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A novel broadband impedance method for detection of cell-derived microparticles

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ABSTRACT

A novel label-free method is presented to detect and quantify cell-derived microparticles (MPs) by the electrochemical potential-modulated electrochemical impedance spectroscopy (EIS). MPs are present in elevated concentrations during pathological conditions and play a major role in the establishment and pathogenesis of many diseases. Considering this, accurate detection and quantification of MPs is very important in clinical diagnostics and therapeutics. A combination of bulk solution electrokinetic sorting and interfacial impedance responses allows achieving detection limits as low as several MPs per μL. By fitting resulting EIS spectra with an equivalent electrical circuit, the bulk solution electrokinetic and interfacial impedance responses were characterized. In the bulk solution two major relaxations were prominent—β-relaxation in low MHz region due to the MP capacitive membrane bridging, and α-relaxation at ~10 kHz due to counter ions diffusion. At low frequencies (10–0.1 Hz) at electrochemical potentials exceeding −100 mV, a facile interfacial Faradaic process of oxidation in MPs coupled with diffusion and non-Faradaic double layer charging dominate, probably due to oxidation of phospholipids and/or proteins on the MP surface and MP lysis. Buffer influence on the MP detection demonstrated that a relatively low conductivity Tyrode's buffer background solution is preferential for the MP electrokinetic separation and characterization. This study also demonstrated that standard laboratory methods such as flow cytometry underestimate MP concentrations, especially those with smaller average sizes, by as much as a factor of 2–40.

1. Introduction

This study describes a novel electrochemical medical diagnostic technology to detect, quantify and characterize cell-derived microparticles (MPs). MPs are membrane bound vesicles ranging in size from 0.05 to 2 μm that bud off cells in response to stimulation and/or apoptosis. MPs are roughly spherical and compositionally similar to the cells of origin (Mallat et al., 2000; Blum, 2009). They are formed through an active process resulting in budding of selective regions of the cell membrane and in loss of membrane asymmetry, such that anionic phospholipids like phosphatidyl serine (PS) that are normally present on the inner leaflet of the cell membrane become exposed on the outer leaflet of MP membrane. MPs also carry surface antigenic markers specific to their cellular origin that can be detected with specific antibodies (Ghosh et al., 2008).

Although initially thought to be insignificant or even artifacts, it is known that MPs are specific, biologically active structures that participate in important physiological and pathological processes, including hemostasis, thrombosis, tumor progression, inflammation, and atherosclerosis (Cocucci et al., 2008; Furie et al., 2005; Leroyer et al., 2008; VanWijk et al., 2003; Van Doormaal et al., 2009). Proteins expressed on MPs target them to specific sites, such as inflammatory lesions, atherosclerotic plaques, developing thrombi, inflamed joints, and malignant tumors, where they regulate many homeostatic and pathologic events. Low concentrations of MPs can be detected in the blood of normal subjects but their number increases significantly in association with many acute and chronic diseases, including sickle cell anemia, coronary disease, aortic aneurysm, venous thrombosis, hypertension, sepsis, cancer and diabetes. Elevated levels of specific circulating MPs may thus serve as biomarkers for disease activity and also for specific disease complications, particularly thrombosis (Puccin et al., 2008; Tesselaar et al., 2007).

Despite an impressive amount of clinical research linking MPs to disease, MP detection, characterization and quantification have not yet become a diagnostic standard in medical care due to the absence of a simple and reliable detection system. Although MPs

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can be present at high levels in the circulation and offer an opportunity for a simpler analysis due to their spherical shapes and specific redox reactions involving membrane proteins, the concentrations of specific MPs are still extremely low comparing to normal blood components. Analytical techniques with detection limit of ~10 MP/μL and capability of detecting particles with sizes as low as 50 nm are required. Furthermore, since clinical correlations and risks are often related to the specific cell of origin of the MP, it is necessary to be able to characterize complex populations of MPs with respect to their cells of origin. Current state-of-the-art methods include fluorescence-activated flow cytometry (using light scattering for detection and immunofluorescence for characterization) and "Coulter" principle particle counting based on impedance. These techniques are expensive, operator-dependent, and unable to accurately detect particles smaller than 0.2–0.4 μm (Furie et al., 2005).

In this manuscript, we describe development of an electrochemical method for diagnostic, detection and characterization of 4 major types of cell-derived MPs—platelet, red blood cells (RBC), monocyte and endothelial cells. The method is label-free and capitalizes on the inherent sensitivity, selectivity, and rapid response of electrochemical impedance spectroscopy (EIS) analysis. EIS has exceptionally high resolving nature based on a combination of AC frequency and electrochemical potential modulation. EIS also allows integrating the electrochemical detection and bulk solution dielectrophoresis (DEP) "trapping" in a single measurement cycle (Ehret et al., 1997; Houssin et al., 2010; Wang, 2009; Gagnon et al., 2008). We show the potential to capture and quantify specific MP using DEP, examine buffer solution and interfacial kinetics effects on the EIS response, and develop a comprehensive equivalent circuit (EC) representation of MP suspensions.

EIS- and DEP-based technology is appropriate for this application because of the inherent differences among MPs based on their cell of origin. Different types of cells possess diverse osmotic pressures, internal ionic strengths, size, geometry, action potentials, and membrane protein profiles and densities, resulting in differences in their responses to electrochemical perturbation (DiBiasio and Cametti, 2007). Schwan was one of the pioneers to propose a complete model of biological cells dielectric dispersion according to the Maxwell-Wagner theory (Bothwell and Schwan, 1956). Since then, EIS has been applied to analysis of colloids (Schwarz, 1962) employed for characterization of different types of cells, proteins, and bacteria (Gimsa, 2001; Jones, 2003; Asami et al., 1996) with respect to their concentration, size, diffusion coefficient, and chemical changes (Yang and Zhang, 2007; Qiu et al., 2009; Smiechowski et al., 2007). Cells such as platelets, monocytes, endothelial cells, cancer cells, and RBCs show significant variability in shape (regular spherical vs. highly irregular), size (ranging from 2 to 20 μm), size distribution, cytoplasm composition (for example, presence of negatively charged hemoglobin in RBC), presence and shape of nuclei, presence and amount of RNA, presence of various ionic exchange pumps, and thickness and composition of cell membranes. We thus hypothesized that MPs derived from these cells would show significant differences in their electrochemical properties.

2. Materials and methods

2.1. MPs generation

MPs from platelets and RBCs were isolated from healthy normal subjects as previously described (Ghosh et al., 2008). For platelet-derived MPs, platelet rich plasma (PRP) was prepared by low speed centrifugation. Washed platelets were then treated with Ca ionophore A23187 (10 μm) in the presence of 2.5 mM $CaCl_2$ for 1 h at 37 °C or with *t*-BuOOH (500 μm) for 2 h followed A23187 for 1 h. Intact platelets were then removed by centrifugation at 2000 × *g* for 15 min. The supernatants were then subjected to a high-speed centrifugation at 100,000 × *g* to pellet MPs. To generate RBC-derived MPs, the RBC layer remaining after the removal of PRP was diluted and washed three times with PBS and treated as above with A23187 or *t*-BuOOH plus A23187. Intact RBCs were then removed by centrifugation at 2000 × *g* for 15 min and MPs were pelleted and re-suspended as above. To generate endothelial cell (EC)-derived MPs, human umbilical vein endothelial cell cultures (Jaffe et al., 1989) were incubated with 100 ng/ml TNF-α (R&D Systems) and 50 μg/mL cycloheximide (Sigma–Aldrich) for 24 h. Culture supernatants were collected and non-viable cells and large cell fragments were removed by centrifugation at 4300 × *g* for 5 min. The supernatants were then centrifuged at 100,000 × *g* for 90 min at 10 °C to pellet MPs. THP1 cells (Acute human monocytic leukemia cell line) were stimulated with 12 μM A23187, to generate MPs according to a previously published protocol (Ghosh et al., 2008).

All MPs were re-suspended in PBS (pH 7.1) or Tyrode's buffer (pH 7.4; 137 mmol/L NaCl, 2.8 mmol/L KCl, 1.0 mmol/L $MgCl_2$, 12 mmol/L $NaHCO_3$, 0.4 mmol/L Na_2HPO_4). The MPs were detected by flow cytometry using light scatter (forward and side scatter) and quantified using known amount of standard 0.3 and 3 μm latex beads (Sigma–Aldrich) spiked into the solutions (Ghosh et al., 2008). Stock solutions of 5×10^5 MP/μL were prepared for each of the 4 MP types and were successively diluted by factors of 10^1 to 10^5 for further analysis. Surface PS was detected and quantified on the MPs using flow cytometry with PE-conjugated annexin V and the cell of origin was confirmed using antibodies to endothelial (CD105 and CD144), platelet (CD41) and monocyte (CD14) and RBC (CD235) specific markers.

2.2. Electrochemical analysis

EIS testing was performed using an Impedance/Dielectric Analyzer (Novocontrol GmbH, Hundsangen Germany) with ZG4 cell adapter. The sampling frequencies ranged from 20 MHz to 5 mHz with the AC perturbation of 10 mV. The EIS data was initially collected in a "dielectric regime" at rest potential on two 2 cm diameter disc parallel plate gold-plated copper electrodes with a spacing of 0.1 mm. Subsequently the impedance response at applied potentials of 0, +0.5, −0.2, −0.5 and −0.8 V (vs. Ag/AgCl) was studied using the same gold-plated electrodes as working and counter electrodes with an Ag/AgCl reference electrode (BAS Analytical) following an initial stabilization period. Plotting, analysis, and modeling of the data were performed with the WinFIT software package (Novocontrol GmbH).

The cyclic voltammetry (CV) testing was performed using a 660A Electrochemical Workstation (CH Instruments, Austin, TX). For the CV testing the working electrode was a 50 μm diameter gold wire (Alfa Aesar, 99.9% pure) sealed in soft glass and physically polished. The counter electrode was a 1 cm^2 platinum foil and the reference electrode was a standard Ag/AgCl electrode. Voltammetry scans were recorded at scan rates of 100 and 10 mV s^{-1} between +1.0 and −1.0 V (vs. Ag/AgCl).

For the CV and EIS testing a single compartment glass electrochemical cell was constructed using 25 g glass vials (Kimble Glass, Inc.). Prior to the experiment the electrodes were rinsed in acetone, polished, and rinsed in deionized water before being inserted in the cell. All solutions were deoxygenated before the analysis by nitrogen gas bubbling through the cell for 10 min with all measurements being conducted at a controlled temperature of 25 °C at least in triplicate. Thus the values plotted on figures were calculated from at least three measurements.