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Research paper

The impact of various preanalytical treatments on the phenotype of small extracellular vesicles in blood analyzed by protein microarray

Rikke Bæk *, Evo K.L. Søndergaard, Kim Varming, Malene M. Jørgensen

Department of Clinical Immunology, Part of Extracellular Vesicle Research Center Denmark (EVsearch.dk), Aalborg University Hospital, Aalborg, Denmark

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ABSTRACT

The research field of extracellular vesicles (EVs) is increasing immensely and the potential uses of EVs seem endless. They are found in large numbers in various body fluids, and blood samples may well serve as liquid biopsies. However, these small membrane-derived entities of cellular origin are not straightforward to work with in regard to isolation and characterization.

A broad range of relevant preanalytical issues was tested, with a focus on the phenotypic impact of smaller EVs. The influences of the i) blood collection tube used, ii) incubation time before the initial centrifugation, iii) transportation/physical stress, iv) storage temperature and time (short term and long term), v) choice of centrifugation protocol, vi) freeze-thaw cycles, and vii) exosome isolation procedure (ExoQuick™) were examined. To identify the impact of the preanalytical treatments, the relative amounts (detected signal intensities of CD9-, CD63- and/or CD81-positive) and phenotypes of small EVs were analyzed using the multiplexed antibody-based microarray technology, termed the EV Array. The analysis encompassed 15 surface- or surface-related markers, including CD9, CD63, CD81, CD142, and Annexin V.

This study revealed that samples collected in different blood collection tubes suffered to varying degrees from the preanalytical treatments tested here. There is no unequivocal answer to the questions asked. However, in general, the period of time and prospective transportation before the initial centrifugation, choice of centrifugation protocol, and storage temperature were observed to have major impacts on the samples. On the contrary, long-term storage and freeze-thawing seemed to not have a critical influence. Hence, there are pros and cons of any choice regarding sample collection and preparation and may very well be analysis dependent. However, to compare samples and results, it is important to ensure that all samples are of the same type and have been handled similarly.

1. Introduction

In recent years, the interest in extracellular vesicles (EVs) has increased immensely (Lötvall et al., 2014) and several studies have shown the potential of utilizing them in a clinical setting, as diagnostic, prognostic and as therapeutic agents, as reviewed by Revenfeld et al. (Revenfeld et al., 2014) and György et al. (György et al., 2015). Furthermore, it has proven relevant to investigate immune cell-derived EVs, as they appear to be important in several immunological relations (Robbins and Morelli, 2014; Pugholm et al., 2016). EVs are small membrane-derived entities produced from a diverse range of cell types throughout the human body and, therefore, they are accessible in various body fluids (Caby et al., 2005; Admyre et al., 2007; Ogawa et al., 2008; Gonzales et al., 2009). These vesicles can be divided into several subgroups according to specific characteristics such as cellular origin, size, protein/RNA composition, and biogenesis. When sorting using the latter characteristic, the major subgroups are exosomes (30–100 nm in diameter), microvesicles (MVs, 100–1000 nm) and apoptotic bodies (500–4000 nm) (Pugholm et al., 2015), although MVs are also often denoted microparticles (MPs) (Colombo et al., 2014). Each of these subgroups possess numerous biological functions, and it is of interest to determine them thoroughly to fully understand and utilize the vesicular biology (Mulcahy et al., 2014).

A major challenge when working with EVs is the pronounced impact that the preanalytical treatment has on the analysis outcome. Many investigators have highlighted the importance of a consistent protocol for sample collection and preparation of EVs (Lacroix et al., 2010; György et al., 2011; Yuana et al., 2011; Lötvall et al., 2014); however, it is also relevant to consider which protocols are the best suited for the research question addressed (Witwer et al., 2013). The choice of anticoagulant in the blood collection tube has a considerable influence on the MV/MP count (Jayachandran et al., 2012; György et al., 2014), as has the incubation time between blood collection and centrifugation, with an up to 80% increase in the MP count after 4 h (Lacroix et al., 2012). One very

* Corresponding author at: Department of Clinical Immunology, Aalborg University Hospital, Urbansgade 32-36, DK-9000 Aalborg, Denmark.
E-mail address: rikke.baek@rn.dk (R. Bæk).

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well-described preanalytical factor is the centrifugation procedure. A standardized protocol for the preparation of platelet-free plasma (PFP) has been suggested and it has shown to be of great importance when analyzing MVs/MPs (Lacroix et al., 2012). However, it is hard to obtain strictly platelet-free plasma and consequently it is urged that the same protocol is applied to all samples that are to be compared (Witwer et al., 2013).

Factors such as freezing, storage temperature and time, freeze-thaw cycles, and transportation have been examined as well (György et al., 2014; Jayachandran et al., 2012; Dey-Hazra et al., 2010; Lacroix et al., 2012). Nevertheless, these features are somewhat influenced by the method of isolation and subsequent choice of analysis. However, all of these factors have primarily been investigated regarding MVs/MPs, whereas there is little knowledge of the impact of the smaller vesicle types (exosomes and exosome-like vesicles), especially in regard to protein load.

Analysis of biobank samples is typically an ongoing process years after collection; hence, a challenge is that the material is often quite old. Other issues are that the samples may have been exposed to several freeze-thaw cycles, and/or that the blood has been collected in tubes that were most optimal for the first analysis in mind. Therefore, it is critical to determine the preanalytical impact as thoroughly as possible and optimally for all types of analyses and for all types of EVs.

To determine the phenotypes of small EVs (sEVs), we have established a protein microarray-based analysis, which is termed the EV Array. This analysis platform, described by Jørgensen et al. (Jørgensen et al., 2013), is optimized to catch and detect the smaller types of EVs, such as exosomes and exosome-like vesicles, with diameters up to ~150 nm. The detection is performed by utilizing a cocktail of antibodies against the tetraspanins CD9, CD63, and CD81, which are found on exosomes (Vlassov et al., 2012). The detection antibodies are easily exchangeable, as are the capture antibodies, which can be combined as desired and target up to 60 different markers simultaneously in the same well (Jørgensen et al., 2015). The analysis is performed in a 96-well setup and consumes only 10 μL of plasma, which makes the platform very cost-efficient, multiplexed and high-throughput. It has already demonstrated great diagnostic potential in non-small cell lung cancer (NSCLC), where cancer patients were distinguished from non-cancer lung-diseased patients with up to 75.3% accuracy (Jakobsen et al., 2015; Sandfeld-Paulsen et al., 2016).

As Choi et al. (Choi et al., 2013) previously noted, a number of limitations and challenges prevent EVs from being used diagnostically at present. The goal is a rapidly performed and low-cost analysis platform, which meets the limited decision time of the clinician. However, aspects such as preanalytical standardization and development of a reliable technique must be addressed initially. Here, we aim to complement the current knowledge of preanalytical factors with a focus on phenotyping sEVs using an antibody-based platform designed to include 15 relevant markers. Using four different types of blood collection tubes, blood from five healthy volunteers was analyzed and a broad range of preanalytical factors was tested, as illustrated in Fig. 1.

2. Materials and methods

2.1. Blood sampling

All research involving samples from human subjects was approved by the local ethics legislation. Each person signed a written consent form allowing for the use of their blood for research purposes. Venous blood samples were obtained from five healthy volunteers and collected into four different types of Vacuette® blood collection tubes (Greiner Bio-One GmbH, Germany): CPDA (citrate phosphate dextrose adenine), EDTA (K3EDTA, ethylenediaminetetraacetic acid), heparin (Lithium Heparin) and serum (Z Serum Clot activator) tubes. The same number of tubes was collected for each Vacuette® type from the same donor at once within the same laboratory where the samples were to be handled afterwards. Subsequently, the preanalytical treatments and tests were performed simultaneously on all four Vacuette® types as specified in the following.

Unless otherwise specified, the blood samples were centrifuged once (1800*g* for 6 min at room temperature, RT) 1 h after collection, before aliquoting and storage at −40 °C. Furthermore, the samples were analyzed within a few days of sample collection. The same experienced operator carried out the handling of all samples and analyses.

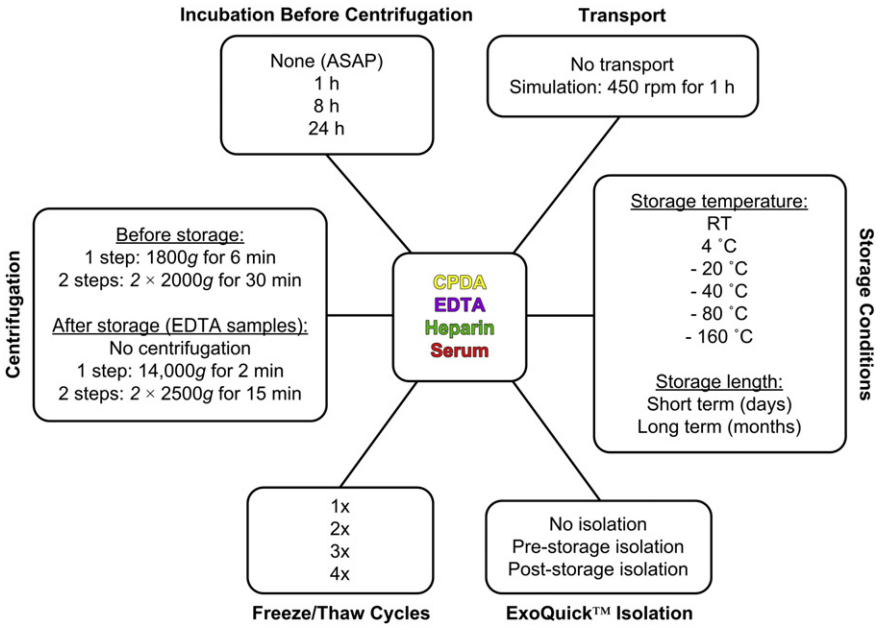

Fig. 1. Schematic overview of the preanalytical factors tested. Five healthy volunteers were included in the study and samples were assembled using the Vacuette® blood collection tubes CPDA, EDTA, heparin, and serum. Unless otherwise specified, blood collection tubes were centrifuged once at 1800*g* for 6 min 1 h after collection and sample aliquots were stored at −40 °C. ASAP, as soon as possible; h, hour; RT, room temperature; rpm, rounds per minute.