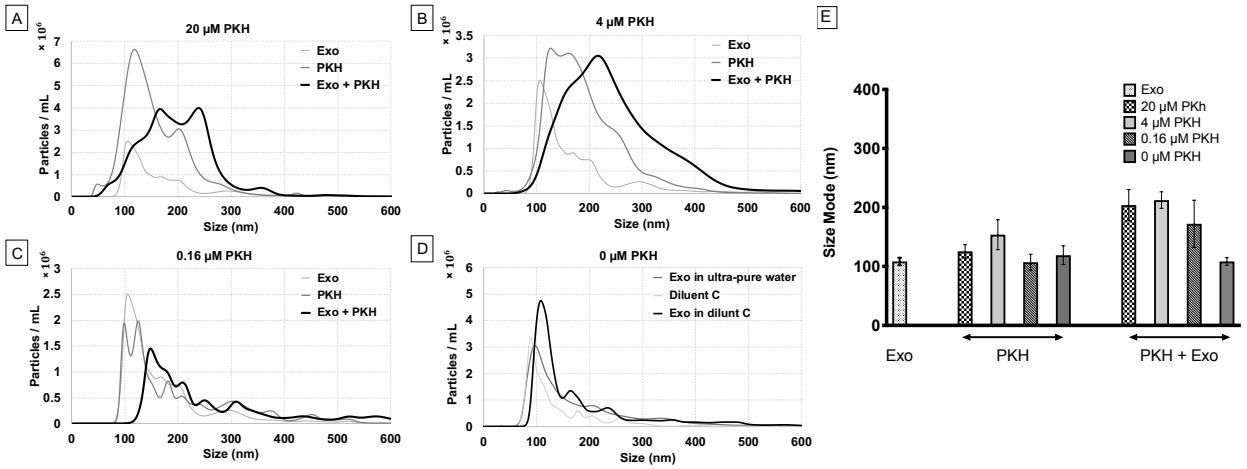

Fig. 4. Effect of PKH concentration on the size distribution of particles in PKH-labelled exosome samples by Nanoparticles Tracking Analysis (NTA). A-D) Size distribution of PKH-labelled exosome samples with different PKH concentrations (20, 4, 0.16 and 0 µM respectively) with 0.3 µg/mL of exosomes (n=7). E) Particles size mode for exosome-only control, PKH-only controls, and PKH-labelled exosome samples (Error bars represent the 95% confidence interval of the mean).

Taken together, labelling exosomes with different concentrations of PKH, even for the PKH concentration below the level of fluorescent detection, showed the size distribution shift indicative of the generation of larger species. This finding suggests that minimizing the formation of larger species by reducing the concentration of PKH used for labelling exosomes, while maintaining the fluorescent detectability may not be feasible.

Further confirmation of PKH induced larger species was done by varying the concentration of exosomes while holding the PKH concentration constant. The PKH concentration used was the level shown to generate fluorescently detectable PKH-labelled exosomes (Figure 2B). Representative examples of particles size distribution measured by NTA for different concentration of exosomes labelled by PKH dyes can be seen in figure 5. Additionally, quantitative determination of NTA results was conducted by comparing the mode of the nanoparticles size (Fig. 5E). As expected, generation of larger species was observed by size distribution as well as the shift in the size mode regardless of the exosome concentration.

Labeling exosomes with PKH caused a shift in the size of exosomes from ~100 nm for unlabeled exosomes to ~200 nm for PKH labelled exosomes. Studies have found that using inorganic particles, a similar size shift from 100 nm to 200 nm decreases the cellular uptake efficiency and kinetics. Different types of nanoparticles and cells have been investigated such as mesoporous silica nanoparticles on Hela cells by Lu et al. [30], fluorescent latex beads on B16 cells by Hökstra et al. [38], polystyrene nanoparticles on Caco-2 and MDCK cells by Kulkarni et al. [25]. In addition to cellular uptake efficiency, Hökstra et al. [38] have shown that nanoparticles smaller than 200 nm can be taken up by clathrin-coated pits, while larger particles tend to be internalized by caveolae-mediated processes. This suggests the size shift due to the PKH labelling may change both the cellular uptake level and mechanism of endocytic internalization of PKH labelled exosomes.

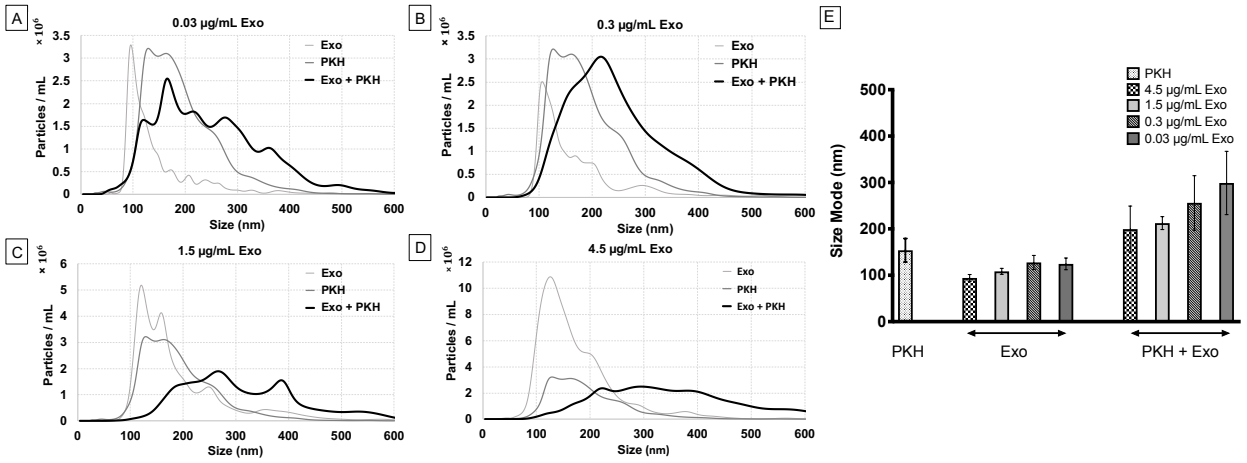

Fig. 5. Effect of exosome concentration on the size distribution of particles in PKH-labelled exosome samples evaluated by Nanoparticles Tracking Analysis (NTA). A-D) Size distribution of PKH-labelled exosome samples with different exosome concentrations (0.03, 0.3, 1.5, 4.5 µg/mL respectively) with 4 µM of PKH (n=7). E) Size mode of exosome-only control, PKH-only controls, and PKH-labelled exosome samples (Error bars represent 95% confidence interval of the mean).

Furthermore, Kulkarni et al. [25] studied the biodistribution of intravenously injected polystyrene nanoparticles in different rat's organs. They found that 200 nm nanoparticles showed higher accumulation in both liver and spleen compared to 100 nm nanoparticles. Again, this raises the possibility that increasing the size of exosomes by labelling with PKH may affect the exosomes' biodistribution. Gangadaran et al. [30], examined the biodistribution of extracellular vesicles (EVs) including exosomes using DiR (a similar lipophilic dye as PKH) and found that lipophilic labelling of EVs increased the localization of EVs in liver and spleen. This change in the biodistribution may be due to the increasing in size of EVs after labelling by lipophilic dyes. In summary, the size shift towards larger particles caused by PKH labelling of exosomes is likely to change the cellular uptake level and internalization mechanism as well as the biodistribution of exosomes, reducing its validity as an exosome tracer.

As opposed to lipophilic dyes which may generate larger species, potentially through PKH nanoparticles fusion or PKH dyes intercalation with exosomes, it is anticipated that direct protein labelling of exosomes with fluorescent compounds will not generate these larger exosome species. Consistent with this hypothesis Morales-Kastresana et al. [22] and Pospichalova et al. [23] found protein binding fluorescent compounds did not increase exosome size. To confirm this finding in our system, the protein binding dye 5-(and-6)-Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) was used to label exosomes. CFSE dyes are membrane permeable chemical compounds which covalently bind to proteins and fluoresce after esterification in the lumen of the exosome [39,40].

Exosomes were labelled with CFSE dyes using a previously established protocol [22] and the size of particles was explored using NTA (Fig. 6). Size distribution and quantitative determination of the size mode showed no significant change in CFSE-labelled exosomes compared to unlabeled exosomes which was in agreement with findings of Morales-Kastresana et al. [22] and Pospichalova et al. [23]. As opposed to PKH labelling of exosome which increases the size, this result suggests that CFSE dye labelling maintains the normal size of exosomes which precludes any size related cellular uptake and biodistribution aberrancies.

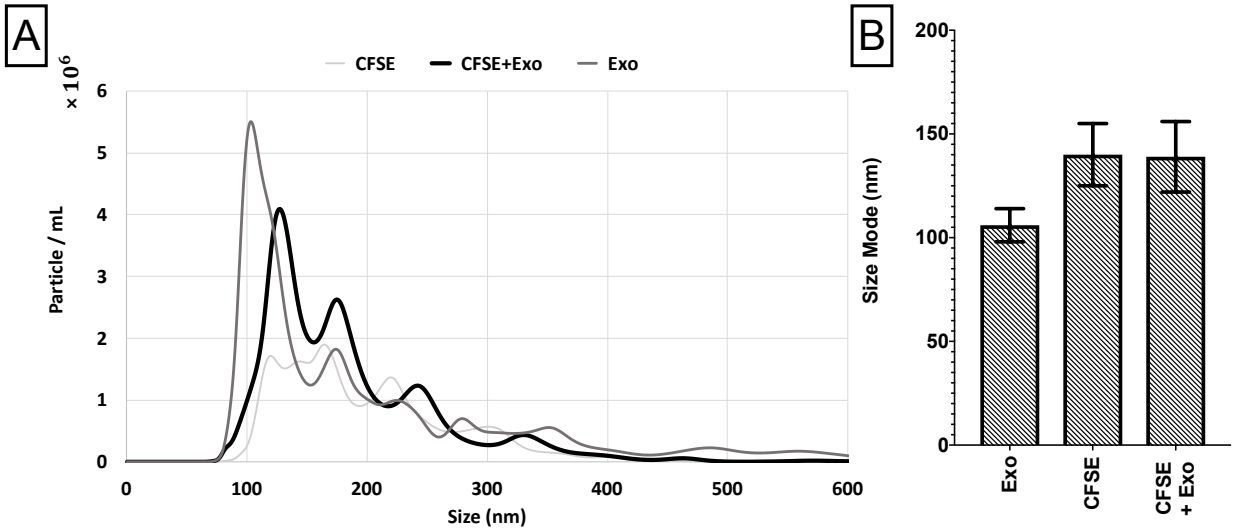

Fig. 6. Size characterization of CFSE exosome labelling by Nanoparticles Tracking Analysis (NTA). A) Size distribution of exosome-only control, CFSE-only control, and CFSE-labelled exosome sample (n=7). B) Size mode of exosome-only control, CFSE-only control, and CFSE-labelled exosome sample (Error bars represent 95% confidence interval of the mean).

Conclusion

Lipophilic labelling of exosomes has been shown to have multiple drawbacks. First, non-specific labelling of non-exosomal biofluid components such as microvesicles, lipoprotein particles, and proteins [41]. Second, the *in vivo* biodistribution of EVs has been shown to be altered by lipophilic labelling [25]. Third, the formation of PKH nanoparticles was observed which can potentially cause false positive results in exosome cell uptake and biodistribution studies. Fourth, a size shift in the size of exosomes was shown after labelling, most likely due to the PKH nanoparticles fusion and PKH dyes intercalation with exosomes. These larger species formed after PKH labelling of exosomes may cause aberrancies in cellular uptake, biodistribution and half-life circulation.

Here, the relative ratio of PKH to exosomes was systematically studied in order to minimize the exosome size shift towards larger particles by PKH labelling while maintaining the fluorescent detection of labelled exosomes. In all conditions tested, formation of larger species after labelling was detected, even in the conditions where the PKH level is below the fluorescent detection level. In contrast to lipophilic dyes such as PKH, protein binding dyes like CFSE did not cause a size shift in labelled exosomes, suggesting that CFSE may be a better labelling option for exosomes.

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