Chapter 5

Extended Large Area Heterojunction Biosensing Device

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5.1 Outline

Neurons are highly polarized cells that exhibit a characteristic shape of a single axon with several dendrites [236]. Dendrites are in charge of receiving incoming signals at the synapse and relay them to the soma (cell body) [237]. The signals received during the process of synaptogenesis generates action potentials within the cell body that traverse the length of axon and propagate to target cells at the presynaptic site [238]. Therefore, it is of vital importance to have intact neuronal circuit-connectivity for proper maintenance of normal organ and organism function [239]. The primary goal of modern neuroscience it to understand the relationship between signal processing of neuronal circuitry and its physiological and pathological functions. Accordingly, a collection of electrophysiological parameter recordings such as action potential, resting potential, excitatory and inhibitory postsynaptic potential etc., is required to gain an accurate and deep understanding into the current issues of brain research.

Neurons transfer information using electrical and chemical signals [240], [241]. The above mentioned electrophysiological parameters are the results of these signals that help to decipher the inter and intracellular activities of a neuronal network. The somal and axonal membranes of the neuron are electrically conductive due to the presence of voltage-gated ion channels. These ion channels allow the movement of calcium, sodium, potassium and chloride ions, because of which electrical signals are produced that is propagated to the axons [242]. The commonly used contemporary methodologies for monitoring neural activity and morphology in vitro are stimulation by patch-clamp electrodes [243], optical imaging and stimulation by fluorescent indicators [244], microelectrode arrays [245], [246], genetically encoded markers [247], optogenetics [248], [249] etc. However, all these methods are laborious, expensive and damaging to

the cells that offers only a snapshot of the whole action. Thus, a much sensitive, non-destructive and real-time monitoring readout system is the demand of the hour that is able to investigate the neuronal circuit properties in a monolayer of neuron cell culture. Conventionally, non-invasive *in vitro* cell-substrate impedance sensing techniques are being used for recording extracellular activity of the neuronal cells without any potential cell damage through the developed neuro-electrode junction [250]–[252]. The impedance posed by the neuronal membrane regulates the alterations in membrane potential in response to input currents and therefore the synaptic outputs of the neuronal circuitry [253].

Adhesion and differentiation of neuron and neural stem cells are highly influenced by the underlying substrate's physicochemical structural properties of the extracellular microenvironment, which activates the signaling pathways to perform the cell functions [254]. Ciofani G *et al.* investigated the interaction of electrically excitable neuronal (PC12) cells, and muscle (H9C2) cells seeded on ZnO nanowire arrays, to evaluate their potential application towards minimal invasive sensing and stimulation using the piezoelectric property of ZnO nanostructure [255]. Thrivikraman G *et al.* reported an increase in growth, orientation, and fusion of mouse myoblast (C2C12) cells when myoblast cells is seeded on the conductive substrate without any external stimulation [256]. Robinson J.T *et al*. reported the fabrication of vertically aligned nanowire electrode arrays to record and stimulate the neuronal activity of rat cortical neurons in dissociated cultures to map multiple individual synaptic connections [257].. Serb A *et al*. have reported the fabrication of a memristor paired with the array of microelectrode, to recording excitatory postsynaptic potentials of the rat hippocampus neuron [258]. However, none of them reported any cell-induced changes in the electrical characteristics under the progression of cellular functional behaviour.

Typically, the thin film nanomaterials based junction devices namely diodes and transistors are used as a non-linear rectifier, switching device and also as transistors [259]–[261]. Wherein, heterojunction diode with extended larger area was used as a solar cell, photodiode, and gas sensing applications [262]–[267]. However, none of researchers have reported the extended larger area heterojunction device as biosensor. Hence, in the present study, we report the fabrication of an extended larger area heterojunction device for assessing the dynamic behaviour of primary neuronal cells by evaluating the cell-induced change in electrical characteristic property as the function of progression of cellular behaviour. The ZnO nanomaterial was synthesised via sol-gel technique. The surface of n-type ZnO thin film formed on p-type Si substrate was modified with poly-L-lysine to improve the cellular adhesion. We used neurons from embryonic Charles Foster rat cortex as a model cell for this study. The poly-L-lysine functionalized ZnO thin film based extended larger area heterojunction device was further characterized for assessing the cell-induced electrical characteristic property change.

5.2 Experimental details

5.2.1 Sensor fabrication procedure

The process of ZnO sol-gel preparation can be referred in **Section 4.2.1.** The ZnO sol-gel spin coated on the cleaned p-Si substrate and glass substrate are used to fabricated heterojunction biosensor and for surface imaging respectively. The process of fabricating an extended larger area heterojunction device is shown in the Figure 5.1. In brief, single-side polished, moderately doped p-type silicon (p-Si) substrate was cut with following dimensions 20 × 15 mm^2 (as in Figure 5.1a) and cleaned thoroughly using chemical wet cleaning procedures. Some portion of cleaned p-Si substrate was covered using polyimide Teflon tape, to develop an ohmic contact from p-Si substrate (Figure 5.1b). The

uncovered regions of p-Si substrate were coated using filtered ZnO sol-gel solution through the spin coating technique with rotation cycle having 3000 rpm for 30 sec (Figure 5.1c). To obtain the film thickness of ~200 nm, the spin coating process was repeated several times and the ZnO film coated p-Si substrate was heated at 140 °C for 10 min at each steps to remove organic residuals. However, the obtained ZnO thin film coated p-Si substrate were post-annealed at 450 °C [268] for 2 h in the inert (Argon) atmosphere with the gas flow rate of ~30 sccm in the muffle furnace to obtain crystallized ZnO TSMO thin film. Finally, to obtain the ohmic contact, research grade Aluminium (Al, 99.99%) metal was deposited using thermal evaporation chamber (Hind High Vac, model smart coat 3.0A) with a thickness ≈100 nm through a mask having an electrode dimension of about 5 x 10 mm^2 at each end (i.e., on p-Si and spin coated ZnO thin film (Figure 5.1d). Hence, an extended larger area heterojunction of ~13 mm was obtained [226].

Typically to hold the culture medium, the head position of 1.5 mL Cliklok Micro-centrifuge (Tarsons Product Pvt Ltd, India) tube was used as described in **Section 2.22**. This structure is termed as 'culture device or heterojunction biosensor' as shown in Figure 5.1e. ZnO spin coated glass substrate was also shown in Figure 5.1f**.**

5.2.2 Primary cortical neuron cell culture procedure

Cerebral cortical neurons from embryonic (E18) Charles Foster rats were used for all the experiments. Cortices were removed from the brains after decapitation. The meningeal layer was removed and the cortices were collected in a tube with trypsin for enzymatic dissociation. A solution of single neurons was obtained after trituration and centrifugation at 1500 rpm for 3 min [269]. Neurons were plated onto 150 µg/mL poly L-lysine coated Si/ZnO covered glass substrates at a density of 2×10^4 cells/well. Cultures were grown in serum-free Neurobasal media, supplemented with 2% B27, 0.5 mM L-glutamine, 25

μM Glutamic acid and 40 μg/ml gentamycin at optimum conditions of 37°C inside a 5% CO_2 incubator (Galaxy® 170 S, Eppendorf, Germany) in a humidified environment [270].

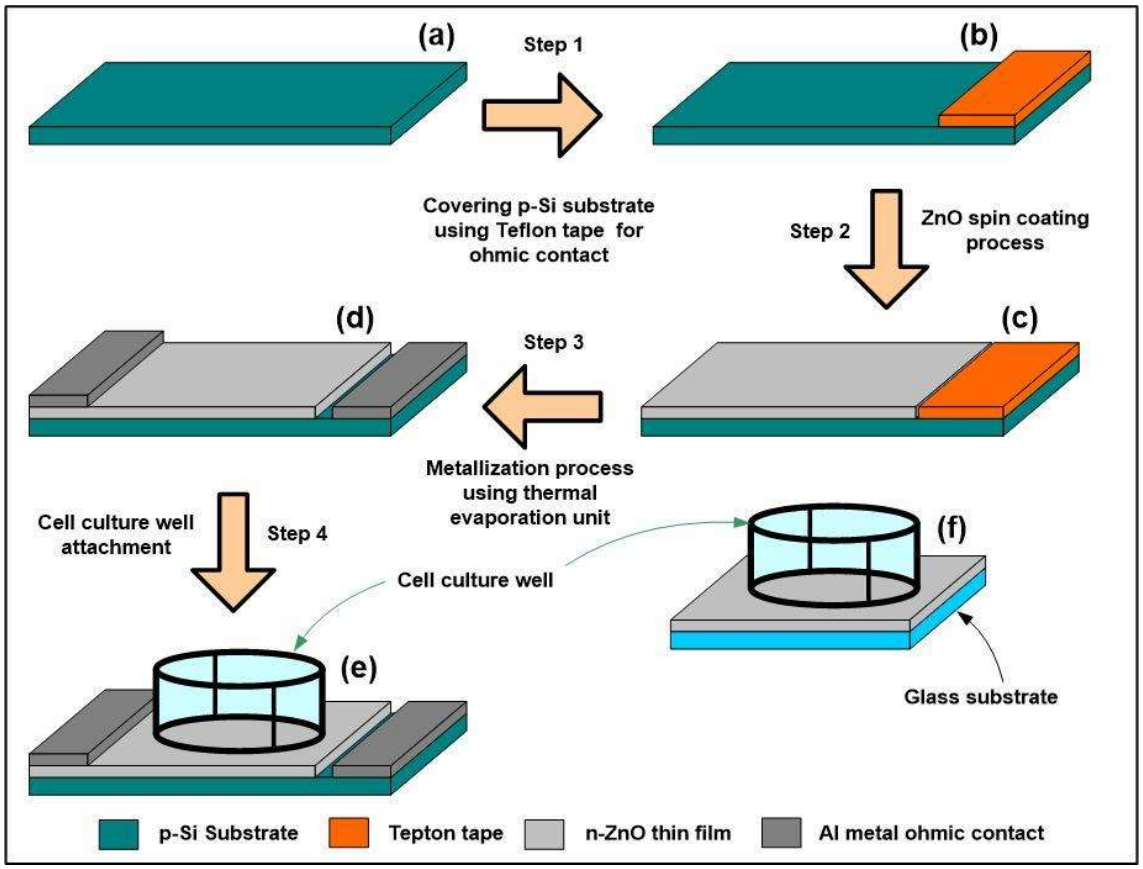

Figure 5.1: An extended larger area heterojunction device with cell culture well fabrication process.

Animals were sacrificed as per the guidelines of the animal ethical committee of the Institute of Medical Sciences (IMS), Banaras Hindu University (BHU), Varanasi, India.

5.2.3 Surface modification

Electrical coupling between a neuron and n-type ZnO thin film is a prerequisite for the analysis of morphological and electrophysiological characteristics through an in vitro cell-substrate sensing technique. The primary goal is to cover the sensing area by dissociated neurons entirely and firmly to establish a superlative degree of neuron-thin film contact [271]. Therefore, to improve the tightness in adhesion of neuronal cells, a

surface coating of poly-L-lysine (polycation) is generally used on the substrates [251]. Neurons are negatively charged cells that exhibit enhanced cellular adhesion on positively charged surfaces. Because of their distinctive and polarized morphology neurons possess a much smaller somata leading to the presence of a few anchoring points that introduces additional challenges for in vitro primary neuron cultures [272].

5.2.4 Fluorescent staining of nuclei and actin cytoskeleton of neuronal cells

After 3 and 5 DIV, the cultured cells were fixed with 4% paraformaldehyde (HiMedia) for 20 min at a room temperature (RT) followed by washing with phosphate buffer saline (PBS). Subsequently, cells were permeabilized with 0.5% Triton X-100 (HiMedia) in 1% BSA/PBS for 10 min at RT. The cells were then blocked with 1% BSA (HiMedia) in PBS for 30 min at RT followed by a PBS wash. It was followed by the fluorescent staining of the cells with rhodamine-conjugated phalloidin (1:1000 in 1% BSA/PBS, AAT-Bioquest) for 1 h at RT to stain actin cytoskeleton. After washing, 1 µg/mL DAPI (4,6-diamidino-2-phenylindole, HiMedia) solution in 1% BSA/PBS was added and kept for 45 min at room temperature to counterstain the nuclei, and then a final washing was given to the cells before imaging under a fluorescence microscope (Nikon, Ti-U).

5.3 Results and discussion

The spin coated ZnO thin films were characterised to determine its structural, optical and elemental properties using X-ray diffraction (XRD), Fourier transform infrared (FT-IR) spectroscopy, UV-*Vis* spectroscopy, High resolution scanning electron microscopy (HR-SEM), energy-dispersive X-ray spectroscopy (EDX), and atomic force microscope (AFM) methods can be referred in **Section 4.3**. Further, the functionality of the culture device was validated using electrical and optical characterisation techniques.

5.3.1 Electrical characterisation of fabricated extended larger area heterojunction device

The energy band level in the Si/ZnO heterojunction is shown in Figure 5.2a. It is observed that the majority carriers in Si and ZnO move due to difference in carrier density across the junction. The depletion of the carriers forms a potential barrier (depletion layer) at the junction of Si and ZnO. The developed potential barrier resulted in the formation of rectifying Si/ZnO heterojunction device. The fabricated Si/ZnO heterojunction device was characterized for the electrical performance using semiconductor parameter analyser (B1500A, Keysight, USA). The heterojunction was characterized to analyse the developed capacitance due to storage charge carriers across the junction. It was found that the capacitance was modulated under the bias potential as shown in Figure 5.2b. The measured current-voltage (*I-V*) characteristic of the heterojunction is shown in Figure 5.2c. It is found that the fabricated heterojunction device had a rectification ratio of ~26 for the operative voltage of ±1 V. The obtained rectifying characteristic of the fabricated heterojunction is verified by simulating identical three dimensional (3D) device structure in the ATLAS software (Silvaco, USA). The various parameters and dimension considered for the simulation are listed in Table 5.1 [235]. The black line denotes the simulated results whereas the red colour stars denotes experimental results in Figure 5.2c.

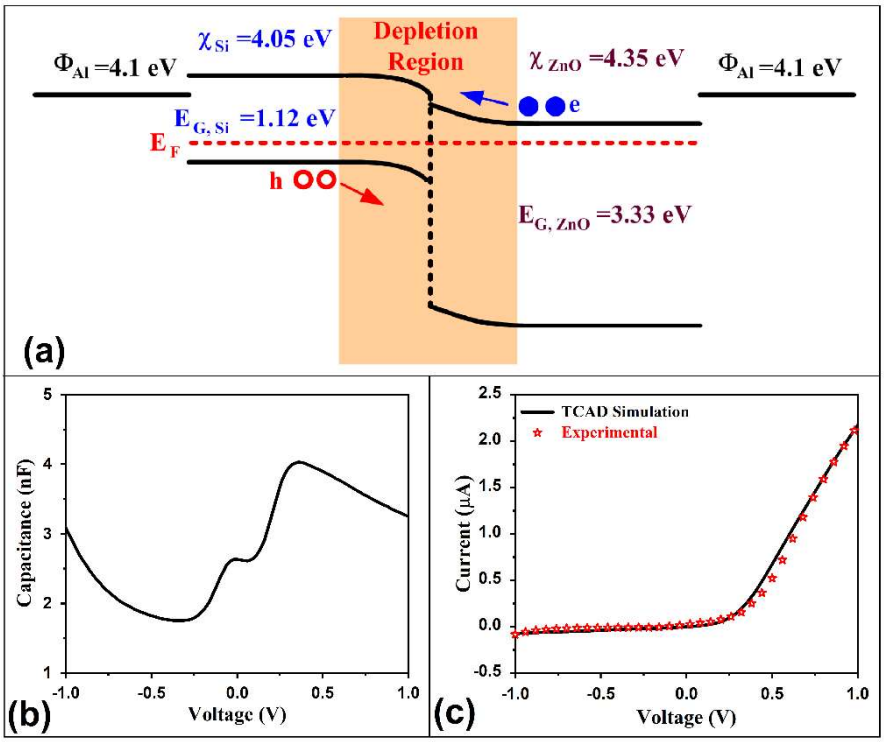

Figure 5.2: (a) Energy band diagram for the Si/ZnO heterojunction structure, (b) C-V characteristics of the fabricated Si/ZnO heterojunction, and (c) *I-V* characteristics of fabricated and simulated Si/ZnO heterojunction.

Table 5.1: Parameters used for the simulation of Al/Si/ZnO/Al heterojunction.

Parameters	**Si** [235]	**ZnO** [235]
Effective density of states in the conduction band (N_C) [cm^{-3}]	2.8×10^{19}	2.2×10^{18}
Effective density of states in the valance band (N_V) [cm^{-3}]	1.04×10^{19}	1.8×10^{19}
Electron affinity (χ) [eV]	4.05	4.35
Bandgap of ZnO [eV]	1.12	3.33
Recombination lifetime (τ_n and τ_h) [s]	2.5×10^{-3}	2.1×10^{-9}
Donor concentration (N_D) [cm^{-3}]	-	2.5×10^{16}
Acceptor concentration (N_A) [cm^{-3}]	7×10^{15}	-
Dielectric constant (ε)	11.9	9
Electron mobility (μ_n) [cm^2/Vs]	1500	100
Hole mobility [cm^2/Vs]	450	25

5.3.2 Optical and electrical characterisation of fabricated cell culture attached extended larger area heterojunction device

By the decade of 1940, neuroscience had acknowledged that neuronal membranes possess impedance properties and had started to identify the cellular mechanisms that contribute to impedance, capacitance and inductance. The phospholipid bilayer membrane was responsible for capacitance and membrane permeability determined resistance. Soon after, it was learned the neuronal kinetics such as cell adhesion, dendritic morphology, membrane channels also play a major role in shaping the electrical properties of neurons [253].

The cell-membrane is considered as insulator, as they are selectively permeable for the flow of ions in and out. This insulating property is modulated during the cellular function such as cell adhesion, proliferation, migration, differentiation and apoptosis. We also reported the change in the impedance during cellular process, where it increased when the cells adhered on the sensory region of the device and it decreased when the cells detached due to space restriction or cell apoptosis from that surface using mouse myoblast C2C12 cells [9]. Whereas, in case of primary neuronal cells, they do not proliferate (i.e., the number of live cells remains constant) but the morphology of the cells changes with respect to time. Hence, we performed the cellular study by seeding cells of 2×10^4 cells/well into the culture chamber, to analyse the effect induced after cell-substrate interaction and due to flow of ions through their cell-membrane [254], to regulate and maintain its physiological behaviour which causes a change in the characteristic electrical property i.e., conductivity increases with a decrease in magnitude of impedance of the device shown in Figure 5.3a, b. We performed the electrical measurements at different

time interval such as Day 1, 3 & 5, to avoid disturbance in the cells growing biological environment.

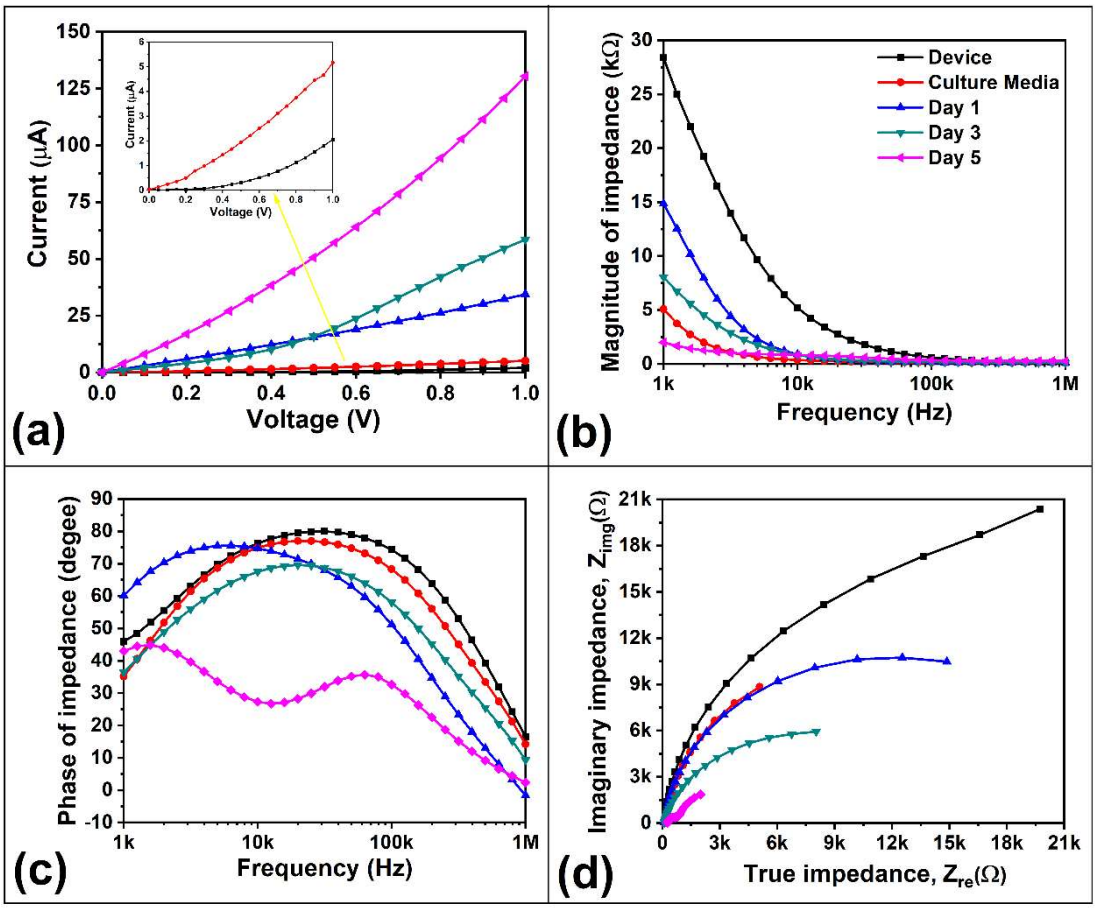

Figure 5.3: Change in electrical (resistance, conductive) properties of the spin coated ZnO thin film with respect to change in various cell functionality. (a) *I-V* characteristics plot; (b) Magnitude of impedance plot; (c) Phase of impedance plot; and (d) Nyquist plot.

We also analysed the morphological changes through fluorescent, SEM and optical imaging. Figure 5.4 and Figure 5.5 shows neuronal cells grown on the fabricated extended lager area heterojunction device using a fluorescent microscope (the process of fluorescent staining is given in supplementary file) and SEM images of Day 3 & 5 respectively. While, Figure 5.6 shows neuronal cells grown on the poly-L-lysine modified ZnO coated glass substrate using a bright field microscope. From Figure 5.6, it is inferred

that neurons have adhered and initiated networking with surrounding neurons, leading to a dense forest of neuronal circuits by day 5 thus turning into a confluent monolayer culture of neurons. The insulating properties of cells causes a change in impedance values. Whereas, mouse myoblast cells displayed a tight intracellular space in a two-dimensional monolayer culture that results in an increase in impedance measurement as they proliferate over the time [9]. However, neurons are specialized cells that do not undergo cell division hence manifest far less tight cellular junctions compared to other mammalian cells [273], thus causing a decrease in impedance during the course of *in vitro* cell culture (as in Figure 5.3a). Figure 5.4 depicts that neurons are distributed homogeneously over the poly-L-lysine coated surface and tend to cluster by day 5. From Figure 5.5 it is gathered that there is flourishing cell-cell contact by day 5 as adjacent neurons are more likely to get wired through neurite contact.

After 24 h (Day 1), it was observed that, neuronal cells became flat (as in Figure 5.6a), which implies that the neuronal cells started to grow on the substrate, due to which an increase in the magnitude of impedance (Figure 5.3a) with a decrease in the conductivity (Figure 5.3b) was observed. Further, we also observed that the change in morphology of the neuronal cells alters the phase angle (as shown in Figure 5.3c). The change in morphology is due to the cell-substrate interaction and also due to the formation synaptic cleft on the extracellular matrix (thin film). The magnitude of Nyquist plot (i.e., true *vs.* imaginary impedance) was highly effected due to formation of synaptic junction after neuronal differentiation between the neuronal cells across the surface (as shown in Figure 5.3d**)**, due to which the resistivity or magnitude of impedance get decreased (as in Figure 5.3b) with an increase in conductivity (as in Figure 5.3a) of the fabricated device as the function of progression of cellular function on extended larger area heterojunction device, which overall contribute to decrease the Nyquist characteristic plot. Therefore, the change

in characteristic electrical properties of the fabricated extended larger area heterojunction device are due to the change in the progression of cellular processes that is being exhibited by the neuronal cells.

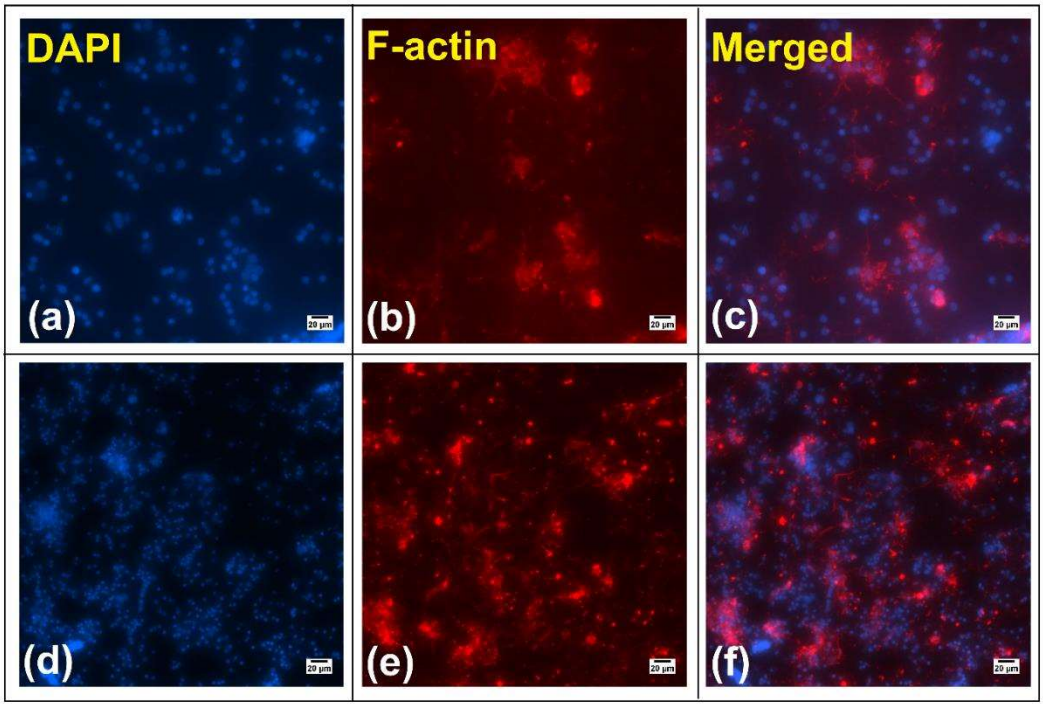

Figure 2.4: Fluorescent image shows morphological changes of cortical neuronal cells (a-c) Day 3, and (d-f) Day 5 ; Scale bar: 20 μm.

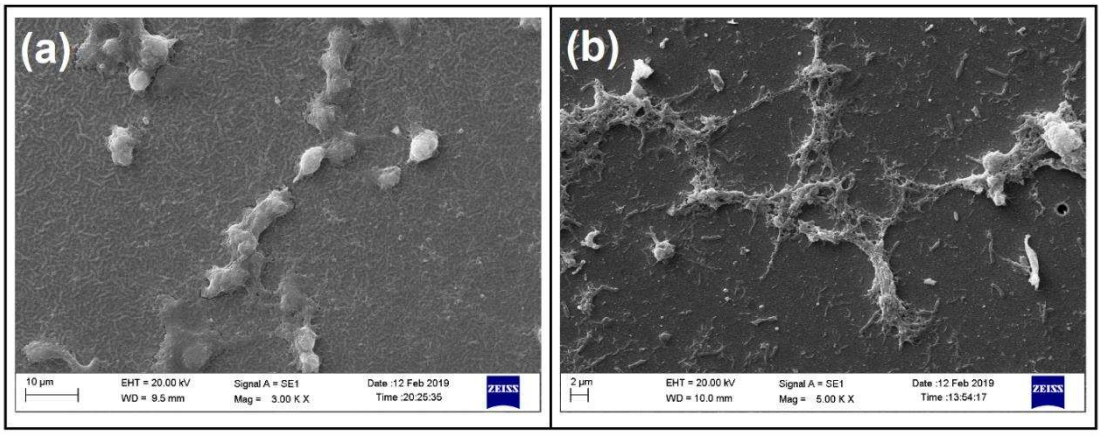

Figure 5.5: SEM image shows morphological changes of cortical neuronal cells (a) Day 3, and (b) Day 5.

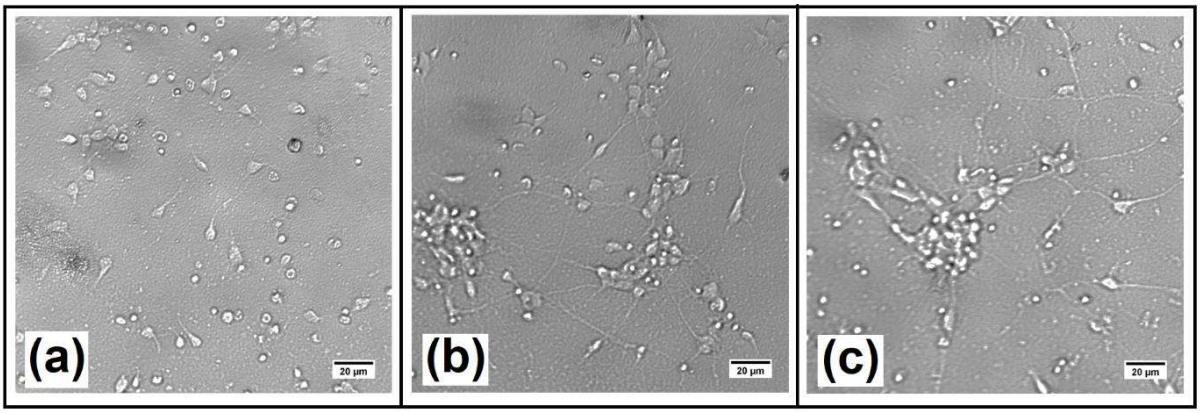

Figure 5.6: Micrograph shows morphological changes of cortical neuronal cells at various time point (a) Day 1, (b) Day 3, and, (c) Day 5.

5.4 Conclusion

The work exposes the demonstration of change in characteristic electrical properties of the poly-L-lysine coated extended large area heterojunction device, due to the change in the progression of cellular processes that is being exhibited by the neuronal cells. We analysed that an increase in the conductivity with a decrease in the magnitude of impedance is due to the formation of cell-substrate interaction and also due to the formation of synaptic junction after neuronal differentiation between the neuronal cells across the surface. Further, we also found that the characteristic electrical properties was well in correlation with the observed microscopic and SEM images. Hence, we anticipate that the fabricated extended large area heterojunction device could be used in the *in vitro* cell-substrate sensing application where the recording of the dynamic behaviour of the mammalian neuronal cells is adequately important.