Impedimetric Characterization of Bioelectronic Nano-antennae

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The merging of electronics with biology at the nanoscale holds considerable promise for sensing and modulating cellular behavior. Advancing our understanding of nano-bioelectronics will facilitate development and enable applications in biosensing, tissue engineering and bioelectronic medicine. However, studies investigating the electrical effects when merging wireless conductive nanoelectrodes with biology are lacking. Consequently, a new tool is required to develop a greater understanding of the bioelectrical effects of merging conductive nanoparticles with biology. Herein, this

challenge is addressed by developing an impedimetric method to evaluate bipolar electrochemical systems (BESs) that could act as nano-antennas. A theoretical framework is provided, using impedance to determine if conductive nanoparticles can be polarized and used to drive current. It is then demonstrated that 125 nm Au nanoparticle bipolar electrodes (BPEs) could be sensed in the presence of biology when incorporated intracellularly at 500 $\mu\text{g/ml}$, using water and PBS as electrolytes. These results highlight how nanoscale BPEs act within biological systems and characterize their behavior in electric fields. This research will impact on the rational design of using BPE systems in biology for both sensing and actuating applications.

1. Introduction

We are entering a new era where bioelectronic tools enable us to harness and modulate cellular electricity. This allows us to control and sense cell behavior for various applications, from biosensing (Mehrotra, 2016), microbial fuel cells (Rabaey and Verstraete, 2005) and to the treatment of disease (Olofsson and Tracey, 2017). One of the remaining challenges to be overcome to further advance this area is to develop new nano-bioelectronics, capable of interfacing with cells at an equivalent spatial level of cellular electronic components, which can be operated remotely in a non-invasive manner. Also key is the development of new sensing systems capable of measuring cellular bioelectronic inputs and outputs. In doing so, this will shed light on how bioelectricity underpins cell behavior. To date, the rational design of wireless nano-bioelectronic systems has been limited due to the lack of label-free analytical techniques to study electrical effects on conductive nano-sized objects when combined with cells. Therefore, one of the aims of this work was to address this particular scientific challenge and to further explore how electric fields interact with nanoscale bioelectronic systems (Figure 1).

Figure 1. Interaction of electric fields with nanoscale bioelectronic systems. Schematic showing a bipolar electrochemical cell (A); applications of optimizing bipolar electrochemistry in vivo (B) and the experimental set-up used herein to evaluate nano-bioelectronic systems (C).

We have recently reported on the development of a nano-bioelectronic system which was based on using nano-bipolar electrodes (nano-BPEs) in the presence of biological cells for the first time (Sanjuan-Alberte *et al.*, 2019a). This work was based on a ‘bipolar electrochemical system’ (BES) which, consists of bipolar electrodes (BPEs) and an aqueous electrolyte solution (Fosdick *et al.*, 2013; Karimian *et al.*, 2019; Koefoed *et al.*, 2017; Loget *et al.*, 2013). Upon the application of an external electric field from feeder electrodes (FEs), the bipolar electrode (BPE) becomes polarized. This means that the BPE has an induced positive and negative pole generating free energy that results in the ability to mediate redox reactions (Figure 1A). This

ability to control redox reactions in cells has the potential for considerable therapeutic impact in diverse fields (Figure 1B). We demonstrate in this work that Au nanoparticles (AuNPs) act as nano-antennae, due to an ability to sense external electric fields and then act as reporters via changes in electronic properties, which we sense by impedance spectroscopy (Figure 1C).

The use of BPEs in electrochemistry is widespread (Backhurst et al., 1969; Cervera-March et al., 1988; Ellis and Jansson, 1981; Lee et al., 1989; Manji and Oloman, 1987; Smotkin et al., 1986; Wiesener et al., 1999). Over the last decade, new interest has revitalized the field with exciting applications at the micro/nanoscale (Bradley et al., 1997b, 1997a; Loget et al., 2012; Loget and Kuhn, 2011a; Perro et al., 2005). However, the field has been largely neglected for cellular applications and recent conflicting observations at the nanoscale, raises questions about the underpinning theory. For example, previously the assertion was that to polarize nanoscale BPEs, a relatively high voltage was required in the kV region (Loget and Kuhn, 2011b). However, most recent research has indicated that nano-antennae that act as bipolar electrodes are affected at much lower voltages (~ 10 V) (Sanjuan-Alberte et al., 2019a). It is therefore clear that a further understanding of bipolar electrode characteristics is required to optimize applications at the nanoscale and within biological systems. This previous body of work demonstrated the use of direct current to wirelessly drive current at an intracellular BPE. We believe moving towards using alternating current could allow for better prospects of merging with biological systems due to the greater flexibility in tuning potentials by altering frequencies. Hence, the work herein aims to better understand the effect of alternating current on BESs.

Other approaches exist to understand BPEs and optimize their use by facilitating carefully designed criteria to allow current flow through the BPEs. One approach includes equivalent circuit modelling using data from split BPEs or bespoke bipolar cells (Kusakabe et al., 1986, 1982; Sudoh et al., 1988). Translating these methods to the nanoscale would be extremely difficult, due to the need to manufacture electronics to connect to individual nano-

BPEs. Another possible method is electrochemically-modulated fluorescent probes (Scida et al., 2019). However, as this is an indirect method it is difficult to produce quantitative information and only BPEs are analyzed, thus neglecting other cell elements (electrolyte/feeder electrodes). This approach also adds the process of fluorescently tagging electrodes and typical fluorescence imaging issues such as photobleaching and subsequent irreversibility. Hence, an alternative analytical method is required that can be easily applied to nano scale BESs. We hypothesized that Electrochemical Impedance Spectroscopy (EIS) would be suitable for studying the electrical components of nano-BPE systems as it allows for the assessment of a BES in terms of its electrical circuit behavior, without the need for physical connections to each component in the system.

EIS is a powerful tool capable of deconstructing total impedance into its capacitive, resistive and inductive contributions (Macdonald, 2006), and has been extensively used to study corrosion (Epelboin et al., 1972). Other applications include material characterization (Chen et al., 2017; Loveday et al., 2004), battery and fuel cell development (Gomadam and Weidner, 2005; He and Mansfeld, 2009; Selman and Lin, 1993) and bipolar membrane characterization (Blommaert et al., 2019). In more recent years the tool has drawn interest in the life sciences field due to its ability to reveal electrochemical properties such as diffusion coefficients, electron transfer rate constants, adsorption mechanisms and charge transfer resistances (Randviir and Banks, 2013). These applications are dominated by EIS based biosensors, for example for label-free sensing of DNA (Kafka et al., 2008) and tuberculosis (Kekomaki et al., 1998), possible identification of osteoporosis (Bhardwaj et al., 2018), and identification of thyroid cancer using ‘EIS on a needle’ (EON) (Yun et al., 2018, 2016). EIS has also been widely applied to living cells in order to non-invasively count, identify or monitor cellular functions (Sun and Morgan, 2010); state-of-the-art instrumentation is now available allowing the monitoring of cell adhesion, morphology, proliferation, and motion of cells (electrical cell-substrate impedance sensing – ECIS®) (Lukic and Wegener, 2015). EIS is extremely practical

and easy to carry out, whilst also being quickly repeatable. As seen by the wide array of applications, this makes EIS an attractive technique for analytical purposes. Importantly, to the best of our knowledge, it has not been used to study cellular electrochemical systems as proposed here (Figure 1).

Herein, we report on a label-free EIS tool for direct modelling of the equivalent circuits of BESs. As seen in Figure 1C, this was performed using 125 nm AuNP BPEs, in electrolytes including water and phosphate-buffered saline (PBS). These nano-BPEs were then combined with biological systems to establish if they could act as bipolar nano-antennae within cells. The developed assay sheds light on how electrical input behaves with biology when using BPEs as electrical antennae. This indicates that electrical input parameters can be tuned to drive electrical polarization of nanoparticles, which could be used to design systems to manipulate cellular biochemistry in a wireless manner.

2. Results and Discussion

Our first step in designing an analytical tool that could be used to help us understand how BPEs behave under an electric field was to model them using a simplified equivalent circuit with three circuit elements. These elements are the impedance of the feeder electrodes (Z_F), the impedance of the BPEs (Z_B) and the ‘bypass impedance’ of the electrolyte (Z_E). Figure 2 (inset) shows the configuration of these components for samples with BPEs, without cells.

Figure 2. Basis of EIS method to evaluate bipolar electrochemical systems, by evaluating the ratio of BPE impedance and electrolyte impedance. Total impedance (Z_T), normalized by BPE impedance (Z_B), is plotted against efficiency parameter η (defined in Equation 1). Equivalent Circuit can be seen in the inset, with impedance components relating to the feeder electrodes Z_F , the electrolyte Z_E , and the BPEs Z_B .

An impedance-based parameter was then introduced with the ability to quantitatively-describe the efficiency of these BESs in driving current. η is defined as the ratio of the impedance of the BPEs (Z_B) to the bypass impedance of the electrolyte (Z_E), as described by (Equation 1). This parameter does not include the impedance of the feeder electrode (Z_F) as its effect on the overall cell impedance is negligible, exerting a low impedance in series with the overall impedance of the bipolar system.

$$\eta = \frac{Z_B}{Z_E} \quad (1)$$

$$Z_T = \frac{Z_E Z_B}{Z_E + Z_B} \quad (2)$$

In order to investigate the influence of the efficiency parameter on the total cell impedance, we can model a BES with a fixed Z_B and varying Z_E . As seen in Figure 2, by calculating total impedance (Z_T), using Equation 2, and normalizing using Z_B , we can plot normalized total impedance against the efficiency parameter, giving three distinct regions of η .

In the range where $\eta \geq 10$, Z_T is dominated by Z_E ; this case occurs when a highly conductive electrolyte is used. Current will favor the electrolyte, meaning little to no current would pass through the BPEs, therefore being undesirable in driving electrochemical reactions. As Z_E increases, falling within the range of $0.1 < \eta < 10$, Z_T begins to reflect both Z_E and Z_B . This is due to impedance values becoming more similar and hence current tends towards equally splitting between the two components. Finally, when η falls below 0.1, the overall impedance is then limited only by Z_B . This shows that when a highly resistive electrolyte is used, current would then favor the BPEs with little to no current passing through the electrolyte. In order to

drive electrochemical reactions at the BPE, and to probe any changes in the BPEs, current is required to pass through the BPEs. Hence, we can conclude that $\eta \leq 10$ must be satisfied when using this analytical method. A similar concept has previously been described, though not using impedance spectroscopy as in this study (Loget and Kuhn, 2011b). Previous studies required physical connection or labelling of the BPEs to assess electrical behavior (Kusakabe et al., 1982; Scida et al., 2019; Sudoh et al., 1988). Utilizing impedance spectroscopy is label-free and therefore allows a much simpler method, but also allows for the analysis of nano-BPEs for the first time.

2.1. Nano BPE systems

Before exploring how BESs behave in the presence of cells, we first validated the above theoretical framework with FEs, BPEs and varying electrolytes only. The BES consisted of gold feeder electrodes with spherical gold nanoparticle (AuNP) BPEs placed in between. AuNPs are commonly used for *in vivo* applications (Cabuzu et al., 2015). Furthermore, we have recently developed bioelectronic systems using AuNPs in which we observed that they could act as electrical antennae within cells (Sanjuan-Alberte et al., 2019a). They would therefore be suitable for establishing an EIS analytical protocol to study the effects of nano-sized BPEs in biological systems. A larger diameter particle is used herein (125 nm) to allow for higher charge density and compatibility with other techniques, such as assessing plasmonic effects (Sanjuan-Alberte et al., 2019a). In order to test the effect of varying Z_E and hence experimentally validate the above model (Figure 2), samples were tested in high and low impedance electrolytes: deionized water and PBS respectively. Furthermore, a range of concentrations of PBS were also studied to further test the hypothesis that increasing Z_E allows for the increased efficiency of the system (η) (Figure S1). It is important to note that this is not to simulate *in vivo* conditions, but to assess how these nano BESs behave in high and low impedance electrolyte examples.

Subsequently, we then analyzed the resulting frequency impedance plots by least squared fitting to construct equivalent circuits (Figure 3). Warburg impedance was also

included in series with the feeder electrode to account for diffusion in the system and to allow for better fitting. However, it was found that these values were not significantly different between repeats and hence are not discussed. All results did however satisfy the Kronig-Kramer's test, showing that the system is linear and stable with time, which is a pre-requisite for circuit modelling. Both FEs and BPEs are adequately described by a capacitor only, in the absence of charge transfer across the interface, and therefore $Z_{F/B}$ are modelled as $C_{F/B}$. When deionized water is used as an electrolyte, the impedance of AuNPs is lower in comparison to Z_E , resulting in current flow through the BPEs. As the current passes through BPEs capacitively, an additional capacitor appears in the equivalent circuit accounting for this effect (Figure 3A). In contrast to deionized water, when PBS is used current flows through the electrolyte, the impedance of BPEs is relatively high and therefore behaves as an open circuit (Figure 3B). As PBS is more physiologically relevant to intracellular space than deionized water, BPE concentration will be extremely important in order to drive current in the presence of cells. Due to the practicality of this method, equivalent circuits are much easier to produce when compared to previous methods for probing BPEs (Kusakabe et al., 1986, 1982; Sudoh et al., 1988).

Figure 3. Electrical characterization plots and example circuit fittings of Au feeder electrodes with AuNP bipolar electrodes in water (A) and PBS (B). Circuit diagrams can be seen in the inset.

Warburg impedance was also included in series with the feeder electrode to account for diffusion in the system and to allow for better fitting. With Z_E being modelled as a resistor (R_E) and Z_B modelled as a capacitor (C_B), C and D show the average magnitudes of R_E and C_B respectively in samples using water (blue) and PBS (red). Error bars show standard error of mean ($n = 3$). Efficiency parameter (η) of 125 nm AuNP BPEs in PBS (blue) and deionized water (red) can be seen in E.

Bode plots shown in Figure 3A and 3B for deionized water and PBS respectively, confirm that the BESs do behave differently with varying electrolyte impedance. In water, there are three distinct sections of the impedance spectrum. At low frequencies, the impedance is limited by the capacitive nature of the feeder electrodes. This shows a typical frequency dependent behavior, described in Equation 3, where impedance drops by increasing frequency. The second region is characterized by a constant impedance occurring within the frequency

range $\sim 100 - 3000$ Hz. This component arises from the resistance of charge flow (i.e. Z_E , electrolyte impedance). When this is described in terms of the theoretical framework, previously described in Figure 2, the parameter $\eta \geq 10$ for this region. This means, within this region, the electrolyte impedance is still relatively lower than the BPE impedance and therefore is limiting the overall impedance. As the BPE is also behaving as a capacitor, its impedance is also frequency-dependent; hence, the impedance of the BPEs becomes less than the impedance of the electrolyte for the frequency range $\sim 3000 - 10,000$ Hz. This means current flows through the BPE when $\eta \leq 10$ is satisfied. It can therefore be concluded that in order to probe these nano-BPEs using impedance spectroscopy within deionized water, measurements should be performed at frequencies higher than ~ 3000 Hz when relatively low voltages are applied. Although the reported frequency band is only suited to the BES described above, the concept can be adapted to probe remotely-induced electrochemical reactions. To achieve this, a suitable frequency region should be identified since the electrochemical impedance of BPE-electrolyte interface changes with redox charge transfer. It is worth noting that there is a possibility of driving current at low frequencies if higher voltages are applied, as we have observed previously (Sanjuan-Alberte et al., 2019a). Within PBS, Figure 3B, we only observe two disparate sections of the curve relating to: (i) the capacitive effect of the FEs at lower frequencies; and (ii) plateauing to the resistive effect of the electrolyte at high frequencies. As expected, the addition of BPEs does not significantly change the impedance spectra, indicating we are working within the region of $\eta \geq 10$ with little to no current flowing through the BPEs. As within deionized water, it is expected that at much higher frequencies the BPEs may then be able to be sensed due to their decreasing impedance.

$$Z = \frac{1}{j\omega c}$$

$$\text{where } j = \sqrt{-1}, \omega = \text{angular frequency or } 2\pi f \text{ and } c = \text{capacitance} \quad (3)$$

We were then interested in determining how the different BES components are behaving, which would yield knowledge to better understand and design systems. The average of the equivalent circuit components from three independent repeats is plotted in Figure 3C and D. These plots present the electrolyte resistance (R_E) and the BPE capacitance (C_B) respectively. FE behavior is not discussed here as it has low impedance in series with the BES and does not affect the BPEs. As seen in Figure 3C, the resistance of deionized water is in the order of $M\Omega$, as expected due to the low concentration of ions; values are reasonable as the dH₂O resistance is rated at 18.2 $M\Omega$.cm and there is a 1 mm gap between electrodes. PBS impedance is in the order of $k\Omega$, also reasonable for this concentration (Ribeiro et al, 2020). The results presented show that the electrolyte impedance is not affected by the presence or absence of the BPEs. This observation further confirms the robustness of the technique in extracting the components of the BES. As anticipated from the theoretical framework in Figure 2, the BPEs are detected in the deionized water, rather than in PBS as presented in Figure 3D. The BPE component was not included when modeling samples in PBS and with no nano-BPEs, due to markedly better fitting being achieved (this can be seen in Figure S2). AuNP BPEs were used in two concentrations to determine if the method was sensitive to such differences: 50 $\mu\text{g/ml}$ and 12.5 $\mu\text{g/ml}$. Although this was not the case between these concentrations, it is expected that a higher magnitude of difference would show a change in capacitance values; this will be pertinent when applying nano-BPEs with living cells.

Given the capacitance of BPEs calculated from equivalent circuits in Figure 3D (~ 2.5 pF), we then calculated the efficiency of these BPEs in different electrolyte systems by calculating η (using Equation 1 and Equation 3). It is important to note that the capacitance of the BPEs would change in PBS and an estimate of this value was not possible due to no current flowing through the BPEs. However, when applying Equation 1 and Equation 3, even a magnitude difference in capacitance will have little effect on η , therefore this will allow a

rudimentary comparison between water and PBS. This comparison, seen in Figure 3E, shows that η falls below 10 when in the water at frequencies higher than ~ 3000 Hz, again suggesting that we are able to drive current through these BPEs under these conditions and not when using PBS.

2.2. Living Cells with Nano BPEs

Development of bioelectronic systems that use conductive NPs to sense and actuate cellular behavior would potentially be an extremely important advancement for cancer diagnosis and treatment (Sanjuan-Alberte et al., 2018.; Sanjuan-Alberte and Rawson, 2019b). Consequently, we used the assay to study how AuNP BPEs behave when placed within U251 malignant brain cancer cells. To determine whether current would flow through BPEs when cells are present, U251 cells were seeded onto samples without nano-BPEs, as well as with intracellular nano-BPEs. Again, high and low impedance electrolytes of water and PBS respectively, were used. We have previously shown that the short time frame of these experiments (< 1 min) using water does not affect cell viability (Sanjuan-Alberte et al., 2019c).

To provide intracellular nano-BPEs, cells were incubated with AuNPs, before seeding cells onto FEs. As seen in Figure 4, cellular uptake of AuNPs was confirmed using confocal microscopy. To achieve this, we covalently conjugated fluorescent Zinc(II) 5-(4-aminophenyl)-10,15,20-(tri-4-sulfonatophenyl)porphyrin triammonium (zinc porphyrin) to these AuNPs (Figure S3). For comparison purposes, two concentrations of AuNPs were tested along with two incubation times: 50 or 25 $\mu\text{g/ml}$ and 6 or 24 hours. Both the higher concentration (50 $\mu\text{g/ml}$) and the longer time frame (24 h) showed significantly higher AuNPs inside cells and hence, 50 $\mu\text{g/ml}$ for 24 hours was used for experiments herein. We also performed inductively coupled plasma mass spectroscopy (ICP-MS) for cellular uptake analysis of AuNPs, to support the obtained confocal data (Figure S4). Considering both confocal imaging and ICP-MS results, using porphyrin functionalized and bare AuNPs respectively, we can be confident that AuNPs are being taken up by the cells.

Figure 4. Cellular uptake analysis of AuNP BPEs comparing two concentrations of AuNPs and two incubation times. Confocal microscopy was used to image U251 cells incubated with AuNPs conjugated with Zn porphyrin (A). All scale bars represent 50 μm . Mean cell fluorescent intensity as a function of cellular uptake and SD was plotted for at least 20 cells (B and C). All the values were normalized with fluorescent intensity obtained with AuNP concentration of 50 $\mu\text{g/ml}$ (B) or AuNP incubation time of 24 hours (C). 50 $\mu\text{g/ml}$ AuNP concentration was taken forward from B to perform the incubation time study in C.

Cell experiments were initially performed between the frequencies of 10 - 10,000 Hz and a current limit of 1 mA. Introducing living cells produced a non-stable system within this range, due to the system changing over time. Due to this, the systems did not pass Kronig-Kramer's tests and cannot be reliably fitted to electrical circuits. We include this data as it is an important example of design rules when analyzing NP effects and without analyzing higher frequencies, data can be misunderstood.

As seen in Figure 5, the intracellular nano-BPEs appear to show a difference from cells alone within water, increasing to $> 100 \text{ k}\Omega$. However, without being able to fit equivalent circuits we are unable to investigate whether this is in fact due to the nano-BPEs being sensed.

Figure 5. Intracellular BPE impedance spectra, in water (A) and PBS (B), in comparison with cells alone. Comparison of the data to data from previous section (nano BPE systems) can be seen in Figure S5. Intracellular BPEs were incorporated in two different concentrations, 25 and 50 $\mu\text{g/ml}$.

Despite EIS being applied to many biological applications, to the best of our knowledge, only one previous study exists comparing the impedance of tissue with and without the presence of conductive objects (AuNPs) (Ostovari et al., 2018). As measurements are also taken at low frequencies, circuits are not fitted, so the behavior of such AuNPs cannot be quantified. Therefore, to better understand how the individual components in the systems are behaving, impedance measurements were taken covering a higher frequency range of $0.1 - 10 \times 10^7 \text{ Hz}$. This fully covers the range of frequencies usually used to study cells using EIS (Wegener et al., 2000) and allowed us to fit circuits to better explain the systems. Furthermore, the higher frequencies are believed to be more affected by cell membranes, whereas lower frequencies are more affected by solution channels under and between adjacent cells (Wegener, Keese, & Giaever, 2000). Therefore, there is a better scope to probe intracellular BPEs at these higher frequencies. Moreover, unlike raw spectra, resistance, and capacitance values from fitting, are normally distributed, passing a Shapiro-Wilk normality test ($p > 0.01$), allowing us to discern

significant differences more easily. As low frequency did not appear to show differences between the two concentrations of AuNPs, the higher concentration was chosen to be studied at high frequencies (50 $\mu\text{g/ml}$). A ten-fold higher concentration was also studied intracellularly to maximize the chance of sensing intracellular BPEs (500 $\mu\text{g/ml}$).

As performed with samples without cells, circuit fittings were carried out in order to better understand how the systems are behaving. Upon analysis of the samples in water, we elucidate that we have two-time constants, represented by the two sem-circles in the Nyquist plots, and an increase in the phase angle at higher frequencies (Figure 6A and B). These phenomena are not present in the PBS data and hence a different circuit was required for the different electrolyte samples. We hypothesise that this is because, within PBS, the method is less sensitive so we are unable to separate the two RC circuits during fitting; however, the combined RC circuit is still sensitive to the presence of the BPEs inside cells. As seen in Figure 6C and D, these circuits included FE capacitance (C_F), in series with an RC circuit with electrolyte resistance R_1 , and cell membrane capacitance C_1 . Water samples included a second RC circuit with a second resistance and capacitance (R_2 and C_2).

Figure 6. Example data of samples with AuNPs and cells present, using water as an electrolyte. Nyquist plots (A) and Phase angle (B) are plotted. C and D show circuits fitted to water and PBS respectively.

The above simple circuits were fitted to the experimental data and the circuit components are presented in Figure 7. Sample fitting can be seen in Figure S6. As expected, C_F values were not significantly different across all samples and hence will not be discussed. R_1 values were also not significantly different within the electrolytes, suggesting the main contributor to this is the resistance of the electrolyte used. We do however observe a significant difference in C_1 values when comparing the highest concentration of AuNPs to the case of low concentration or absence of AuNPs. Due to this, we can conclude that the capacitance C_1 is associated with the intracellular ionic differences and therefore the reported analytical method is sensitive to the presence of AuNPs in the intracellular space. This is supported by the evidence we provided that AuNPs are inside the cells, which was demostarted using ICPM-MS and fluorescent studies. If the capacitor was linked to the cellular component alone then the AuNP concentration would have no effect; therefore this importantly indicates that current can be directed through the AuNPs inside the cells in both water and PBS. These findings show that

measurements can be performed in PBS, which will provide a better mimic of physiological conditions and allow for longer term studies. It is reported that the impedance of the cell membrane drops significantly at MHz frequencies and it effectively acts as a short circuit (Hart et al. 2018). This results in an increase in current flow that enhances the sensitivity of EIS in detecting BPEs inside cells. This is the first evidence that AuNPs can act as nanoantennae and therefore could potentially be used to wirelessly drive electrochemistry. This exciting finding suggests that the proposed impedimetric characterization can be used to detect cell-NP interfacing and probe the flow of therapeutic current through these BPEs.

Figure 7. Comparison of fitting values from samples with cells and AuNPs at two concentrations: 50 and 500 µg/ml. Capacitance (C) and resistance (R) values from circuit fittings can be seen using water (blue) and PBS (red) as electrolytes. Circuits used can be seen in Figure 6. Error bars show standard

error of mean ($n = 3$). Significant differences (*) were assessed using two way ANOVA and Tukey's multiple comparison test.

Considering water samples only, we witness a second RC circuit. Again R_2 is similar in magnitude to the electrolyte resistance and so this is likely to be the main contributor. As for C_2 , we believe this to be linked to the cells themselves acting as BPEs, due to the presence of the AuNPs having no effect on the capacitance. The reason this is not seen when using PBS is due to the ratio η being too high when comparing electrolyte and cell impedance, meaning current favours the electrolyte and hence little current flows through the cells. These results show that within these conditions, nano-BPEs can behave as nano-antennae when in the intracellular space, which could have useful sensing applications. Further optimisation could allow the generation of useful currents at the BPEs for actuation applications. This could then allow for the use of nano-BPEs in targeting or enhancing electric fields, growing *in vivo* electronics and sensing/actuating cellular functions (Figure 1B I, ii and iii).

3. Conclusion

This work provides advancing knowledge on the application of nanoscale BPE systems in biology, which until now was lacking. As applied herein, EIS allows a simple way to evaluate a BPE system and model its equivalent circuit. Such a method is both simpler than existing methods (Kusakabe et al., 1986, 1982; Scida et al., 2019; Sudoh et al., 1988) as well as made possible with nano-BPEs for the first time.

Here we propose an impedimetric method of evaluating the efficiency of a BPE and electrolyte combination. By modelling total impedance in relation to BPE and electrolyte impedance, we propose the efficiency term η . We then validate this model experimentally using AuNP BPEs and high and low impedance electrolytes: deionized water and PBS respectively. This confirms that within these conditions, these BPEs can be probed in water at high frequencies, but not in PBS.

Further to this, we introduced cells to the system to aid in determining if AuNP BPEs can be used as bioelectronic antennae. The addition of cells provides an unstable system. To combat this, high frequency measurements were taken to allow for circuit fittings. Within this system, intracellular BPEs at a concentration of 500 μ g/ml show a significant difference from cells alone, in both water and PBS. This suggests that current is being driven through them. This is a key finding suggesting that nano-BPEs could be used as nano-antennae with biology when in the intracellular space. Further measurements of BPEs behavior *in vivo* are required to determine whether they may be used for bioelectronics purposes.

4. Experimental Section

Electrode Manufacture: All electrodes were manufactured in house. Feeder electrodes were fabricated using a Peltier cooled dual-target sputter coater (Emitech k575x) and 3D printed polylactic acid (PLA) masks (printed using an Ultimaker). As seen in Figure 8, electrodes were approximately 2 mm in width with a 1 mm gap between them. All sputter coating consisted of first coating with 20 nm of ITO (at 30 mA), as an adhesion layer, before coating with 100 nm (at 120 mA) of gold. Accurate™ spherical citrate stabilized gold nanoparticles (AuNPs) were purchased from Nano Partz™ with a diameter of 125 nm. Nano bipolar electrodes were prepared using an electrostatic coating of AuNPs in between the feeder electrodes at two concentrations: 50 and 12.5 μ g/ml. Firstly 20 μ l of potassium hydroxide solution (1 M KOH in 60% ethanol) was pipetted between feeder electrodes and left for 1 hour. Following this step, two synthetic polymers with opposite charge, Poly (allylamine hydrochloride) (PAH) and Poly (sodium 4-styrenesulfonate) (PSS), were deposited for 30 minutes each. Polymer solutions were both 0.1 M in dH₂O. Finally 20 μ l of AuNPs was left to drop coat overnight. Between all steps, the electrodes were washed with dH₂O and dried with nitrogen.

Figure 8. Au feeder electrode setup. Feeder electrodes were approximately 2 mm in width, with a 1 mm gap between them. 20 μ l of electrolyte was used to bridge the gap between electrodes, the approximate boundary of this droplet can be seen in blue. Samples with cells were produced using Ibidi $\text{\textcircled{R}}$ sticky slides, creating an approximate area of cell coverage of 1 cm^2 , this can be seen in red. 20 μ l of electrolyte was also added, however this spread across the entire cell surface.

EIS Measurements: All impedance measurements were carried out using an Autolab potentiostat (Metrohm) with an applied AC amplitude of 0.01 V_{RMS} in potentiostatic mode. A two-electrode system was used, with working electrode and reference/counter electrodes combined. Impedance was measured between 10 and 10,000 Hz, with a current limit set at 1 mA. For high frequency measurements a high frequency module, attached to the potentiostat, was used (ECI10M). Ag conductive paint (RS components) was used to create larger contact areas to attach samples to the potentiostat via crocodile clips. A single droplet of 20 μ l of electrolyte was used, as this was enough to just bridge the gap between feeder electrodes.

Surface Functionalization of Gold Nanoparticles: Zinc(II) 5-(4-aminophenyl)-10,15,20-(tri-4-sulfonatophenyl)porphyrin triammonium (Zinc porphyrin) was covalently attached on gold nanoparticles (AuNPs) using a EDC/NHS coupling chemistry. In the first step, AuNPs were functionalized with 11-Mercaptoundecanoic acid (MUA) to introduce carboxylic groups. Briefly, 1 mL of MUA (100 μ M) in 1:9 ethanol/water was added to 5 mL of citrate capped AuNPs (100 μ g/mL). The solution was stirred overnight at room temperature under N_2

atmosphere. Later, the AuNPs were washed three times with water and centrifuged at 2500 rpm for 20 min to obtain MUA-capped AuNPs. In the second step, freshly prepared 1 mL EDC (100 mM) and 1 mL NHS (200 mM) was added to the MUA capped AuNPs to activate the carboxyl groups. The reaction was allowed to continue for 4 h under an N₂. Then, 1 mL of zinc porphyrin (100 μM) was added to this mixture and the solution was stirred at room temperature for 24 h under dark. Finally, the AuNPs were washed 3-4 times with water and centrifuged at 2500 rpm for 20 min to obtain AuNPs conjugated with zinc porphyrin.

Cell Culture and Cell Experiments: A human glioblastoma cell line (U251s) was cultured and expanded under standard cell culture conditions, using culture medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine. The cells were passaged using trypsin, with all cells used in experiments having a passage number of <50. Cells were cultured onto microelectrode samples using Ibidi sticky slides® at 500,000 cells and left for 4-6 hours to attach before running EIS measurements. For EIS measurements sticky slides were removed, all traces of media were first washed away by submerging samples in deionized water and then 20 μl of electrolyte was added. During all experiments deionized water (18.2 MΩ.cm) and sterile PBS (0.01M, ThermoFischer) was used.

For cellular uptake analysis, 1.5×10^4 U251 cells were seeded on 13 mm glass viewing area of a 35 mm glass-bottom dish (ThermoFisher SCIENTIFIC) and incubated at 37°C for 24 h. After 24 h, the culture medium was replaced with fresh medium containing 50 or 25 μg/ml AuNPs (functionalized with zinc porphyrin) for 6 or 24 h. After each incubation period, the media containing the AuNPs was removed and cells were washed thrice with PBS. The plasma membrane of the cells was stained using CellMask™ Green Plasma Membrane Stain (Thermo Fisher SCIENTIFIC) for 10 min. Later the cells were fixed with 4% formaldehyde for 5 min and subsequently washed 3 times with PBS. Afterward, cells were treated with DAPI for 5 min at RT under dark and washed again thrice with PBS. Finally, the cells were immersed in PBS and imaged using a Zeiss Elyra confocal microscope. Cellular uptake of AuNPs functionalized

with zinc porphyrin was quantified by measuring fluorescent intensity of at least 20 different cells using ImageJ. The fluorescent intensity values were normalized to the total number of cells per field and expressed as corrected total cell fluorescence (CTCF) obtained by applying Equation 7.

$$CTCF = Integrated\ density - (Area\ of\ selected\ cell \times \\ Mean\ fluorescence\ of\ background\ signal)$$

(7)

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Supporting Information

Impedimetric characterization of bioelectronic nano-antennae

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Nano BPEs

In order to further validate the theoretical framework outlined in Figure 2, impedance of systems with and without AuNP bipolar electrodes (BPEs) was measured in varying concentrations of electrolyte between deionized water and phosphate-buffered saline (PBS). Dilutions were made in 10 fold using a PBS stock (0.01 M Thermo Fischer). When looking at samples without BPEs, we saw no change in shape of the nyquist plot. All concentrations exhibiting a 45° slope which is characteristic of Warburg impedance due to diffusion limits. When BPEs are present we saw a change in the graphs shapes at high frequencies as you approach low PBS concentrations and deionized water. This semicircle that begins to form is due to a capacitance being introduced from the BPEs. This validates the theoretical framework showing that increasing impedance of electrolyte allows for increasing current driven though the BPEs shown by the increasing semicircle size.

Figure S1: Varying PBS concentrations were tested in systems with (B) and without (A) AuNP bipolar electrodes. Real impedance was normalized to allow Nyquist plots to be overlaid. Insets show expanded lower frequency portion of graphs.

Circuits with the BPE component were first fitted to PBS data to determine if they were contributing to the impedance of the system due to current being driven through them. As seen in Figure S2, better fitting was produced by removing the BPE component, suggesting that no current is being driven through the BPEs.

Figure S2: Raw data of bipolar system with AuNP BPEs in PBS. Fitting lines show fitting with (red) and without (green) the BPE component.

Cells with nano-BPEs

Figure S3. Fluorescent emission spectrum of AuNPs functionalized with zinc porphyrin monitored upon excitation with a wavelength of 422 nm.

To further study the cellular uptake of AuNPs, inductively coupled plasma mass spectroscopy (ICP-MS) was carried out (iCAPQ Thermo Fischer) with bare AuNPs. Using the same methods as described for confocal microscopy, ICP-MS samples were prepared by digesting samples in 5% aquaregia overnight, before then being diluted with milliQ water to reach a final acid concentration of 2% (Jiang et al., 2015). As seen in Figure S4, both cells (blue) and supernatant (red) were collected from incubation studies. Comparing concentrations, within the same time frame, it can be seen that the increase in Au is present in the supernatant and not the cells. This suggests that increasing concentration does not allow for increased cellular uptake within these conditions. There is however a significant difference between 6 and 24 hours of the same concentration. Au in cells increases, whilst Au in supernatant decreases, suggesting the increase in time allows for further cellular uptake from the supernatant. Due to both ICP-MS and confocal microscopy results, 50 $\mu\text{g/ml}$ of AuNPs and an incubation time of 24 hours was used.

Figure S4. Inductively coupled plasma mass spectrometry (ICP-MS) results for 25 and 50 $\mu\text{g/ml}$ of AuNPs incubated with U251 cells for 6 or 24 hours.

Figure S5. Impedance spectra of samples with cells, in comparison to samples without. ‘Cells’ and ‘AuNPs’ samples consist of cells/AuNPs with the corresponding electrolyte.

Figure S6. Graphs showing sample fitting of raw data of systems with living cells, in water (A) and PBS (B). Such fitting was used to determine resistance and capacitance values seen in Figure 7.