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Impedance spectroscopy assisted by magnetic nanoparticles as a potential biosensor principle for breast cancer cells in suspension

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Abstract

Breast cancer (BC) is the leading cause of cancer death in women worldwide, with a higher mortality reported in undeveloped countries. Ideal adjuvant therapeutic strategies require the continuous monitoring of patients by regular blood tests to detect circulating cancer cells, in order to determine whether additional treatment is necessary to prevent cancer dissemination. This circumstance requires a non-complex design of tumor cell biosensor in whole blood with feasibility for use in poor regions. In this work we have evaluated an inexpensive and simple technique of relative bioimpedance measurement, assisted by magnetic nanoparticles, as a potential biosensor of BC cells in suspension. Measurements represent the relative impedance changes caused by the magnetic holding of an interphase of tumor cells versus a homogenous condition in the frequency range of 10–100 kHz. The results indicate that use of a magnet to separate tumor cells in suspension, coupled to magnetic nanoparticles, is a feasible technique to fix an interphase of tumor cells in close proximity to gold electrodes. Relative impedance changes were shown

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to have potential value as a biosensor method for BC cells in whole blood, at frequencies around 20 kHz. Additional studies are warranted with respect to electrode design and sensitivity at micro-scale levels, according to the proposed technique.

Keywords: breast cancer, magnetic nanoparticle, bioimpedance, biosensor

(Some figures may appear in colour only in the online journal)

1. Introduction

Breast cancer (BC) is the most commonly diagnosed cancer and the leading cause of cancer death in women worldwide (Farlay *et al* 2010). In Mexico, BC was the second leading cause of death in 2010. The mortality observed represents approximately 13 deaths per day (DGIS 2013). In general, mortality from BC is higher in poor regions as compared to industrialized countries (Farlay *et al* 2010, IHME 2012). The treatment of BC depends on factors related to the location and spread of the tumor as well as its histological and immunological features. In most cases, surgical resection is effective for local treatment while reducing the risk of relapse; however, many patients have recurrences at distant sites, probably as a result of the spread of the tumor that is undetected at the time of initial treatment. Identification of occult micrometastases in patients with localized BC could play an important role in establishing the prognosis, treatment decisions and monitoring therapy effectiveness. Ideally, assessment of the presence and number of circulating tumor cells (CTCs) would be of major benefit before macroscopic signs are evident. The ideal treatment condition would be one in which patients who have received treatment for localized BC are monitored with regular blood tests for the detection and characterization of CTCs to determine whether any additional treatment is necessary to prevent recurrence.

The presence of CTCs was first described in 1869 by Thomas Ashworth, an Australian physician (Ashworth 1869). CTCs have been associated with survival and disease progression in patients with metastatic BC and have been used to test new treatment therapeutic strategies (Cristofanilli *et al* 2004, Weigelt *et al* 2003, Ignatiadis and Piccart 2012). The number of CTCs in the blood of a cancer patient can range from 0 to 50 ml^{-1} (Allard *et al* 2004), i.e. 0–50 CTCs/10 billion blood cells (Liberti *et al* 2001). Techniques for identifying CTCs have been broadly divided into two approaches: cytometry and nucleic acids (Ring *et al* 2004). Because of the very low levels of CTCs, immunomagnetic cell separation techniques lack the ability to separate CTCs directly from whole blood (Berois *et al* 1997, Berry *et al* 2003, Mapara *et al* 1997, Molday and MacKenzie 1982). However, in 2011 Xu *et al* reported the technical feasibility of performing such whole blood separation (Xu *et al* 2011). CTC identification is usually based on the expression of epithelial lineage markers such as cytokeratins (cytoskeletal proteins present in epithelial cells) or EpCAMs (epithelial cell adhesion molecules), absence of common leukocyte marker (CD45) and/or putative tumor-specific antigens (MUC1, HER-2 and/or others) (Witzig *et al* 2002). Other detection methods have involved developments such as EpCAM selection/RT-qPCR, filtration and dielectrophoresis (Choi *et al* 2013, Chung *et al* 2011, Han *et al* 2006). In addition, some authors have used bioimpedance spectroscopy as a cell analysis tool to extract the pathological characteristics from the BC cells. The technique is potentially a useful tool to differentiate malignancy based on the variation of electrical resistance and reactance when cells are excited at different frequencies (Qiao *et al* 2012).

Nanoparticles play an important role in current cancer research, and the interaction of cells with nanoparticles is of particular interest. In 2005, De Nardo *et al* tested a method

for designing bio-reagent probes, which were formed by the coupling of a ferromagnetic nanoparticle complex and a chimeric monoclonal ChL6 (DeNardo *et al* 2005). In 2011, our group reported a study showing the technical feasibility of marking MCF-7 BC cells with ferromagnetic nanoparticles bioconjugated with monoclonal anti c-erbB-2 (Silva *et al* 2011). Recently we have expanded this study to three cancer cell lines, which differentially express the antigen c-erbB-2. The results indicate the technical feasibility of differentially marking cancer cells with magnetic nanoparticles as a function of an antigen–antibody reaction (Silva *et al* 2013). Magnetic nanoparticles attached to antibodies or other ligands provide a versatile tool for isolation and labeling of BC cells.

Ideal BC adjuvant therapeutic strategies in poor regions demand monitoring of patients with regular blood tests to detect CTCs in order to determine whether additional treatment to prevent cancer dissemination is necessary; it requires simple and inexpensive CTC biosensors in whole blood, applicable for use in undeveloped countries. We have proposed that monitoring based on measurements of relative electrical impedance spectroscopy assisted by magnetic nanoparticles could be effective. In this paper we evaluate an inexpensive technique of relative bioimpedance measurements, assisted by magnetic nanoparticles as a potential biosensor for the investigation of BC cells in suspension. The proposed approach is based on the use of a magnet to separate tumor cells once they were coupled to magnetic nanoparticles, to fix an interphase of tumor cells in the close neighborhood of gold electrodes as bioimpedance sensors. Measurements represent relative impedance changes given by the interphase of tumor cells–homogenous condition.

2. Methods

2.1. Experimental design

The experimental concepts of tumor cells in suspension incubated with 'magnetic nanoparticle–monoclonal antibody' bioconjugate and of bioimpedance measurement assisted by magnetic nanoparticles are shown in figures 1 and 2. Two substantially different suspensions for incubation were used: phosphate buffered saline (PBS) and female whole blood (WB) with ethylene diamine tetraacetic acid (EDTA) as an anticoagulant. To observe differences given by the BC cell presence, relative bioimpedance estimations were developed in both suspensions with bioconjugate only, and a second condition incubated with tumor cells. Thus, the evaluated experimental conditions were (a) PBS or WB + bioconjugate and (b) PBS or WB + bioconjugate + tumor cells. Relative bioimpedance measurement assisted by magnetic nanoparticles is based on the use of a magnet to separate tumor cells once they are incubated with the bioconjugate, the idea being to fix an interphase of tumor cells in the close neighborhood of gold electrodes as bioimpedance sensors. Impedance of the interphase of tumor cells was homogenized with respect to measurements in the homogenous solutions without the influence of the magnet. Thus, the measurements represent relative impedance changes given by the interphase of tumor cells–homogenous condition.

2.2. Targeting of tumor cells by magnetic nanoparticles

2.2.1. Tumor cell cultures. The BT-474 (ATCC® cat: HTB 20™) BC cell line characteristically expresses the surface protein c-erbB-2 (Hathaway *et al* 2011). This cell line was cultured in a 75 cm^2 cell culture flask (Nunclon Surface™) with ATCC Hybri-Care medium (ATCC® catalog No 46-X™). BC cells were incubated in a CO_2 incubator (Thermo Scientific™) at 37 °C and a carbon dioxide (CO_2) atmosphere at 5%. The number and cell

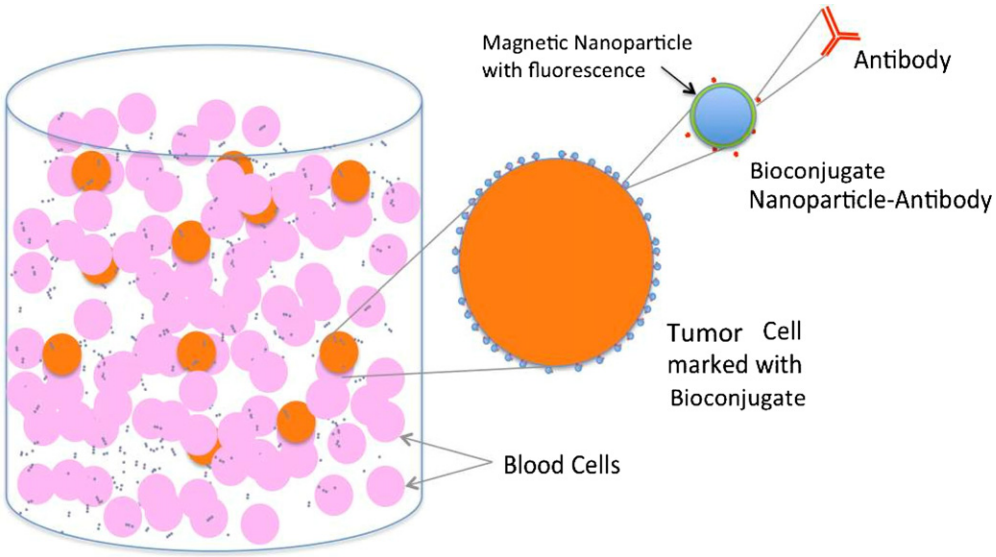

Figure 1. Schematic representation of the experimental concepts of tumor cells in suspension incubated with 'magnetic nanoparticle–monoclonal antibody' bioconjugate (not to scale). The figure represents incubation in whole blood.

viability were evaluated using trypan blue staining and an automatic cell counter (Countess model, Invitrogen®).

2.2.2. Magnetic nanoparticle–antibody bioconjugate. Nanoscreen MAG/G-ARA ferromagnetic nanoparticles (Chemicell) were used, having an average size of 100–300 nm, labeled with fluorescein isothiocyanate (FITC), and coated with a layer of silica; the mouse monoclonal antibody c-erbB-ab15 (Neomarkers, catalog No MS-599P) was coupled with the ferromagnetic nanoparticles. The bioconjugate was formed by means of the carbodiimide method, following the covalent coupling procedure described on fluid MAG-ARA (protocol A10, Chemicell)[6].

2.2.3. Tumor cell bioconjugate incubation. Experimental conditions, as described in the experimental design subsection, were developed accordingly following procedure. The first condition involved 1 ml of PBS or WB incubation with only 40 μl of bioconjugate, and in the second incubation 2.7×10^5 BT-474 tumor cells were added. Incubations were carried out for 1 h in the dark at room temperature. Every experiment was run in triplicate. To observe tumor cells marked by the bioconjugate, 10 μl of incubated tumor cells were placed on a glass slide covered with a coverslip and observed under a fluorescence microscope at 40 $\times$ (Olympus TM, model BX4).

2.3. Impedance measurements

2.3.1. Analytical impedance estimation. We utilized the electrical configuration shown in figure 3, with an alternating current $I\cos(\omega t)$ flowing through the circuit. The presence of $\boldsymbol{Z}$ (a sample of tumor cells in suspension) and R (a resistive reference) as complex impedance

[6] CHEMICELL at www.Chemicell.Com.

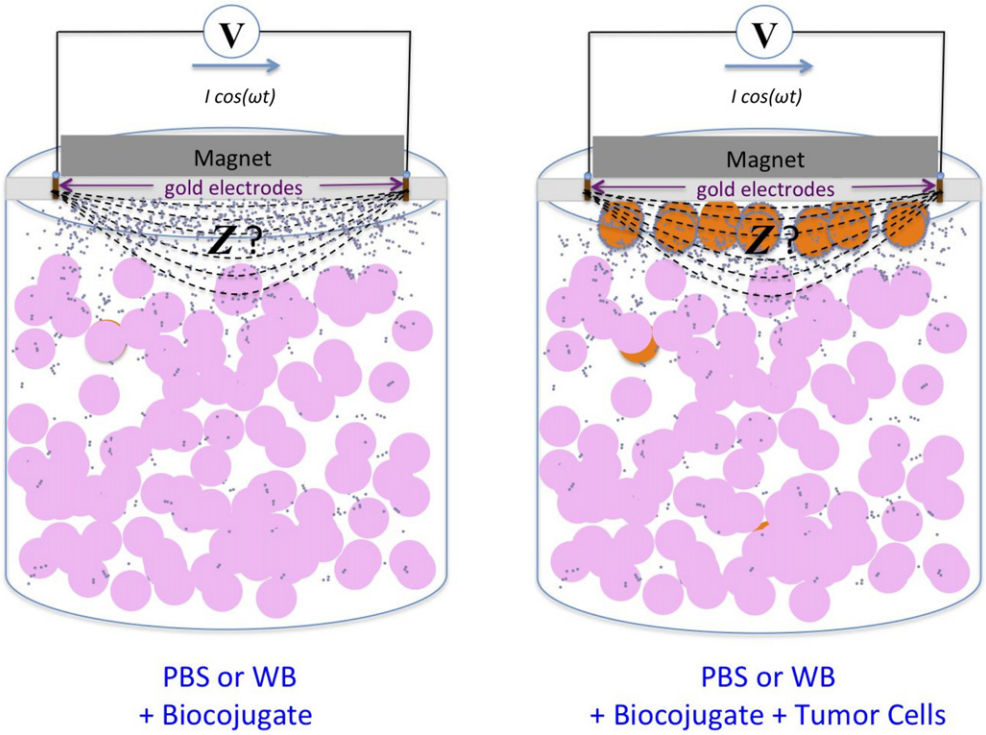

Figure 2. Schematic representation of the relative bioimpedance measurement concept (not to scale). A magnet placed at the top generates an interphase of magnetic nanoparticles and/or tumoral cells in close proximity to gold electrodes. Two experimental conditions are shown: (a) PBS or WB + bioconjugate and (b) PBS or WB + bioconjugate + tumor cells.

elements produces two specific complex voltages $\boldsymbol{V}_{\mathrm{A}}$ and $\boldsymbol{V}_{\mathrm{B}}$ related according to Kirchoff's and Ohm's laws:

$$\frac{V_{\mathrm{A}}}{V_{\mathrm{B}}} = \frac{Z+R}{R}. \tag{1}$$

Thus, the complex impedance $\boldsymbol{Z}$ is given by

$$Z = R\left[\left(\frac{|V_{\mathrm{A}}|}{|V_{\mathrm{B}}|}(\cos(\theta_{\mathrm{A}}-\theta_{\mathrm{B}})) + j(\sin(\theta_{\mathrm{A}}-\theta_{\mathrm{B}}))\right) - 1\right]. \tag{2}$$

Here, the complex impedance $\boldsymbol{Z}$ is a function of the electrical properties of the sample studied, and in this study we estimated it in terms of amplitude and phase as a relative measurement of the complex ratio ($\boldsymbol{V}_{\mathrm{A}}/\boldsymbol{V}_{\mathrm{B}}$) and difference ($\boldsymbol{\theta}_{\mathrm{A}} - \boldsymbol{\theta}_{\mathrm{B}}$) respectively.

2.3.2. Experimental impedance estimation. An experimental relative impedance measurement system was designed and constructed on the basis of a gain and phase detector. Figure 3 shows a block diagram of the experimental implementation. The system consists of five modules: the function generator, electrode system, gain and phase detector, data acquisition and data processing modules. The function generator was implemented by an HP3314A signal generator (Hewlett-Packard, Palo Alto, CA, USA), which supplied a signal, $I\cos(\omega t)$, of approximately 10 mA in the range of 0.001–50 MHz. The electrode system consisted of

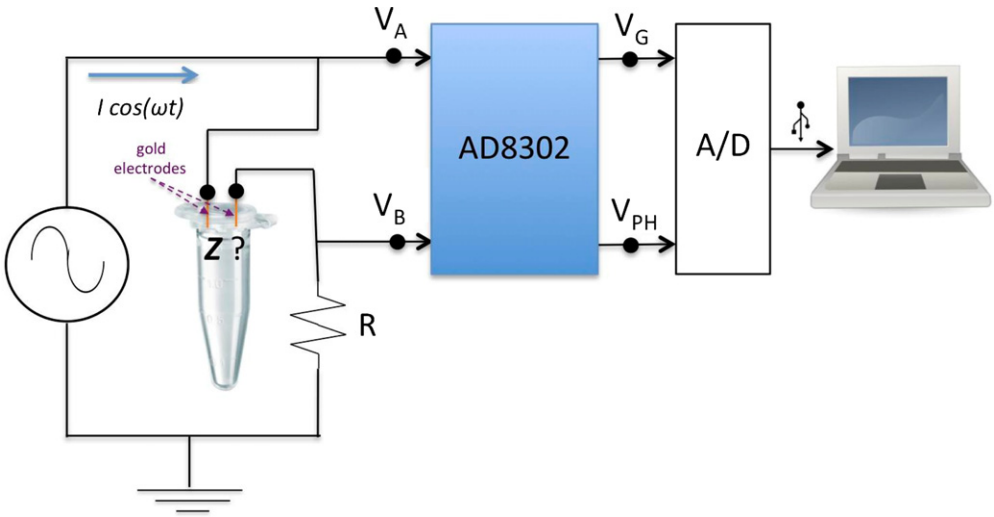

Figure 3. Electrical and block diagram of the experimental configuration to estimate relative bioimpedance of tumor cells in suspension ($\boldsymbol{Z}$) given a real reference (R) as a function of measurements of the complex ratio $\boldsymbol{V}_A/\boldsymbol{V}_B$.

two electrodes positioned to close the electrical circuit through the sample of tumor cells in suspension. Both electrodes were made of gold wire held by a plastic circle with radius $r = 9$ mm in such a way that it was used as the top of an Eppendorf tube. The gold electrodes were centered on the plastic holder at a distance of $d = 7$ mm (figure 5(b)). The gain and phase detector was designed on the basis of the AD8302 (Analog Devices, Norwood, MA, USA). The AD8302 is a fully RF integrated circuit for measuring differences in amplitude (V_G) and phase (V_{PH}) between the two measured signals, with a resolution of 30 mV dB^{-1} and 10 mV/degree respectively. Thus; the magnitude and phase of the related complex potentials $\boldsymbol{V}_A$ and $\boldsymbol{V}_B$ described in (1) are given by

$$V_G = \frac{|V_A|}{|V_B|}[30 \text{ mV dB}^{-1}]. \tag{3}$$

$$V_{PH} = (\theta_A - \theta_B)[10 \text{ mV/degree}]. \tag{4}$$

The data acquisition module used the USB-1408FS A/D converter (Computing Measurements, Norton, MA, USA) with a sample rate of 48 kS s^{-1}. The data processing system was controlled by a Dell Precision 490 personal computer (Dell, Round Rock, TX, USA). Measurements were developed in the frequency range of 10–100 kHz in 20 steps spaced logarithmically, and changes in gain and phase of the ratio $\boldsymbol{V}_A/\boldsymbol{V}_B$ were recorded. The concept behind the system is that the measured potential shifts V_G and V_{PH} are representative of the changes in electrical conductivity and permittivity inside the Eppendorf tube; this means the complex impedance $\boldsymbol{Z}$ of the sample with or without tumor cells in suspension.

3. Results

Figure 4 shows a confocal microscopy image of a representative BT474 BC cell after incubation in PBS with a 'magnetic nanoparticle–monoclonal antibody' bioconjugate. The observed fluorescence is given by the presence of magnetic nanoparticles on the surface cell. Figure 5

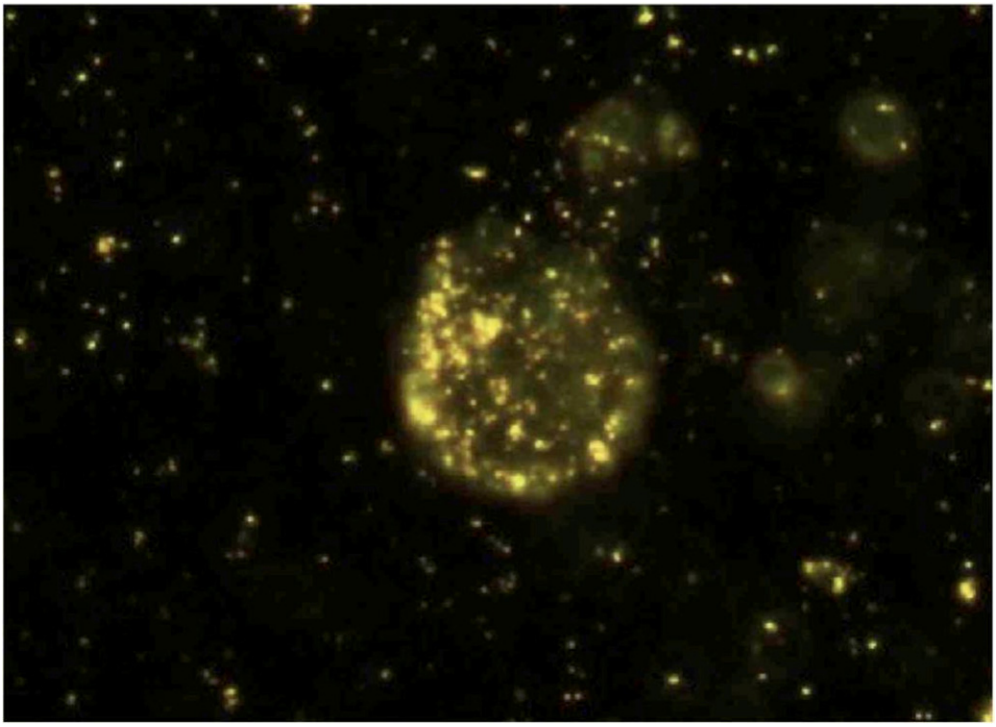

Figure 4. Confocal microscopy image of a representative BT474 BC cell after incubation in PBS with 'magnetic nanoparticle–monoclonal antibody' bioconjugate. Fluorescence is given by the presence of magnetic nanoparticles (image at 40 ×).

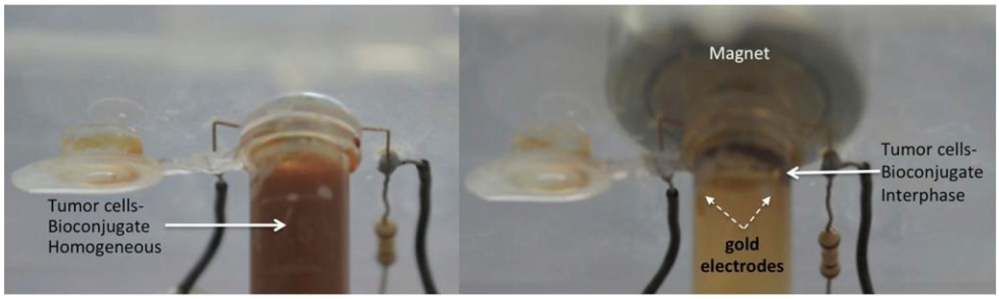

Figure 5. Experimental setup of relative bioimpedance measurement assisted by magnetic nanoparticles. For schematic purposes: (a) tumor cells incubated with bioconjugate in homogenous PBS solution and (b) tumor cells separated under the influence of the magnet and fixing an interface in close proximity to the electrodes (electrodes are roughly visible in this condition only). It is assumed that the interface is given by the magnetic attraction of ferromagnetic nanoparticles and coupled tumor cells.

shows the experimental setup of the relative bioimpedance measurement assisted by magnetic nanoparticles: (a) tumor cells incubated with bioconjugate in a homogenous PBS solution, and (b) tumor cells separated by the influence of the magnet and holding an interphase in close proximity to the electrodes. Figure 6 shows spectra of the amplitude (upper) and phase

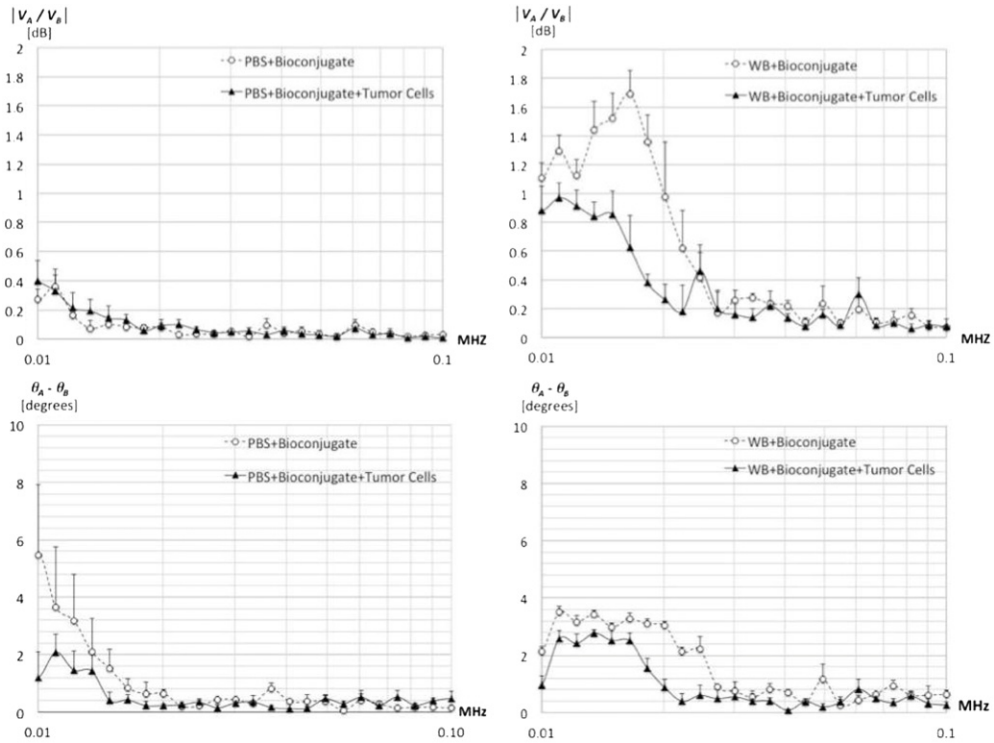

Figure 6. Spectra of the amplitude (upper) and phase (lower) of the $\boldsymbol{V}_{\mathrm{A}}/\boldsymbol{V}_{\mathrm{B}}$ ratio and phase differences $\boldsymbol{\theta}_{\mathrm{A}} - \boldsymbol{\theta}_{\mathrm{B}}$ as a function of the relative impedance changes in the interphase of tumor cells–homogenous condition for both PBS (left) and WB (right). Standard errors of three samples measured are shown. Gain changes are evident for the WB incubation condition at frequencies centered around 20 kHz, while phase observations are relevant below 20 kHz for both the PBS and WB conditions.

(lower) of the $\boldsymbol{V}_{\mathrm{A}}/\boldsymbol{V}_{\mathrm{B}}$ ratio as a function of the relative impedance changes in the interphase of tumor cells–homogenous condition for both PBS (left) and WB (right). Amplitude changes are evident for the WB incubation condition at frequencies centered around 20 kHz, while phase observations are relevant below 20 kHz for both PBS and WB suspensions.

4. Discussion

The ratio ($\boldsymbol{V}_{\mathrm{A}}/\boldsymbol{V}_{\mathrm{B}}$) and difference ($\boldsymbol{\theta}_{\mathrm{A}} - \boldsymbol{\theta}_{\mathrm{B}}$) for PBS or WB shown in figure 6 represent the bioimpedance measurement in terms of amplitude and phase respectively. Thus, the observation of no difference in the gain spectra of the ratio $\boldsymbol{V}_{\mathrm{A}}/\boldsymbol{V}_{\mathrm{B}}$ for PBS incubation represents no variation in the bioimpedance amplitude of the evaluated experimental condition; this can be explained because PBS is a water-based salt solution with similar osmolarity to the human body, so the electrical conductivity of structural components of tumor cells seems equivalent to the ionic concentration of PBS. Minimal and non-significant phase differences $\boldsymbol{\theta}_{\mathrm{A}} - \boldsymbol{\theta}_{\mathrm{B}}$ were observed for PBS incubation in the frequency range below 20 kHz; such an observation seems to reflect the initial emergence of the capacitive effect of the membrane of tumor cells. In contrast, important differences in bioimpedance amplitude were observed

for the WB incubation at frequencies centered around 20 kHz; such an observation might be explained because the influence of structural components of the blood content such as plasma, red and white cells, and platelets, as well as the contribution of WB structural components that might be carried by the magnet as heme iron, could have an important effect on the observed sensitivity. Phase differences were observed for the WB experimental conditions in virtually the same frequency range as for PBS, but this seems to be significant for the WB condition only; this might represent an evident emergence of the capacitive effect of the structural components of tumor cells. In addition, the explored frequency range in this study corresponds to a part of the α-dispersion bandwidth; it has been reported to be caused by the relaxation in the counter-ion atmosphere surrounding the charged cell membrane surface (Grimnes and Martinsen 2000). It is expected that the bioimpedance changes measured in this experimental study were influenced by the counter-ion atmosphere surrounding the formed interphase of tumor cells, the interaction of ion currents with the structural electrical conductivity of the ferromagnetic nanoparticles coupled to tumor cells, and the zeta potential at the shear plane of the ferromagnetic nanoparticles related to both surface charge and the local environment (Zhang *et al* 2009).

The ratio of CTCs in the blood of a cancer patient can range from 0 to 50 for every 10 billion blood cells per ml (Xu *et al* 2011). The ratio of tumor cells evaluated and detected in this experiment was around 5000 times greater, thus the findings are not related directly to clinical applications, and the experiment was not designed in such a way. In any case, the purpose of this experiment was to evaluate a volumetric detection approach to detect tumor cells in suspension and to determine the technical feasibility of using simple and inexpensive instrumentation as a biosensor tool. In addition, the electrode polarization effect should be considered in the frequency range where the highest sensitivity was observed (below 20 kHz), thus the following microelectrode system should be designed in such a way that tumor cells could be measured surrounded by large electrodes to minimize such an effect and to reveal small changes of the impedance caused by individual cells between the electrodes. The observations suggest the possibility for detecting CTCs in suspension by relative impedance spectroscopy measurements, and further studies are now warranted regarding microelectrodes and the involved instrumentation design to detect small tumor cell populations in whole blood, as well as a study of the zeta potential effect in normal and tumor cells marked with ferromagnetic nanoparticles under the proposed experimental conditions.

5. Conclusion

The use of a magnet to separate tumor cells in suspension coupled to magnetic nanoparticles is a feasible technique to fix an interphase of tumor cells in the proximity of gold electrodes. Relative impedance changes given by the differences of interphase of tumor cells–homogenous condition showed their potential value as a biosensor method for evaluating BC cells in suspension, at frequencies below 20 kHz, and particular sensitivity in the WB condition has been observed. The method described seems suitable for implementation to detect CTCs in WB, and further studies are warranted involving electrode design at the micro-scale level as well as instrumentation to detect small tumor cell populations under experimental conditions analogous to the above.

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