Electrochemical Impedance Spectroscopy as a Tool for Monitoring Cell Differentiation from Floor Plate Progenitors to Midbrain Neurons in Real Time

Aya Elghajiji, Xin Wang, Stephen D. Weston, Guenther Zeck, Bastian Hengerer, David Tosh, and Paulo R. F. Rocha*

1. Introduction

Central nervous system (CNS) injuries, particularly, spinal cord injury (SCI) and hypoxic-ischemic brain injury lead to motor/sensor deterioration for patients. Traumatic SCI has been related to tissue loss, including forfeiture of neurons and oligodendrocytes, development of glial scars, and demyelination. Emerging therapies seek to implant neural progenitor cells (NPCs) aided by electrical stimulation[1] to promote neural repair subsequent to neural injury.[2–5] Upon implantation into the region of injury, NPCs are expected to migrate toward injured spots and, upon adhesion, differentiate and integrate into localized neural lineages toward their prime goal, that is, neural repair.[6–13]

Electrical impedance spectroscopy (EIS) characterization has been emerging as a promising tool in the fields of impedance-based biosensing and neuro-prosthetics. Cell growth and locomotion,[18] bacterial growth[19] and even antigen–antibody reactions[20] have been characterized by EIS. Addressing the physiological state of cells in real-time and without the hurdle of fluorescence dyes is a major advantage of EIS. Yet, although EIS is gaining traction as a low cost non-invasive and label free sensing tool, there is a lack of reports on the development of a non-invasive real time tool to probe and quantify cell differentiation. Here, we accomplish this goal by monitoring the differentiation from human-relevant midbrain floor plate progenitors (mFPPs) to midbrain neurons on Au electrodes coated with human laminin. The electrical data and equivalent circuit modeling are consistent with standard microscopy analysis and reveal that within the first 6 hours progenitor cells sediment and attach to the electrode within 40 hours. Between 40 and 120 hours, midbrain progenitor cells differentiate into midbrain neurons, followed by an electrochemically stable maturation phase. The ability to sense and characterize non-invasively and in real time cell differentiation opens up unprecedented avenues for implantable therapies and differentiation strategies.
analysis and equivalent circuit modeling reveal the ability to non-invasively monitor cell adhesion and differentiation during 240 h.

2. Methodology Cell Culture and Differentiation

2.1. Human iPSC Culture and Embryoid Body Formation

We utilized the human induced pluripotent stem cell (hiPSC) line termed Munich cell line (iPSC-MUC). Name of the ethics committee is Ethikkommission der Medizinischen Fakultät der Ludwig-Maximilians-Universität München, with the project number 141080. hiPSCs were maintained in mTESR plus medium according to manufacturer’s instructions (Stem Cell Technologies). 2mg mL\(^{-1}\) collagenase (Sigma) was used for dissociation and cells were resuspended in induction medium composed of basal medium containing DMEM/F12 (Fisher) and Neurobasal medium (Fisher) (1:1), with 1:100 B27 supplement without vitamin A (Fisher), 1:200 N2 supplement (Fisher), 1% penicillin/streptomycin (Sigma), and 1% l-glutamine (Sigma). Induction medium also included 10 \(\mu\)M SB-431642 (Millipore), 0.1 \(\mu\)M LDN193189 (Sigma), 3 \(\mu\)M CHIR99021 (Tocris), and 0.5 \(\mu\)M smoothened agonist (SAG; Millipore). Supplements were reconstituted according to manufacturer’s instructions and were added to the medium prior to addition to the cell culture. Resuspended cells were plated onto low attachment plates in order to form NPC embryoid bodies (EB) formation.

2.2. Derivation of smNPCs

In order to derive small molecule neural progenitor cells (smNPC), EBs were disaggregated and plated as a monolayer on 5 \(\mu\)g mL\(^{-1}\) of human LN521 laminin (BioLamina) (as schematically shown in Figure 1a). Their derivation was according to the protocol by Reinhardt et al.[22] Basal medium was used with the addition of 3 \(\mu\)M CHIR99021, 200 \(\mu\)M ascorbic acid (Sigma), and 1 \(\mu\)M SAG. Media were changed every other day, up to 7 days.

2.3. Patterning of smNPC into mFPP

Protocol was based on Reinhardt et al.[22] with minor modifications as described by others.[22,23] Once a confluent layer of smNPCs was obtained, Accutase (Sigma) was used to dissociate the cells. The cells were then deposited onto a fresh plate covered with 5 \(\mu\)g mL\(^{-1}\) of LN521. The patterning medium at this stage consisted of basal medium with 0.5 \(\mu\)M SAG, 200 \(\mu\)M ascorbic acid, 100 ng mL\(^{-1}\) fibroblast growth factor 8b (FGF8b; R&D Systems) (as suggested by refs. [22,23], 1 ng mL\(^{-1}\) glial cell line-derived neurotrophic factor (GDNF; Peprotech) and 2 ng mL\(^{-1}\) brain-derived neurotrophic factor (BDNF; Peprotech). The media were changed every other day for a total of 6 days.

2.4. Differentiation into Midbrain Neurons

All differentiation experiments were conducted with smNPCs of passage 10 and above. The differentiation protocol was based on protocols previously published by Kriks et al.[22] and Nolbrant et al.[23] Following dissociation using Accutase, 7 \(times\) \(10^6\) cells cm\(^{-2}\) were deposited on 10\(\mu\)g mL\(^{-1}\) of LN521 on the transducer. Maturation medium was used which included basal medium supplemented with 20ng mL\(^{-1}\) GDNF, 20ng mL\(^{-1}\) BDNF, 200 \(\mu\)M ascorbic acid, 10 \(\mu\)M N\(^{6},2^{'},O\)-Dibutyryl adenosine 3’,5’-cyclic monophosphate sodium (dCAMP; Sigma), 1ng mL\(^{-1}\) transforming growth factor-\(\beta\) (TGF-\(\beta\); Tocris), and 10 \(\mu\)M N-[3,5-difluorophenylacetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester (DAPT; Tocris). Maturation medium was changed every day.

2.5. Immunocytochemistry and Microscopy

Cells cultured on either glass coverslips (VWR), chamber slides (Nunc Lab-Tek II Chamber Slide), or glass electrodes were fixed in 4\% (v/v) paraformaldehyde in sterile PBS (PFA; Fisher) for 15 min. 0.1\% Triton X-100 (diluted in PBS; Sigma) was used for permeabilization, followed by blocking for 1 h in 2\% blocking buffer (Roche). Following blocking, the fixed cells were incubated with primary antibodies (diluted in 2\% blocking buffer) overnight at 4 \(\degree\)C, as shown in Table 1. On the next day, the cells were washed three times in PBS for 5 min each and were then incubated with the corresponding secondary antibodies (diluted in 2\% blocking buffer) for 2 h in the dark at room temperature and were then washed two times with PBS for 5 min each as to remove any residual secondary antibodies. This was followed by an incubation with 0.5\mu\g mL\(^{-1}\) 4’,6-diamidine-2-phenylindole (DAPI; Sigma) in PBS for 10 min, if nuclei counterstaining was required. The coverslips were mounted on microscope glass using fluorescent mounting medium (Dako). Images were acquired using a Zeiss LSM880 confocal microscope as a Z-stack and were processed using the maximum intensity projection (MIP) parameter in Zen Black Software (Zeiss). Zen Blue was used to obtain the 3D representation and orthogonal projection of laminin dynamics.

3. Differentiation Process of the hiPSC Line

Figure 1a is a diagrammatic representation of the protocol used in this study. The differentiation process starts with the derivation and expansion of neural progenitor cells (NPCs) as described by Reinhardt et al.[24] In order to derive NPCs, hiPSC colonies (70\% confluent well of a 6-well plate) were collected and deposited into low attachment plates in order to form NPC embryonic bodies (EBs). The first stage of Embryoid bodies (EB) formation, was performed using the Munich iPSC line (iPSC-MUC). NPCs were disaggregated and cultured as a monolayer. Following the formation of EBs and their disaggregation, small-molecule neural precursor cells (smNPC) were induced. We note that human LN521 laminin is reliably used for midbrain dopaminergic differentiation, and therefore LN521 was used for smNPC propagation and differentiation. Multiple passages of NPCs (MUC), ranging from p2 to p15, were also cryopreserved. More than ten passages occurred to achieve homogeneity in our populations. These cells were then exposed to supplemented basal media allowing for their patterning into midbrain floor progenitors (mFPPs) and
their subsequent differentiation and maturation into midbrain and dopaminergic neurons. The protocol is a highly orchestrated process that involves the coordinated manipulation of several signaling pathways including sonic hedgehog (shh), Wnt, transforming growth factor β pathway (TGFβ), Notch, and bone morphogenetic protein pathway (BMP). The stage-specific activation or inhibition of these pathways ensures correct specification, differentiation, and maturation.

As shown in Figure 1b, smNPCs were characterized by immunostaining for the NPC markers SOX2, PAX6, and Nestin. These experiments were performed with p > 10 NPCs grown on LN521, as suggested by Reinhardt et al. [21]. All three markers were expressed. Given that SOX2 is also a pluripotency marker, cells were also stained for the pluripotency marker OCT4. During the first stages of neural differentiation, downregulation of OCT4 occurred concomitantly with the upregulation of neural progenitor Nestin, SOX2 and Pax6 gene expression, as shown in Figure 1b. Cells showed homogenous expression of these markers and the absence of OCT4 suggesting efficient differentiation from PSCs to NPCs.

Figure 1. Differentiation of the hiPSC line to midbrain neurons. a) Schematic diagram of the differentiation process of the hiPSC line. The process is divided into three stages. Stage 1 refers to derivation and expansion of neural progenitor cells (NPCs). Stage 2 refers to patterning of NPCs into midbrain floorplate progenitors (mFPP). Stage 3 refers to the passage of mFPP cells onto the transducer and corresponding stages of i. sedimentation and attachment, ii. differentiation, and iii. maturation into midbrain neurons. Figure created with Biorender.com. b) The upregulation of the expression of neural progenitor markers Sox2, Pax6, and Nestin with the downregulation of the pluripotency marker, Oct4, following the differentiation of iPSC–MUC to neural progenitor cells. c) Following a 14-day maturation, NPCs were differentiated into cells expressing FoxA2, a floor-plate midbrain specification marker while also expressing markers of early midbrain dopaminergic neurons such as TH and the immature neuron marker Tuj. d) Bright field images of mFPP cells differentiating into midbrain neurons, over different time points.
NPCs were then patterned into midbrain floorplate progenitors (mFPP), deposited into our transducer, and differentiated into midbrain neurons. We note that, following dissociation using Accutase, $7 \times 10^4$ mFPP cells cm$^{-2}$ were deposited on the LN521 coated transducer. Maturation medium was applied and changed every day, up to day 10. Differentiated cells display FOXA2 transcription factor crucial for the specification, differentiation, and maintenance of dopaminergic neurons during embryonic development\cite{23}. To confirm the presence of midbrain neurons, we therefore immunostained for the presence of the floor-plate FOXA2 transcription factor as shown in Figure 1c. To investigate the presence of midbrain dopaminergic cells, immunostaining of the rate-limiting enzyme, tyrosine hydroxylase (TH), was determined. TH is involved in dopamine synthesis by facilitating the conversion of tyrosine into L-dihydroxyphenylalanine (L-DOPA) and subsequently into dopamine by the action of the DOPA decarboxylase. TH-positive cells also express markers for immature neurons (TuJ) and as the differentiation protocol proceeds, the cells also express markers of mature neurons, for example, microtubule associated protein 2 (MAP2)\cite{22–24}.

To investigate the time dependence of mFPP cell differentiation into midbrain neurons, $7 \times 10^4$ mFPP cells cm$^{-2}$, were deposited on a LN521 coated cell culture dish, for brightfield imaging recording, for up to 12, 40, 96, and 120 h. Figure 1d shows that the time evolution can be categorized in three distinct stages, for example, i. Attachment within the first 40 h of cell passaging, ii. Differentiation between 40 and 120 h and iii. Maturation, after 120 h. During maturation, we confirm using fluorescence microscopy, the existence of TH-positive neurons representing around 20% of the total cell population and 50% of the neuronal population\cite{47}.

In summary, NPCs are derived from hiPSC, patterned into mFPP, and passed onto the transducer and onto cell culture plastic, for differentiation into midbrain neurons. In this first section, the differentiation from mFPP to midbrain neurons was monitored over time using brightfield imaging and showed three distinct stages, for example, i. Attachment within the first 40 h of cell passaging, ii. Differentiation between 40 and 120 h and iii. Maturation, after 120 h. During maturation, we confirm using fluorescence microscopy, the existence of TH-positive neurons representing around 20% of the total cells. In the next section, EIS is described and used to monitor the differentiation of mFPP cells into midbrain neurons, as depicted in Figure 1a.

### 4. Electrochemical Impedance Spectroscopy (EIS)

#### 4.1. Transducer

EIS is an emerging tool to non-invasively monitor the biological interactions between cells adherent to the electrode. Here we apply EIS to monitor mFPP cells differentiation into midbrain neurons. As depicted in Figure 2a, this is accomplished by using two electrodes, where one electrode is used for sensing and the other one is a counter electrode\cite{28}. An input sinusoidal current flows from the measurement electrode to the counter electrode. The measured impedance reflects the cell membrane impedance, cell–cell connections and cell–substrate interactions.

The transducer, schematically depicted in Figure 2a, is made from commercially available components. The transducer
was fabricated on thermally oxidised Si wafers. The substrate was first cleaned with acetone and isopropanol sonication for 15 min each at room temperature. Thermal evaporation was used to deposit 10 nm of chromium (Cr) followed by 50 nm of gold (Au), following placement of a shadow mask, using an Edwards 306 thermal evaporator with a quartz crystal growth rate (thickness) monitor (Intellemetrics IL150). The electrodes had an area of 10.6 mm², with an inter-electrode distance of 6 mm. The gold electrodes were located inside the well and connected with a small strip-line of 0.2 mm, included in electrode area, to the contact pad outside the well. Metal evaporation was followed by a 20 min UV-Ozone exposure (Bioforce UV/Ozone ProCleaner) and a 10 min oxygen-plasma treatment at 100 W and 1 mbar. 10 µg mL⁻¹ of human LN521 laminin (BioLamina) was deposited following manufacturers’ instructions. Before passing the mFPP cells, a poly(methyl methacrylate) (PMMA) well was used to contain the maturation medium and mFPP cells over the electrodes. A cell density of 7 × 10⁴ cells cm⁻² was passaged onto the laminin coated Au electrodes. Cell media were changed essentially every 24 h.

The sensing electrodes are immersed using a homemade mechanical holder depicted in Figure 2b. Details are presented in the methods. In Figure 2b, (1) depicts the metallic connection to the impedance analyser input, (2) represents the Au plated springs to electrically connect the sensing pads to the metallic connection of (1, 3, 4). 3, 4 represents the solution holder. The Au plated contact pins outside the well were purchased from Distrelec (Bremen, Germany), with a length of 24.64 mm and type SPA-2D, allowing the connection of the chip with the measurement equipment. The solution holder incorporates a rubber to press and contain the cell maturation medium, within the well. 5, 6) represents the connections to fix the whole mechanical part inside a Faraday box and enable electrical connection to a Solartron 1260 impedance/gain-phase analyzer.

The impedance measurements were conducted across the frequency range 1 Hz–1 MHz with an AC voltage of 50 mV. The transducer with the cell maturation medium and the cells were kept inside an incubator with 37 °C and 5% CO₂ during the EIS measurements. EIS measurements during differentiation were taken as a function of time, up to 240 h.

### 4.2. Cell Adhesion and Morphological Growth in the Transducer

Figure 2c shows four brightfield images over time during which the mFPP cells differentiate into midbrain neurons. Cells were viable and behaved similarly to those on a traditional cell culture dish, as can be inferred from Figure 1d. To determine the number of cells sedimented and adherent to the electrode over time, we have randomly selected three equal regions in each time series of Figure 2c and counted the number of cells within the region. The brightfield images were taken at 0.5, 10, 14, and 250 h after the mFPP cells were passaged onto the transducer. We estimate the number of adhered cells as a function of time using the cell segmentation plugin within ImageJ.

| Time [h] | Cells cm⁻² | Mean | σ |
|----------|-------------|------|---|
| 0.5      | 50 000      | 52 300 | 1730 |
| 10       | 63 600      | 62 200 | 9480 |
| 14       | 66 300      | 65 500 | 2020 |
| 250      | 35 300      | 32 400 | 6000 |

Table 2. Adherent cell density as a function of time.
is in good agreement with the initial mFPP cell density onto the transducer. Cell differentiation from mFPP to midbrain neurons is often accompanied by cell death, which explains the lower cell number of cells observed after 250 h. We note that after 120 h, the morphology of the adherent cells was consistent with that of neurons.

4.3. Electrochemical Characterization During Cell Differentiation

The impedance as a function of frequency, between 1 Hz and 1 MHz of a bare Au and a laminin coated electrode, immersed in the maturation medium are presented in Figure 3a. The inset represents the equivalent electrical circuit that consists of an electrical double layer capacitance, $C_{dl}$, a spreading resistance of the maturation medium, $R_{sol}$, and a charge transfer resistance $R_{CT}$.

At any electrode-medium interface, an electrical double layer is formed. The corresponding capacitance is described in the Gouy–Chapman–Stern model as a series capacitance of the Helmholtz double layer capacitance and the Gouy–Chapman diffuse layer capacitance. The double layer capacitance, $C_{dl}$, is best described by a constant phase element, CPE. Hence, using the IUPAC reference, the impedance of the CPE is given as:

$$Z_{CPE} = \frac{1}{Q(1+\jmath \omega)^n} \quad (1)$$

where the fractional exponent, $n$, varies from 0 to 1, and $Q$ represents a frequency independent, phenomenological parameter. The CPE is hence described by $Q$, related with the magnitude of the capacitive effect, and $n$, which translates its resemblance with an ideal capacitor. When $n$ is 1, the impedance resembles a discrete linear capacitor, and the equivalent circuit becomes identical to Randles equivalent circuit. Discussions on the origin of CPE are out of scope for this investigation.[20-34]

To estimate the double layer capacitance, $C_{dl}$ from the CPE values, we use the approximation of Hsu:  

$$C_{dl} = \frac{(QR_{CT})^\frac{1}{n}}{R_{CT}} \quad (2)$$

The spreading resistance is due to the spreading of current from the working electrode to the counter electrode. For a circular electrode of radius $r$, the spreading resistance can be calculated as:

$$R_{sol} = \frac{\rho}{4r} \quad (3)$$

where $\rho$ is the specific resistivity of the solution.

At equilibrium, equal and opposite reduction and oxidation currents flow across the electrode–medium interface. For an ideally polarizable, or blocking electrode, this exchange current density is zero, and for an ideally non-polarizable electrode, this current density tends to infinity. The equilibrium exchange current density depends exponentially on applied bias. However, at low bias the exchange current density follows Ohms law. The Faradic redox processes can then be characterized by a frequency independent, linear charge transfer resistance, $R_{CT}$.

The impedance as a function of frequency is fitted to the equivalent circuit by using the fitting routine of the electrochemical software programme ZView. For the bare Au and laminin coated electrodes, the fitted impedance as a function of frequency are presented in Figure 3a as the fully drawn black and blue line respectively. A good agreement is obtained. The extracted values for the charge transfer resistance, double layer capacitance, and spreading resistance are presented in Table 3.

The spreading resistance agrees with the independently measured specific conductivity of the maturation medium of $\approx 10$ mS m$^{-1}$, cf.

$$\rho = \frac{\mu}{\sigma} \quad (4)$$

where $\mu$ is the specific conductivity and $\sigma$ the specific resistivity of the solution.

At equilibrium, equal and opposite reduction and oxidation currents flow across the electrode–medium interface. For an ideally polarizable, or blocking electrode, this exchange current density is zero, and for an ideally non-polarizable electrode, this current density tends to infinity. The equilibrium exchange current density depends exponentially on applied bias. However, at low bias the exchange current density follows Ohms law. The Faradic redox processes can then be characterized by a frequency independent, linear charge transfer resistance, $R_{CT}$.

The impedance as a function of frequency is fitted to the equivalent circuit by using the fitting routine of the electrochemical software programme ZView. For the bare Au and laminin coated electrodes, the fitted impedance as a function of frequency are presented in Figure 3a as the fully drawn black and blue line respectively. A good agreement is obtained. The extracted values for the charge transfer resistance, double layer capacitance, and spreading resistance are presented in Table 3.

The spreading resistance agrees with the independently measured specific conductivity of the maturation medium of $\approx 10$ mS m$^{-1}$, cf.

$$\rho = \frac{\mu}{\sigma} \quad (4)$$

where $\mu$ is the specific conductivity and $\sigma$ the specific resistivity of the solution.
impedance was measured as a function of time. A 3D representation of the measured EIS spectra is presented in Figure 3b. The EIS spectra were fitted using the same equivalent circuit, as presented in Figure 3a. In all cases, a good agreement between measured and fitted impedance spectra was obtained. Several attempts were made to include additional components and circuits. Introduction of additional elements to electrically describe the cells did not improve the fitting, and, therefore, was disregarded. The existing electric model cannot substantiate, in detail, all changes in the dynamic biostructure. Yet, by lumping the complex dynamic biostructure into the present electrical parameters, we show an extremely clear correlation between changes in the electrical parameters with changes occurring at the biostructure, during cell differentiation and maturation, from mFPPs to midbrain neurons. The extracted fit parameters are presented in Table 2.

The trend in the impedance becomes clear when we plot the impedance over the whole differentiation period, both at low frequency (10 Hz) and at high frequency (1 kHz), as presented in Figure 3c. Experiments were repeated three times and presented with the corresponding mean and standard deviation, in Figure 3c. Low frequency data are presented by red symbols and high frequency data are represented by blue symbols. The dashed lines are guides to the eye. In the first 40 h, the impedance decreases; at 10 Hz from 4000 to 2600 Ω cm² and at 1 kHz, from 65 to 45 Ω cm². The impedance remains relatively stable from 40 to 96 h. From 96 to 120 h, the impedance increases, and after 120 h, the impedance stabilises.

The temporal change in the impedance spectra is partly due to the change in the equivalent parallel capacitance. A 3D representation of the measured capacitance as a function of frequency over 240 h is presented in Figure 4a. At low frequency, the capacitance is dominated by that of the double layer. The capacitance is not constant but slightly increases with decreasing frequency as the double layer is not a linear capacitor but a constant phase element. At high frequency, the capacitance is dominated by that

### Table 3. Extracted equivalent circuit parameters for cell differentiation over time, from mFPPs to midbrain neurons, on laminin coated Au electrodes.

| Electrode   | Time [h] | \( R_{ct} \) [Ω] | \( Q \) [Ω\(^{-1}\) cm\(^{-2}\)] | \( n \) | \( R_{int} \) [Ω] | \( R_{ct} \) 5 [Ω cm\(^{-2}\)] | \( C_{el} \) [μF cm\(^{-2}\)] |
|-------------|----------|-----------------|-----------------|------|-----------------|-----------------|-----------------|
| Au          | -        | 5.20 × 10⁶      | 1.52 × 10⁻⁶     | 0.92 | 225             | 2.75 × 10⁵      | 8.6             |
| Au/LN       | 0        | 1.26 × 10⁶      | 7.82 × 10⁻⁷     | 0.92 | 366             | 6.64 × 10⁴      | 3.7             |
| Au/LN/cells | 0.5      | 1.30 × 10⁶      | 6.22 × 10⁻⁷     | 0.93 | 302             | 6.88 × 10⁴      | 2.9             |
| Au/LN/cells | 1        | 1.14 × 10⁶      | 6.44 × 10⁻⁷     | 0.93 | 295             | 6.05 × 10⁴      | 3.0             |
| Au/LN/cells | 2        | 1.07 × 10⁶      | 6.58 × 10⁻⁷     | 0.92 | 300             | 5.66 × 10⁴      | 3.0             |
| Au/LN/cells | 10       | 463 900         | 8.70 × 10⁻⁷     | 0.91 | 276             | 2.45 × 10⁴      | 3.8             |
| Au/LN/cells | 12       | 369 600         | 8.97 × 10⁻⁷     | 0.91 | 260             | 1.95 × 10⁴      | 3.8             |
| Au/LN/cells | 14       | 348 600         | 9.32 × 10⁻⁷     | 9.1  | 272             | 1.84 × 10⁴      | 3.9             |
| Au/LN/cells | 16       | 287 300         | 9.10 × 10⁻⁷     | 9.2  | 270             | 1.52 × 10⁴      | 3.8             |
| Au/LN/cells | 34       | 192 400         | 9.10 × 10⁻⁷     | 0.95 | 275             | 1.02 × 10⁵      | 3.8             |
| Au/LN/cells | 46       | 145 600         | 9.14 × 10⁻⁷     | 0.95 | 288             | 7.70 × 10⁴      | 3.8             |
| Au/LN/cells | 96       | 102 800         | 9.15 × 10⁻⁷     | 0.95 | 252             | 5.44 × 10⁴      | 3.8             |
| Au/LN/cells | 120      | 106 200         | 8.43 × 10⁻⁷     | 0.95 | 290             | 5.61 × 10⁴      | 3.5             |
| Au/LN/cells | 168      | 101 300         | 7.88 × 10⁻⁷     | 0.95 | 299             | 5.36 × 10⁴      | 3.2             |
| Au/LN/cells | 216      | 94 800          | 7.70 × 10⁻⁷     | 0.95 | 298             | 5.01 × 10⁴      | 3.1             |
| Au/LN/cells | 240      | 87 300          | 7.75 × 10⁻⁷     | 0.94 | 286             | 4.62 × 10⁴      | 3.1             |

**Figure 4.** a) 3D representation of the measured equivalent parallel capacitance as a function of frequency, over 240 h. b) Capacitances at 10 Hz and 1 kHz over time extracted from Figure 4a over three different experiments. A guide to the eye is presented by the dashed lines. c) The time evolution of the Maxwell–Wagner relaxation frequency, \( f_r \), which corresponds to the peak in the dielectric loss. Inset represents the measured (symbols) and fitted (solid lines) dielectric loss, \( C_{dl} / \omega \) in blue and capacitance in red, at 96 h.
of the maturation medium, which can be ignored. In all cases, a good agreement between measured and fitted capacitance spectra was obtained. The trend in the capacitance becomes clear when we plot the capacitance over the whole differentiation period, both at low frequency (10 Hz) and high frequency (1 kHz), as presented in Figure 4b. We understand that EIS in cell studies is routinely made at high frequency regimes.[38] Yet, we also understand that the biological production of an extracellular matrix affects the electrode/medium interface, and for that reason, we decided to study both low and high frequencies regimes. At 10 Hz, capacitance increases from \( \approx 4–7 \) \( \mu \)F cm\(^{-2}\). Similarly, at 1 kHz, capacitance increases from \( \approx 1.7–2.6 \) \( \mu \)F cm\(^{-2}\). Thereafter, capacitance stabilizes around \( \approx 7–2.6 \) \( \mu \)F cm\(^{-2}\) for 10 and 1 kHz, respectively. Finally, capacitance at 10 Hz decreases to \( \approx 5.5 \) \( \mu \)F cm\(^{-2}\) and capacitance at 1 kHz reduces to \( \approx 2.2 \) \( \mu \)F cm\(^{-2}\). Thereafter, the capacitance remains relatively stable until the end of recordings, for example, 240 h.

The inset of Figure 4c shows, as a typical example, the dielectric loss, \( \varepsilon' / \varepsilon'' \) as a function of frequency measured after 96 h. The loss is low at both low and high frequency and equal to \( 1 / \omega R_{CT} \) and \( 1 / \omega R_{sol} \), respectively. The maximum loss is obtained at the Maxwell–Wagner frequency, \( f_r \), which is given by:[39,40]

\[
f_r = \frac{1}{2\pi} \left( \frac{R_{CT} - R_{sol}}{R_{CT} + R_{sol}} \right)^{-1/2} = \frac{1}{2\pi} (C_{dl} R_{sol})^{-1/n}
\]

where we have used that the charge transfer resistance is orders of magnitude larger than the spreading resistance. The change of the Maxwell–Wagner relaxation frequency with time is presented in Figure 4c. The change in relaxation frequency indeed mimics the inverse change in equivalent parallel capacitance.

To analyse in detail where the temporal change in impedance is coming from, we plot the extracted capacitance parameter, \( Q \), and the charge transfer resistance, \( R_{CT} \), as presented in Table 2, as a function of time in Figure 5a,b respectively. The other parameters, viz. the spreading resistance and the fractional exponent \( n \), hardly change with time.

Figure 5a shows that in the first 40 h, \( Q \) increases from an initial value of \( 5.8 \times 10^{-7} \) to \( 9.2 \times 10^{-7} \) \( \Omega^{-1}s^{n} \). The capacitance reaches a plateau and remains stable until 96 h. Thereafter, \( Q \) slowly decreases and levels off near \( 7.9 \times 10^{-7} \) \( \Omega^{-1}s^{n} \), after about 120 h. On the other hand, the charge transfer resistance depicted in Figure 5b monotonically decreases with time, reaching a stable value after 120 h. The dotted red line is a phenomenological fit to the data according to:

\[
R_{CT}(t) = R_{CT_{\text{max}}} + \frac{R_{CT_{\text{min}}}}{1 + \left( \frac{t}{\tau} \right)^{\beta}}
\]

where \( t \) is the time, and \( R_{CT_{\text{max}}} \) and \( R_{CT_{\text{min}}} \) represent the charge transfer resistance at time zero and infinity respectively. To be discussed later, the fit constant \( \alpha \) of about 6 h maybe correlated with the sedimentation time while the fit constant \( \beta \) about 1.5 maybe correlated with the differentiation process.

We note that organic residues on the transducer surface can contribute to Faradaic currents, which decreases the charge transfer resistance and polarizes the electrode.[41] Since cells are essentially efficient organic residues manufactures, \( R_{CT} \) is therefore an important variable to monitor. The laminin coating appears to counterintuitively facilitate charge transfer instead of impeding reactions. The facilitation of charge transfer is, so far, phenomenological and consistent with other investigations.[37] Figure 3a shows the monitored and extracted \( R_{CT} \) parameter (using the equivalent circuit) as a function of time. The extracted equivalent circuit parameters are presented in Table 3.

In summary, the EIS spectra show that in the first 40 h after passing mFPP cells on laminin coated Au electrodes, the impedance decreases with immersion time and the capacitance increases with immersion time. The EIS spectra could quantitatively be fitted with Randle's equivalent circuit. The spreading resistance, \( R_{sol} \), and the exponent of the CPE, \( n \), describing the double layer capacitance, remain about constant. The major change in the first 40 h is an increase with time of the capacitance parameter, \( Q \), and an exponential decrease with time of the charge transfer resistance, \( R_{CT} \), with a time constant of about 6.5 h. Then from \( \approx 40 \) h–96 h, the EIS spectra, and the extracted parameters, remain constant. After about 96 h, the impedance goes slightly up and the capacitance down. After about 120 h, the EIS spectra are stabilised and do not significantly change anymore.

Figure 5. a) Time evolution of extracted parameter \( Q \) as a function of time up to 240 h, over three different experiments. b) The extracted charge transfer resistance as a function of time. The dashed red line represents a phenomenological fit, for the average of three different repeats.
5. Discussion

We determined the utility of using EIS as a non-invasive approach to measure cellular differentiation in a time-dependent manner. To date, this approach has not been used in the context of potentially treating spinal cord injuries. The different phases extracted from the EIS spectra are interpreted as (1) the attachment phase during the first 40 h, which includes an initial process of sedimentation, a (2) differentiation phase from 40 to 120 h and, lastly (3) a maturation phase, after 120 h. This distinction is supported by the optical investigation which confirms the same three stages. The differentiation process of mFPP cells shows an increasing number of sedimented cells, within 10 h of passaging the mFPP cells onto the transducer. The number of sedimented cells over time matching the seeding cell density further reveals the biocompatible nature of our transducer. Cells sediment and attach to the electrodes. After sedimentation and attachment, neurite outgrowth is observed. The optical investigation shows a consistent morphological change, including neurite outgrowth in the differentiation phase. Finally, Figure 1c shows a stable maturation phase occurring after 120 h, which is complemented by the expression of FoxA2, TH, and TuJ1, confirming the presence of midbrain and dopaminergic neurons.

5.1. Cell Sedimentation and Attachment

The attachment of cells to the extracellular matrix and the differentiation process, for both in vitro and in vivo scenes, is a dynamic process rather than a static one. The cellular responses to environmental cues, include orientation of lipid molecules and changes in their morphology mediated through molecular bonding between cell-surface receptors and their ligands or counter-receptors on the other surfaces in the extracellular matrix. After sedimentation and attachment, neurite outgrowth is observed. The optical investigation shows a consistent morphological change, including neurite outgrowth in the differentiation phase. Finally, Figure 1c shows a stable maturation phase occurring after 120 h, which is complemented by the expression of FoxA2, TH, and TuJ1, confirming the presence of midbrain and dopaminergic neurons.

The impedance parameters, and with medium only, have been measured and remain stable over time, in agreement with others. On the other hand, the impedance measured with cells, departs from the impedance measured with medium only. From the moment mFPP cells are transferred onto the transducer, the capacitance increases from that of the laminin capacitance (about 3 µF cm⁻² as shown in Table 2) to near 4 µF cm⁻². The time evolution analysis shown in Figure 1d reveals that progenitor morphology occupies nearly 1/10 of space in comparison to mature and differentiated neurons, in agreement with previous reports. Of relevance is the area of the somatic region which is known to increase significantly within the time scale of our experiment. The somatic increase and fractional progenitor attachment should lead to a capacitance per cell in between 0.001–0.1 µF cm⁻². Within 40 h, cells adhere and sediment and consequently the capacitance increases up to a plateau dependent on the adherent cell number.

We note that the laminin layer decreases after cell adhesion. The coating thicknesses are reflected in the equivalent circuit parameter . represents the interface capacitance and is therefore inversely proportional to the laminin/dielectric thickness. This means that a thicker coating reduces and vice-versa. The extracted value of as a function of time is depicted in Figure 5a. In the first 48 h, in the sedimentation and attachment phase, increases from an initial value of 5.8 × 10⁻⁷ to 9.2 × 10⁻⁷ Ω⁻¹s⁻¹. Apparently, the laminin extraction process by the seeded cells, makes the coated layer thinner and therefore increases . Hence, in order to investigate the arrangement pattern of the extracellular matrix, laminin, during the differentiation process, immunostaining was utilized to examine the expression of the laminin subunit γ1. Laminin coverage was observed at 12 and 34 h using maximum intensity and orthogonal projection. At 12 h, a homogenous distribution and thickness of Laminin γ1 can be observed throughout the section (Figure S1a,c. Supporting Information). Following 34 h of culture, the distribution and thickness seem to be altered with the latter showing a decrease of 40% (Figure S1b,d, Supporting Information), during cell attachment.

For laminin, there seems to be a consensus that cell adhesion occurs after 6 h. The adhesion time reported in literature is in close agreement with our adhesion time represented by and with our cell counts depicted in Figure 2c. Our recordings suggest that cell differentiation rate from mFPPs to midbrain neurons, is directly dependent on the parameter which reflects the decay slope from about 6 to 120 h.

5.2. Cell Differentiation

In the second stage, the differentiation stage, there are two different patterns detected. A constant impedance and capacitance between 40 and 96 h and a slowly varying pattern between 96 and 120 h, translating into an increase in impedance and decrease in capacitance. After 40 h, the capacitance spectra reach a plateau and remain stable until 96 h. The mFPP cells are differentiating into midbrain neurons, and consequently, their morphology is changing, evidenced by the increase in somatic area and neurite outgrowth, as depicted in Figure 1b–d and in Figure 2c. Inevitably, during cell differentiation, cell death occurs. Yet, we note although less cells are adherent to the electrode, the morphology of the cells is changing. This means that the lower adherent cell number is counterbalanced by the concurrent increase in somatic area and neurite outgrowth, occupying therefore a larger area. This balance is reflected by the relatively stable of 9 × 10⁻⁷ Ω⁻¹s⁻¹ until 96 h. After 96 h, this balance is no longer verified, and the wider spread of insulating membranes and concomitant dendrites from midbrain neurons cover the electrode surface, leading to an increase in impedance until about 120 h.
Concomitantly, from 96 to 120 h, the parameter $Q$ drops to $8 \times 10^{-7} \, \Omega^{-1}$, where it stabilises into the maturation stage. The drop in capacitance could be partially linked with a decrease in adherent cell density, due to cell death during differentiation. In addition, the membrane of dead cells is leaky or becomes permeable, which significantly affects the ability to impede ions flux and concomitant surface reactions. This leads to a conductivity resembling that of the extracellular maturation medium, of $\approx 10 \, \text{mS m}^{-1}$.[45,46] Importantly, the decrease in capacitance should be influenced by the extracellular matrix being produced by matured midbrain neurons. Note that cells secrete and alter the matrix continually leading to the remodeling of its composition and topography.

The time scale of differentiation is therefore orders of magnitude larger than the time to measure a full EIS spectrum. Hence, we argue that the collection of EIS spectra and analysis can be done in quasi real-time. The impedance data collection can be synchronously processed to determine the state of adherent cells.

5.3. Cell Maturation

After 120 h, during the third stage, the maturation phase, the impedance stabilizes. Maturation occurs after differentiation and it involves a change in specificity or function but not substantially, in morphology. In our case, the differentiated dopaminergic neurons, displayed in Figure 1c, start expressing tyrosine hydroxylase, while other Tuj1 neurons do not despite having a very similar morphology. This constant behaviour is shown in Figure 4b, at 10 Hz and 1 kHz, when capacitance stabilizes. Maturation occurs after differentiation, based on the fit constant $\beta$, of Equation (5), and complemented by both the onset for rise in impedance and decrease in capacitance.

6. Conclusion

EIS has been applied to monitor cell adhesion and differentiation from midbrain floor plate progenitors into midbrain neurons, on Au electrodes coated with human laminin.

The EIS data and equivalent circuit modeling were consistent with standard microscopy analysis and reveal that (1) within the first 6 h, progenitor cells sediment to the electrode, then attach and then from 40–96 h, midbrain neurons emerge and after 120 h, maturation is likely to develop. The ability to sense, characterize, and mathematically predict, non-invasively and in real time cell differentiation, opens new avenues for implantable therapies and in-vitro differentiation strategies.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

For technical support, the authors would like to acknowledge Dr. Michael Zachariadis, Dr. Sivapathasundaram Sivaraaya, Michael Linham, and David Chapman at University of Bath, and Hans Peter Reich from the Max Planck Institute for Polymer Research, Mainz, Germany. The authors would also like to thank Dr Susanne Zach from Boehringer Ingelheim Pharma GmbH & Co. KG. P.R.F.R. acknowledges the support and funding from the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme (grant agreement No. 947897). A.E., P.R.F.R., and D.T. acknowledge funding from the Schumacher Foundation Faculty for the Future Fellowship.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Research data are not shared.

Keywords

bioelectronics, biophysics, cell differentiation, electrochemical impedance spectroscopy, spinal cord injuries

Received: February 11, 2021
Published online: April 7, 2021

[1] C. Chen, X. Bai, Y. Ding, I.-S. Lee, *Biomater. Res.* 2019, 23, 25.
[2] J. E. Shin, K. Jung, M. Kim, K. Hwang, H. Lee, I.-S. Kim, B. H. Lee, I.-S. Lee, K. I. Park, *Exp. Mol. Med.* 2018, 50, 39.
[3] M. L. Carter, *Anaesth. Intensive Care* 2004, 32, 11.
[4] J. Caylor, R. Reddy, S. Yin, C. Cui, M. Huang, C. Huang, R. Rao, D. G. Baker, A. Simmons, D. Souza, S. Narouze, R. Vallejo, I. Lerman, *Bioelectron. Med.* 2019, 5, 12.
[5] J. P. Miller, S. Eldabe, E. Buchser, L. M. Johanek, Y. Guan, B. Linderoth, *Neuromodulation: Technol. Neural Interface* 2016, 19, 373.
[6] F. H. Gage, S. Temple, *Neuron* 2013, 80, 588.
[7] M. M. Daadi, A. S. Davis, A. Arac, Z. Li, A.-L. Maag, R. Bhatnagar, K. Jiang, G. Sun, J. C. Wu, G. K. Steinberg, *Stroke* 2010, 41, 516.
[8] K. I. Park, Y. D. Teng, E. Y. Snyder, *Nat. Biotechnol.* 2002, 20, 1111.
[9] J. Imitola, K. Raddassii, K. I. Park, F.-J. Mueller, M. Nieto, Y. D. Teng, D. Frenkel, J. Li, R. L. Sidman, C. A. Walsh, E. Y. Snyder, S. J. Khoury, *Proc. Natl. Acad. Sci. U.S.A.* 2004, 101, 18117.
[10] K. I. Park, M. A. Hack, J. Ourednik, B. Yandava, J. D. Flax, P. E. Stieg, S. Guillas, F. E. Jensen, R. L. Sidman, V. Ourednik, E. Y. Snyder, *Exp. Neurol.* 2006, 199, 156.
[11] K. I. Park, B. T. Himes, P. E. Stieg, A. Tessler, I. Fischer, E. Y. Snyder, *Exp. Neurol.* 2006, 199, 179.
[12] O. Lindvall, Z. Kokaia, *J. Clin. Invest.* 2010, 120, 29.
[13] F. Barnabe-Heider, J. Frisen, *Cell Stem Cell* 2008, 3, 16.
[14] Y.-S. Chen, H.-J. Harn, T.-W. Chiou, *Cell Transplant.* 2018, 27, 407.
[15] J. Silver, J. H. Miller, *Nat. Rev. Neurosci.* 2004, 5, 146.
[16] N. B. Skop, F. Calderon, C. H. Cho, C. D. Gandhi, S. W. Levison, *Mol. Cell Ther.* 2014, 2, 19.
[17] T. Führmann, R. Y. Tam, B. Ballarin, B. Coles, I. E. Donaghue, D. van der Kooy, A. Nagy, C. H. Tator, C. M. Morshead, M. S. Shoichet, *Biomaterials* 2016, 83, 23.
[18] J. Wegener, C. R. Keese, I. Giaever, Exp. Cell Res. 2000, 259, 158.
[19] L. Yang, C. Ruan, Y. Li, Biosens. Bioelectron. 2003, 19, 495.
[20] V. M. Mirsky, M. Riepl, O. S. Wolfbeis, Biosens. Bioelectron. 1997, 12, 977.
[21] P. Reinhardt, M. Glatza, K. Hemmer, Y. Tsytstyura, C. S. Thiel, S. Höing, S. Moritz, J. A. Parga, L. Wagner, J. M. Bruder, PLoS One 2013, 8, e59252.
[22] S. Kriks, J.-W. Shim, J. Piao, Y. M. Ganat, D. R. Wakeman, Z. Xie, L. Carrillo-Reid, G. Auyeung, C. Antonacci, A. Buch, Nature 2011, 480, 547.
[23] S.-L. Wu, M. E. Orazem, B. Tribollet, V. Vivier, J. Electrochem. Soc. 2009, 156, C214.
[24] J.-B. Jorcin, M. E. Orazem, N. Pèbère, B. Tribollet, Electrochim. Acta 2006, 51, 1473.
[25] Z. Kerner, T. Pajkossy, J. Electroanal. Chem. 1998, 448, 139.
[26] W. Franks, I. Schenker, P. Schmutz, A. Hierlemann, IEEE Trans. Biomed. Eng. 2005, 52, 1295.
[27] C. H. Hsu, F. Mansfeld, Corrosion 2001, 57, 747.
[28] R. Pethig., S. Smith, Introductory Bioelectronics: For Engineers and Physical Scientists, John Wiley & Sons, New York, NY 2012, pp. 29–72.
[29] W. Franks, I. Schenker, P. Schmutz, A. Hierlemann, IEEE Trans. Biomed. Eng. 2005, 52, 1295.
[30] S. Arndt, J. Seebach, K. Psathaki, H.-J. Galla, J. Wegener, Biosens. Bioelectron. 2004, 19, 583.
[31] R. Hirshorn, M. E. Orazem, B. Tribollet, V. Vivier, I. Frateur, M. Musiani, J. Electrochem. Soc. 2010, 157, C458.
[32] P. R. F. Rocha, P. Schlett, U. Kintzel, V. Mailänder, L. K. J. Vandonatte, G. Zeck, H. L. Gomes, F. Biscarini, D. M. de Leeuw, Sci. Rep. 2016, 6, 34843.
[33] E. Yeager, Electrochim. Acta 1984, 29, 1527.
[34] R. P. McEver, C. Zhu, Annu. Rev. Cell Dev. Biol. 2010, 26, 363.
[35] S. Kang, X. Chen, S. Gong, P. Yu, S. Yau, Z. Su, L. Zhou, J. Yu, G. Pan, L. Shi, Sci. Rep. 2017, 7, 12233.
[36] G. Zeck, P. Fromherz, Langmuir 2003, 19, 1580.
[37] T. Gerasimenko, S. Nikulin, G. Zakharova, A. Poloznikov, V. Petrov, A. Baranova, A. Tonevitsky, Front Bioeng. Biotechnol. 2020, 7, 474.
[38] H. Fricke, J. Gen. Phys. 1924, 6, 375.