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WIRED FOR SUCCESS: PROBING THE EFFECT OF TISSUE-ENGINEERED NEURAL INTERFACE SUBSTRATES ON CELL VIABILITY

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Abstract

This study investigates the electrochemical behaviour of GelMA-based hydrogels and their interactions with PC12 neural cells under electrical stimulation in the presence of conducting substrates. Focusing on indium tin oxide (ITO), platinum, and gold mylar substrates supporting conductive scaffolds comprised of hydrogel, graphene oxide, and gold nanorods, we explored how the substrate materials affect scaffold conductivity and cell viability. We examined the impact of an optimized electrical stimulation protocol on PC12 cell viability. According to our findings, substrate selection significantly influences conductive hydrogel behaviour, affecting cell viability and proliferation as a result. In particular, the ITO substrates were found to provide the best support for cell viability with an average of at least three times higher metabolic activity compared to platinum and gold mylar substrates over a 7-day stimulation period. The study offers new insights into substrate selection as a platform for neural cell stimulation and underscores the critical role of substrate material in optimizing the efficacy of neural interfaces for biomedical applications. In addition to extending existing work, this study provides a robust platform for future explorations aimed at tailoring the full potential of tissue-engineered neural interfaces.

Key words: Conductive substrates, electroactive hydrogels, electrochemical dynamics, biomaterial design, bioelectric modulation, stimulative substrate-hydrogel synergy

Introduction

Neural cell behaviour under electrical stimulation is a key area of study in biomedical engineering, given that it directly contributes to advancements in tissue engineering, neurostimulation and biomedical sensing technology. In this context, the choice of a conductive substrate for electrical stimulation becomes crucial, as its conductive properties have a direct impact on cellular responses such as cell proliferation, differentiation and gene expression ^[1]. Another common practice in the field is to combine the conductive substrate with a scaffold such as hydrogels containing additional conductive materials. The addition of conductive hydrogels onto different substrates adds an additional layer of complexity and potential to this research field by enabling precise control over electrical conductivity and cell response. The interaction between the unique properties of the different substrates and the conductivity of the hydrogels opens new research pathways for applications in neural tissue engineering, bioelectronics, and neuroprosthetics ^[2].

While numerous studies have explored the effects of various substrates and hydrogel materials on neural cell behaviour, many have focused on a limited range of substrates or lacked comprehensive characterization. For instance, some studies have investigated neural cell responses to common substrates like silicon or glass, but often overlook the unique electrochemical properties of materials like ITO, platinum, and gold mylar ^[3]. Moreover, while the integration of conductive polymers containing polypyrrole (PPy) and poly(3,4-ethylenedioxythiophene) (PEDOT) into hydrogels has been explored, the specific interactions and synergistic effects of conductive hydrogels with different substrates remain underexplored ^[4, 5]. While valuable, these studies often provide a limited view of the complex interplay between neural cells, hydrogels, and substrates, particularly in terms of long-term viability and proliferation under electrical stimulation. Additionally, previous research has predominantly focused on a singular aspect of the substrate-hydrogel-cell interface, such as biocompatibility or conductivity, without considering the overall impact on neural cell behaviour ^[6, 7]. This results in a fragmented understanding of how these variables collectively influence neural tissue engineering applications. The purpose of this study is to address these deficiencies by examining GelMA-based hydrogels in combination with conductive substrates, referred to as "substrates" hereafter. Through detailed electrochemical and cellular response analyses, we provide a more complete understanding of the substrate-hydrogel interactions, setting our work apart from previous studies in terms of depth and scope. This approach not only reinforces the

rationale behind our substrate selection but also positions our study to significantly contribute to the field, offering insights that can guide the design of more effective neural interfaces.

In our previous studies, we investigated the properties of conductive hydrogels, highlighting their potential in mimicking the extracellular matrix and enhancing cellular activities alongside exploring the impact of electrical stimulation protocols on neuronal cell survival and proliferation [8]. These studies have expanded the understanding of conductive hydrogels and their prospective applications in neural tissue engineering. Previously, we have focused on tailoring hydrogels biocompatibility and matrix structure for neural cell support, as well as improving its electrical properties for neural interfacing. We have also demonstrated enhanced neuromodulation and potential for advanced neural regeneration techniques [9]. There is, however, a critical gap in understanding how conductive hydrogels interact with underlaying conductive substrates. These substrates, chosen for their distinct surface properties, conductivity and chemical composition can introduce diverse electrical and chemical cues that intricately modulate the interaction of neuronal cells with hydrogels. To optimize their performance and translate them into biomedical applications, it is essential to explore the intricate relationships between these substrates and conductive hydrogels [10, 11].

This study aims to fill this gap by investigating the electrochemical properties of gelatin methacryloyl (GelMA) based hydrogels when interfaced with different substrates such as indium tin oxide (ITO), platinum (Pt), and gold (Au) mylar. Due to their importance in the field and the lack of previous comparative studies, we aim to directly compare these materials in terms of their impact on neural cell viability and functionality. By assessing these materials for neural applications, this approach seeks to standardize research and improve comparability. Our methodology includes electrochemical investigation and an examination of substrate-hydrogel interactions under electrical stimulation protocol previously optimized to enhance the survival and proliferation of neuronal cells [8]. The impact of the optimized electrical stimulation protocol will determine how the substrates impact cell viability of PC12 cells. This step is crucial for the further applications of our findings in the design of neural interfaces.

Our study defines the most effective hydrogel-substrate combinations, essential for developing neural interfaces with specific functionalities. Modeling platforms and cellular stimulation are investigated in this study in an effort to advance the existing knowledge base. This is crucial for guiding the evolution of innovative biomaterials in biomedical engineering, offering strategic insights to shape the future of neural interface technologies, and contributing

significantly to the field with new perspectives and methodologies in neural tissue engineering and biomaterial design.

Experiment section

Materials

GelMA (type 1) with a methacrylation degree of $82\% \pm 4$ was obtained from Translational Research Initiative for Cell Engineering and Printing (TRICEP - University of Wollongong, Wollongong, AUS) and synthesized as previously described ^[12]. Graphene oxide (GO) was supplied by the Australian National Fabrication Facility (ANFF) Materials Node at the University of Wollongong (NSW, AUS). AuNRs was provided by Nanopartz (Loveland CO, USA). Lithium-acylphosphonate (LAP) photocrosslinker was provided by Tokyo Chemical Industry Co., Ltd. (South Australia, AUS). Reagents used in covalent conjugation were provided by Sigma-Aldrich Pty Ltd (New South Wales, AUS), being EDC (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride), sulfo-NHS (salt sodium N-hydroxysulfosuccinimide) and 14 k molecular weight cut off. The electrolyte solution used for the electrochemical experiment was phosphate-buffered saline (PBS – pH 7.4), also provided by Sigma-Aldrich Pty Ltd. For cell culture, the reagents used were Dulbecco's modified Eagle's medium high glucose (DMEM) 1×, qualified fetal bovine serum (FBS), horse serum (HS), trypsin-EDTA and penicillin- streptomycin (P/S); all provided by Gibco Thermo Fisher Scientific (Victoria, AUS). For live and dead assays, the reagents used were calcein AM and ethidium homodimer-1, also obtained from Thermo Fisher. CellTiter-Blue obtained from Promega (Victoria, AUS) was used for metabolic activity assay. For the DNA extraction assay, the reagent used was papain extraction buffer prepared with NaH_2PO_4 monohydrate, Na_2HPO_4 dodecahydrate, PBS, cysteine, EDTA and papain, all from Sigma-Aldrich Pty Ltd (New South Wales, AUS). Triton X-100 was obtained from BDH (Victoria, AUS) and the Quant-iT PicoGreen dsDNA test kit from Thermo Fisher (Victoria, AUS). All reagents used were analytical grade chemicals. The substrates used in this study were indium tin oxide (ITO) (Part N° CB-90IN-S111, Delta Technologies – Limited, Loveland, CO), platinum coated microscope slides (here after referred to as platinum) and gold mylar provided by University of Wollongong (Wollongong – AUS).

Hydrogel synthesis

Scaffold fabrication and cell encapsulation have been previously described ^[9], in summary GO-AuNRs composite was been synthesised via covalent conjugation at a final stock concentration of (GO 1.25; AuNRs 0.25 mg.mL^{-1}), and the concentration used for the analysis was 0.30;0.06 mg.mL^{-1} with the first number representing the GO concentration and the second number the

AuNRs. Controls of GO were also prepared in an aqueous solution at the same concentration. GelMA stock at 12 % w/v was prepared by dissolving freeze-dried GelMA in PBS, which was diluted to 5 % w/v for the analysis.

Electrochemical characterization

Electrochemical measurements were recorded in PBS (pH 7.4) using CHI430A Electrochemical Analyzer (CH Instruments, Inc.) with a three-electrode cell configuration. The working electrode was either indium tin oxide (ITO), platinum or gold mylar, Ag|AgCl (KCl 3 M) was the reference electrode, and a platinum wire was the counter electrode. Cyclic voltammetry (CV) readings were taken within the potential range of 0.1 to 0.8 V at a scan rate of 100 mV.s⁻¹ over thirty cycles, and the electrochemical impedance spectroscopy (EIS) was performed at open circuit potential using a ± 10 mV alternating voltage over the frequency range of 10⁻¹ to 10⁴ Hz.

The electrochemical recordings focused on GelMA–GO–AuNRs hydrogel crosslinked onto the three different substrates: ITO, platinum, and gold mylar. Samples were prepared by depositing 100 μ L of each hydrogel solution onto the substrates inside the electrochemical chamber and subsequently photo-crosslinked in situ using a 405 nm light source (BioLambda LEDBox, São Paulo, Brazil) at 20 mW.cm⁻² for 210 seconds (see Figures S1 and S2 for electrochemical chamber and hydrogel forming process). Following the crosslinking process, 1 mL of PBS (pH 7.4) was added to the electrochemical chamber for recordings.

PC12 cell culture

PC12 cell line, derived from rat pheochromocytoma, was provided by ATCC (Catalog N° CRL-1721). Cells were maintained in a controlled environment at 37 °C with 5% CO₂. The growth medium used was DMEM, enriched with 10 % horse serum, 5% fetal bovine serum, 2 mM L-glutamine, and 1% penicillin/streptomycin. Once the cells reached confluency, they were detached using trypsin-EDTA (1 $\times$) for passaging and further used in the study. The use of rat pheochromocytoma (PC12) cells offers several advantages aligned with our aims to explore neuronal proliferation and functionality under electrical stimulation. The ease of culturing and genetic stability of PC12 cells enhances the reproducibility of results. Moreover, their known response to electrical stimuli further underscores their suitability for evaluating the biocompatibility and effectiveness of neural substrates, enabling direct comparisons with established literature.

Substrates surface and cell-laden hydrogels for electrical stimulation

PC12 cells were encapsulated within the scaffold at a cell density of 5×10^4 cells.mL⁻¹, then samples were photocrosslinked onto the substrate inside the custom-made electrochemical chamber previously described [8], at a cylindrical shape 9 mm diameter and 1.5 mm height ($\approx$ 100 μ L per sample), using a 405 nm source of light exposure (BioLambda LEDBox, São Paulo, Brazil) at 20 mW.cm⁻² for 210 seconds and Lithium phenyl-2,4,6-trimethylbenzoyl phosphinate (LAP) at 0.1% v/v as a photoinitiator. Once crosslinked, 600 μ L of growth media was added into the same chamber and samples were incubated for the periods of 1, 3 and 7 days. For the 2D experiments (cells onto substrates), PC12 cells were resuspended in 600 μ L in proliferation media and applied directly to the substrate surfaces within the electrochemical chambers, at a final concentration of $\approx 1.6 \times 10^4$ cells.cm⁻². The cells were given an overnight period for adhesion prior to initiating the experiments. Substrates used in this study were ITO, platinum and gold mylar.

Electrical stimulation protocol

The selected electrical stimulation (ES) protocol used to electrically stimulate neural cell lines has previously reported [13-16]. Briefly, the ES protocol features a charged-balanced biphasic current pulse of 100 ms, with a 20 ms interphase at 250 Hz (3.78 ms short circuit - the time between pulses). This protocol was implemented using a Digital Stimulator DS8000 and A365 Isolator units from World Precision Instruments, Australia, and was interfaced with an e-corder system (eDAQ, AUS). The analysis of capacitance density and impedance values were conducted at stimulation current of ± 2.5 mA, with a selected stimulation duration of 8 hours per day over three days. The capacitance density values were determined through the application of Equation (1);

$$\text{Capacitance density} = Q_{inj} / ((V_T - V_P) \times A) \quad (1)$$
$$Q_{inj} = I \times t_{pulse}$$

Where Q_{inj} represents the injected charge, V_T is the voltage peak, V_P is the voltage drop extracted from voltage transient measurements (VTM) (Figure S3), A is the electrode area, I is the current amplitude, and t_{pulse} is the period (Figure 2A).

Impedance values were computed using Ohm's law, as outlined in Equation (2) below:

$$Z = V_T / I \quad (2)$$

where Z represents impedance, V_T refers to voltage (extracted from VTM), and I is the applied current.

Electrical stimulation setup

The electrical stimulation (ES) of cells cultured onto the substrates or within cell-laden hydrogels was conducted using the custom-made chamber (Figure S1) and stimulation protocol detailed above for electrochemical characterizations. In summary, cells underwent stimulation for 8 hours a day (i.e., each stimulation round involved 8 hours of electrical stimulation followed by 16 hours of incubation without stimulation) over three days, with four consecutive days of incubation (non-stimulated) following the stimulation rounds. Proliferation analyses during electrical stimulation were performed at three distinct time points: 24 hours, three days (following the last stimulation round), and seven days (after the final incubation period). Throughout the analyses, electrochemical chambers were kept incubated at 37°C, 5% CO₂, containing growth media. The stimulation system consists of a WPI digital stimulator, isolator unit, e-corder, and electrochemical chambers. Our experimental design, involving an 8-hour electrical stimulation period followed by a 16-hour rest period, is based on optimizing conditions for neuronal stimulation. This regime aims to mimic physiological conditions, ensuring cellular recovery and viability while minimizing thermal and electrical stress. As continuous stimulation can cause cellular stress or desensitization, it balances the benefits of electrical stimulation with the need to maintain cell health ^[17]. Our other published studies ^[8] validated this approach, demonstrating its effectiveness in promoting neurite outgrowth and enhancing neuronal marker expression, thereby optimizing the functional response of PC12 cells to electrical cues.

Cell viability and cytotoxicity

Live and dead

To evaluate the viability of cell-laden hydrogels (3D study) after electrical stimulation, a fluorescent live/dead assay (Calcein-AM/ethidium homodimer-1, Thermo Fisher 0053scientific, AUS) was employed. Briefly, the scaffolds were extracted from the chamber, placed into fresh 24-well plates, and immersed in a solution containing 1 mM Calcein-AM and 2 mM Ethidium homodimer-1 for 30 minutes at 37°C. Next, samples underwent two rinses with fresh PBS, and images were taken using an EVOS FLoid Cell Imaging Station (wavelengths 524/24 nm and 593/40 nm from Thermo Fisher Scientific, AUS). For the cells seeded onto the substrate (2D study), first the media was removed from the electrochemical chamber then the substrate was transferred to a glass bottom dish for cell imaging (Thermo Fisher Scientific, AUS). Subsequent image processing with ImageJ software was used to eliminate background noise.

Metabolic activity

CellTiter-Blue Reagent manufactured by Promega (Madison, Wisconsin, USA) was used according to the guidelines provided by the manufacturer. In brief, growth media was removed from the electrochemical chambers and 600 μL of cell culture medium supplemented with CellTiter-Blue reagent in a 1 : 5 ratio was added directly to the wells. Then scaffolds ($n = 3$) were incubated for 2 hours at 37 °C and 5 % CO_2 . Subsequently, the solution was collected in triplicate in an Opti Plate 96F and measured using a CLARIOStar plate reader at an excitation wavelength of 550–15 nm and an emission wavelength of 600–20 nm, with a consistent gain applied for all readings. A control without cells was included to correct for background fluorescence originating from the media. The blank-corrected values were then employed to generate graphs using GraphPad Prism 10 software. Once supernatant was collected for analysis scaffolds were transferred to 1.5 mL centrifuge tubes and stored at -80 °C for further analysis. Fold change was determined by taking the logarithm of the ratio between the values observed on day 7 and those from day 1 for each sample.

DNA extraction and Pico green assay

The DNA extraction technique employed in this study is adapted from O'Connell et al. ^[18] and further refined by Mendes et al. ^[8]. The samples for DNA extraction analysis were the same scaffolds used for the metabolic as mentioned before. Therefore, samples were retrieved from the -80 °C freezer, thawed at room temperature, and weighed to standardize the DNA amount per scaffold for each tested condition. The papain extraction buffer, previously prepared in accordance with the method outlined by Mendes et al. ^[8], was supplemented with 1% v/v Triton X-100, functioning as a detergent to disrupt the cell membrane and release DNA from the GO flakes. A 0.5 mL volume of the mixture was added to each sample and left to incubate overnight at 60 °C. Samples were occasionally vortexed during incubation period. Following incubation, samples underwent centrifugation at 10,000 g for 10 minutes, and the supernatant was collected for DNA quantification.

Quantification of DNA content was carried out using the Quant-iT PicoGreen dsDNA assay kit. Initially, a standard curve was prepared using Lambda DNA from the kit. Subsequently, 100 μL of papain extraction was combined with 100 μL of PicoGreen, and 100 μL of this mixture was added to an Ultravision 96-well plate (Cellstar – Sigma Aldrich). After a 5-minute incubation at room temperature, fluorescence was measured using a plate reader (Clariostar – BMG Labtech) configured for PicoGreen with excitation at 483–15 nm and emission at 530–30 nm, maintaining a focal plate height of 4.6 and a gain of 1500. Fold change was determined

by taking the logarithm of the ratio between the values observed on day 7 and those from day 1 for each sample.

Contact angle

Contact angle was measured using a Dataphysics OCA 25 tensiometer coupled with a 5 mm diameter syringe. Water droplets (7 μ l) were dispensed onto clean substrates. Bidirectional (left and right) angle measurements were taken after 5 second post droplet dispensing and images were processed using Dataphysics software. The results were plotted as average and standard deviation using GraphPad Prism 9. Sample substrates, sized approximately 15 mm x 25 mm, included ITO, platinum, and gold mylar with five replicates each ($n = 5$).

Four point probe

Sheet resistance measurements were conducted using a Jandel RM3000 four-point probe station. A constant current of 10 mA was applied, and the voltage difference was measured to calculate the sheet resistance in ohms per square (Ω/sq). Sample substrates, sized approximately 15 mm x 25 mm, included ITO, platinum, and gold mylar with five replicates each ($n = 5$).

Statistical analyses

Statistical analyses were performed using the academic software version of GraphPad Prism 9. Data are represented as mean and standard deviation. The values of n represent the number of replicates for each analysis. The comparison between samples and controls was analysed using a one-way ANOVA, where statistical significance was established as * at $p < 0.05$, ** at $p < 0.01$, and *** at $p < 0.001$.

Results and discussion

Electrochemical characterization

Cyclic voltammetry (CV) was undertaken to investigate the electrochemical behaviour of the conductive hydrogels formed onto different substrates. Samples were crosslinked in-situ onto the substrates inside the electrochemical chambers and CVs recorded in PBS electrolyte solution following our published approach [8, 9]. Samples consisted of gelatin methacryloyl (GelMA) based hydrogels, GelMA with graphene oxide (GO), and GelMA with the composite previously designed containing graphene oxide and gold nanorods (GO-AuNRs) and the substrates were Indium Tin Oxide (ITO), platinum, and gold mylar. Figure 1 presents the CV profiles recorded in PBS (pH 7.4) of conductive hydrogels on various substrates.

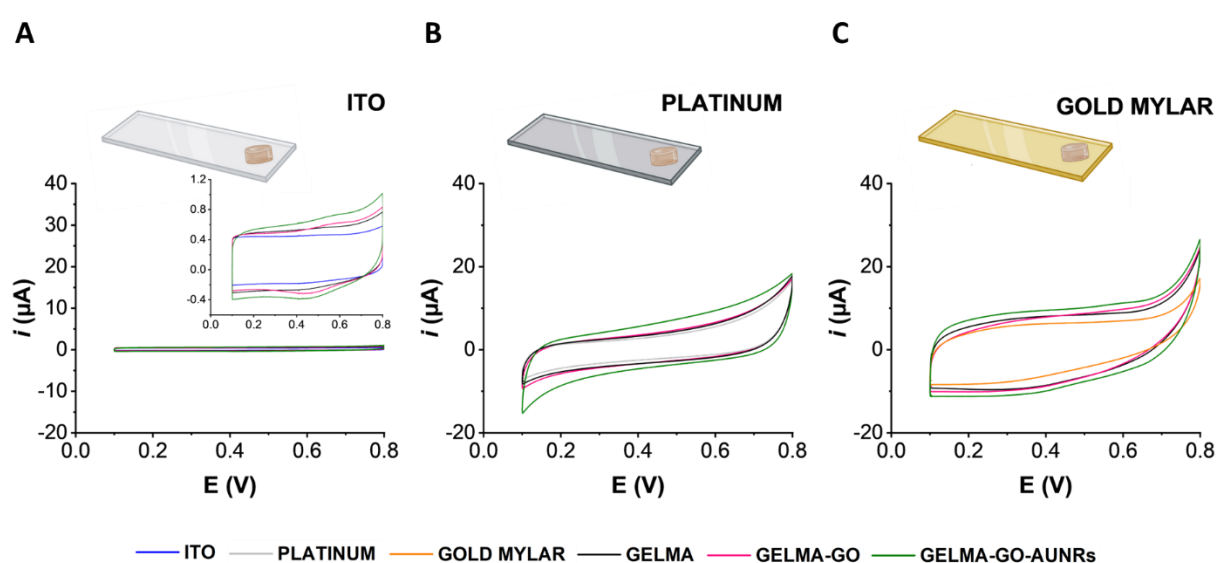


Figure 1 – CV profiles recorded in PBS of conductive hydrogels on various substrates within the potential range of 0.1 to 0.8 V at a scan rate of 100 mV.s^{-1} . A) CVs for hydrogels crosslinked on B) ITO; C) platinum; and C) gold mylar substrates.

In Figure 1A, ITO substrate (blue line) presents a capacitive behaviour. GelMA formed on the ITO (black line) increased capacitive current and displayed no redox peaks, consistent with its non-electroactive nature [19]. Conversely, the conductive GelMA samples containing GO (pink line) and GO-AuNRs (green line) presented notable redox activity, with oxidation and reduction peaks occurring at $\approx 0.6 \text{ V}$ and $\approx 0.5 \text{ V}$ respectively. Oxygen functional groups, particularly carboxylic groups, within the GO structure account for the presence of redox peaks in the conductive hydrogels [20]. The overall increase in capacitive current observed upon adding AuNRs to the scaffold could be attributed to enhanced surface area, improved electrical conductivity, electrochemical activity, catalytic effects, decreased charge transfer resistance, and modified interface properties [21]. This redox activity suggests potential applications for

controlled electrical stimulation or sensing, since these properties could be tailored to interact with biological systems in a predictable and beneficial manner ^[22]. These results corroborate with findings in our previous study ^[23].

Platinum substrate (Figure 1B – grey line) is a highly conductive material and results in high current presented in its CV when compared to the ITO. GelMA (black line) and GelMA-GO (pink line) also present high currents like platinum substrate (grey line), where no increase in the capacitive current in relation to the platinum only was observed. A high capacitive current of platinum may have masked the presence of redox peaks for the functional groups of GO. This indicated that both platinum and hydrogels primarily exhibit charging and discharging of their electrical double layers during the CV ^[24]. Conductive hydrogels GelMA-GO-AuNRs (green line) present a higher capacitive current also with a lack of distinct redox peaks for the GO functional groups. The electron transfer process might be too vigorous for sensitive redox-based applications but could be beneficial for high conductivity applications ^[25]. The subtle variation in current observed between the conductive hydrogels GelMA-GO and GelMA-GO-AuNRs could be attributed to the difference in electrochemical properties presented by the GO and the GO-AuNRs, these properties include enhanced electrical conductivity, improved electrocatalytic activity, and alterations in electron transfer dynamics. Consequently, the presence of AuNRs increased the electrode surface area and electrical conductivity, contributing to the observed capacitive current increase ^[26, 27].

Figure 1C shows the electrochemical behaviour of the hydrogels on gold mylar. Similar to platinum substrate, a high conductivity of gold mylar substrate (orange line) is suggested by the high current response observed in the CV. The properties of the substrate suggest it could provide a highly conductive environment, potentially enhancing cellular processes that benefit from strong electrical cues ^[28]. The hydrogels GelMA (black line), GelMA-GO (pink line) and GelMA-GO-AuNRs (green line) on gold mylar substrate (orange line) present the highest current response among the tested substrates, indicating superior conductivity but similar uptake of redox peaks as observed with platinum (Figure 1B – grey line). While there are no major differences among the current response of the hydrogels, a notable increase is observed when these hydrogels are interfaced with the gold mylar substrate (orange line). This pattern could suggest an enhanced capacitive interaction between the hydrogels and the gold mylar, indicating the substrate potential to enhance charge storage capabilities within the hydrogel matrices ^[29]. A significant influence of substrate conductivity on electrochemical behavior of conductive hydrogels was found in the data, having implications for their use in electrically

stimulated environments such as tissue regeneration, biosensor development, or wound healing. ^[30].

Overall, the substrates platinum and gold mylar presented high conductivity, as indicated by the substantial capacitive current observed in their CV profiles. Moreover, there is a potential risk that their elevated conductivity might cause overstimulation when there are cells introduced in the system, potentially impacting cell viability or functionality negatively ^[31]. Finally, ITO substrate presented the lowest overall capacitive currents, but clear redox peaks from the GO functional groups could be observed, offering a balanced electroactive environment. As a result, electrochemical processes are stable and efficient, potentially supporting cell viability and function without causing overstimulation ^[32-36].

Capacitance density in the context of electrodes is an important parameter that provides insights into the performance and stability of the electrode in certain applications, particularly in bioelectronic devices such as cardiac pacemakers or in electrochemical systems ^[37]. Here, the capacitance density of the electrodes under stimulation, in this case substrates, was calculated over the stimulation period using equation (1 under electrical stimulation protocol (Experiment section). Each substrate/hydrogel combination was stimulated for 8 hrs a day over three days and remaining incubated reaching a 7-day period. Square wave stimulation, a standard in bioelectronic applications, plays a crucial role in assessing electrode performance. This stimulation mimics standard square wave charge balance current stimulation used in many devices such as cochlear implant ^[38, 39] and deep brain stimulators ^[40]. Here voltage transient measurements (VTM) were employed to analyse the capacitance density of electrodes, essential for understanding their efficiency and stability. Therefore, VTM was collected daily before each stimulation cycle or recess period after the three cycles of electrical stimulation. Figure 2 shows the capacitance density of the hydrogels onto the various substrates over the stimulation period.

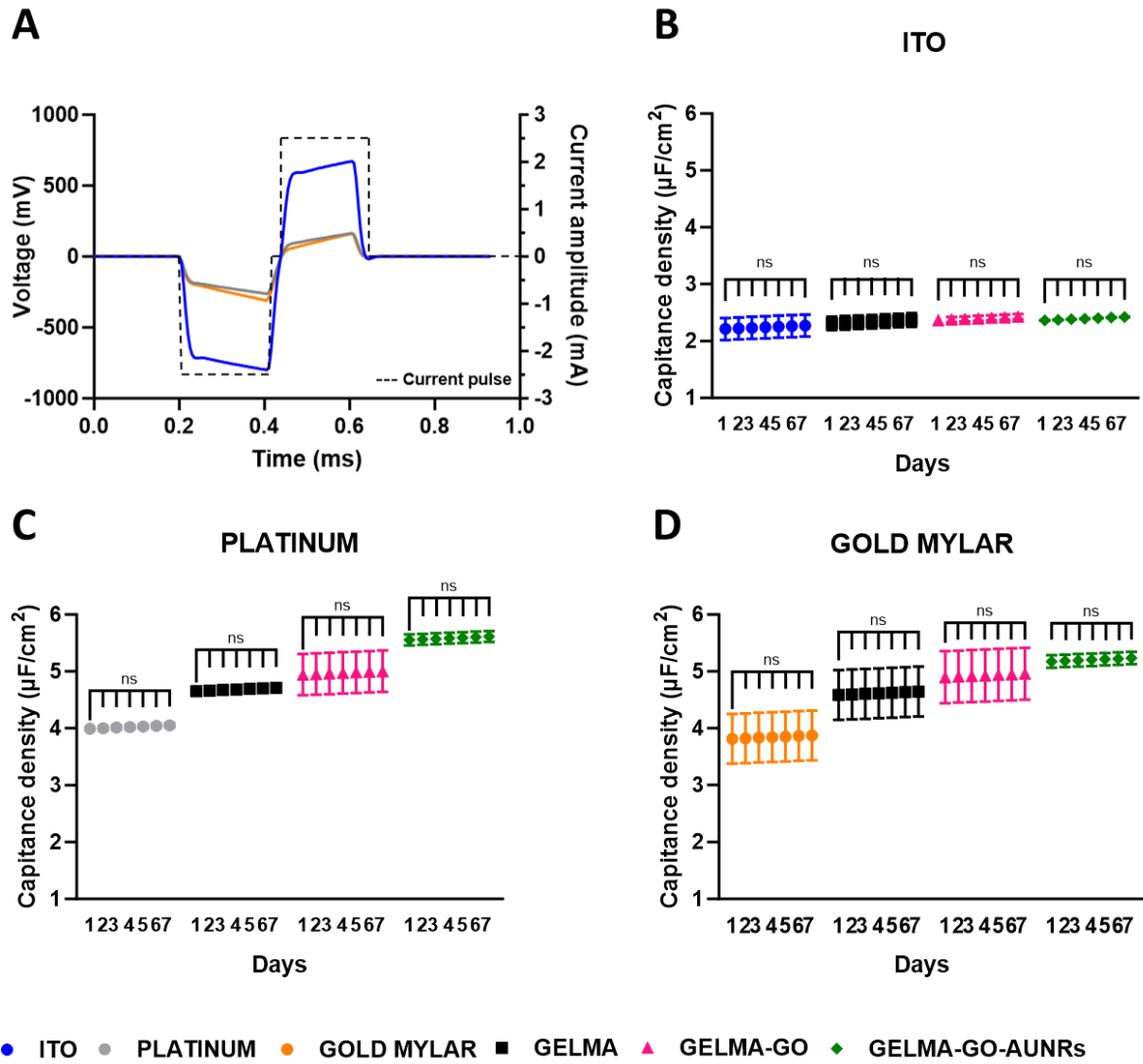


Figure 2 - Capacitance density of the substrates and hydrogels onto ITO, platinum and gold mylar under electrical stimulation. A) Example of voltage response waveform when a charge biphasic current pulse is applied to the system. Capacitance density measurements on B) ITO; C) platinum; and D) gold mylar substrates. ANOVA was used for all statistical analysis, and the error bars represent the standard deviations of the average measurements of three samples ($n = 3$).

Figure 2A shows a VTM example, illustrating the changes in voltage over time when a charge biphasic current pulse is applied to the system. Figure 2B shows the capacitance density of hydrogels onto ITO substrate (blue) starting at $\approx 2 \mu\text{F}.\text{cm}^{-2}$ and slightly increasing with the addition of the hydrogels GelMA (black), GelMA-GO (pink) and GelMA-GO-AuNRs (green) onto the substrate. This observation suggests that the hydrogel may be minimally enhancing the electrochemical properties of the ITO electrode (blue), potentially by increasing its surface area or improving the ion mobility within the electrode material ^[41]. Hydrogels are highly porous materials with a large surface area, therefore, once added onto the ITO substrate (blue)

it could increase the overall surface area by providing more sites for charge storage, which can lead to an increase in capacitance ^[42]. Hydrogels are also known for their high-water content, which can improve the mobility of ions within the substrate. This improved ion mobility may facilitate faster charging and discharging of the substrate, which can also lead to an increase in capacitance. Although the addition of the hydrogels onto the substrate could increase its capacitance density, even slightly, there is no significant difference among the samples over the stimulation period. In the context of bioelectronic devices such as neural interfaces or cardiac pacemakers, a capacitance density of $2 \mu\text{F}\cdot\text{cm}^{-2}$ would be considered low due to applications typically requiring much higher capacitance densities to efficiently store and deliver energy ^[43]. The focus is often on stability and biocompatibility rather than high energy storage in biomedical sensors and devices, such as those used to monitor physiological signals or for drug delivery systems ^[44]. Moreover, for electrochemical sensors a capacitance density of $2 \mu\text{F}\cdot\text{cm}^{-2}$ could be sufficient, especially if the sensor is designed for detecting small changes in chemical concentrations as the key factor here is the sensitivity of the sensor rather than its energy storage capacity ^[45]. Therefore, when evaluating capacitance density specific applications requirements must be considered ^[46].

Figure 2C presents the capacitance density of hydrogels on platinum. As expected, the capacitance density of platinum substrate (grey) is higher than ITO (Figure 2A - blue). Platinum has a higher work function than ITO, meaning that due to its electron configuration it has more electrons in its outermost shell, which makes it more attractive to electrons and gives it a stronger binding force ^[47]. As a result, platinum requires more energy to lose an electron compared to ITO, resulting in a higher work function meaning the energy required to liberate an electron from the solid surface into the surrounding space ^[48]. Moreover, having a higher work force allows platinum to form a stronger electrical double layer at the substrate surface resulting in a higher capacitance density ^[49]. A stronger electrical double layer allows efficient charge storage, making platinum a better choice for applications such as electrochemical drug delivery systems, where high capacitance is essential for achieving high energy and power density ^[50, 51]. The addition of the hydrogels GelMA (black), GelMA-GO (pink) and GelMA-GO-AuNRs (green) onto the substrate enhances its capacitance density due to reasons previously discussed. During the stimulation period, there was no significant difference between samples in terms of capacitance density when hydrogels were added to the surface of platinum substrate. In fact, electrode stability is often evaluated by examining how capacitance density changes over multiple charge and discharge cycles. Therefore, a stable capacitance density presenting no significant changes indicates that the electrode material can endure

repeated cycling without significant degradation. Stability and electrode integrity are crucial for the long-term performance of energy storage devices ^[52].

Figure 2D shows the capacitance density measurements of hydrogels on gold mylar substrate. Initially, the gold mylar (orange) demonstrates a capacitance density similar to platinum, which could be attributed to the high work function characteristic of its surface ^[53]. Over the stimulation period, the capacitance density remains relatively stable with minor fluctuations that are not statistically significant. According to the stability in capacitance density, gold mylar provides a consistent electrochemical environment for the hydrogels. The minor variations observed do not affect the overall trend, reinforcing its suitability for applications where stable electrical properties are desirable ^[54]. Adding hydrogels to the substrate causes a slight increase in capacitance density, which was previously discussed.

Overall, capacitance density plays a crucial role in defining the efficacy of electrodes, especially for neural recording and stimulation applications ^[55]. A higher capacitance density suggests a more extensive surface area for charge storage, which enhances the signal-to-noise ratio (SNR) and reduces impedance in neural recording ^[56]. It also facilitates the delivery of a higher charge per pulse during neural stimulation, crucial for effectively activating neural tissues ^[57]. Ongoing research continues to explore strategies to further optimize the capacitance density and performance of electrodes through careful material selection, design refinements, or by integrating nanostructures, aiming to boost both the performance and stability of electrodes in various energy storage roles paving the way for even more efficient and powerful electrochemical devices ^[58]. For further discussion on how adding hydrogel to substrates enhances their capacitance density see SI (Increased capacitance density from hydrogel-substrate integration section). The capacity density the different substrates has also been investigated with PC12 cells loaded within the hydrogels. In theory, cells could introduce additional interfaces and boundaries within the hydrogel, which could potentially increase the capacitance density by providing more sites for charge storage ^[59]. In this context, the capacitance density exhibited a slightly increase when cells were loaded within the hydrogels, yet this change was not statistically significant.

Cell viability and proliferation assays under electrical stimulation protocol

Cellular behaviour has been investigated under electrical stimulation to provide a deeper understanding of PC12 cells encapsulated within the hydrogels crosslinked onto various substrates. In the 2D experiments, cells were seeded directly on substrates ITO, platinum, and gold mylar. For the 3D experiments, PC12 cells were encapsulated within hydrogels GelMA,

GelMA-GO, and GelMA-GO-AuNRs that were formed on the ITO, platinum and gold substrates. This approach aims for a comprehensive comparison of cell behaviour on different substrates and within various 3D hydrogel environments. The proliferation assays under electrical stimulation were conducted at three distinct intervals: 24 hours, 72 hours (after last stimulation round), and seven days (concluding the final incubation period). Every stimulation round consisted of 8 hours of electrical stimulation phase followed by 16 hours of resting period without stimulation. During the analysis, electrochemical chambers were kept under incubation at 37 °C, 5 % CO₂, using proliferation media. Following these initial experiments, the next phase of the study investigated cellular responses to electrical stimulation.

Electrical stimulation can mimic the natural electrical environment of neural cells, allowing a direct investigation into how cells react and adapt to electrical signals ^[60]. We can therefore use this study to develop more accurate and relevant applications for tissue engineering and regenerative medicine based on a realistic assessment of cell behavior in natural environments ^[61].

Figure 3 illustrates the various substrate configurations for PC12 cell culture alongside the detailed timeline of the electrical stimulation protocols chosen for this study.

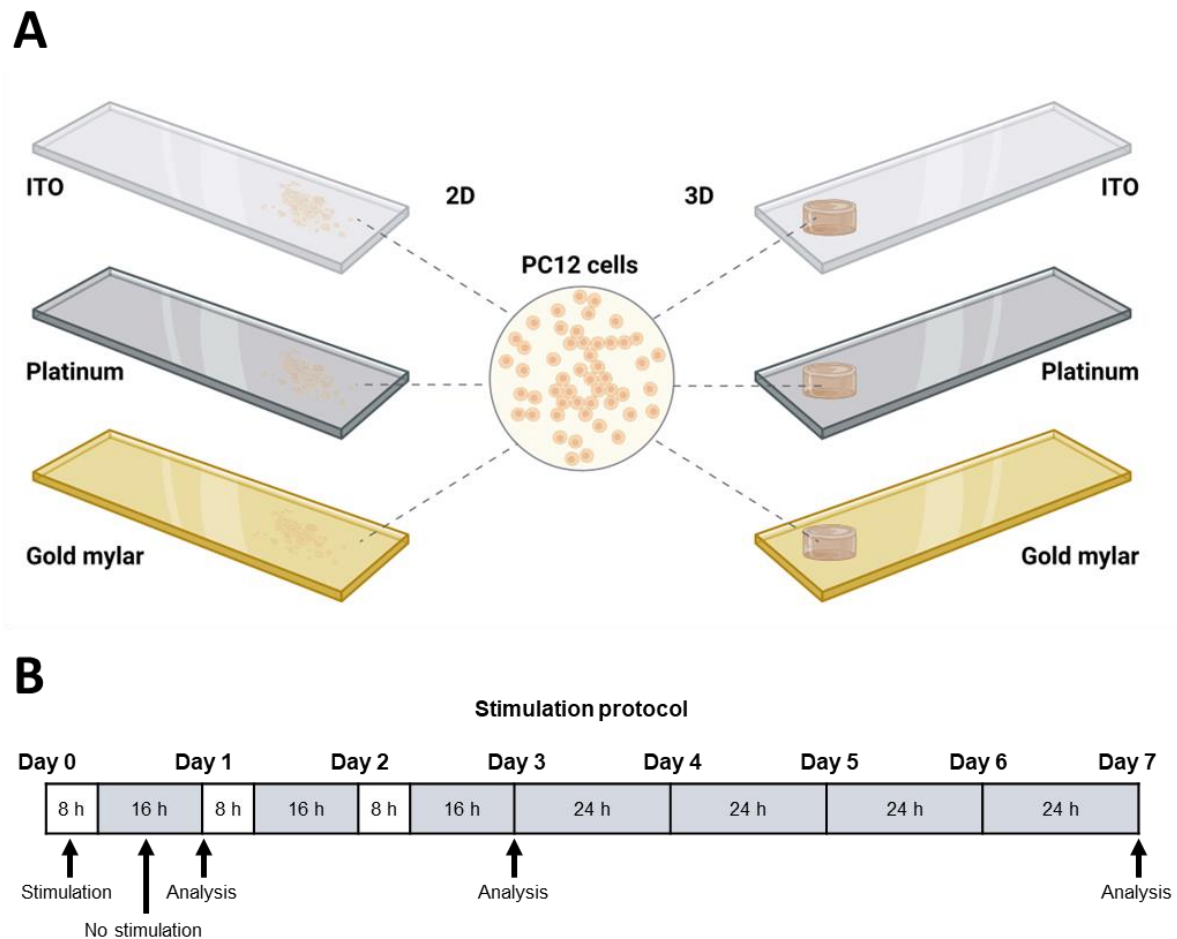


Figure 3 – A) Comparative schematic of PC12 cell culture setups on various substrates for electrical stimulation. B) Timeline of electrical stimulation protocols.

PC12 2D Culture

Figure 4 shows our investigation into the metabolic activity, DNA extraction and live/dead staining of PC12 cells at a cell density of 1.6×10^4 cells.cm⁻² cultured on ITO, platinum, and gold mylar substrates, providing valuable insights into cell behaviour and viability in 2D.

SUBSTRATES

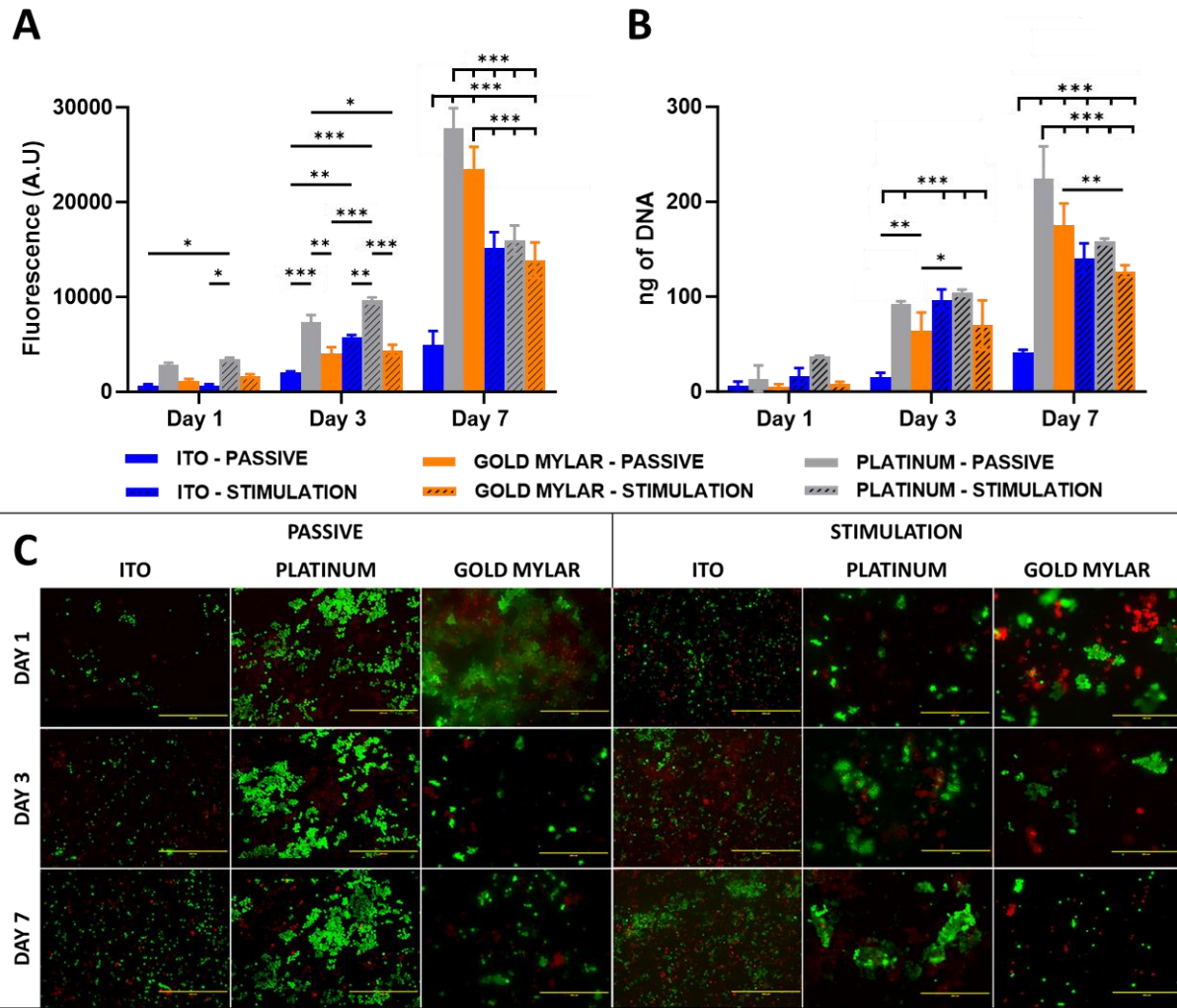


Figure 4 - Cell viability and proliferation in 2D on various substrates under electrical stimulation. A) The bar graph shows the metabolic activity measured with Cell Titer blue assay and reported as absolute fluorescence units for all the conditions listed at the bottom of the graph along the 7 day time course analysis. B) The bar graph shows the DNA amount extracted and quantified with PicoGreen and reported as ng of DNA from the same samples of A. C) Representative images of the Live and Dead staining performed on the samples reported along a 7 day time course analysis. The images have been taken with an epifluorescence microscope using 20× objective and the merged signals of Calcein staining (Live-green channel) and of the ethidium homodimer staining (Dead-red channel) are presented. The scale bar denotes 400 μm . All statistical analyses have been performed with ANOVA where the errors bars show the standard deviations of the average measurements of three samples ($n = 3$), * indicates a significant difference ($p < 0.05$), and ** ($p < 0.01$).

Figure 4A displays the relative metabolic activity of PC12 cells, serving as an indicator of their viability and proliferation^[62]. The metabolic activity is a measure of the cells ability to process

energy sources that directly correlates with their health and growth potential ^[63]. In this context, changes in metabolic activity in PC12 cells, a commonly used neuronal model, provide insights into their response to the selected experimental conditions ^[64, 65]. For each substrate ITO (blue), platinum (grey) and gold mylar (orange) the data is shown for cells with electrical stimulation (striped bars) and passive without electrical stimulation (solid bars).

At Day 1, there are no significant differences in metabolic activity between the different substrates and conditions, indicating a steady response of the cells under both passive and stimulated conditions ^[66, 67]. It is important to note that these initial metabolic activity levels under passive conditions establish a control for understanding the impact of electrical stimulation on these substrates over the course of the study ^[68]. By Day 3, the metabolic activity increases on ITO (striped blue) under stimulation suggests a enhancing effect on cell viability. Meanwhile, platinum and gold mylar show no significant metabolic changes, indicating a lack of response to electrical stimulation ^[69]. By Day 7, there is a divergence in metabolic activity among the substrates; while ITO demonstrates enhanced activity, platinum (grey) and gold mylar (orange) show a decrease under stimulation conditions, indicating varied cellular responses and adaptations to the stimulation ^[70]. Moreover, the decreased metabolic activity could indicate cell death, reduced cell metabolism due to stress or adaptation, or inhibition of key signalling pathways by electrical stimulation ^[71, 72].

Figure 4B highlights the significant impact of substrate material on PC12 response to electrical stimulation regarding its DNA content. ITO (blue) emerges as a favourable substrate, enhancing metabolic activity under stimulation, while platinum (grey) and gold mylar (orange) substrates appear less supportive, as evidenced by decreased DNA count. Those results are consistent with metabolic activity observations and emphasize the importance of substrate selection for optimizing cell-material interactions in bioelectrical applications ^[73].

Figure 4C presents the comparison between live and dead PC12 cells cultured on ITO, platinum, and gold mylar substrates over the course of 1, 3, and 7 days, both under passive and stimulated conditions. This data provides valuable insights into the cellular response to different substrates and time points, enhancing our understanding of their viability and behaviour ^[74]. More live cells are observed on platinum compared to ITO under passive conditions, however, under stimulated conditions, ITO shows an increased number of cells but also exhibits a higher cell death. Platinum shows cell clumping under stimulation, indicating altered cell adhesion or response to stimulation ^[75]. Gold mylar cell count decreases over 7

days, with no major changes between passive and stimulated conditions in the end. Live and dead staining findings corroborates with the metabolic data, reinforcing our overall findings.

The data provided above outlines a multifaceted relationship between the substrate and cells. Other physicochemical properties of the substrates were obtained to provide some insight into these interactions (Table 1). As a result of these data, we can gain insights into the disparity between the behaviour of mylar substrates made of gold, platinum, and ITO in the context of substrate-cell interactions.

Table 1 – Physicochemical properties of the conductive substrates.

Substrate	Contact angle (°)	Sheet resistance (Ω/sq)	Coating thickness (nm)	Fold change	
				Met. Activity	DNA ext.
ITO	82.7 ± 1.5	79.29 ± 0.42	15 – 30	3.7	1.5
Platinum	55.9 ± 1.0	7.65 ± 1.22	10 – 50	4.4	2.3
Gold mylar	22.6 ± 3.0	2.13 ± 0.02	10 – 50	4.4	2.3

Contact angle measurements serve as a key indicator of substrate hydrophilicity, a property that significantly influences cellular adhesion and subsequent proliferation ^[76]. Figure 5 shows the contact angle investigation performed on the substrates.

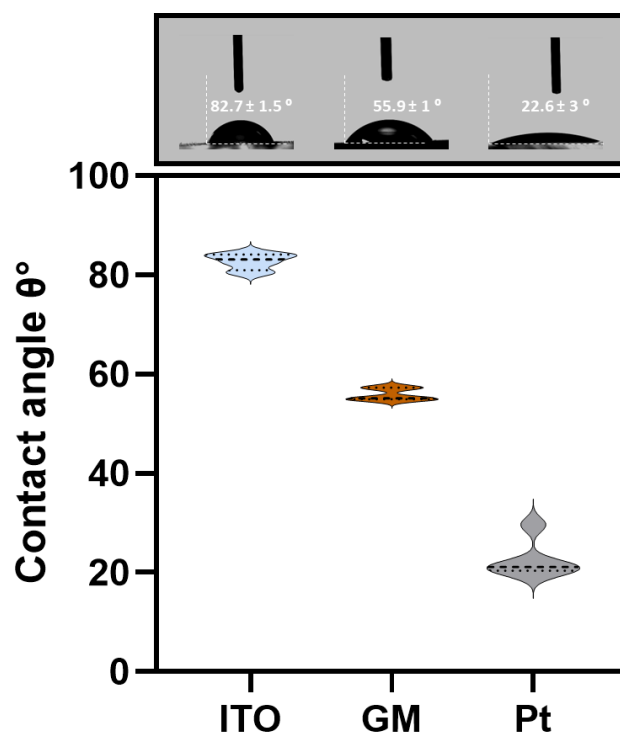


Figure 5 - Contact angle comparison across ITO, gold mylar (GM) and platinum (Pt) substrates.

Our study demonstrates that ITO exhibits a higher contact angle of 82.7 ± 1.5 degrees, suggestive of a moderately hydrophilic surface. It is known that this level of hydrophilicity is conducive to protein adsorption, which is essential for successful cell adhesion and spreading. In contrast, gold mylar presents lower contact angle of 55.9 ± 1 degrees indicates a more hydrophilic surface, which can sometimes lead to excessive protein adsorption, potentially resulting in an environment less favourable for cell adhesion. The significantly lower contact angle of platinum, at 22.6 ± 3 degrees, suggests a highly hydrophilic surface, which can be detrimental to cell attachment due to the formation of a protein layer that may impede cell-surface interactions [77].

Sheet resistance is an important parameter in determining the electrical properties of substrates, which is known to affect cell-material interactions, particularly in the context of electroactive tissues and devices [78]. The sheet resistance of our substrates was measured using a four-point probe method, showing significant differences. ITO showed a sheet resistance of 79.29 ± 0.42 , platinum recorded 7.65 ± 1.22 , and gold mylar presented the lowest resistance at 2.13 ± 0.02 all in $\Omega.\text{sq}^{-1}$. These variations in sheet resistance can influence the electrical signalling environment of cells, which is crucial for applications such as neural interfaces and cardiac patches, where electrical cues guide cell behaviour. A moderate sheet resistance, as seen with ITO, may support the optimal electrical stimulation required for cellular processes without causing detrimental effects like Joule heating or altered signal transduction, which can occur at both high and low extremes of resistance. Given the sensitivity of cells to their electrical environment, as evidenced by their response to the electric field (Figure 4) and material EIS data presented in Figure S4 (Supporting Information), the distinct sheet resistance values of our substrates likely contribute to the observed differences in cell adhesion, proliferation, and function. This is particularly relevant in the context of tissue engineering, where precise control over electrical properties is essential for the desired tissue function and integration with host tissues [79].

Coating thickness is another determinant of the interfacial properties between the substrates and the GelMA base hydrogels, which could present implications for cell behaviour. According to the measurements provided by the manufactures, it indicates a coating thickness range of 15-30 nm for ITO, 10-50 nm for platinum, and 10-50 nm for gold mylar. In general, thinner coatings tend to be more flexible, which could potentially affect the mechanotransduction pathways of cells. For instance, cell fate decisions, such as differentiation, can be guided by substrate stiffness, which a cell is known to be sensitive to. For example, a thicker coating can provide a stiffer substrate that may promote osteogenic differentiation, while a thinner, more

flexible surface could support softer tissue integration, such as neuronal growth or skin tissue engineering. Furthermore, the uniformity of the coating affects not only the mechanical integrity but also the homogeneity of cell distribution and growth. Thicker coatings within the range we observed for platinum and gold mylar may provide a more robust platform for cell attachment and growth, given a uniform distribution of thickness. Therefore, the observed coating thicknesses on our substrates could significantly influence the physical microenvironment of cells, thereby affecting their adhesion, morphology, and function. The moderate coating thickness of ITO, coupled with its other favourable properties, may contribute to its superior performance as a substrate for biomedical applications, as seen in the higher metabolic activity and DNA extraction efficiency demonstrated in our findings.

The fold change calculated based on the seven-day period investigation of the PC12 cells coated onto the substrates under passive regime (previously showed in Figure 4), this data provides valuable insights into the temporal dynamics of cell viability and activity across different substrates. While ITO showed a lower fold change in metabolic activity (3.7) compared to platinum and gold mylar (both at 4.4), it ultimately supported cell viability more effectively. This suggests that while platinum and gold mylar may initially induce a higher metabolic response, the cellular viability is better sustained on ITO over time. Similarly, for DNA extraction, ITO showed a fold change of 1.5, and platinum and gold mylar both showed a higher fold change of 2.3. The fold change data for metabolic activity and DNA extraction indicate that while platinum and gold mylar substrates induce an initial increase in cell proliferation, ITO provides a stable environment that supports sustained cell viability over a seven-day period.

These observations highlight the importance of considering not just the immediate cellular responses to substrate materials but also the longer-term viability and stability of the cell-substrate interaction. The initial detrimental effects observed with platinum and gold mylar suggest a dynamic cellular response that adjusts over time, pointing to a possible period of cellular adaptation to the conductive properties of these materials.

PC12 3D Culture

Following from the characterisation of the GelMA hydrogels formed on the substrates and evaluating the cell-substrate interaction, a detailed evaluation of PC12 cultured within these GelMA structures, formed on the substrates, was undertaken (Figure 6, Figure 7, and Figure 8). The data reports the metabolic activity, DNA content and live/dead staining of PC12 cells

encapsulated within the hydrogels and provides valuable insights into cell behaviour and viability in 3D.

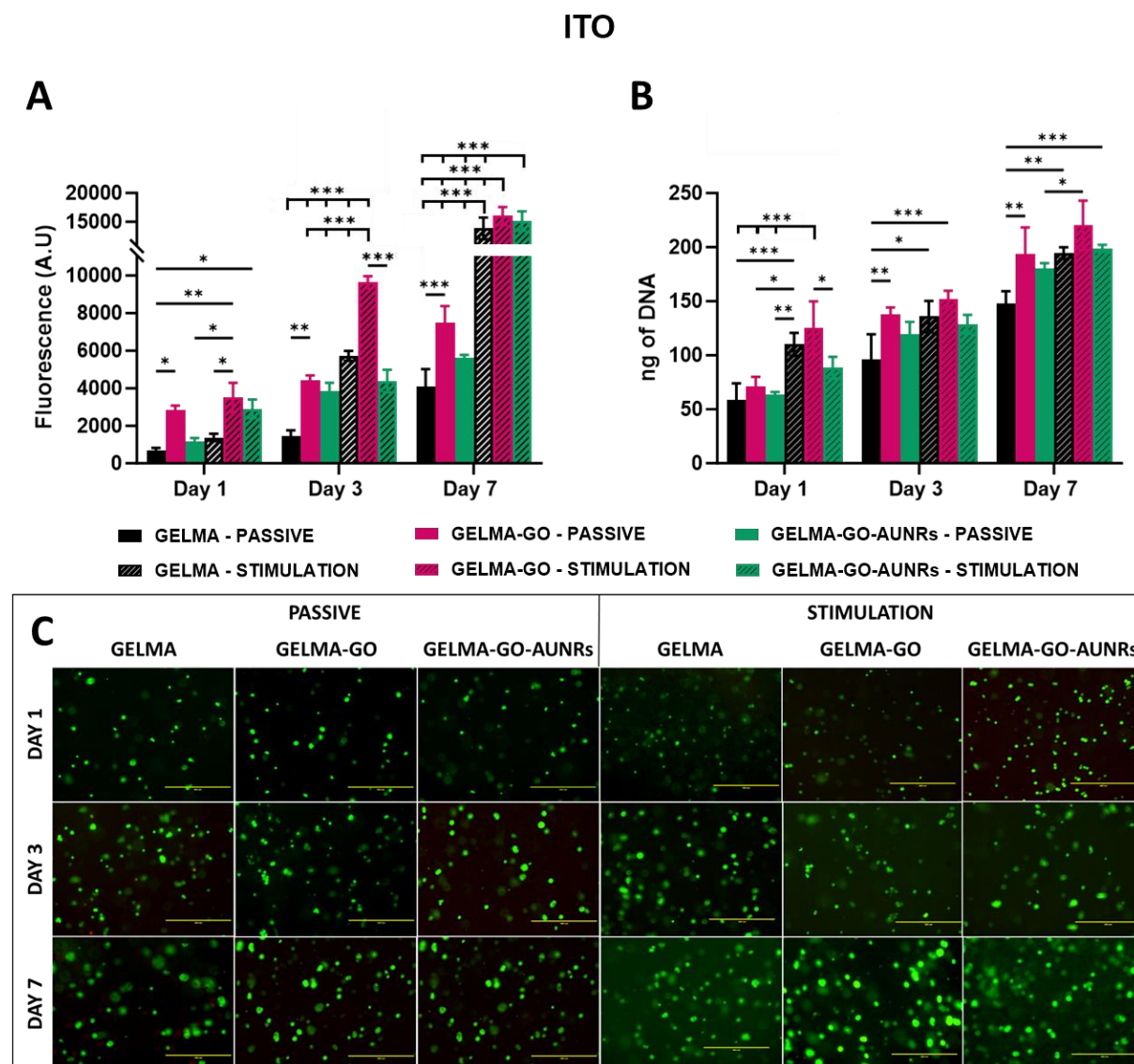


Figure 6 - Cell viability and proliferation of PC12 cells in hydrogels on ITO substrates under electrical stimulation. A) The bar graph shows the metabolic activity measured with Cell Titer blue assay and reported as absolute fluorescence units for or all the conditions listed at the bottom of the graph along the 7 day time course analysis. B) The bar graph shows the DNA amount extracted and quantified with PicoGreen and reported as ng of DNA from the same samples of A. C) Representative images of the Live and Dead staining performed on the samples reported along a 7 day time course analysis. The images have been taken with an epifluorescence microscope using 20× objective and the merged signals of Calcein staining (Live-green channel) and of the ethidium homodimer staining (Dead-red channel) are presented. The scale bar denotes 400 μ m. All statistical analyses have been performed with ANOVA where the errors bars show the standard deviations of the average measurements of

three samples ($n = 3$), * indicates a significant difference ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

Figure 6A shows the metabolic activity of PC12 cells encapsulated at a cell density of $\approx 5 \times 10^5 \text{ cells.mL}^{-1}$ within hydrogels onto ITO substrate under passive and stimulation conditions over a period of 7 days. Initially, Day 1 shows steady metabolic activity between passive and stimulated conditions across all hydrogel types. However, the addition of GO to GelMA enhances this response, with GelMA-GO (striped pink) showing the most significant increase under both passive and stimulation regimes indicating a consistent enhancement independent of stimulation^[80]. By Day 3, cells across all hydrogels show enhanced metabolic activity under stimulation, suggesting either enhanced cell proliferation or increased metabolic functioning^[81]. On Day 7, the overall increase in metabolic activity under stimulation remains consistent, indicating a potential stimulation effectiveness over prolonged periods^[82]. Overall, it has been observed that incorporating GO and GO-AuNRs into GelMA hydrogels increases their metabolic activity on ITO substrate^[83].

Figure 6B presents the critical influence of hydrogel composition on PC12 cell response to electrical stimulation. The GO and GO-AuNRs hydrogels present an increase in DNA content under stimulated conditions, maintaining this increase consistently over the 7-day period.

Figure 6C shows the live and dead staining of the PC12 cells encapsulated within the hydrogels onto ITO substrate, under both passive and stimulation conditions, taken at intervals over 7 days. On Day 1, GelMA samples stimulated present moderate cell number compared to the passive ones, a trend also seen in GelMA-GO and GelMA-GO-AuNRs with increased fluorescence under stimulation. By Day 3, while the fluorescence in passive samples decreases, indicating lower viability or activity, the stimulated samples across all hydrogels maintain higher fluorescence, suggesting continued activity due to stimulation^[84]. By Day 7, overall, stimulated hydrogels consistently exhibit greater fluorescence, implying enhanced metabolic activity or cell viability, particularly for GelMA-GO and GelMA-GO-AuNRs. The live and dead staining, alongside DNA and metabolic assays, provides a comprehensive view of cell behaviour, although the observations should be interpreted cautiously as fluorescence intensity alone does not offer quantitative data^[85, 86].

Overall, literature corroborates that ITO substrates significantly influence cellular behaviour in terms of adhesion, proliferation, and differentiation particularly in response to electrical stimulation. For instance, a study involving cardiomyocyte cultures have highlighted the role

of ITO in improvement of cell function and growth under electrical stimulation [87]. Furthermore, research involving mesenchymal stem cells and human dermal fibroblasts has used ITO for detailed cell surface charge mapping, demonstrating its use in advanced cellular function analyses [88]. These findings demonstrate the capability of ITO to modulate cell behaviour across various cell types, reinforcing the significance of our findings within the broader context of tissue engineering and regenerative medicine [89].

Figure 7 shows the ratio of metabolic activity, DNA content and live/dead staining of PC12 cells encapsulated within hydrogels onto platinum substrate under passive and stimulation conditions over a period of 7 days. Platinum is a noble metal with excellent conductivity and biocompatibility, which could influence cell behaviour differently compared to ITO.

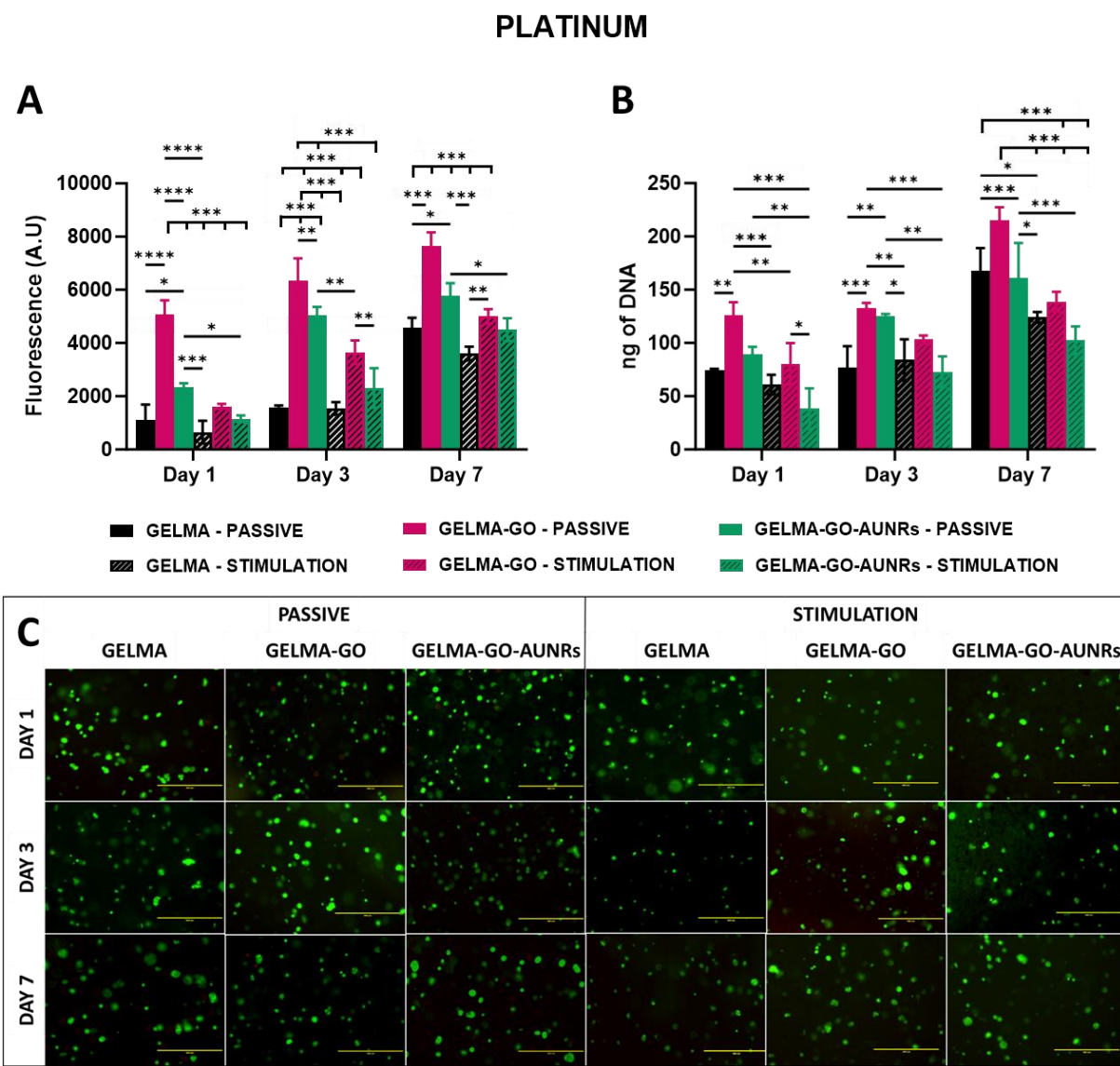


Figure 7 - Cell viability and proliferation of PC12 cells in hydrogels on platinum substrates under electrical stimulation. A) The bar graph shows the metabolic activity measured with Cell Titer blue assay and reported as absolute fluorescence units for or all the conditions listed at

the bottom of the graph along the 7 day time course analysis. B) The bar graph shows the DNA amount extracted and quantified with PicoGreen and reported as ng of DNA from the same samples of A. C) Representative images of the Live and Dead staining performed on the samples reported along a 7 day time course analysis. A 20x epifluorescence microscope has been used to capture these images, which show merged signals of Calcein staining (live-green channel) and Ethidium homodimer staining (dead-red channel). The scale bar denotes 400 μm . All statistical analyses have been performed with ANOVA where the errors bars show the standard deviations of the average measurements of three samples ($n = 3$), * indicates a significant difference ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

Initially, in Figure 7A on Day 1 most of the hydrogels shows no response to electrical stimulation, with metabolic activity similar to its passive state, indicating no enhancement under stimulation^[90]. GelMA-GO (pink) exhibit decreased metabolic activity when stimulated, suggesting that platinum's conductivity does not effectively improve cellular response to electrical stimuli^[91]. Under electrical stimulation, all hydrogels maintain this pattern by Day 3, indicating a complex and potentially unbeneficial interaction with platinum^[92]. The overall decline in metabolic activity across all hydrogel compositions on Day 7 indicates that this stimulation protocol was ineffective for these hydrogel compositions.

Aligning with the metabolic activity findings, the DNA extraction results from the hydrogels, displayed in Figure 7B, reveal a decrease in DNA content under stimulated conditions. The negative impact of electrical stimulation could be attributed to specific underlying mechanisms likely dependent on various conditions such as the nature of the electrical stimulation, cell type, and hydrogel properties, indicating a level of cellular resilience or adaptation in the genetic material to the stimulation protocol^[93].

Figure 7C shows the live and dead staining of the PC12 cells encapsulated within the hydrogels onto platinum substrate, under both passive and stimulation conditions, taken at intervals over 7 days. On Day 1, both passive and stimulated GelMA samples show a moderate number of live cells, with stimulated samples indicating potentially lower activity or cell count. GelMA-GO and GelMA-GO-AuNRs exhibit lower fluorescence under stimulation, aligning with metabolic activity and DNA extraction data. By Day 3, GelMA shows reduced fluorescence intensity under stimulation, suggesting decreased viability or metabolic activity, while unstimulated samples maintain intensity. GelMA-GO and GelMA-GO-AuNRs passive samples show high fluorescence, but the stimulated samples dim, indicating reduced activity under stimulation over time. On Day 7, GelMA's brightness decreases under stimulation,

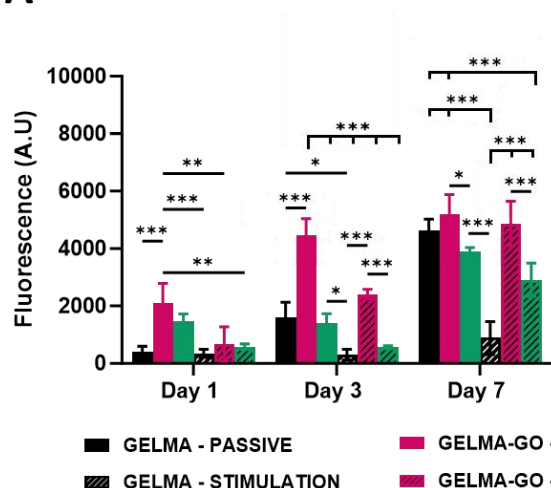
pointing to possible cell death or reduced metabolic activity ^[94]. Both GelMA-GO and GelMA-GO-AuNRs exhibit similar intensities for passive and stimulation conditions.

The observed decline in cellular activity on the platinum substrate is likely due to multiple interacting factors. Its high surface area and reactivity can trigger oxidative stress in cells, disrupting vital functions and metabolic processes ^[95]. Additionally, the inherent positive charge of platinum surfaces might hinder optimal cell adhesion and signalling ^[96]. Furthermore, metal ion leaching from platinum under specific conditions could disrupt cellular viability and function through protein and enzyme interactions ^[97]. These combined effects could explain the observed decline in metabolic activity. To ensure safe and effective neural interface applications, future research should explore alternative conductive materials with minimal detrimental effects on cellular behaviour, focusing on strategies to mitigate oxidative stress, control surface charge, and prevent metal ion leaching ^[98].

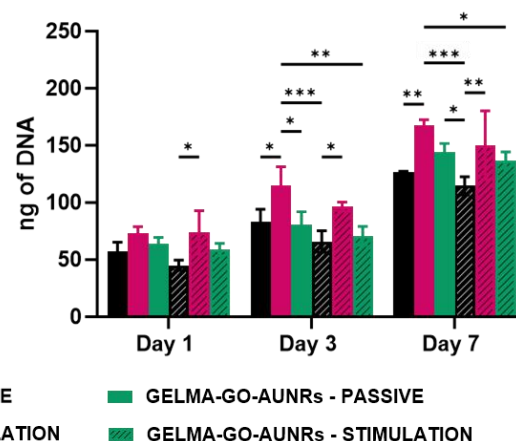
Figure 8 illustrates the metabolic activity, DNA content and live/dead staining of PC12 cells encapsulated within hydrogels on a gold mylar substrate under passive and stimulation conditions over a 7-day period. Gold mylar unique surface properties include conductivity and surface energy, hold potential for influencing cell behaviour under electrical stimulation ^[99].

GOLD MYLAR

A



B



C

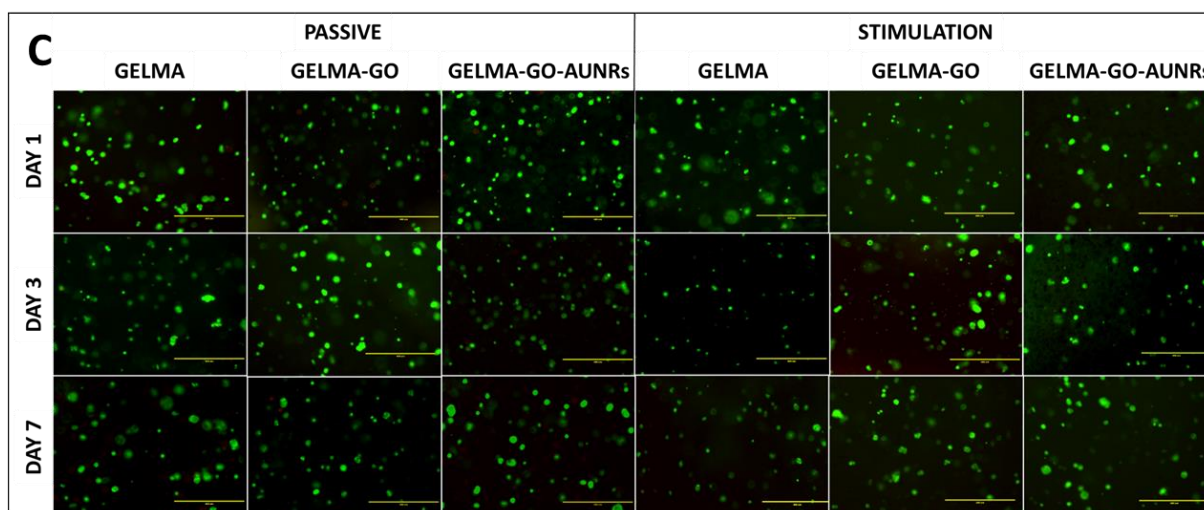


Figure 8 - Cell viability and proliferation of PC12 cells in hydrogels on gold mylar substrates under electrical stimulation. A) The bar graph shows the metabolic activity measured with Cell Titer blue assay and reported as absolute fluorescence units for or all the conditions listed at the bottom of the graph along the 7 day time course analysis. B) The bar graph shows the DNA amount extracted and quantified with PicoGreen and reported as ng of DNA from the same samples of A. C) Representative images of the Live and Dead staining performed on the samples reported along a 7 day time course analysis. The images have been taken with an epifluorescence microscope using 20 $\times$ objective and the merged signals of Calcein staining (Live-green channel) and of the ethidium homodimer staining (Dead-red channel) are presented. The scale bar denotes 400 μ m. All statistical analyses have been performed with ANOVA where the errors bars show the standard deviations of the average measurements of three samples ($n = 3$), * indicates a significant difference ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

Figure 8A shows the metabolic activity PC12 cells encapsulated within hydrogels onto gold mylar substrate under passive and stimulation conditions over a period of 7 days. Under both conditions, hydrogels on gold mylar show similar metabolic activity on Day 1, but GelMA-GO (striped pink) presenting a decrease in metabolic activity under stimulation suggesting a unsuitable environment for cell growth ^[100]. Compared to passive conditions, all hydrogels display a significant metabolic decline by Day 3, suggesting an unsuitable growth environment for cells ^[101]. On Day 7, the same trend is observed, indicating that electrical stimulation fails to enhance cellular activity over the long term. This trend suggests a non-beneficial, or even inhibitory ^[102], effect of stimulation of GelMA based hydrogels on gold mylar. Contrasting with metabolic activity, DNA content (Figure 8B) remains steady across conditions, suggesting that while stimulation affects metabolic processes, it does not significantly impact cellular DNA content.

Figure 8C shows the live and dead staining of the PC12 cells encapsulated within the hydrogels onto gold mylar substrate, under both passive and stimulation conditions, taken at intervals over 7 days. Initially, on Day 1, all hydrogels show similar live cell counts under both passive and stimulation conditions, consistent with the metabolic activity data. However, by Day 3, GelMA exhibit decreased cell number under stimulation, indicating reduced metabolic activity. In contrast, GelMA-GO maintains stable fluorescence, suggesting unchanged cell viability. On Day 7, GelMA and GelMA-GO continuing to show reduced fluorescence under stimulation, while GelMA-GO-AuNRs remains consistent. These findings from the live and dead assay confirm that electrical stimulation does not improve, and may reduce, metabolic activity and cell viability in GelMA-based hydrogels on gold mylar over 7 days, highlighting that gold mylar's properties do not enhance cell survival in this context.

Similar to platinum, gold ion leaching might disrupt cellular homeostasis by interfering with vital enzymatic processes and membrane integrity, potentially contributing to the observed metabolic decline and cell death ^[103]. Additionally, the combination of electrical stimulation and gold mylar properties might create a synergistic detrimental effect. Electrical fields themselves can induce stress on cells ^[104]. The specific interactions of gold mylar surface charge and potential ion leaching could lead to the observed decline in cellular health ^[105].

Conclusion

Under electrical stimulation, GelMA-based hydrogels show complex interactions between substrate characteristics and the electrochemical properties, offering new insights into substrate selection as a platform for neural cell stimulation. The findings underscore the significant impact of substrate selection on the behaviour of conductive hydrogels and, consequently, on cell viability and proliferation. Different substrates, including ITO, platinum, and gold mylar, influenced the electrochemical behaviour of the hydrogels and the metabolic activity of PC12 cells in distinct ways. Our findings suggest that the substrate material plays a pivotal role in optimizing the efficacy of neural interfaces for specific biomedical applications such as tissue engineering and neural interface devices.

The different responses of PC12 cells cultured on these substrates, as well as within various hydrogel environments, under electrical stimulation protocols, provide valuable data for advancing tissue engineering and regenerative medicine. Notably, comparing the conductive hydrogels to 2D groups across all substrates revealed a protective effect against cell death, underscoring their potential to improve 3D cell viability. Our findings on cell viability, proliferation assays, and metabolic activity indicate that the choice of substrate can either enhance or inhibit cellular responses to electrical stimuli. During the 7-day stimulation period, ITO showed better cell viability than platinum or gold mylar in the 3D culture system. While platinum and gold mylar initially exhibited a detrimental effect on cells, they stabilized by Day 7, indicating a dynamic response of cells to these conductive metals. Moreover, the conductive hydrogels provide a protective effect against cell death to cells encapsulated within the conductive hydrogels. This is crucial for the practical application design of neural interfaces.

Furthermore, a crucial gap has been identified and bridged in understanding the interaction between substrates and conductive hydrogels, and how this affects cellular behaviour as a result. In the future, future research could focus on optimizing substrate-hydrogel combinations and underlying mechanisms for the observed effects. The implications of these findings are significant for the advancement of tissue engineering, biosensor technology, and the broader field of biomedical engineering. This study not only extends the existing body of work but also provides a robust platform for future explorations aimed at tailoring the full potential of tissue-engineered neural interfaces.

Author contributions

A.T.N, S.M.S and S.E.M conceived and designed the experiments. A.T.N, analysed the data, and wrote the manuscript. L.C.A performed the contact angle. A.X.M., S.D, D.D, A.F.Q,

R.M.I.K, D.R.N, P.R.S, S.M.S and S.E.M supervised the project, suggested experiments, and revised the manuscript.

Conflicts of interest

There are no conflicts to declare.

Supplementary information

Provides additional experimental details, materials, methods, and further electrochemical characterization of the substrates.

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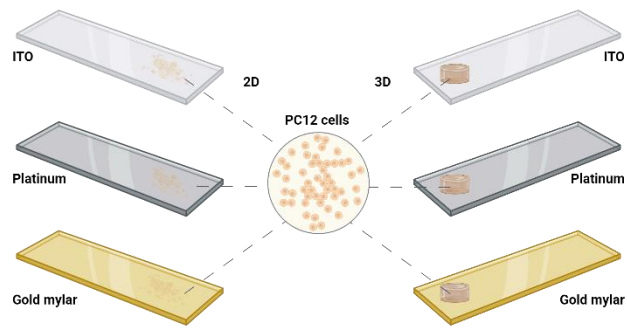
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