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Oil Red O based method for exosome labelling and detection

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ABSTRACT

With the realization of the role of exosomes in diseases, especially cancer, exosome research is gaining popularity in biomedical sciences. To understand exosome biology, their labelling and tracking studies are important. New and improved methods of exosome labelling for detection and tracking of exosomes need to be developed to harness their therapeutic and diagnostic potential. In this paper, we report a novel, simple and effective method of labelling and detecting exosomes using Oil Red O (ORO), a dye commonly used for lipid staining. Using ORO is a cost effective and easy approach with an intense red coloration of exosomes. Further, the issues faced with commonly used lipophilic dyes for exosome labelling like long-term persistence of dyes, aggregation and micelle formation of dyes, difficulty in distinguishing dye particles from labelled exosomes, and detection of large aggregates of dye or dye-exosome, are also resolved with ORO dye. This method shows good labelling efficacy with very sensitive detection and real-time tracking of the cellular uptake of exosomes.

1. Introduction

Exosomes are vesicles of 40–150 nm diameter released by all cells into the extracellular milieu. They are of endosomal origin and are formed by the fusion of multivesicular bodies with the plasma membrane. Structurally, exosomes are lipid bilayer vesicles with the inclusion of nucleic acids, proteins, amino acids, lipids, and metabolites [1]. They are stable entities with characteristic functions and activity [2]. Initially exosomes were thought to be cellular waste products, but now they are considered as important component of cellular processes. One of the most important biological function of exosomes is their role as mediators of intercellular cell communication. They carry cargo from donor cells and release it into recipient cells, thus facilitating cell communication.

Exosomes are now emerging as potential diagnostic and therapeutic tools for the treatment of various diseases, including cancer. They are emerging as one of the best biomarkers of cancer diagnosis because of their role in tumorigenesis, tumour metastasis and targeted therapy [3]. However, detection, purification and analysis are the major challenges for detailed study of their function and characterization. A number of labelling and tracking approaches have been used to examine exosome uptake into target cells and *in vivo* bio distribution [4].

Exosome detection to study their bio distribution and target cell interaction often involves labelling of lipid bilayer membranes using the fluorescent lipid membrane (lipophilic) dyes like PKH26, PKH67, DiI and DiD [5] However, these dyes have certain drawbacks, such as the formation of dye aggregates without exosomes, indistinguishable dye particles and labelled exosomes and/or dye-exosome aggregates, which are taken up by cells with lesser efficiency than smaller exosomes [6].

In the present study, we have used Oil Red O(ORO) a fat-soluble fluorescent dye usually used to stain neutral lipids, cholesterol esters and lipoproteins. ORO is cell permeable and has been used for a long time for intracellular lipid staining and for tissue staining. ORO is an azo dye; its chemical structure contains two azo groups attached to three aromatic rings. Using oil red O, neutral lipids (mainly triglycerides) are stained with an orange-red tint [7,8]. The principle of staining lipids with ORO is based on the higher solubility of ORO in lipids compared to the dye solvents. Therefore, ORO solves the issue of long-term persistence of commonly used lipophilic dyes. Further, ORO also gives an intense red coloration which helps in the easy detection of lipids. Therefore, in the present study, we evaluated the lipid staining property of ORO for staining exosomes.

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2. Materials and methods

2.1. Materials

RPMI (Cat. No. AL028A), Trypsin-EDTA (Cat. No. TCL007) and Penicillin-streptomycin (Cat. No. A018) were purchased from Himedia. Fetal Bovine Serum (10%) (Gibco, Cat. No.10270106), Oil RED O Stain (Merck, Cat No.09755), NaCl (Merck, Cat. No. 1064040500), KCl (Merck, Cat. No. P3911), Na_2HPO_4, KH_2PO_4, Acridine orange (Cat.No.158550) and bisBenzimide H 33342 trihydrochloride (Cat. No. B2261) were purchased from sigma.

2.2. Cell culture and exosome isolation

The human A549 lung cancer cell lines were obtained from National Centre for Cell Science, Pune. The cells were grown in RPMI culture media with 5% CO_2 at 37 °C in a humidified incubator. Exosomes were isolated by ultracentrifugation method using protocol described by Thery et al. [9]. Briefly, 40% confluent cells were washed three times with 1x PBS and then 10 ml of exosomes depleted media was added. After 48 h of incubation, media were collected and filtered through 0.22-μm syringe filter. To remove dead cells and debris supernatant was subjected to several centrifugation and filtration steps. Exosomes were pelleted down by ultracentrifugation at 100000×*g* for 70min and resuspended in 1x PBS. All the samples were pooled together in one tube and ultracentrifuged for a second round of centrifugation at 100000×*g* for 70 min. The supernatant was discarded and pellet was dissolved in 50 μl of 1x PBS and stored at −20 °C.

2.3. Characterization of exosomes

For characterization studies, fresh exosomes were used as repeated freeze and thaw cycles damaged and affected the quality and quantity of exosomes.

Transmission Electron Microscopy (TEM): Exosome samples were spotted on a TEM (HR-TEM JEOL 2100F) copper grid for ~2 min, and then washed with water. Samples were stained with 2% (w/v) aqueous uranyl acetate solution for ~10 min followed by another washing step. Air-dried grids were then examined at an accelerating voltage of 120 KV.

Atomic Force Microscopy (AFM): The surface morphology of the exosomes was visualized by AFM using the WITec AFM system, Germany. The samples were diluted (10 folds) in ultrapure water and 20 μl samples were smeared on freshly cleaved mica sheet and air dried. The dried samples were then washed thoroughly with ultrapure water and air dried again. Images were taken immediately using tapping mode (NC-AFM) with a resonance frequency of 300 Hz.

Dynamic Light Scattering: The hydrodynamic size of exosomes was determined by DLS. The measurements were performed by spectrosize300 (Nano Bio chem Technology, Hamburg) equipped with an inbuilt Peltier controller unit. Samples were suspended in PBS before analysis.

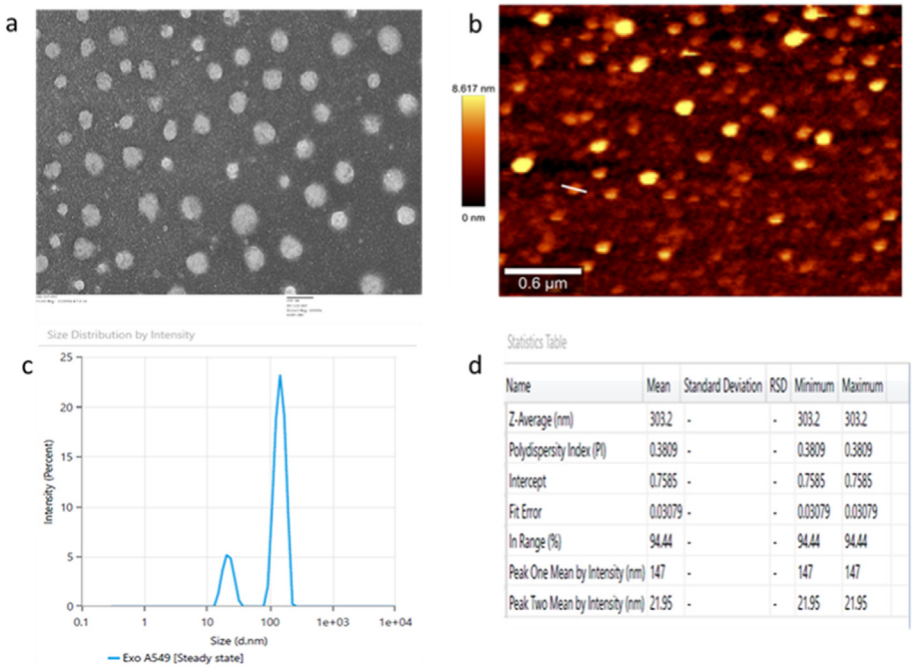
2.4. ORO preparation and it's binding with exosome

2.4.1. Dye preparation

ORO stock-solution was prepared by dissolving 5 mg of dye powder in 1 ml of 100% isopropanol and left overnight at room temperature. Next day, the dye was filtered with a 0.2 μm pore size membrane filter. A working solution was made by mixing 350 μl ORO stock-solution with 150 μl double distilled water. The working solution was again filtered using a 0.1 μm pore size filter.

2.4.2. Fluorescence spectrophotometey

Binding of ORO dye to exosomes was monitored by a fluorescence spectrophotometer (Shimadzu RF-5300, Japan). The ORO was excited at 560 nm and emission maxima was at 690 nm. The fluorescence intensity was measured using arbitrary units. Cells



Name	Mean	Standard Deviation	RSD	Minimum	Maximum
Z-Average (nm)	303.2	-	-	303.2	303.2
Polydispersity Index (PI)	0.3809	-	-	0.3809	0.3809
Intercept	0.7585	-	-	0.7585	0.7585
Fit Error	0.03079	-	-	0.03079	0.03079
In Range (%)	94.44	-	-	94.44	94.44
Peak One Mean by Intensity (nm)	147	-	-	147	147
Peak Two Mean by Intensity (nm)	21.95	-	-	21.95	21.95

Fig. 1. Characterization of isolated exosomes from A549 cells. (a) Transmission electron microscopy image of the isolated particles (b) Atomic force microscopy images showing topography (c) &(d) DLS result for the size distribution of exosomes.

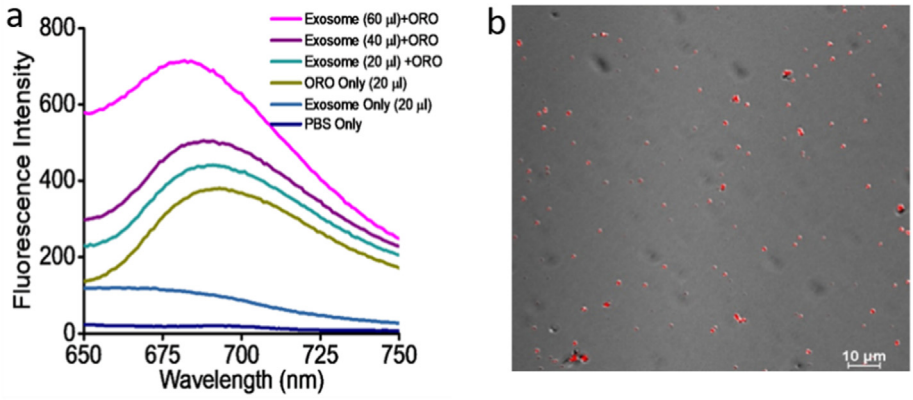


Fig. 2. Staining of exosomes by ORO a) Fluorescence emission spectra of Exosomes–Oil Red O complex. Fluorescence intensity increases with an increase in exosome concentration (l_{em} = 690 nm and l_{ex} = 560 nm). b) Isolated exosomes stained by ORO showing fluorescence under confocal microscopy.

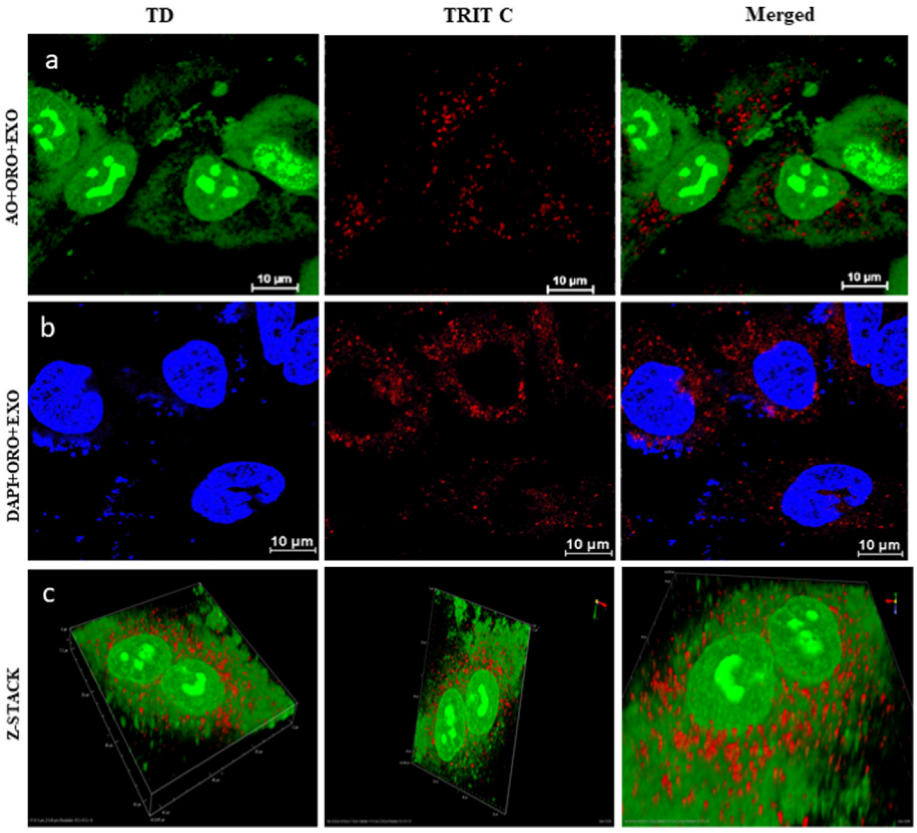


Fig. 3. Exosome localization and uptake by A549 cells incubated with ORO stained exosomes. a) Cells stained green (acridine orange). b) Nucleus stained blue (Hoechest). c) Representative Z-stack confocal image in different orientations and zoomed view of 3D reconstruct. All confocal microscopy images were captured at100 X magnification (Scale: 10 µm). Mark the red stained exosomes.

were incubated with the same concentration of ORO working solution and incubated for given time to check the binding of dye to cells.

2.5. Uptake of ORO stained exosomes by cells

Confocal Microscopy Imaging:Nikon AIR HD Fluorescent Confocal Microscopy was used to visualize ORO stained exosomes and their uptake by the cells. Live cell imaging was used to monitor the uptake. ORO-stained exosomes were incubated with the cells, at different time points. Hoechst33342 (10 µg/ml) and acridine orange (50 µg/ml) were used for nuclear and cell staining respectively. Cells were grown on coverslips, mounted on glass slides, and Z-stack images of the exosome uptake, were captured at 100x. NIS element software was used for data acquisition and analysis of images.

3. Results & discussion

3.1. Characterization of isolated exosomes

Transmission electron microscopy images of the isolated exosomes showed spherical morphology, indicating the membrane

integrity of the exosomes isolated by the ultracentrifugation method. The lipid bilayer membrane could also be seen. The exosomes were smaller than 100 nm in size (Fig. 1a), which is in agreement with earlier studies. Exosomes of similar sizes and morphology have been reported in other studies in A549 as well as in other cancer cell lines [10,11,12]. The surface topography of the exosomes was analysed using Atomic Force Microscopy. This further confirmed the spherical shape and the membrane integrity of the exosomes.

The size of the exosomes measured in terms of hydrodynamic size using Dynamic Light Scattering showed an average height of 8.617 nm (Fig. 1b). The Z average, or overall mean size of the exosomes, was found to be 303 nm (Fig. 1c and d). The PDI was 0.3, which is within the acceptable range.

Binding of ORO dye to exosomes was monitored by a fluorescence spectrophotometer (Shimadzu RF-5300, Japan). The excitation and detection wavelengths were 560 nm and 690 nm respectively. The fluorescence intensity of dye increased significantly after binding to exosomes. Fluorescence intensity was measured using arbitrary units. Increasing the amount of exosomes increased the fluorescence further (Fig. 2a). Fig. 2b shows images of ORO-stained exosomes. ORO may bind to exosome cargo like lipids, proteins, RNA or even dsDNA to give red fluorescence [13].

To monitor the uptake of labelled exosomes by cells, stained exosomes were incubated for 3 h with the cells grown on cover slips. Only exosomes stained with ORO showed fluorescence under the TRIT C channel, while no fluorescence was detected in cells stained with the same concentration of dye. (Supplementary Figs. 1a and 1b). Incubation of cells with ORO stained exosomes for up to 3 h showed intracellular fluorescence. The cells stained with Acridine Orange for whole cell staining or Hoechst33342 for nuclear staining showed exosomes stained red with ORO scattered in the cytoplasm and not inside the nucleus (Fig. 3a and b).

To further check the binding of dye to cells, cells were incubated with the same concentration of dye and incubated for the same time points without exosomes. Cells treated with this concentration of ORO did not stain any cell components (Supplementary Figs. 2a and 2b). The intracellular cytoplasmic localization of ORO stained exosomes was further confirmed by the 3D Confocal Microscopy Z slicing images (Fig. 3c).

In conclusion, staining of exosomes using ORO dye was successful, with good sensitivity of detection using fluorescence microscopy. This method did not show any dye aggregate and dye exosome aggregates were also negligible. Since the binding of the dye to exosomes increased the fluorescence significantly, it was easy to detect and track the exosome uptake by the cells. The concentration of dye required to stain exosomes does not stain any cell components, making it easier to localize them in live cells. The uptake of the stained exosomes by live cells was easy to monitor because of the distinct red fluorescence. Thus, the ORO staining method can be used as a good alternative to the commonly used fluorescent dyes for exosome labelling and uptake studies.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.04.087.

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