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Nucleic acid loading and fluorescent labeling of isolated extracellular vesicles requires adequate purification

Stephan Stremersch[a,b], Toon Brans[a,b], Kevin Braeckmans[a,b], Stefaan De Smedt[a,*], Koen Raemdonck[a,*]

[a] Laboratory of General Biochemistry and Physical Pharmacy, Faculty of Pharmaceutical Sciences, Ghent University, Ottergemsesteenweg 460, 9000 Ghent, Belgium
[b] Center for Nano-and Biophotonics (NB-Photonics), Ghent University, 9000 Ghent, Belgium

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ABSTRACT

Extracellular vesicles (EVs) are nanosized vesicular structures released by cells to communicate with one another. The growing interest in the (patho)physiological function and potential pharmaceutical application of these vesicles is accompanied by a vast number of new research groups entering this research field and a plethora of different protocols to separate EVs from non-vesicular components. This lack of uniformity often generates conflicting or difficult-to-compare results. Here we provide a comparative analysis of different EV isolation strategies, discussing the purity of the final isolate and highlighting the importance of purity on downstream experimental readouts. First, we show that ultracentrifugation (UC) of B16F10 melanoma cell-derived conditioned medium co-purifies proteins or protein complexes with nuclease activity. Such contaminants should be taken into account when aiming to apply EVs as delivery carriers for exogenous nucleic acids. Second, three commonly used purification strategies (i.e. precipitation, UC and density-gradient centrifugation) were evaluated for their ability to remove non-incorporated fluorescent dye (i.e. the lipophilic PKH67 dye), important when probing EV interactions with cells. For both types of impurities, endogenous and exogenous, density gradient purification outperforms the other evaluated methods. Overall, these results demonstrate that the implementation of stringent purification protocols and adequate controls is of pivotal importance to draw reliable conclusions from downstream experiments performed with EV isolates.

1. Introduction

Since their discovery in the late 1960s, extracellular vesicles (EVs) such as exosomes and ectosomes have been linked to many physiological processes and are scrutinized for potential pharmaceutical applications in different areas, including biomarker discovery (Skog et al., 2008), nucleic acid delivery (Alvarez-Erviti et al., 2011), immunotherapy (Zitvogel et al., 1998) and as cell surrogate for regenerative therapy (Lai et al., 2010). Especially the last couple of years, since the identification of EVs as nature's own RNA transporters (Valadi et al., 2007), interest to exploit these vesicles as bio-inspired nanocarriers for exogenous nucleic acids, e.g. siRNAs, has been growing exponentially (Stremersch et al., 2016a).

One of the major bottlenecks still hampering the development of EVs as nanosized drug carriers is the inability to efficiently load them with exogenous synthetic RNAs (Mateescu et al., 2017; Vader et al., 2014). A first reported approach applied electroporation (EP) to introduce transient pores in the EV membrane through which the therapeutic cargo can passively migrate (Alvarez-Erviti et al., 2011; Wahlgren et al., 2012). However, this technique appeared to be much less efficient as previously believed due to EP-induced unspecific aggregation of the siRNA, independent of the presence of EVs (Kooijmans et al., 2013). As a result other methods have been evaluated. The use of cholesterol-modified siRNA was explored by us and others to associate siRNA to the vesicular membrane by means of hydrophobic insertion (Didiot et al., 2016; O'Loughlin et al., 2017; Stremersch et al., 2016b). Alternatively, Fuhrmann et al. induced pores in the EV membranes using a saponin treatment to allow cargo influx (Fuhrmann et al., 2015). Bryniarski and colleagues reported that antibody-coated EVs released by B1a cells could take up free, unconjugated miR-150 after simple co-incubation at 37 °C and functionally deliver this miRNA to effector T-cells (Bryniarski et al., 2013, 2015). The latter three approaches are all based on the co-incubation of EVs and a (nucleic acid) cargo. Therefore, the method that is used to separate EVs from the free, non-associated cargo is of critical importance to reliably asses the true loading efficiency.

* Corresponding authors.
E-mail addresses: Stefaan.Desmedt@Ugent.be (S. De Smedt), Koen.Raemdonck@Ugent.be (K. Raemdonck).

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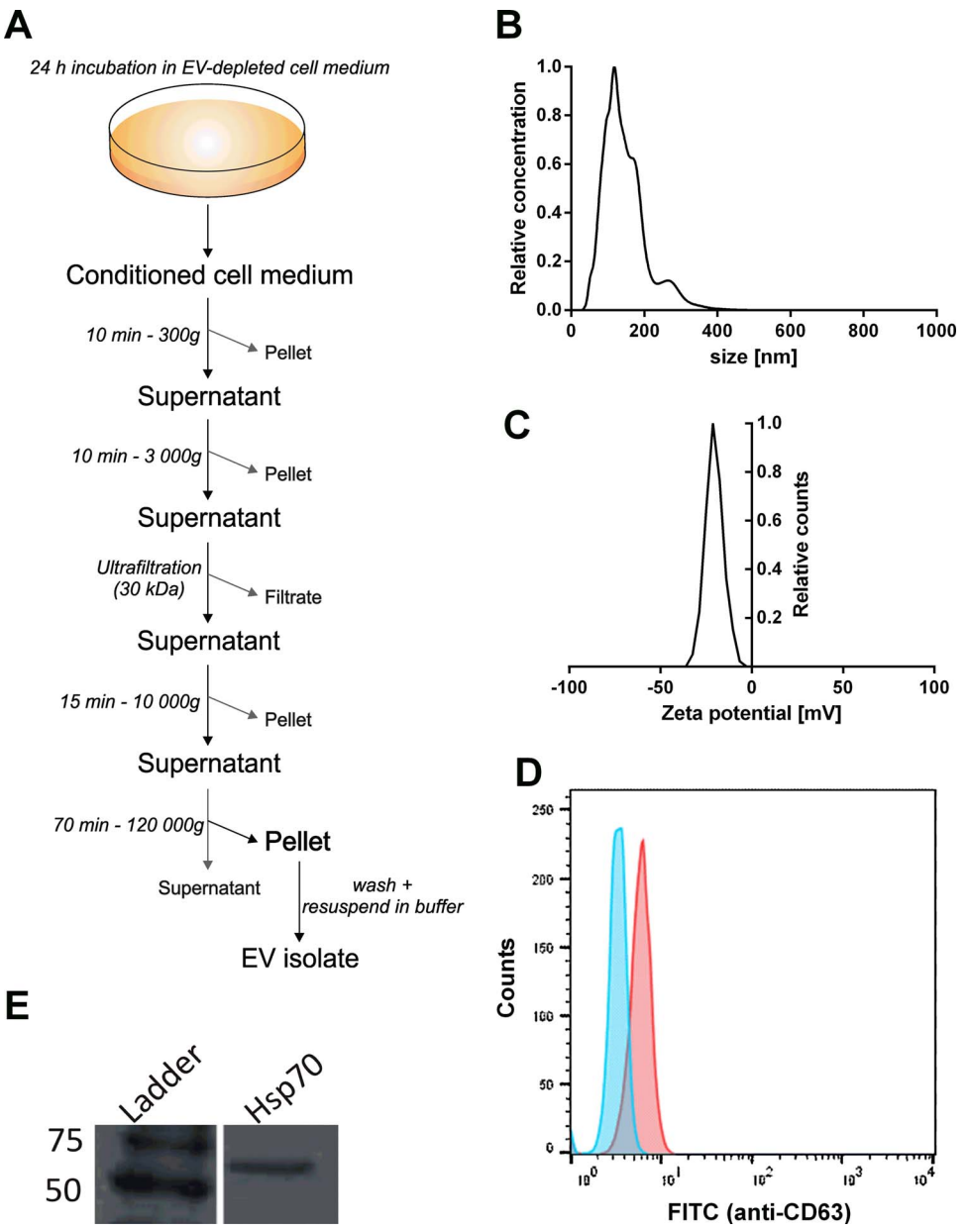

Fig. 1. Purification and characterization of extracellular vesicles (EVs) isolated from B16F10 melanoma cells. [A] Schematic overview of the UC-based purification protocol. [B] Representative size and [C] zeta potential distribution of purified B16F10-derived EVs. [D] Flow cytometry analysis of anti-CD63 coated dynabeads® incubated with B16F10-derived EVs and FITC anti-CD63 (red) or FITC control antibody (blue), respectively. [E] Immunoblotting of isolated EVs using an anti-Hsp70 antibody. The molecular weight of the reference ladder proteins is indicated in kDa. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Besides methods to load EVs with therapeutics, robust protocols to study their interaction with cells are highly desired. In this respect, fluorescence microscopy is an important tool to investigate the cellular internalization, subsequent intracellular trafficking and nucleic acid delivery potential of EVs (Mulcahy et al., 2014). In analogy with the cell-like architecture of EVs, many cell labeling strategies are adopted for EVs as well. The type of dyes most often used throughout the literature are equipped with a lipophilic tail allowing insertion in the lipid membrane of EVs (e.g. PKH26 (Christianson et al., 2013), PKH67 (Ekstrom et al., 2012; Feng et al., 2010; Svensson et al., 2013; Zhu et al., 2015), R18 (Montecalvo et al., 2012; Parolini et al., 2009), DiI (Obregon et al., 2006)). Alternatively, EVs have been labeled with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE), which is a membrane permeable molecule responsive to esterase activity present in the lumen of (a subtype of) EVs. After cleavage of the acetate group the molecule can bind covalently to amino acids present inside the EV lumen, after which it becomes fluorescent (Temchura et al., 2008). Finally, the nucleic acid cargo of EVs can be labeled using the membrane permeable acridine orange (Waldenstrom et al., 2012) and SYTO RNASelect dyes (Li et al., 2014). Although the above mentioned dyes can effectively label certain EV components, protocols to wash away unbound or non-EV associated labels are insufficiently characterized, which might lead to incorrect interpretation of downstream experimental data.

In the past, the use of stringent purification protocols was mainly emphasized in a diagnostic context where co-purification of non-vesicular RNAs or proteins impedes the identification of reliable biomarkers (Van Deun et al., 2014). Here, we demonstrate that non-vesicular contaminants present in EV isolates can equally lead to misinterpretation of downstream data regarding EV post-formation loading with nucleic acid based therapeutics and fluorescent dyes. Consequently, we postulate that stringent purification strategies, such as density gradient isolation, are essential to unambiguously investigate the value of EVs as cellular drug delivery vehicles.

2. Experimental section

2.1. Materials

Nucleic acids are listed in Table S1. Exoquick-TC was purchased