Tracking of Extracellular Vesicles in *In Vivo* Models using Acoerela's Lipophilic Probes

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Abstract

Red Blood Cell-derived Extracellular Vesicles (RBCEVs) hold significant promise for therapeutic interventions due to their ability to deliver modulatory cargoes. *In vivo* monitoring of Extracellular Vesicles (EVs) provides a means to study and assess their interactions with target cells. Considering the limitations of existing optical probes, there is a clear need for a robust fluorogenic probe that offers precision and effective illumination while minimising the risk of false positive signals. We describe here the use of Acoerela's Aco-490, a water-soluble membrane dye, which fulfils these criteria and is emerging as a reliable tool for tracking EV distribution *in vivo*. Aco-490 has proven effective in analysing trafficking patterns of RBCEVs, revealing their preferential uptake by specific cells. This discovery offers insights into potential therapeutic applications of RBCEVs for multiple disease states including atherosclerosis and inflammation.

Keywords: *Extracellular Vesicles (EVs), biodistribution, lipophilic, fluorogenic, membrane dye, water-soluble dye, flow cytometry, confocal microscopy, histology*

Introduction

Red Blood Cell-Derived Extracellular Vesicles

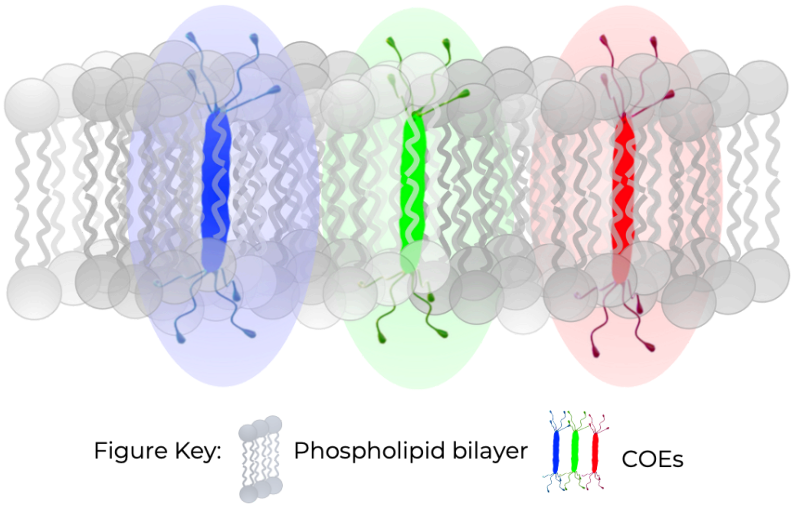
Red Blood Cell-derived Extracellular Vesicles (RBCEVs) are crucial for intercellular communication and play a vital role in various physiological and pathological processes [1]. Serving as carriers of cargoes that can alter cell behavior, RBCEVs have emerged as potential candidates for therapeutic applications [2]. *In vitro* approaches to studying Extracellular Vesicles (EVs) may not fully encapsulate the intricate dynamics of living organisms' physiological environments. Therefore, conducting *in vivo* studies becomes imperative to study the biodistribution, cellular uptake mechanisms, and cargo delivery kinetics of EVs, which are crucial for guiding the development of impactful therapeutic interventions.

Fluorescent probes are invaluable tools for tracking EVs *in vivo*, enabling visualisation and quantification of their interactions with target cells and tissues [3]. Unfortunately, most fluorescent dyes are extremely hydrophobic, often necessitating reconstitution in organic solvents such as DMSO or ethanol, which can introduce artifact changes due to their lack of biocompatibility. Additionally, certain dyes, such as carbocyanine dyes like PKH26, tend to form nanoaggregates during serial dilution of their solvent stocks [4, 5], potentially generating false positive signals when analysing small biological particles such as EVs.

Conjugated Oligo-Electrolytes

Conjugated Oligo-Electrolytes (COEs) are organic compounds with distinctive chemistry that enables them to intercalate into lipid bilayer membranes spontaneously, as depicted in Figure 1. These molecules feature a hydrophobic backbone composed of π-delocalised repeat units and pendant ionic side chains, which make them soluble in water and facilitate specific interactions with charged targets [6]. Their affinity to negatively charged phospholipid bilayers makes them useful probes, particularly for detecting EVs.

Figure 1. COE intercalation within the lipid bilayer membrane



COEs light up the brightest when embedded within the lipid bilayer, and their unique structure enables them to span the entire lipid bilayer.

Acoerela presents COEs under the trade name AcoDyes™, which are the world's first full bilayer-spanning lipophilic membrane dyes, ideal for tracking EVs *in vivo* with several advantages. These dyes are highly water-soluble and do not form micelles or nanoparticles in aqueous environments, mitigating the risk of false positive signals and ensuring accuracy and reliability of experimental results. They are fluorogenic, increasing in fluorescence emission exclusively upon binding to a lipid bilayer, thereby reducing background fluorescence. Furthermore, they are available in multiple fluorescent wavelengths and are customisable, offering users a range of choices tailored to individual requirements. Collectively, these features position Acoerela's dyes as indispensable tools for advancing EV research.

Materials and Methods

Preparation of Aco-490-Labelled RBCEVs

RBCEVs were prepared at a concentration of 0.5 µg/µL (based on vesicular haemoglobin content), and incubated with 2 µM Aco-490 (Excitation = 405 nm, Emission = 458 – 508 nm) at room temperature for 1 hour.

Dye Removal and Washing

Unbound Aco-490 was removed by centrifugation. The labelled RBCEVs at a concentration of 1 µg RBCEVs/µL were washed three times with PBS. Each wash step involved centrifugation at 21,000 x g for 30 minutes. Supernatant from the final wash step served as a flowthrough control.

RBCEV Injection and Tissue Collection

RBCEVs (25 mg/kg) labelled with Aco-490 were injected intravenously into 8-10 week old C57BL/6 mice (Invivos, Singapore). After 8 hours, the mice were euthanised, and their liver, lungs, bones, and spleen were collected, fixed in 10% formalin overnight at 4°C, and processed for imaging.

Tissue Preparation and Staining

The organs were washed with PBS followed by incubation in PBS containing 15% sucrose and then in PBS containing 30% sucrose. The tissues were embedded in optimal cutting temperature (OCT) compound and frozen on dry ice. Sections of 7 µm thickness were prepared using superfrost slides. The sections were initially blocked with blocking buffer (PBS with 2% FBS) for 40 minutes and then with blocking buffer containing TruStain (BioLegend, Cat# 101319) at a dilution of 1:1000 for 5 minutes. The slides were incubated with antibodies against the surface markers for resident macrophages: mouse F4/80 (BioLegend, Cat# 123105) or CD169 (BioLegend, Cat# 142417) at a 1:500 dilution for 1 hour at room temperature and subsequently washed with a wash buffer (PBS with 2% FBS). Staining with biotinylated anti-mouse F4/80 antibody required the slides to be further incubated with Streptavidin AlexaFluor 647 for 1 hour at room temperature followed by a wash with the wash buffer. Nuclei were labelled with NucSpot488 (Biotium, Cat# 40081), diluted with PBS at a 1:2000 dilution. Finally, slides were washed with PBS and water, mounted using Vectashield Vectashield antifade medium (Vector laboratories, Cat# H-1000-10) and imaged with a confocal microscope (Olympus FV3000).

Results

Representative flow cytometry scatter plots of Aco-490-labelled RBCEVs alongside appropriate controls are shown in Figure 2. The V525 fluorescent channel of the CytoFLEX LX flow cytometer (Beckman Coulter) having the violet side scatter (Violet SSC) configuration was used to detect Aco-490 fluorescence. Aco-490 positive signal is exclusively observed in labelled RBCEVs (Figure 2c), with nearly no signal detected in unlabelled RBCEVs (Figure 2b) and negligible background fluorescence in the dye-only control (Figure 2a). This illustrates the dye's fluorogenic property and minimal false positive rate, showcasing the exceptional specificity and reliability of AcoDyes™ for detecting EVs.

Figure 2. Flow cytometry signals of Aco-490

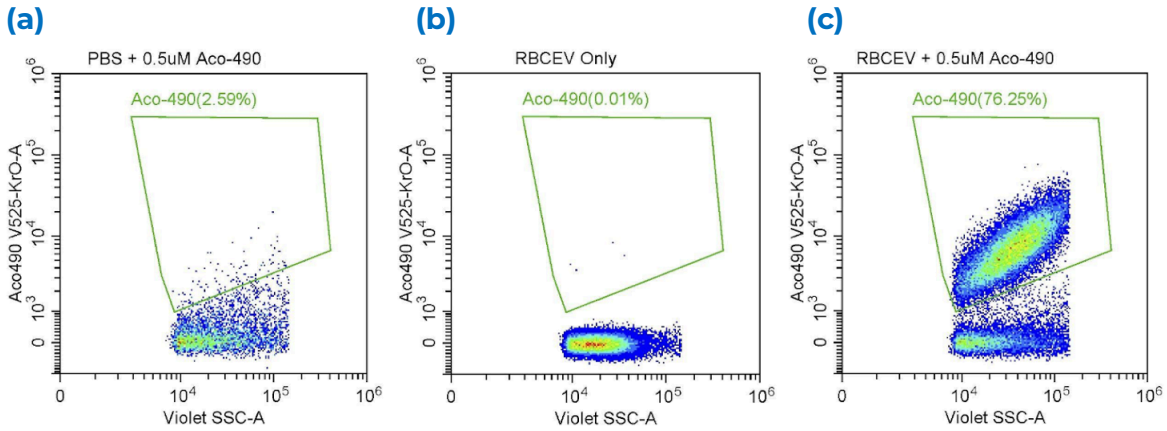
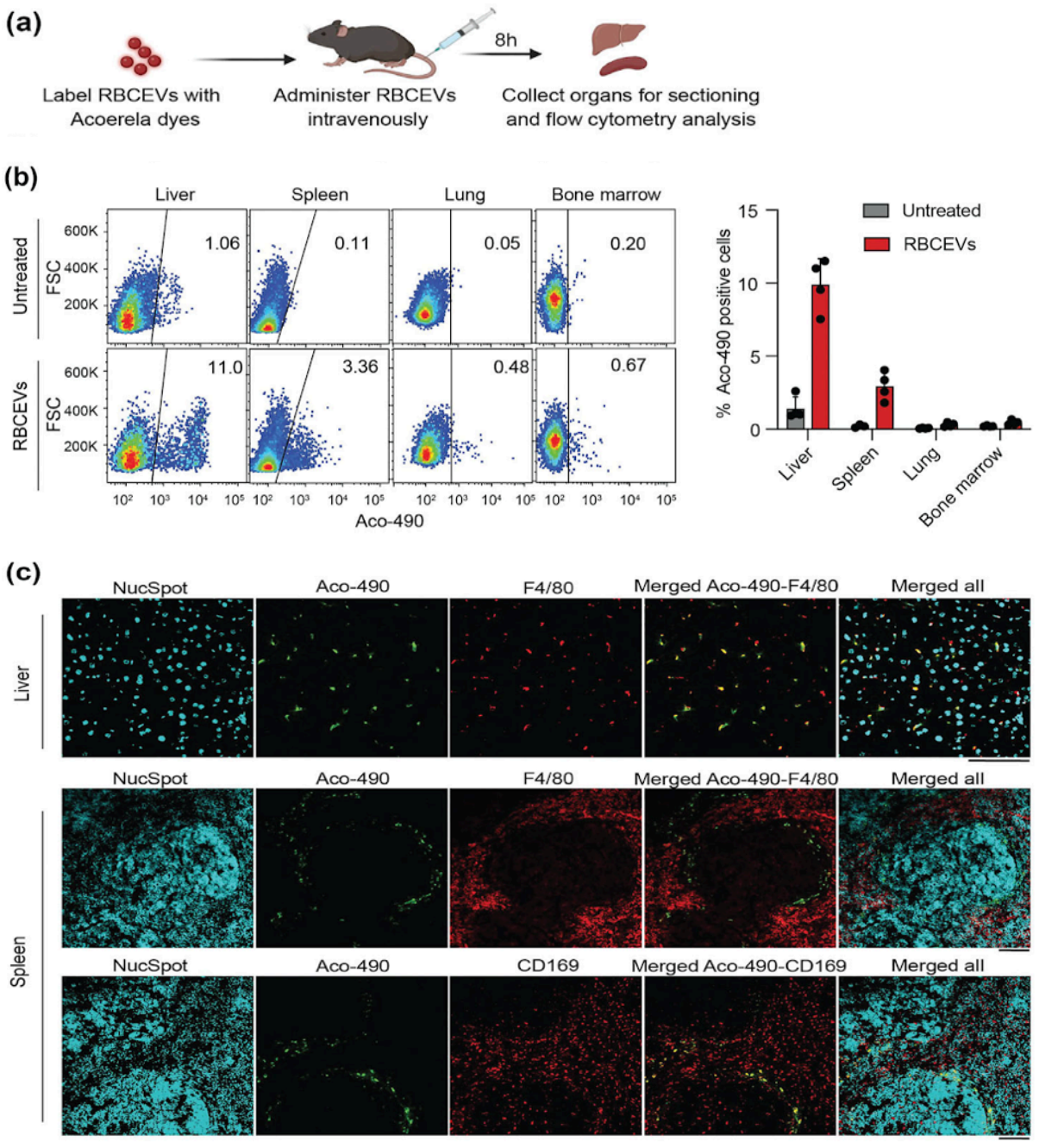
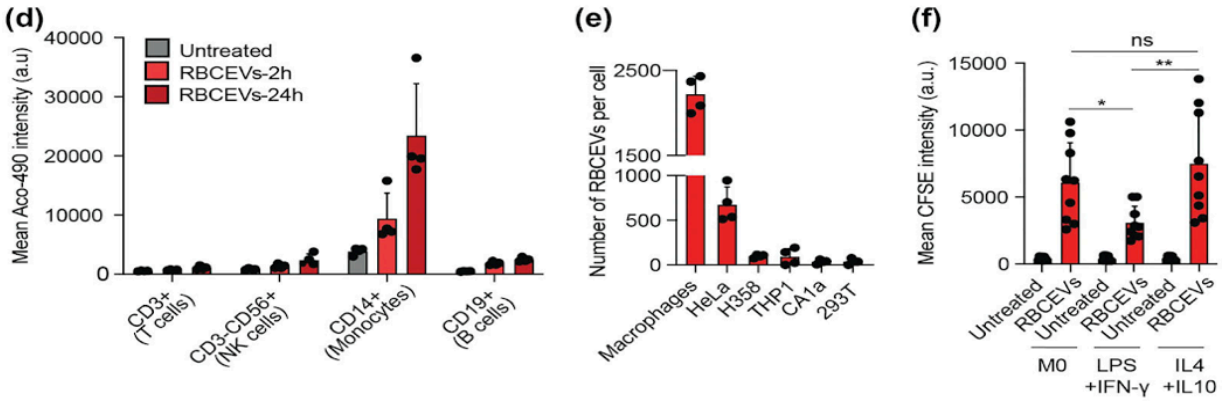


Figure 3 demonstrates the efficacy of Aco-490 in *in vivo* tracking of RBCEVs. The experiment was designed (Figure 3a) to investigate the distribution of RBCEVs at the cellular level. Flow cytometry analyses (Figure 3b) displayed pronounced Aco-490 signals in the liver and spleen, highlighting the preferential accumulation of RBCEVs in these organs over others. Confocal microscopy images of liver and spleen sections (Figure 3c) showed immunolabeling of macrophages with F4/80 or CD169 antibodies (in red) and nuclei labelled with NucSpot® Live 488 (in cyan). Scale bars indicate 100 µm. In liver tissue sections, Aco-490 signals (in green) were predominantly colocalised with F4/80+ cells, implying significant RBCEV uptake by Kupffer cells. In

contrast, in the spleen, RBCEVs were less associated with F4/80+ cells. Instead, they were primarily located in CD169+ cells at the marginal zone.

Figure 3. *In vivo* tracking of RBCEVs using Aco-490





Findings from the analyses of Aco-490 signals using Flow Cytometry and Confocal Microscopy. Source: [7]

Conclusion

Aco-490 has shown remarkable success as a reliable option for EV staining. It enhances the monitoring of EVs in an *in vivo* model by providing high specificity and low false positives.

As all COEs share a similar chemical structure and fluorogenic properties, the entire array of AcoDyes™ can be utilised for EV tracking. The availability of such innovative dyes opens up novel avenues for thoroughly investigating the full potential of EVs in therapeutic applications.

Guidelines for using AcoDyes™

- AcoDyes™ vary in intensity and staining efficiency. It is recommended to optimise the method for each dye, EV sample and study condition.
- Consider purifying the EV sample using Size Exclusion Chromatography (SEC) to remove any residual particles, such as cryoprotectants, saccharides or remnants from the cell culture medium that might interfere with dye binding.
- For very low volumes, use the reverse pipetting technique.
- AcoDyes™ are not compatible with detergent or detergent-based permeabilisation buffers.

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