Supporting information

The impact of electrical stimulation protocols on neuronal cells survival and proliferation using cell-laden GelMA/Graphene Oxide hydrogels

Alexandre Xavier Mendes [a,b]; Adriana Teixeira do Nascimento [a,b]; Serena Duchi [b,c]; Anita F. Quigley [b,c,e]; Lilith M. Caballero Aguilar [a,b]; Chaitali Dekiwadia [f]; Robert M.I. Kapsa [a,b,c,e]; Saimon Moraes Silva [a,b,g*] ;Simon E. Moulton [a,b,g*]

[a] *ARC Centre of Excellence for Electromaterials Science, School of Science, Computing and Engineering Technologies, Swinburne University of Technology, Melbourne, Victoria 3122, Australia.*

[b] *The Aikenhead Centre for Medical Discovery, St Vincent's Hospital Melbourne, Melbourne, Victoria 3065, Australia.*

[c] *Department of Surgery, University of Melbourne, St Vincent's Hospital Melbourne, Victoria 3065, Australia*

[d] *School of Electrical and Biomedical Engineering, RMIT University, Melbourne, Victoria 3001, Australia.*

[e] *Department of Medicine, University of Melbourne, St Vincent's Hospital Melbourne, Victoria 3065, Australia.*

[f] *RMIT Microscopy and MicroAnalysis Facility (RMMF), STEM College, RMIT University, Melbourne, VIC, 3000, Australia*

[g] *Iverson Health Innovation Research Institute, Swinburne University of Technology, Melbourne, Victoria 3122, Australia.*

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Figure S1 *– Stimulation system based on the digital stimulator, isolator unit, e-corder, electrochemical chambers and incubator.*

The stimulator setup used to electrical stimulate cells comprises five components: digital stimulator, isolator unit, e-corder, electrochemical chamber and incubator. The digital stimulator DS8000 is used to create the waveform output. The isolator unit, through a regulator, controls the current amplitude. E-corder + computer is used to record the response in the system due to secondary connections. Finally, electrochemical chambers hold the samples (hydrogels or ITO surfaces) and supply a structure to receive the electrolyte solution (proliferation media). Thus, the incubator provides the best conditions for cell incubation.

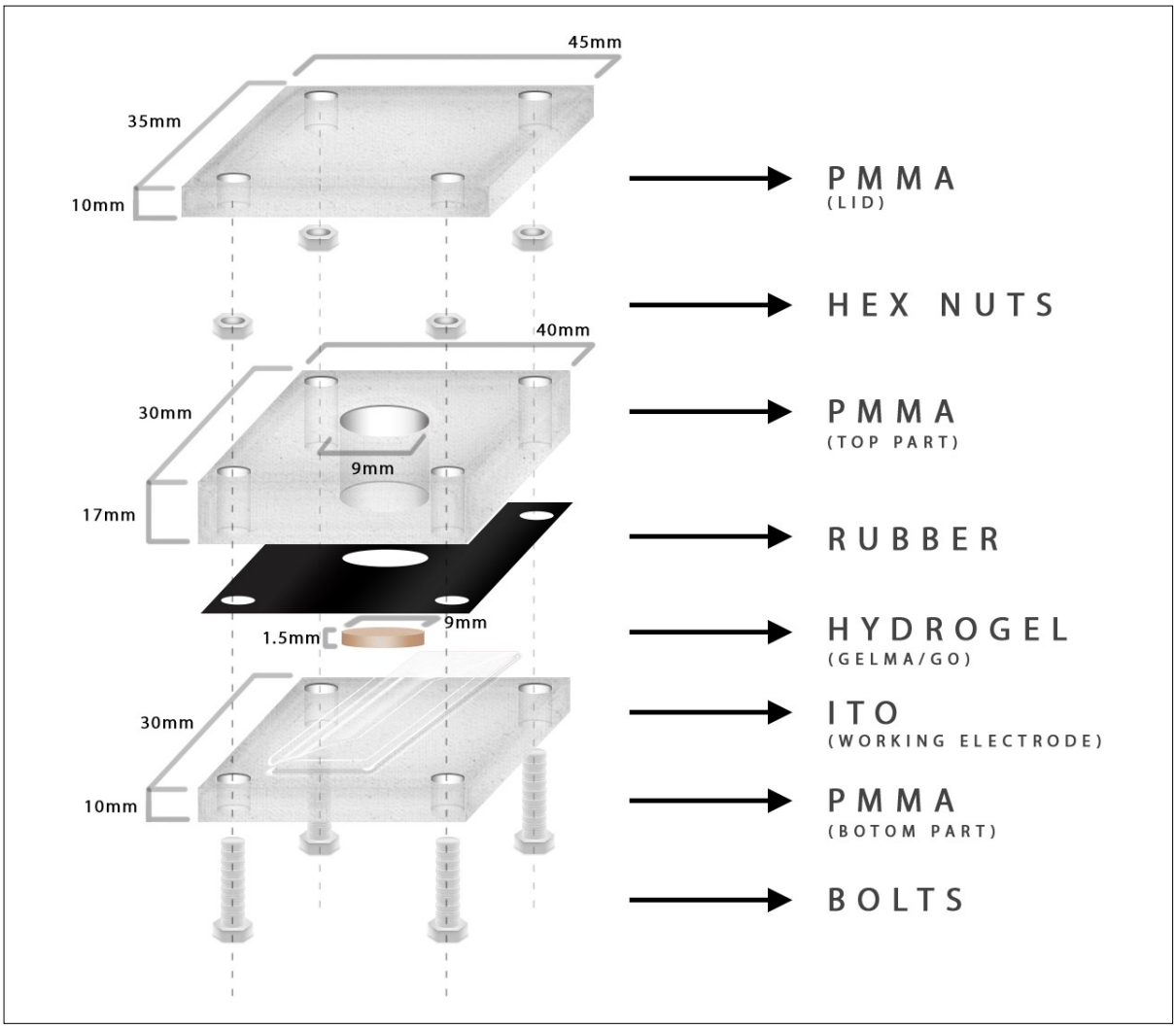

Figure S2 - *Custom-made electrochemical chamber used for electrical stimulation of cells.*

The electrochemical chamber was composed of three parts, the main chamber, a base, and a lid. The main chamber dimensions were 40 mm (length) x 30 mm (width) x 17 mm (height), the base was 40 mm (length) x 30 mm (width) x 10 mm (height) and lid were 45 mm (length) x 35 mm (width) x 10 mm (height). The chamber's main part (for sample loading) presented a hole in a cylinder shape with a diameter of 9 mm. A 1mm-thick Viton ® (DuPont Performance Elastomers L.L.C, USA) seal was used to seal both electrochemical chamber parts and avoid leaks. Four stainless-steel bolts (25 mm) and nuts were used to close the electrochemical chamber. The lid was placed on top of the chamber above the platinum mesh closing the apparatus to avoid contamination during the cell's electrical stimulation

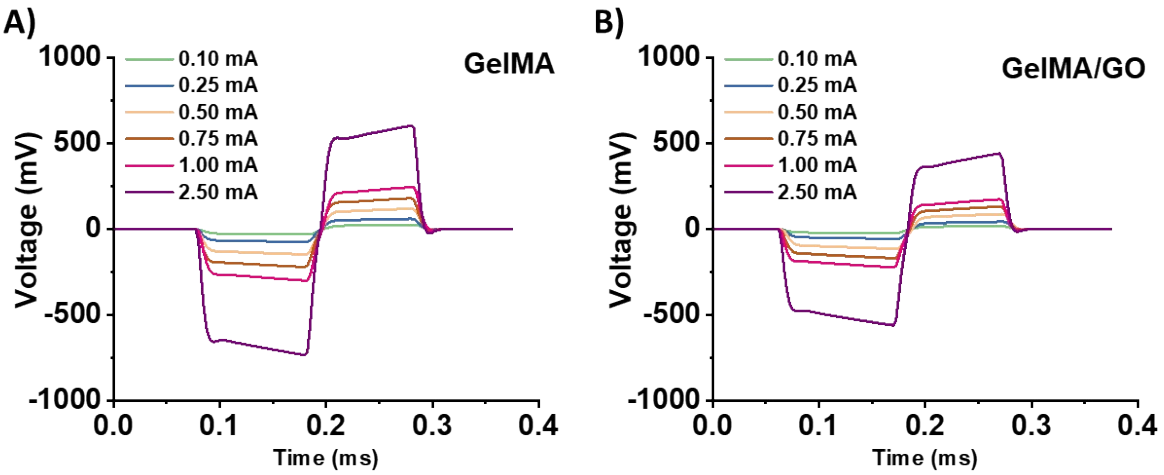

Figure S3 *– A) and B) Voltage Transient Measurements of GelMA and GelMA/GO, respectively, analyses done over different current amplitudes (0.1 to 2.5 mA). All scenarios presented an increase in the voltage response with increased current amplitude. GO samples present a lower voltage response when compared to GelMA hydrogels, which means that GelMA/GO samples need a lower voltage to delivery the same amount of current when compared to GelMA hydrogels. This behaviour might be related to the electrical properties of Graphene oxide.*

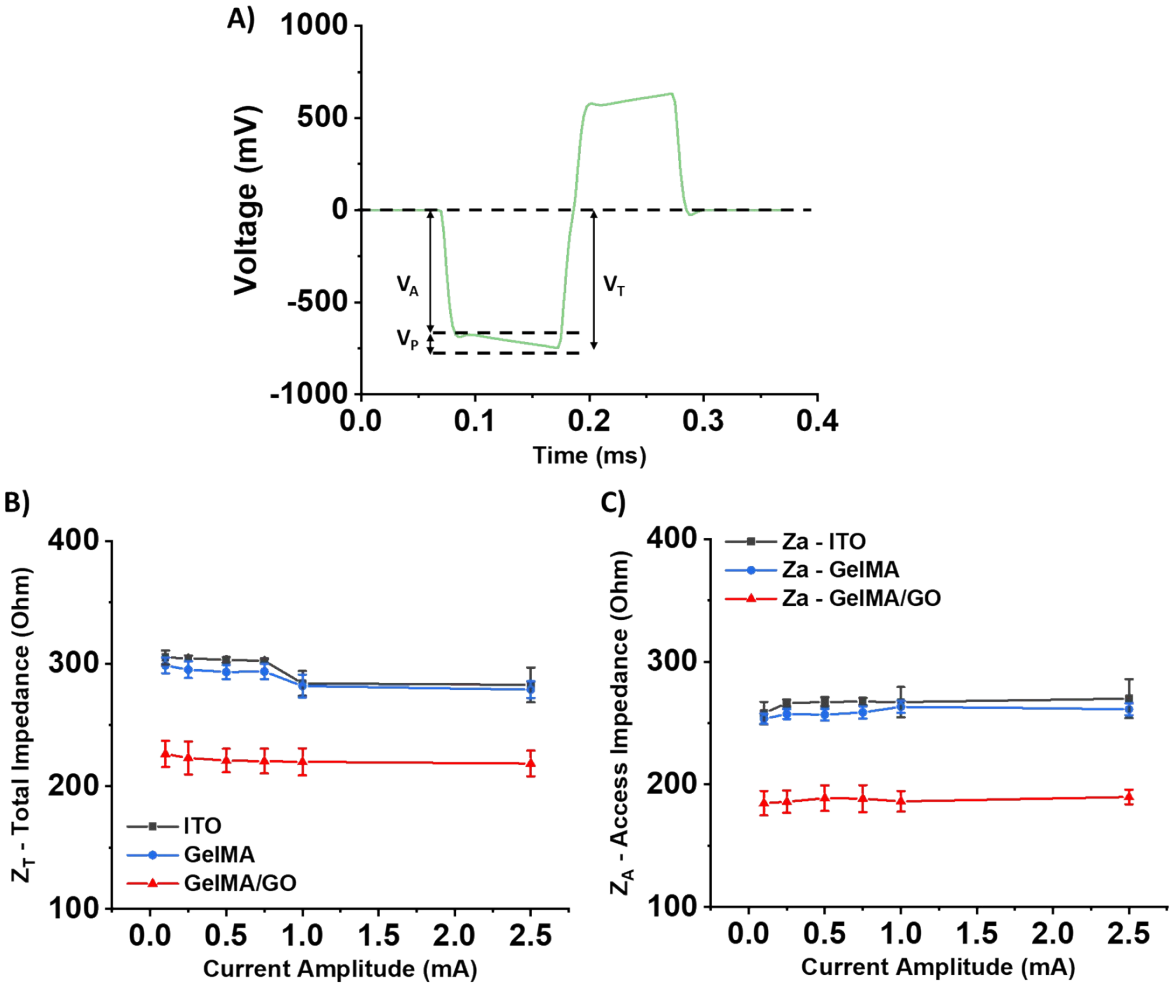

Figure S4 – *Example of voltage response waveform when a charge biphasic current pulse is applied to the system. V_T, V_A and V_P represents the total voltage, access voltage and polarization voltage respectively. Impedance values are calculated based on those voltage values using the Ohm's law. B), C) represent the total impedance and access impedance values of ITO, GelMA and GelMA/GO samples. GelMA and ITO samples present similar values for impedances over all the current amplitudes. Furthermore, GelMA/GO samples show a decrease in the impedance values to all current amplitudes compared to GelMA hydrogels. (n=3)*

The impedance values were calculated using Ohm's law using the equation below:

$$Z = V/I$$

where Z is the impedance, V is the voltage (value extracted from the voltage transient measurements), and I is the current applied.

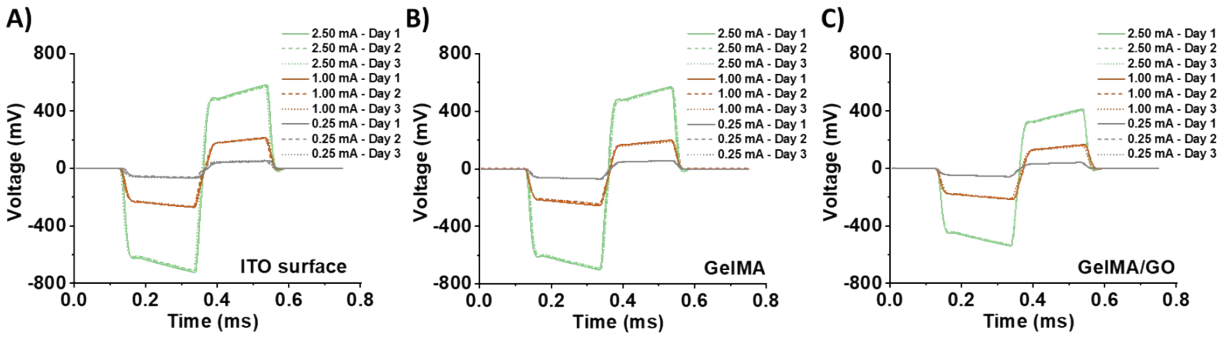

Figure S5 *– Voltage transient measurements of GelMA and GelMA/GO samples over different stimulation times at current amplitudes of 0.25, 1.00 and 2.50 mA. A) ITO surface B) GelMA and C) GelMA/GO. All samples present good stability on the voltage transient measurements over stimulation time even after three days. (n=3)*

Table S1 *– Values of polarization impedance (Zp) for all the samples over three different current amplitudes, 0.25, 1.00 and 2.50 mA and different days of stimulation.*

	0.25 mA	**1.00 mA**	**2.50 mA**
Sample	***Zp***	***Zp***	***Zp***
ITO - Day 1	37.6 ± 4.5	36.4 ± 3.2	35.2 ± 7.8
ITO - Day 2	39.2 ± 4.2	37.3 ± 3.8	35.6 ± 8.1
ITO - Day 3	39.5 ± 3.7	37.6 ± 3.9	35.9 ± 8.1
GelMA - Day 1	35.8 ± 3.8	37.1 ± 4.2	36.6 ± 3.1
GelMA - Day 2	36.1 ± 2.5	36.4 ± 2.7	34.8 ± 2.3
GelMA - Day 3	35.4 ± 3.1	35.5 ± 3.3	35.5 ± 2.8
GelMA/GO - Day 1	24.1 ± 1.4	17.5 ± 0.7	12.9 ± 1.7
GelMA/GO - Day 2	25.4 ± 2.9	19.2 ± 2.3	11.3 ± 2.2
GelMA/GO - Day 3	24.3 ± 2.7	15.8 ± 1.8	9.6 ± 2.2

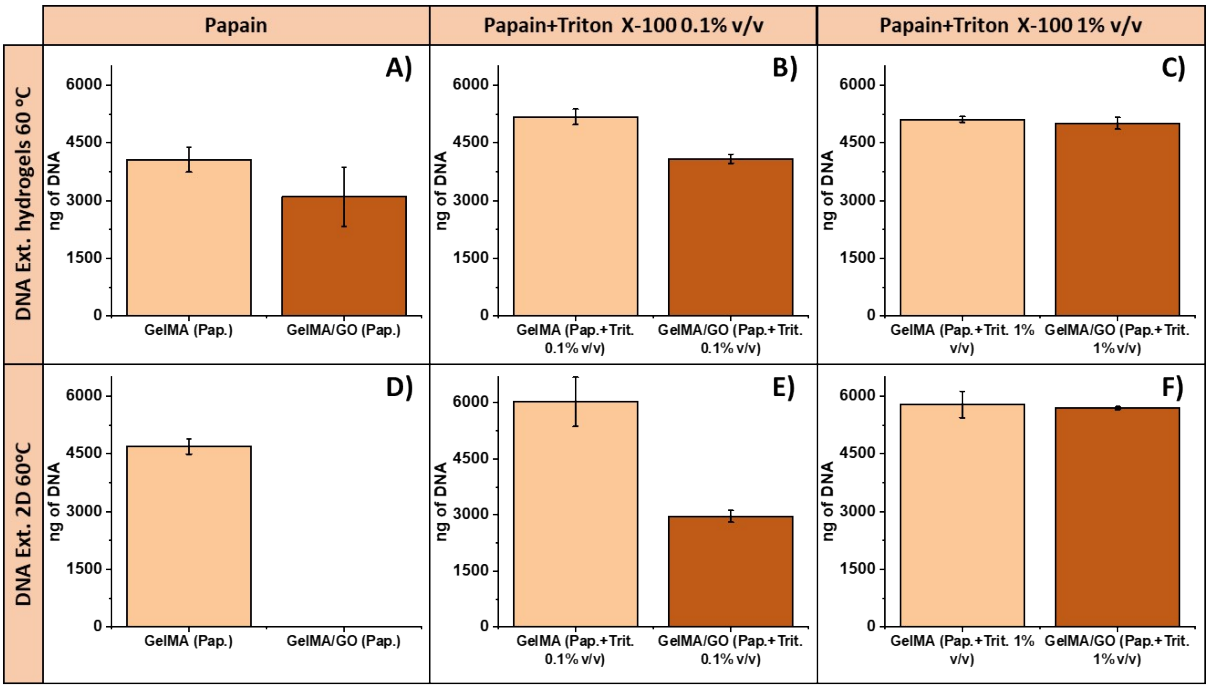

Figure S6 *– Analyses of PicoGreen of 3D scaffolds and 2D tissue plate culture over three different approaches: papain buffer, papain buffer + Triton X-100 0.1% v/v and papain buffer + Triton X-100 1% v/v. DNA extraction was done straight after the crosslinking reaction or seeding of cells on the tissue culture plate, avoiding the proliferation of cells. A) to C) DNA extraction from cell-laden hydrogels at 60 oC. Only on the condition papain + Triton X-100 1% v/v presented similar values of DNA amount for GelMA and GelMA/GO samples. D) to E) DNA extraction for cells seeded on tissue plate culture, where GelMA and GelMA/GO were mixed in solution (no crosslinking reaction). GelMA/GO samples without Triton X-100 and no crosslinking reaction do not disperse into the media, forming big colloids and causing no cell proliferation, which explains the no expression of DNA after the PicoGreen assay. PicoGreen response for both scenarios presented similar behaviour, where Triton X-100 1% v/v showed the best scenario for extraction of DNA. (n=3)*

Triton X-100 acts as a detergent to lyse the cells' membrane, and papain is a peptidase enzyme that will enzymatically break down the cell's proteins. Also, in the extraction of DNA, the Triton-X helps release the DNA from GO sheets (Kim et al., 2015). The efficiency of the papain buffer + Triton X-100 DNA extraction method was investigated prior to the DNA analyses of samples. Two different scenarios, laden cells into hydrogels and cells into 2D surfaces, in three different conditions were analysed: extractions using i) papain buffer, ii) papain buffer + Triton X-100 0.1% v/v, and iii) papain buffer + Triton X-100 1% v/v. DNA content of PC12 from GelMA and GelMA/GO hydrogels was compared with the DNA of the

same amount of cells seeded onto a tissue plate culture. The DNA extraction process was started straight after the crosslinking reaction or seeding of cells, avoiding any proliferation.

Figure S6 shows the results from PicoGreen analyses of DNA extraction. The amount of DNA is expressed as ng of DNA. The first scenario is represented as DNA extracted from encapsulated PC12s from GelMA and GelMA/GO hydrogels and the second from DNA extracted from PC12s seeded on tissue plate cultures in the presence of GelMA and GelMA/GO in solution, which means no crosslink reaction. The same number of cells were used for all the conditions, and DNA was extracted straight after the encapsulation or after seeding cells at tissue plate culture. The main idea was to understand how Triton X-100 could help in the release of DNA that binds to GO during the process of papain buffer extraction. Triton-X acts as a non-ionic surfactant, helping desorption DNA from GO (Kim et al., 2015).

Figure S6A, B and C, show the results to the DNA extracted from encapsulated PC12s using papain, papain + Triton X-100 0.1% v/v and papain + Triton X-100 1.0% v/v respectively. Only the DNA extracted using papain + Triton X-100 1.0% v/v (Figure S6C) shows a similar amount of DNA for GelMA and GelMA/GO samples. A similar analysis was done in Figures S6D, E and F, representing the DNA extracted using the same buffers but from PC12s not encapsulated. In this scenario, cells were suspended into a media solution mixed with GelMA and/or GelMA/GO without exposure to UV light and photocrosslinking reaction. Figure S6D shows no expression of DNA for the samples where GelMA/GO was in suspension; those results might be related to GO not dispersing into the media and forming big colloids, causing no cell proliferation. Also, any DNA traces should be adsorbed into the GO sheets, which explains the no expression of DNA after the PicoGreen assay. In the case of the other two conditions with Triton-X, Figures S6E and F, Triton-X allied the GO to disperse into the solution and helped desorption DNA from GO. Even though the Triton-X helped in this analysis, the amount of 0.1% v/v was not sufficient to achieve the best desorption, and we decided to use a higher concentration of 1% v/v, which expressed similar results of DNA quantification for both scenarios when comparing the results from Figures S6C and F.

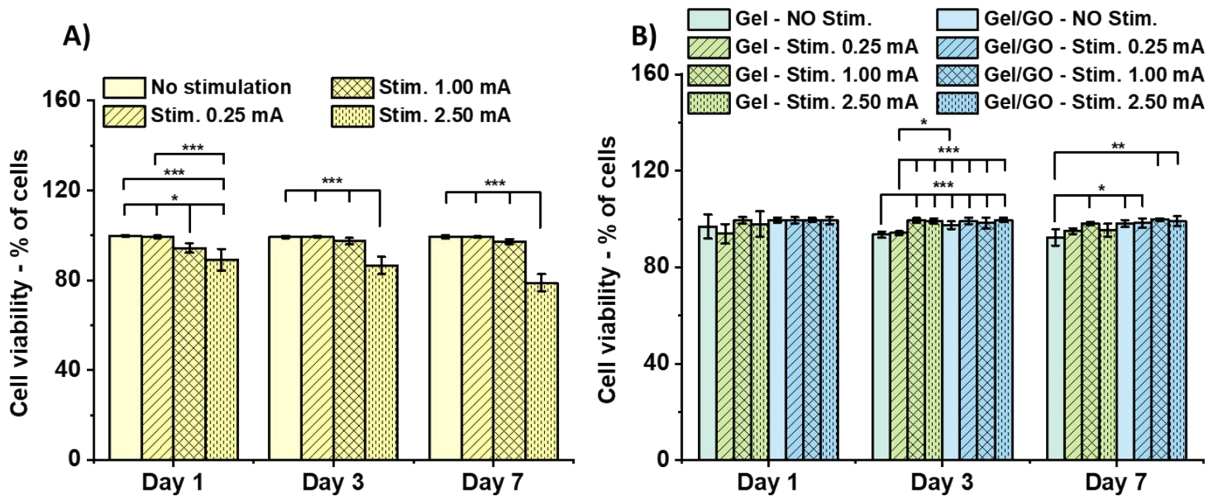

Figure S7 *– Cell viability from live/dead cells staining images. A) Cells seeded into ITO surfaces from unstimulated and stimulated samples over different current amplitudes. On day 1, there was a minimal effect (~1%) in cell viability in unstimulated ITO surfaces and a current amplitude of 0.25 mA. With the current amplitude of 1.00 and 2.50 mA, PC12 cells demonstrated the viability of approximately 94 and 90%, respectively. At three days of stimulation, a decrease in cell viability was observed in the cell at the current amplitude of 2.50 mA compared to unstimulated samples and the other current amplitudes. This effect was also seen on Day 7, with a decrease in the cell viability of ~ 21%. B) Cells encapsulated into GelMA (green bars), and GelMA/GO (blue bars) hydrogels over different current amplitudes of stimulation, a minimal effect (~4-6%) in cell viability of GelMA samples is observed for the current amplitudes of 0.25 mA at day one and day three, and at current amplitudes of 0.25, and 2.50 mA at day 7. (*Indicates significant different ($p < 0.05$) and ** indicates significant different ($p < 0.01$), and *** indicates significant different ($p < 0.001$); if not stated $p > 0.05$; n=3)*

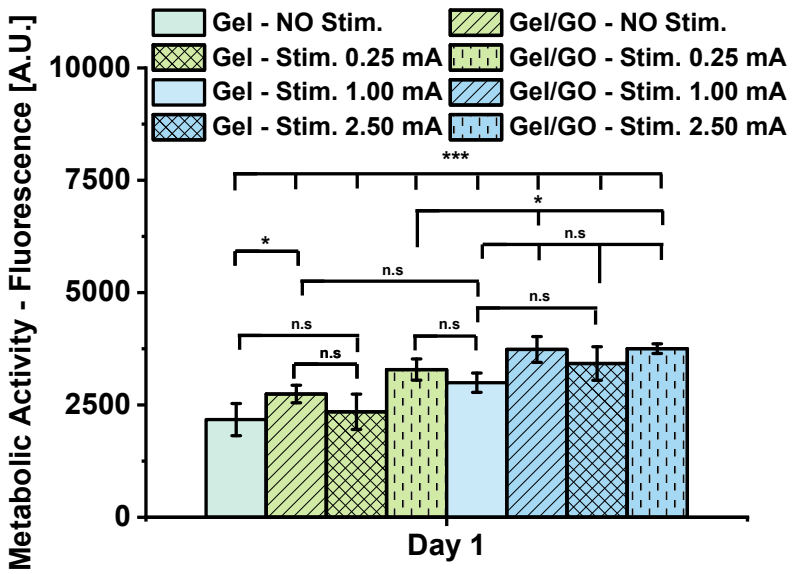

Figure S8 *– Metabolic activity of unstimulated and stimulated PC12 cell-laden hydrogels at day 1. All stimulated samples presented an average higher metabolic activity when compared to the respective unstimulated samples. For all current amplitudes, GelMA/GO presented a higher metabolic activity when compared to GelMA hydrogels at the same current amplitude. Metabolic activity of GelMA/GO stimulated at current amplitudes of 1.00, and 2.50 mA does not show any statistical differences. (*Indicates significant different ($p < 0.05$) and ** indicates significant different ($p < 0.01$), and *** indicates significant different ($p < 0.001$); n.s indicates $p > 0.05$; n=4)*

Table S2 *– Values of metabolic activity times fold*

Sample	Day 1	Day 3	Day 7
GelMA - No stim. *	1.0	1.0	1.0
GelMA - 0.25 mA *	1.1	1.8	1.1
GelMA - 1.00 mA *	1.3	2.8	1.9
GelMA - 2.50 mA *	1.5	3.3	2.4
GelMA/GO - No stim. **	1.0	1.0	1.0
GelMA/GO - 0.25 mA **	1.2	1.5	1.5
GelMA/GO - 1.00 mA **	1.4	2.1	1.9
GelMA/GO - 2.50 mA **	1.4	3.0	2.5
GelMA/GO - No stim. *	1.2	1.8	1.6
GelMA/GO - 0.25 mA *	1.4	2.8	2.4
GelMA/GO - 1.00 mA *	1.6	3.8	3.2
GelMA/GO - 2.50 mA *	1.6	5.6	4.1
ITO - No stim. ***	1.0	1.0	1.0
ITO - 0.25 mA ***	0.9	1.3	1.1
ITO - 1.00 mA ***	0.9	2.0	1.3
ITO - 2.50 mA ***	1.1	0.8	0.8

* Comparison with GelMA no stimulation, ** Comparison with GelMA/GO no stimulation and *** Comparison with ITO no stimulation

Table S3 *– Values of DNA content times fold*

Sample	Day 1	Day 3	Day 7
GelMA - No stim. *	1.0	1.0	1.0
GelMA - 0.25 mA *	1.2	1.4	1.3
GelMA - 1.00 mA *	1.0	2.1	1.9
GelMA - 2.50 mA *	1.2	2.5	2.1
GelMA/GO - No stim. **	1.0	1.0	1.0
GelMA/GO - 0.25 mA **	1.1	1.4	1.2
GelMA/GO - 1.00 mA **	1.4	2.1	1.5
GelMA/GO - 2.50 mA **	1.9	2.4	2.2
GelMA/GO - No stim. *	1.2	1.5	1.7
GelMA/GO - 0.25 mA *	1.3	2.1	2.1
GelMA/GO - 1.00 mA *	1.6	3.0	2.4
GelMA/GO - 2.50 mA *	2.3	3.5	3.6
ITO - No stim. ***	1.0	1.0	1.0
ITO - 0.25 mA ***	0.8	1.0	1.3
ITO - 1.00 mA ***	1.1	1.5	2.0
ITO - 2.50 mA ***	0.8	0.9	0.9

* Comparison with GelMA no stimulation, ** Comparison with GelMA/GO no stimulation and *** Comparison with ITO no stimulation

Table S4 *- Ratio between metabolic activity and DNA amount*

Sample		No Stim.	Stim. 0.25 mA	Stim. 1.00 mA	Stim. 2.50 mA
ITO	*Day 1*	147.1 ± 30.8	169.5 ± 47.5	97.4 ± 19.2	240.0 ± 69.3
	Day 3	153.9 ± 41.8	175.6 ± 20.9	155.7 ± 22.3	132.7 ± 33.9
	Day 7	221.0 ± 44.0	189.3 ± 11.9	197.2 ± 33.2	223.2 ± 50.4
GelMA	*Day 1*	71.2 ± 18.2	60.2 ± 3.2	99.8 ± 24.4	94.5 ± 17.3
	Day 3	480.2 ± 55.5	603.8 ± 66.2	629.9 ± 41.8	592.4 ± 90.0
	Day 7	841.3 ± 33.7	710.5 ± 103.8	831.1 ± 84.1	897.6 ± 103.9
GelMA/GO	*Day 1*	73.6 ± 6.7	80.4 ± 9.7	72.9 ± 6.8	54.1 ± 5.9
	Day 3	616.4 ± 75.7	650.7 ± 71.9	628.8 ± 19.5	765.8 ± 86.6
	Day 7	851.7 ± 60.9	1028.0 ± 105.3	1058.4 ± 74.2	1059.3 ± 124.0

REFERENCES

KIM, S., PARK, C. & GANG, J. 2015. Effect of pH and Salt on Adsorption of Double-Stranded DNA on Graphene Oxide. *J Nanosci Nanotechnol,* 15**,** 7913-7.