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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 to 40.

Keywords

Cell-derived microparticles; electrochemical impedance spectroscopy; dielectrophoresis; clinical diagnostics

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Appendix A and B

Supplementary data associated with this article can be found in the online version.

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 0.05–2 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 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 MPs/ 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, 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 2–20 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 RBC's 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 hr at 37 °C or with *t*-BuOOH (500 μm) for 2hrs followed A23187 for 1hr. Intact platelets were then removed by centrifugation at 2000g for 15 min. The supernatants were then subjected to a high-speed centrifugation at 100,000g 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 2000g 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 $\mu\text{g}/\text{ml}$ cycloheximide (Sigma-Aldrich) for 24 hrs. 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 100,000g 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 $\cdot 10^5$ 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 0V, +0.5V, −0.2V, −0.5V and −0.8V (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 mV s^{-1} and 10 mV s^{-1} between +1.0V and −1.0V (vs. Ag/AgCl).

For the CV and EIS testing a single compartment glass electrochemical cell was constructed using 25 gram 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 minutes with all measurements being conducted at a controlled temperature of 25 $^{\circ}\text{C}$ at least in triplicate. Thus the values plotted on figures were calculated from at least three measurements.

3. Results and discussion

3.1. Buffer solutions without microparticles

The impedance response characteristics of the lower conductive Tyrode's and a highly conductive PBS background solution buffers are very similar (Figure 1). For both buffers, the bulk electrolyte impedance can be represented as a parallel combination of bulk solution resistance and capacitance. The later becomes relevant only in MHz region barely visible at the upper limit of the frequency region of our analysis with the phase angle transition from 90 $^{\circ}$ to 0 $^{\circ}$ (Figure 1). The bulk solution resistances were measured at 31.4 (± 1.64243) Ω and 0.81 (± 0.07772) Ω , and conductivities at 0.10 mS/cm and 4.12 mS/cm , for Tyrode's and PBS buffers, respectively. At low frequencies the overall electrochemical impedance was dominated by double layer capacitive charging at the working electrode. For experiment with PBS, Bode plot showed the capacitive double layer charging behavior up to 100 kHz, and with Tyrode's up to 1 kHz. Since PBS is very conductive, the influence of the electrical double layer on the overall solution impedance and its characteristic frequency were shifted towards higher frequencies, as a more conductive buffer presents more ionic charges at the electrode-electrolyte interface. The equivalent circuit representation for both buffers can be reduced to a solution resistance (R_{SOL}) in series with a double layer charging CPE_{DL} element.

The analysis of electrical properties of the buffers suggested that it would be advantageous to replace PBS saline with a low-conductivity Tyrode's buffer to optimize the suspending media. The higher resistance buffer allows for the current to flow predominantly through the MP and transport the MP towards the working electrode using positive DEP, as has been shown before through enhanced sensitivities for RBC and bacteria detection (Houssin et al., 2010; Gagnon et al., 2008). Cells exhibit long lasting stability with no signs of cell lysis in

such low-conductivity Tyrode's buffer solutions. Our experiments demonstrated that MPs additions had little effect on PBS electrochemical impedance, but decreased Tyrode's buffer bulk solution resistance by 5–10 times. Thus Tyrode's buffer was subsequently selected as supporting media for all experiments.

3. 2. Microparticles suspensions – bulk solution effects

Cellular structures are typically represented as spherical shells composed of internal cytoplasm with conductivity exceeding that of suspending media, surrounded by thin highly resistive membranes (conductivity $\sim 10^{-9}$ S/cm) with capacitance of ~ 1 μ F/cm², permittivity ~ 10 , and thickness of ~ 10 nm. Typical RBCs have cytoplasm conductivity of 3×10^{-3} S/cm, and permittivity ~ 60 (Gagnon et al., 2008). Schwan suggested similar characteristic values for MP (Schwan et al., 1970). The mismatches in the electrical properties of suspended particles and suspending media, effects of cellular surface properties and geometry result in a characteristic pattern of frequency-dependent dielectric relaxations, yielding valuable information on the structural and functional properties of the system.

For the bulk solution relaxation analysis impedance, dielectric, and electrokinetics notations can be employed, allowing developing a stronger evidence for the data interpretation. Electrokinetics has higher resolution than EIS for the electrical parameters of single objects due to its different measurement principle and data representation. Whereas the impedance of a suspension depends on the sum of the current contributions through and around the suspended cells, the forces in AC electrokinetics depend on impedance difference between particles and suspending medium. Thus by choosing a medium of appropriate conductivity and permittivity, particles of similar dielectric properties can be efficiently trapped and identified. The particle and medium properties in impedance studies are qualitatively reflected in an integrative manner, whereas in the AC electrokinetic methods in a differential one. Nevertheless, impedance and the electrokinetic methods describe the same polarization processes.

In the classical theory the DEP force magnitude and direction is determined by a real portion of the AC frequency (ω) dependent Clausius-Mossotti factor (CMF):

$$CMF = \frac{\epsilon_p^* - \epsilon_m^*}{\epsilon_p^* + 2\epsilon_m^*} = \frac{\epsilon_0^2(\epsilon_p - \epsilon_m)(\epsilon_p + 2\epsilon_m) + (\sigma_p - \sigma_m)(\sigma_p + 2\sigma_m)/\omega^2}{\epsilon_0^2(\epsilon_p - 2\epsilon_m)^2 + (\sigma_p - 2\sigma_m)^2/\omega^2} = \frac{Z_m^* - Z_p^*}{Z_m^* + 2Z_p^*} \quad (1)$$

where ϵ_m and ϵ_p , σ_m and σ_p , and Z_m^* and Z_p^* are the real parts of permittivity, conductivity and impedance of the suspending medium and suspended particles, respectively. CMF analysis (Jones, 2003) allows evaluating critical relaxation frequencies, suggests the possibility for MP trapping and separations by DEP force, and determines MP characteristic parameters such as interior fluid permittivity and conductivity, particle sizes, diffusion coefficients, and membrane thickness and capacitance. A positive CMF indicates that the DEP force moves polarized particle toward a local electric field maximum (positive DEP or pDEP), while a negative CMF directs the particle into regions of weak electric field (negative DEP or nDEP). CMF impedance frequency dependence analysis also allows predicting the mechanism of conduction in the media. Positive CMF is an indicator that suspended particles are the main conductors of current as their characteristic impedance is lower than that of supporting media ($Z_m > Z_p$). Negative CMF indicates that the media is offering a path of least resistance to the current ($Z_m < Z_p$). However, the relative concentration of the suspended particles is also important, as in the case of their very low concentration at least part of the current will have to be supported by the migration of other ionic species in the supporting electrolyte despite its higher impedance (Jones, 2003).

Due to the nature of the screening cell membrane, the “single shell” model becomes relevant, exhibiting two relaxations (Jones, 2003). At AC frequencies exceeding the inverse relaxation time of the solution media/interior fluid ($1/R_{INTFL}C_{SOL} \sim 10$ MHz), the resulting dipole will be regulated by permittivity differences between the cell interior fluid (lower) and the bulk solution medium (higher), resulting in a nDEP. If the AC frequency decreases below this limit, but is exceeding the inverse relaxation time of the polarized membrane/solution media interface ($1/R_{SOL}C_{MEMBR} \sim 10$ kHz), the current will be preferentially directed through the highly conductive interior fluid, resulting in a pDEP. At sufficiently low frequencies the conductive charging of the cell membrane with a large capacitance produces a large polarization across the membrane, and the membrane polarization produces a field that opposes and screens the external field, resulting in a nDEP. Thus in this simplified model that does not take into account interior fluid capacitance and effects of membrane conductivity, the low frequency relaxation is governed by the relaxation time of the media/membrane, and one at high frequency is dependent on the relaxation time of the media/interior fluid.

Figure 2 represents experimental results of CMF frequency analysis for the MP suspensions at the stock solution concentrations, which is in agreement with the model described above for membrane covered cellular objects. For all types of MP the two characteristic frequencies are present where CMF changes sign and the conduction mechanism alternates between conduction through the media and through the MP. For all MP types the first relaxation occurs at ~ 10 MHz, and the second in the kHz range. For these analyses, CMF was initially calculated from experimental total impedance values for pure Tyrode's buffer and concentrated MP solutions. This approach provides only initial approximation, as it is not based on direct comparison of the impedances of pure suspended particles and suspending media. The other assumptions were that all MPs are ideal spheres containing homogeneous interior fluid, surrounded by purely capacitive membrane with no ion channels effects and the MP suspensions can be described by a single shell relaxation model. Despite these assumptions, the proposed model is at least qualitatively realistic, allowing estimation of size, membrane thickness and capacitance, interior fluid permittivity and conductivity for the analyzed MPs. The first approximation analysis is based on developing a fit between the experimental and simulated CMF data generated by employing the expansions of the Eq. (1) (Jones, 2003) and using as initial assumptions characteristic MP parameters reported in the literature (Bothwell and Schwan, 1956; Schwan et al., 1970) and progressively adjusting for a better fit. The fit was not ideal at lower frequencies, probably due to the effects of the assumptions such as lack of ion channels effects on transmembrane conduction, but also because of the counterion diffusion relaxation effect in kHz frequencies described below, which is not taken into account by the Maxwell-Wagner relaxation theory in Eq. (1). However, this evaluation allowed for an initial assessment of the characteristic MP parameters (Table 1). The media (Tyrode's buffer) parameters in Eq. (1) are taken as conductivity of 0.10 mS/cm and permittivity of 78.

The data in Table 1 demonstrate that, as expected, RBCs produce the smallest average size MP, while monocyte derived MPs are significantly larger with thicker membranes, lower interior fluid conductivity and higher permittivity, possibly due to the presence of RNA. Some variability in the membrane capacitances is observed; with values for platelet and RBC derived MPs were close to those for the cells at $\sim 1\text{--}3 \mu\text{F}/\text{cm}^2$, while monocyte-derived MPs had lower membrane capacitance ($0.5 \mu\text{F}/\text{cm}^2$). Otherwise interior fluid conductivity and permittivity, membrane capacitance and thickness, and the MP sizes were realistic and close to the literature and anticipated values.

Assuming that MP interior fluid is highly conductive without significant capacitive contribution, and that membrane conductance is very low ($\sim 10^{-3} \text{ Sm}/\text{cm}^2$), the impedance

of a cellular structure can be represented by a series combination of purely capacitive membrane impedance (Z_{MEMBR}) and interior fluid resistance (R_{INTFL}) as $Z_{CELL}=R_{INTFL}+Z_{MEMBR}$. The membrane is very thin with typical capacitance of $C_{MEMBR}=1 \mu\text{F}/\text{cm}^2$, and at sufficiently high AC frequency f the associated impedance

$Z_{MEMBR}=\frac{1}{2\pi f C_{MEMBR}}$ becomes negligible and the current flows through cellular interior fluid with resistance $\sim 1 \Omega$, resulting in a corresponding β_2 -relaxation in the low MHz range. With decrease in frequency the effect of Z_{MEMBR} increases and starts dominating the cell impedance, resulting in the Maxwell-Wagner low kHz β_1 -relaxation, representing a transition from conduction through MP to conduction through suspending solution. However, the α -relaxation process due to the diffusion-controlled process of counterions movement around the polarized cells that present diffusion impedance to counterions was reported as a more likely kHz relaxation mechanism (Schwarz, 1962). The dielectric (Figure 3A) and impedance (Figure 3B) spectra of analyzed MP suspensions in Tyrode's buffer at rest potential revealed two distinct high frequency relaxations in MHz and kHz ranges. The permittivity increase due to the Maxwell-Wagner β_1 mechanism (Eq. 2) and counterion diffusion α -relaxation (Eq. 3) can be compared (Asami et al., 1996) for known MP concentration (N_C in MP/ml), size (R_{MP}) and membrane capacitance (C_{MEMBR}) (Table 1), elementary charge e_0 , dielectric constant in air ϵ_0 , and average charge density on a typical cell surface $q_0 \sim 10^{13} \text{ cm}^{-2}$ (Schwan et al, 1970) as:

$$\Delta\epsilon_\beta = \frac{3\pi R_{MP}^4 C_{MEMBR}}{\epsilon_0} N_C \quad (2)$$

$$\Delta\epsilon_\alpha = \frac{3}{\epsilon_0} \frac{e_0^2 q_0}{kT} \pi R_{MP}^4 N_C \quad (3)$$

The experimentally observed large permittivity increments on the order of 10^3 – 10^5 (Figure 3A) are better described by the counterion relaxation theory and Eq. (3), which predict experimental permittivity increments that are 2–5 orders of magnitude higher than that by the Maxwell-Wagner theory (Eq. 2). In addition, according to the Maxwell-Wagner model one would expect that at low kHz frequencies the impedance becomes almost entirely resistive with phase angle approaching 0° , while the experimental data demonstrates phase angles of 20° – 50° in all suspensions in kHz region (Figure 3B). The phase angle slowly decreased from $\sim 30^\circ$ at 10 MHz to $\sim 20^\circ$ at 10 kHz for RBC- and platelet-derived MP. It is interesting to note a general increase in the phase angle below 100 kHz for endothelial and monocyte-derived MPs, probably indicating a different distribution of media solution conduction versus conduction through interior fluid for these MPs.

Alternatively the measured permittivity increments for the kHz range relaxation can be used to estimate MP concentrations (Table 1) according to Eqs. (2–3) and compare them with the flow cytometry results ($5 \cdot 10^5$ MP/ μL). If Eq. (2) is used, the estimated MP concentrations exceed those reported by flow cytometry by ~ 5 orders of magnitude which is unrealistic even when given the likelihood that light scatter-based flow cytometry underestimates the concentrations of smaller MPs. The MP concentrations estimated from Eq. (3) are more reasonable. The estimated concentrations for smaller MPs exceed those reported by flow cytometry by a factor of 14 for platelet MP and 40 for RBC MP, while for larger MPs that are typically much better quantified by flow cytometry this discrepancy is only a factor of 2. Therefore, we can conclude with some certainty that the counterion diffusion mechanism

described by Eq. (3) presents a realistic description of the kHz relaxation phenomenon, and can be used with a fair degree of certainty for initial quantification of MP.

3.3. Low frequency EIS and CV studies

The CV analysis (Appendix A) for the MP –containing solutions demonstrated no differentiation between the current signal in the pure and MP-containing buffers at the anodic potentials, while some differentiation apparently linked to the interfacial redox kinetics was observed at the cathodic potentials. The details of this electron exchange process at -0.2V , -0.5V and -0.8V (vs. Ag/AgCl) were investigated with a three-electrode impedance probe.

The potential modulated impedance data (Appendix B) revealed low frequency relaxation processes that are strongly dependent on the applied electrochemical potential. At $\sim 1\text{--}10\text{ Hz}$ there appears to be a purely capacitive double layer charging with corresponding double layer capacitance that is increasing slightly with the electrochemical potential. The second process at 0.1 Hz showed significant impedance decrease with increase in the electrochemical potential. At low overpotentials (-0.2 V) the impedance kinetics was more facile for the MP derived from RBC and platelets than from the nuclei-containing cells, while at elevated overpotentials (-0.8 V) the endothelial cells derived MP showed the smallest overall interfacial impedance.

These two interfacial processes can be interpreted as either two separate Faradaic charge transfer processes coupled with the double layer charging, or as a facile charge transfer process at higher frequency coupled with finite diffusion with absorbing boundary at the lower frequency (the Randles model). The first model was intentionally simplified as two parallel combinations of charge transfer resistance R_{CT} and double layer capacitance represented by a constant phase element CPE_{DL} . The resulting data fit produced unconventionally high double layer capacitance value of $C_{DL} \sim 400\mu\text{F}/\text{cm}^2$ (at -0.2V), considering that strong Faradaic pseudocapacitive effect is unlikely. Considering that the thin cell geometry of the impedance electrodes in supported systems typically results in finite absorbing boundary diffusion, the Randles model was then evaluated. This model produced a better fit and more realistic description of the potential dependent interfacial processes represented by the double layer charging capacitance $C_{DL} \sim 50\mu\text{F}/\text{cm}^2$ in parallel with a series combination of charge transfer resistance $R_{CT} \sim 100\Omega$ and finite diffusion impedance of $\sim 1000\Omega$ (at -0.2 V).

This model allows estimating diffusion coefficients D_0 from expression for the finite diffusion impedance Z_{DIFF} (Lvovich and Smiechowski, 2008) using MP concentrations C_0 (Eq. 3), and sizes R_{MP} from the Stokes equation for a fluid of known viscosity η :

$$Z_{DIFF} = \sigma(-j) / \sqrt{\omega} \cdot \tanh(\delta \sqrt{j\omega/D_0}) \quad (4)$$

$$\text{where } \sigma = \frac{RT}{(n^2 F^2 S \sqrt{2})} \left(\frac{1}{C_o \sqrt{D_o}} + \frac{1}{C_R \sqrt{D_R}} \right) \quad (5)$$

$$R_{MP} = \frac{kT}{6\pi D_0 \eta} \quad (6)$$

The results of the particles sizes estimated from the bulk electrokinetic method and the interfacial analysis are very similar (Table 1).

The interfacial impedance detection method offers high selectivity and sensitivity, similar to those reported before in the literature for biological cells and bacteria (Houssin et al., 2010). Figure 4 shows an example of the linear decrease in the low frequency impedance magnitude for five orders of magnitude dilution of the initial stock solution of endothelial derived MP in Tyrode's buffer at -0.2 V, reaching detection levels under 10 MP/ μ L with a resolution ~ 1 MP/ μ L and relative standard deviation of 10%. The resulting impedance changes can be easily converted into MP concentration dependent normalization plot (Figure 4, insert).

3.4. Equivalent Circuit modeling of MP suspension

The bulk solution conduction phenomenon in the MP suspensions can be represented by the β_2 relaxation mechanism due to the membrane capacitive bridging. The time constant of this process is determined by a product of interior fluid resistance and media capacitance, and corresponding switch from conduction through resistive buffer (with corresponding $R_{\text{SOL}} \sim 10 \Omega$), to a current flowing through the MP capacitive membrane ($C_{\text{MEMBR}} \sim 1 \mu\text{F}/\text{cm}^2$) and resistive interior fluid ($R_{\text{INTERFL}} \sim 1 \Omega$). Resulting pDEP effect can be used to manipulate MP suspensions in a microfluidic environment, similar to what has been demonstrated before for the cells suspensions. Even if the cell membrane resistance is very high ($\sim 10^{10} \Omega$) at AC frequencies as low as 10 kHz the membranes are shorted through their

capacitance with $Z_{\text{MEMBR}} = \frac{1}{2\pi f C_{\text{MEMBR}}} \sim 10 \Omega$ which is close to the media resistance.

In the kHz range the β_1 relaxation takes place with a time constant dependent on a product of membrane capacitance and media resistance, as the membrane impedance increases, MP becomes insulating, and nDEP takes place. Our studies demonstrated that Maxwell-Wagner type β_1 relaxation is masked by the counterion diffusion α -relaxation process. That accounts for discrepancies between experimental and estimated CMF in the kHz range for all studied MP types (Figure 2). In the equivalent circuit representation the suspending solution is represented by a parallel resistance-capacitance structure that is placed in parallel with a series combination of the MP membrane capacitance and interior fluid resistance. The counterion diffusion relaxation process is taking place in the suspending solution, with the MP surfaces presenting transport impeding boundary. Therefore, we placed the diffusion element W_S in series with the combined bulk solution and MP impedances (Figure 5).

At low frequencies two interfacial kinetics stages can be represented by the double layer capacitance in parallel with the Faradaic process combining finite diffusion and facile charge transfer processes (Figure 5). The MPs may become transformed into externally polarizable macro ions that can move to the electrode and develop direct molecular connections with the electrode, presumably through phospholipids and extracellular segments of membrane glycoproteins. That results in the interfacial impedance change. Depending on the MP type and the applied potential, the response can be Faradaic, purely capacitive, or a mixture of the two, and allow for an electrochemical potential modulation. Typically as membranes are insulative the “adsorption-type” predominantly capacitive response is developing at the interfaces of the electrodes and the interfacial impedance increases. With potential modulation a significant decrease in the interfacial impedance was observed, in all likelihood due to a process of opening of the membrane channels and leaking of the interior fluid components. Even at low electrochemical potentials of -0.2 to -0.3 V the voltage sensitive “channel proteins” facilitate ionic transport in and out of the extracellular electrolyte, resulting in reduction or oxidation activity of the released ionic

components of the interior fluid. Exposure to even higher electrochemical potentials results in large macromolecules passing in and out of the MP. These species come in contact with the electrode resulting in additional reduction or oxidation activity of ionic and organic components of the interior fluid that are specific for a given cell or MP type.

4. Conclusions

The approach described here can potentially be used to separate various MPs using their variability in CMF frequency dependence and resulting switch between pDEP and nDEP. All MPs can be effectively and separately trapped by applying the AC frequency of 10^6 Hz, as they experience pDEP and are attracted to the surface of the sensing electrode. The selectivity can be enhanced when, for example, the AC frequency of 10^3 Hz is applied and only the endothelial MP experience pDEP and can be trapped in a lower conductivity suspending media. Additionally, some differences in MP electrical and structural properties were made apparent. Such optimization schemes suggest the possibility that novel DEP detection assays can be tailored to specific subgroups of biological systems.

Impedance-based detection and quantification of MPs is based on fundamental interfacial electrochemistry at the sensing electrode, which is driven by electrochemical activities of MPs that can be separated by applied electrochemical potential. Thus interfacial electrochemistry processes can be used to reliably determine concentration and sizes of participating species with higher accuracy, specificity and selectivity than traditional analytical methods. At specific electrochemical potential corresponding to reduction or oxidation of MP interior fluid and membrane components the resulting current measured at a sensing electrode changes proportionally to the concentration of the MP. This model, however, may need a further validation accounting for the variability in surface and volume factors for the cells and counterions, possibility of MP lysis in various physiological solutions, and, especially for solutions with very low numbers of MP, the concentration effects on the current passage and estimated surface area / distance factors.

An important conclusion from this data is that flow cytometry quantification procedures based on light scattering appear to underestimate MP concentrations, especially those with smaller average sizes, by as much as a factor of 2 to 40. Since the predominant source of circulating MPs in most clinical settings is platelets, our data suggest that levels of MPs reported in the literature are inaccurate. The impedance / electrokinetic integrated method that we developed may address this problem and can be adapted to rapidly assessment of microvolume samples. Comparing to flow cytometry procedures, the EIS assay could be integrated as a direct, rapid, easy to use, automated, more reproducible, and accurate MP counting strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Impedance plots: A) Bode plot of pure PBS (— - impedance, □ - phase angle) and Tyrode's (— - impedance, △ - phase angle) buffers.

Figure 2.

Experimental (solid line) and estimated (symbols) Clausius-Mossotti factor AC frequency dependence for blood cells-derived MPs: Platelet (—, Δ); RBC (—, $\square$); Endothelial (—, $\circ$); Monocytes (—, $\diamond$).

Figure 3.

A) Permittivity (solid symbols); B) Total impedance $|Z|$ (solid line) and phase angle (open symbols) plots for MP suspensions at rest potential. Platelet MPs ($\blacktriangle$, — , $\triangle$), RBC MPs ($\blacksquare$, — , $\square$), Endothelial MPs ($\bullet$, — , $\circ$), Monocyte MPs ($\blacklozenge$, — , $\diamond$), respectively.

Figure 4.

Electrochemical impedance detection of endothelial derived MP (concentration in MP/ μ L is shown on the plot) at low frequency and electrochemical potential of $-0.5V$, with resulting normalization plot of conductivity at 0.1Hz vs MP concentration.

Figure 5.
Equivalent Circuit model showing conductive paths in the solution, transcellular path, counterion diffusion path, and typical interfacial impedance

Table 1

Estimated characteristic parameters of microparticles

MP parameters	PlateletMP	RBC MP	EndothelialMP	MonocyteMP
Interior fluid conductivity (± 1), Sm cm^{-1}	4×10^{-3}	3×10^{-3}	2.7×10^{-3}	0.3×10^{-3}
Interior fluid permittivity (± 10)	60	60	70	75
membrane permittivity (± 3)	5	5	7	3
membrane capacitance (± 0.5), $\mu\text{F cm}^{-2}$	2	4	6	0.5
membrane thickness (± 2), nm	2	1	1	10
average MP diameter Eq. (1) (± 100), nm	180	140	300	500
average MP diameter Eq. (6) (± 50), nm	200	150	275	550
concentration estimated Eq. (2), $\text{MP } \mu\text{L}^{-1}$	2.0×10^{10}	2.7×10^{10}	9.0×10^{10}	2.8×10^{10}
concentration estimated Eq. (3), $\text{MP } \mu\text{L}^{-1}$	7.0×10^6	2.0×10^7	1.0×10^6	1.2×10^6