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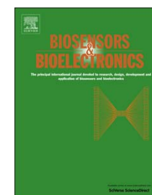

Point-of-care detection of extracellular vesicles: Sensitivity optimization and multiple-target detection

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ABSTRACT

Extracellular vesicles (EVs) are membrane-bound nanovesicles delivered by different cellular lineages under physiological and pathological conditions. Although these vesicles have shown relevance as biomarkers for a number of diseases, their isolation and detection still has several technical drawbacks, mainly related with problems of sensitivity and time-consumed. Here, we reported a rapid and multiple-targeted lateral flow immunoassay (LFIA) system for the detection of EVs isolated from human plasma. A range of different labels (colloidal gold, carbon black and magnetic nanoparticles) was compared as detection probe in LFIA, being gold nanoparticles that showed better results. Using this platform, we demonstrated that improvements may be carried out by incorporating additional capture lines with different antibodies. The device exhibited a limit of detection (LOD) of 3.4×10^6 EVs/μL when anti-CD81 and anti-CD9 were selected as capture antibodies in a multiple-targeted format, and anti-CD63 labeled with gold nanoparticles was used as detection probe. This LFIA, coupled to EVs isolation kits, could become a rapid and useful tool for the point-of-care detection of EVs, with a total analysis time of two hours.

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1. Introduction

Extracellular vesicles (EVs) are considered to be important mediators in intercellular communication. They are delivered by different cell types under physiological and pathological conditions and can be found in different biological fluids (Yáñez-Mó et al., 2015). According to their size, composition, or origin EVs are generally referred to microvesicles (MV, in the range of 1000 nm) and exosomes (40–150 nm) (Théry et al., 2002; György et al., 2011). As current purification methods do not fully discriminate between microvesicles and exosomes, the general term EVs will be used here to refer to the circulating vesicles isolated from biological fluids different from apoptotic bodies. It has been shown that the concentration and composition of EVs is altered in a number of diseases (Melo et al., 2015; Zhang et al., 2015), including vascular disorders (Belting and Christianson, 2015), neurodegenerative diseases (Russo et al., 2012), and cancer (Azmi et al., 2013). Thus, EVs show a great potential for their use as a noninvasive biomedical tool, serving as biomarkers for diagnostic, prognosis and monitoring response to treatment in certain diseases (Properzi et al., 2013; De Toro et al., 2015). This aspect has triggered recent researches on the development of portable analytical platforms (Vaidyanathan et al., 2014; Jeong et al., 2016). Two major challenges for the detection of EVs are the achievement of short real-time detection and sensitivity in bioanalysis. To reduce the time required for target detection, a minimal amount of sample manipulation is essential. Although ultracentrifugation (UC) is generally regarded as the “gold standard”, for isolating EVs, it is time-consuming, requires special equipment and some studies from the literature have demonstrate inconsistencies in reproducibility and low recoveries. Besides UC, other methods such as ultrafiltration, antibody-coated magnetic beads and polymer-based precipitation have been used. Precipitation reagents, which are commercially available, reduce hands-on time and the requirements for specialized expensive equipment (Sunkara et al., 2016). In addition, it has been reported that this method provides a higher yield compared to other isolation methods (Caradec et al., 2014). Challenges exist

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not only in the isolation, but also in the detection of EVs. Immunochromatographic assay, also called lateral flow immunoassay (LFIA), is a powerful tool for rapid and low-cost on-site detection of biomolecules (Posthuma-Trumpie et al., 2009). The overall format of LFIA uses the same rationale than ELISA, where immobilized capture antibody or antigen is bound onto a solid phase nitrocellulose membrane instead of a plastic well. The advantage here is the fact that the entire test can be done in one-step and in a few minutes, unlike the procedure involved at the multiple-step ELISA (Eltzov et al., 2015). The sensitivity of the detection method has to be high enough to eliminate the need for target amplification and enrichment steps, while being able to accurately discriminate between healthy individuals and patients. However, even though LFIA has been successfully applied to numerous systems, it shows limitations in high sensitivity applications (Linares et al., 2012). Thus, great efforts have been made to improve the performance of LFIA using different labels. The choice of the optimal label (e.g. gold nanoparticles, colored latex beads, magnetic particles, carbon nanoparticles, quantum dots, organic fluorophores, enzymes, liposomes, etc.) in the LFIA development process is critical. Therefore, a comparison of the performance and sensitivities of different labels could be used to significantly optimize the detection limit of the end product. Easy conjugation with biomolecules, stability over longer period of time and easy visualization are desirable features for a good label. Markers which give direct signal are preferable in LFIA because of less time consumption and reduced procedure (Eltzov et al., 2015; Huang et al., 2016).

Currently the most commonly used particulate detector reagents in lateral flow systems are colloidal gold (AuNPs) and monodisperse latex. In naked-eye assays, it is often possible to increase sensitivity using AuNPs rather than colored latex particles, because of their optical properties. Due to the smaller size of the AuNPs (typically in the range of 20–40 nm) a higher packing density can be achieved on a test line (O'Farrell, 2009). Additionally, the optical signal obtained from AuNPs can be amplified by deposition of silver, AuNPs and enzymes (Gao et al., 2016; Rodríguez et al., 2016). Despite of these characteristics, in a comparison study performed by Linares et al. (2012) for sensitivities of labels commonly used in LFIA, carbon nanoparticles were ranked above AuNPs and latex beads, exhibiting a limit of detection ten times lower.

Carbon nanoparticles, (Carbon black, CB), are strongly dark colored nanoparticles that exhibit a high signal-to-noise ratio (black signal on a white background), a factor which helps to obtain good parameters of sensitivity, working range and limit of detection. The sensitivity of LFIA employing carbon black is reported to be comparable to ELISA. Other advantages attributed to carbon nanoparticles are the very low cost of the starting material, the ease of preparation, and the stability of the conjugates (Posthuma-Trumpie et al., 2012; Quesada-González et al., 2015; Sajid et al., 2015).

Most LFIA tests using these labels are qualitative by visual interpretation, and only nanoparticles on the top of the membrane contribute significantly to the signal. Consequently, the signal generated beneath the surface is inevitably missed, making them less sensitive. Magnetic nanoparticles (MNPs), as previously mentioned, can be used as labels on LFIA due to their brown color (Liu et al., 2011; Quesada-González et al., 2015). This color produced at the test line can be measured by an optical strip reader, but also the magnetic signals coming from MNPs within the entire volume of membrane could be detected and recorded with a magnetic flow reader (Wang et al., 2009). In addition, MNPs are suitable to achieve preconcentration and purification of the analyte in a sample by means of magnetic separation, which is useful to enhance markedly the sensitivity (Lei and Ju, 2013). Despite these advantages, it is known that signal intensities are usually related to the size of the MNPs, i.e., the larger the MNPs, the higher the responses. Thus, even though nitrocellulose membranes contain pores with widths in the range of micrometers, the biggest MNPs, combined with large analytes such as spores, bacteria or EVs, could block the pores, obstructing the flow of the conjugates across the membrane (Quesada-González et al., 2015).

We have previously demonstrated the potential of LFIA for detection of EVs using AuNP (Oliveira-Rodríguez et al.,2016). However, in the light that the label optimization could yield the sensitivity necessary to be used as a diagnostic tool, herein we evaluated the use of three different labels: gold nanoparticles (AuNPs), carbon black (CB) and magnetic nanoparticles (MNPs). Furthermore, since EVs are highly heterogeneous so that they express different set of proteins on the surface, the use of more than one test line immobilizing different antibodies may allow multiple-targeted detection and consequently increase number of binding sites. Thus, once established the optimal label, the sensitivity of multiple-targeted detection was compared to that obtained from the conventional LFIA based on single-targeted detection.

2. Material and methods

2.1. Reagents and equipment

Anti-tetraspanin antibodies in this study have been previously characterized: anti-CD9 VJ1/20 (Yáñez-Mó et al., 2001); anti-CD63 Tea3/18 (Peñas et al., 2000). Anti-CD81 5A6 was provided by Dr. S. Levy (Oncology, Stanford University School of Medicine, Stanford, CA). Anti-mouse IgG was purchased from Sigma-Aldrich (Spain). 40-nm gold nanoparticles (AuNP) were purchased from BB International (UK). Carbon Black (Special Black 4 Powder) was acquired from Orion Engineered Carbons GmbH (Germany). Magnetic beads of 100-nm with a magnetite core and polyacrylic acid coating were purchased from Chemicell GmbH (fluidMAG-PAS, Germany). Bovine serum albumin (BSA), 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. Other reagents used in this study were of analytical grade. Nitrocellulose membranes (HF07504XSS) and glass fiber sample pads (GFCP001000) were purchased from Millipore, Germany. Other materials used were: backing cards (KN-V1080, Kenoshatapes, Netherlands) and absorbent pads (Whatman, USA).

An IsoFlow reagent dispensing system (Imagene Technology, USA) was used to dispense the detection lines (dispense rate 0.100 μL/mm) and the strips were cut with a guillotine Fellowes Gamma (Spain). A portable strip reader ESE Quant LR3 lateral flow system (Qiagen Inc., Germany) was used to quantify the intensity of the test line by reflectance measurements.

2.2. Enrichment of extracellular vesicles from human plasma

All plasma collection and preparation protocols were approved by the Ethics Research Committee of the *Hospital Universitario Central Asturias (Oviedo, Spain).* 4.5 mL of blood were obtained from healthy volunteers in sodium citrate vacutainer™ tubes. Whole blood was then centrifuged at 1550*g* and subsequently at 3200*g* for 30 mins to separate platelet free plasma.

EV-enriched fractions from 320 μL of plasma were isolated by ExoQuick™ Exosome Precipitation Solution according to the manufacturer's instructions (System Biosciences Inc., Mountain View, CA, USA). The EVs pellet was resuspended in HEPES 10 mM, pH 7.4.

To determine the size distribution and concentration of the

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