Patterned, Transparent and Functionalized Inkjet-Printed Substrates for Neuronal Culture Studies

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Abstract: Many diseases related to the neural network are difficult to study in its natural environment. Due to that fact, the functions and properties of neurons are studied in cultures *in vitro*. This work attempts to manufacture a patterned transparent and biocompatible substrate based on a repulsive background of poly(ethylene) glycol (PEG) and an inkjet-printed adhesive molecule of a collagen/poly-L-lysine (PLL) mixture above it. This ink, totally designed in this study, allows printing specific patterns where neurons will adhere. It was possible to get a characterized repulsive background and a printable adhesive molecule, obtaining biocompatible printed patterns.

I. INTRODUCTION

Many diseases like Alzheimer, Parkinson or epilepsy are related to neural network, the complexity of this system makes difficult to study it in its natural environment, our body. In 1979, Bankers demonstrated that neurons kept their characteristics (neuronal polarity, dendritic growth and synapse formation) in culture [1]. This fact opened the possibility for many researches to deal with neuronal culture *in vitro* to study the functions and properties of neurons.

In order to analyze the properties and evolution of neurons in culture, a biocompatible and functionalized substrate is required. Electrical neuronal activity must be able to be measured in these low-cost substrates, moreover if the substrates are transparent, optical activity can be analyzed too. Early works described how neurons can be fixed in a substrate in order to keep them on certain locations for a period of time. There are several techniques to achieve this, some of them use photolithography [2], modifications of the surface topography [3] or microcontact printing [4], for example. Nevertheless, all this procedures are limited to certain materials and need complicated procedures or expensive facilities. To solve this, inkjet printing is proposed as a method to pattern transparent substrates and deposit some organic molecules where neurons will kept attached. Inkjet printing (IP) is based on an applied voltage that deforms a piezoelectric, this generates a pulse in the ink chamber forcing a droplet to get out of a nozzle [5]. This makes IP a low-cost technique that wastes few material and has a flexible production of different components. Moreover, it provides an easy digital design. There are many works based on inkjet printing and inorganic inks (silver, graphene, boron nitride, etc). However, to assure the biocompatibility between substrate and neurons, the research of organic inks has developed a huge interest.

Turcu et al. (2003) [6] used a piezo-based microdispenser to print line patterns of a polymer based on Viannapas® and laminin above glass, silicon, gold and carbon substrates. In 2004, Sanjana and Fuller [7] developed a method for patterning dissociate neurons in culture depositing by inkjet printing a mixture of collagen and poly-D-lysine (PDL) on neuro-repulsive substrates treated with poly(ethylene) glycol (PEG). A simple microdeposition set-up based on a piezo-dropper system was designed by Macis et al. (2006) [8] to deposit an adhesive solution of laminin and poly-L-lysine (PLL) on microelectrode arrays. Fujie et al. (2012) [9] printed fluoresceine isothiocyanate modified poly-L-lysine (FITC-PLL) through a drop-on-demand ink-jet printer on polymethylmethacrylate (PMMA) nanofilms to promote skeletal muscle cells adhesion.

The aim of this work is to manufacture a patterned transparent and biocompatible substrate formed by a cell-repulsive background and a cell-adhesive molecule above it, to get a neuronal culture adhered only where it is desired. The adhesive molecule ink is totally designed in this study to be able to be inkjet-printed, which allows getting specific patterns.

II. EXPERIMENTAL

The experimental procedure is based on developing a substrate with a repulsive background and an inkjet-printed adhesive molecule to get a neuronal culture on it (*FIG. 1*).

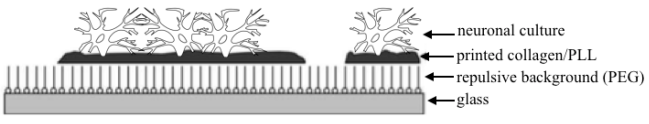

FIG. 1: Scheme (based on [7]) of the patterning process: repulsive coating on a glass coverslip with the inkjet-printed mixture of collagen/PLL above it, where neurons should adhere.

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The background needs to be cell-repulsive so that the neurons tend to avoid those areas, fixing only where the adhesive molecule is printed.

To get the repulsive poly(ethylene) glycol (PEG) background several steps, based on [7], were followed on round coverslips (cover glass, 13 mm diameter, Deckgläser):

1. Coverslips were cleaned in a 10 vol% aqueous solution of KOH overnight so that they become free from contaminants and hydrophilic.

2. Then, coverslips were submerged for 30 minutes in a 2.5 vol% aqueous solution of aminopropyltriethoxysilane (Sigma-Aldrich) to expose reactive amino groups.

3. Coverslips were rinsed in water and ethanol before drying them in a vacuum oven overnight at 80°C.

4. The substrates were soaked for 2 hours in a 2.5 vol% aqueous solution of homobifunctional crosslinker glutaraldehyde (Sigma-Aldrich) in a 0.1M aqueous solution of sodium carbonate (Na_2CO_3), exposing an aldehyde group that is reactive with amino groups.

5. Coverslips were soaked for 1-2 days before printing in a 0.5 wt% aqueous solution of amino-terminated PEG (Sigma-Aldrich).

All this procedure was done in a laminar flow cabinet.

To verify that all the desired molecules were deposited correctly, the substrates were analyzed using Fourier-Transform Infrared Spectroscopy (FTIR) (Thermo Scientific Nicolet iN10 MX, reflection, MCT detector). This method is based on how well a material absorbs light at each wavelength. So we can identify which molecules are present in the coverslips. FTIR uses specular reflection, this means that you only receive the reflected rays that return in the same angle you have sent them, losing the rest of information. However, we could not obtain functional results with this technique due to the glass substrate provided too many reflections causing interferences with the molecular information. So we repeated the measures with Attenuated Total Reflexion-IR (ATR-IR) (Thermo Scientific Nicolet iZ10, ATR diamant, detector DTGS). ATR-IR focuses infrared light through the ATR crystal in a way that it reflects at the internal surface in contact with the substrate. This reflection creates an evanescent wave that penetrates a few micrometers deep on the substrate and returns outside giving us all the information of the superficial substances.

The adhesive molecule ink has to be biocompatible with neuronal cells so they can adhere to it and survive, moreover it has to be able to be inkjet-printed above the repulsive background. We tested several aqueous solutions of a 25:1 mixture of collagen (4 mg/ml) (Sigma-Aldrich) and poly-L-lysine (PLL) (1 mg/ml) (Sigma-Aldrich) (1 wt%, 20 wt%, 50 wt% and 100 wt%). Collagen is used in the study of growth, differentiation, migration of cell lines and tissue morphogenesis during development, while PLL facilitates the attachment of cells to solid surfaces [10]. In Ref. [7] it is used poly-D-lysine (PDL) instead of PLL, the main difference is in their chirality, but they have the same functionality.

The adhesive molecule ink was printed in a Fujifilm Dimatix Materials Printer (DMP-2800 Series). To be inkjet-printed, the ink must have several requirements. The viscosity is one of the most important, it must be between 2-30 cP to assure its printability, but the optimal range is between 10-12 cP [11]. So we analyzed different concentrations of the collagen and PLL solution in water with a viscometer (Brookfield LVDV-I Prime).

The contact angle of the ejected droplets was also measured with a homemade analyzer using imageJ software, in order to see how droplets behaved in time.

To print the chosen ink, a 10 pL droplet ink cartridge was filled with 1.5 ml. The ink was ejected through a single nozzle at 30°C and the printer platen was at room temperature. The drop spacing used was around 30-35 μm.

Different patterns (designed in monochromatic bitmap format) were printed on cleaned glass coverslips using the chosen ink, but anything could be seen in the fiducial camera that provides the printer. One of the further applications of this work is printing the ink above gold electrodes to test electrical neuronal activity. So we printed the ink on an inkjet-printed gold pattern in order to check that the printer was indeed printing the mixture.

Several substrates were prepared (*Table I*) on glass coverslips to test neuronal cultures.

		IP	
Substrate	PEG coating	Pattern	Layers
I	X	-	-
II	X	 3 mm	1
III	-	 3 mm	1
IV	X	 6 mm	10
V	X	 6 mm	10
VI	X	 9 mm	10

TABLE I: Different substrates with different printed patterns.

Cortical neurons came from rat embryos at 18-19 days of development and were plated on our substrates on day *in vitro* 0 (DIV0) employing a plating medium that provides neurons the necessary feeding (45 mL Minimum Essential Medium (MEM), 2.5 mL (5 vol%) Horse Serum (HS), 2.5 mL (5 vol%) Phospate Buffer Saline (FBS), 50 μL B27 supplement). On DIV5, the medium was replaced for the changing medium (45 mL MEM, 5 mL (10 vol%) HS, 200 μL FUDR(Floxuridine)+URIDINE). Finally, on DIV8 there was a second medium changing for the final medium (45 mL MEM, 5 mL (10 vol%) HS). Cultures were kept in an incubator simulating physiological conditions (37°C, 90% humidity, 5% CO_2). Clusters can already be seen on DIV5, while an homogeneous neural network can not be distinguished until DIV7. To check biocompatibility between neurons and the printed solution of collagen-PLL, a virus with a fluorescent protein was injected in a culture on DIV5. This virus (GCAMP (GEGI)) infects the cells attaching to neuronal calcium ions, which then causes fluorescent emissions. This emissions come from spontaneous neuronal activity, showing if the neurons are firing or not.

The culture images were recorded through a high spatial and temporal resolution camera (Hamamatsu ORCA-Flash 4.0) attached to an inverted microscope.

III. RESULTS AND DISCUSSION

ATR-IR results of the repulsive background are shown in *FIG. 2*, where several absorption peaks in different wavelengths can be detected and used to identify molecules. The blue spectrum corresponds to steps 1 and 2 from the repulsive background deposition procedure, where the amino groups of the aqueous solution of aminopropyltriethoxysilane are exposed. The green spectrum corresponds to the result of the deposition after step 4, when the glutaraldehyde has reacted to amino groups. And the red spectrum corresponds to the final step where PEG is fixed. The pink one is from a blank glass coverslip. We can identify the amino groups in blue spectrum through a N-H stretch at 3300 cm^{-1}, a CH_3 group at 2960 cm^{-1} and at 2905 cm^{-1}, and CH_2 around 2900 cm^{-1}. Between 1650-1580 cm^{-1} there are amide I (CO stretch) and amide II (N-H bend) peaks respectively. Green spectrum confirms that there is a glutaraldehyde: at 3000 cm^{-1} there is a C-H and the little peak between 1750-1680 cm^{-1} indicates the group C=O. Finally, PEG can be identified through the band between 3500-3200 cm^{-1} that indicates O-H.

So, we proved that the different steps needed to fix the repulsive background had been deposited properly.

To get a suitable neuronal neuronal ink, it was necessary to prove that the 25:1 mixture of collagen and PLL was able to be inkjet-printed (viscosity must be between 2-30 cP). Different concentrations of the mixture in water were analyzed (1 wt%, 20 wt%, 50 wt% and 100 wt%).

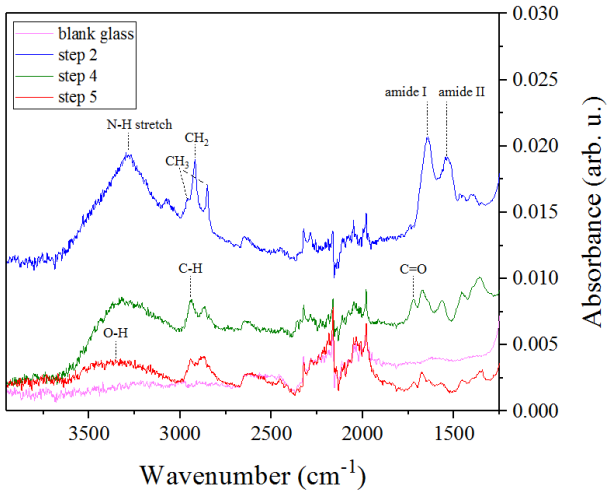

FIG. 2: Absorbance vs. wavenumber of the results obtained by ATR-IR.

The viscosity of solutions up to 50 wt% did not overcome 3.5 cP and the optimal printable range is between 10-12 cP. The best values of viscosity were reached with the 100% solution, that is why it was chosen to work with this solution. In *Table II*, the viscosity of the 100% solution is shown. A non-linear behavior can be seen, which indicates that the solution could be a non-Newtonian liquid. For that we measured the viscosity at different temperatures, obtaining the highest values at 30°C and at low velocities.

	22°C (T_{room})	30°C	35°C
ω (RPM)	η (cP)	η (cP)	η (cP)
12	14.4±0.40	10.2±0.36	7.15±0.33
20	8.75±0.24	13.0±0.28	4.70±0.20
30	7.08±0.17	7.21±0.17	4.75±0.15
50	5.20±0.11	5.76±0.12	3.33±0.09
60	4.75±0.10	4.01±0.09	3.31±0.08

TABLE II: Viscosity (cP) of the 100% solution at different velocities and different temperatures. The error is calculated following viscometer instructions [12].

In the first attempt of printing this ink on cleaned glass coverslips, anything could be seen. This was due to the transparency of the ink and also of the substrates. In order to prove the deposition of the mixture, the solution was printed above an inkjet-printed gold pattern. Due to the contrast that provided gold, we were able to see the printed collagen-PLL solution. So we based the study of the ink deposition onto gold.

The printed droplets were very tiny (5 μm diameter aprox.) and more concentrated in the center, so it was needed to find a suitable drop spacing to get compact patterns for the neurons to adhere. This centered concentration showed that the collagen-PLL solution was not homogeneous enough and two different phases could be distinguished.

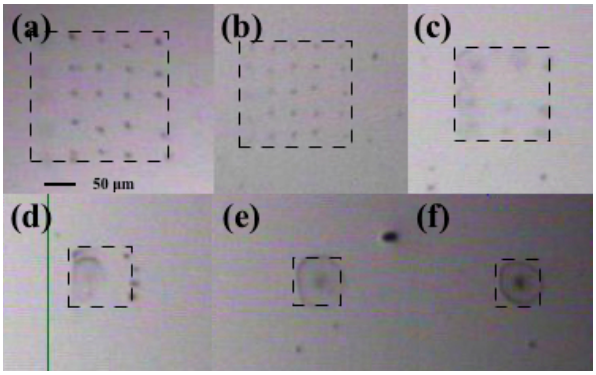

FIG. 3: 5 pixel inkjet-printed square with different drop spacings above inkjet-printed gold substrates: (a) 50 μm, (b) 40 μm, (c) 30 μm, (d) 20 μm, (e) 10 μm and (f) 5 μm.

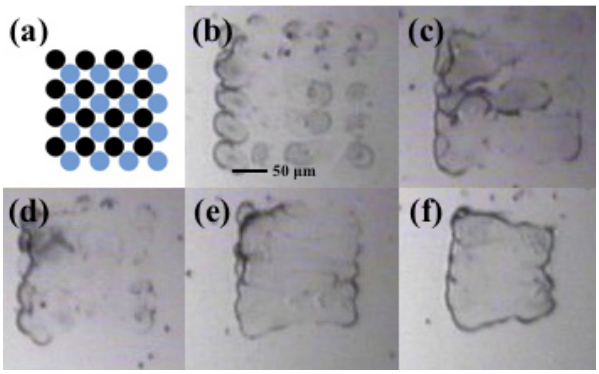

FIG. 4: (a) Pattern of two superposed squares of 4 pixel each. 5 pixel inkjet-printed superposed square with different drop spacings above inkjet-printed gold substrates: (b) 50 μm, (c) 45 μm, (d) 40 μm, (e) 35 μm and (f) 30 μm.

FIG. 3 shows that between 40 and 50 μm of drop spacing, the droplets are still differentiable for separate, so we printed with smaller ones. The problem with lower drop spacings (from 30 μm to 5 μm) was that the droplets joined by surface tension deforming the initial pattern.

We solved this problem by printing two equal superposed patterns (the second one displaced 5 μm in x and y from the first) so that the second pattern droplets could fill up the holes from the first pattern (*FIG. 4 (a)*). This would work if the droplets deposited in the first pattern were more stable in the substrate, so when printing the second, the droplets did not bind by surface tension creating undesired patterns. The contact angle of a droplet was measured above a glass coverslip (with and without PEG coating) in order to see if it decreased in time, what means that the droplet is more fixed on the substrate. The values from *Table III* confirm this behavior, so it may be useful to print superposed patterns to create a compact figure.

	without PEG coating	with PEG coating
0 min	(40.6±2.25)°	(17.2±1.69)°
30 min	(27.2±2.31)°	(15.7±0.87)°

TABLE III: Values of an adhesive ink droplet contact angle on two different glass coverslips and in two different times.

This new methodology allowed us to determine that the suitable drop spacing was around 30-35 μm, achieving the desired compact pattern (*FIG. 4*). Hence, neuronal cultures were prepared using this superposed technique. Moreover, the patterns must have several layers so that there is enough material for the neurons to adhere. So 1-10 layers were printed to test if it was enough for the culture.

The first neuronal culture dealt with two substrates, one with only PEG coating (substrate I) and the other with coating and a printed circle of the collagen-PLL ink (substrate II). On DIV2, no difference could be found between the two substrates. Neurons seem to have a tendency to accumulate in the center where there is the adhesive molecule, but not following the printed pattern at all (*FIG. 5*). If neurons were adhered correctly to the printed molecule, we would see a uniform neural network. Instead of that, neurons have formed clusters as a mechanism of defense. However, it can be demonstrated that neurons remain alive in the substrate, since on DIV5 a virus with a fluorescent protein was injected in the culture and neuronal impulses were recorded (*FIG. 6*).

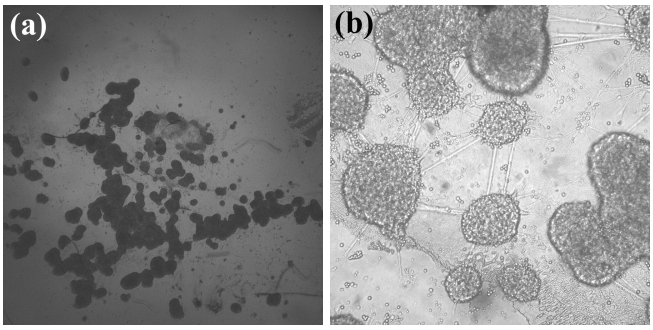

FIG. 5: Image of the first neuronal culture where some neuronal clusters can be seen in a sample with PEG coating and a collagen-PLL circle (1 layer) above glass coverslips: (a) 2.5X (b) 10X.

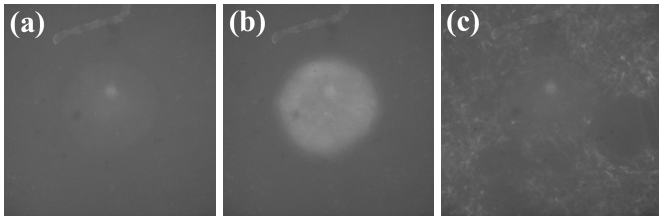

FIG. 6: Images of a neuronal culture with fluorescence: (a) non-firing state (b) firing state of a neuronal cluster (c) firing state of neural network.

Substrates II and III were used in the next culture. No difference could be found between them, obtaining similar images like *FIG. 5*.

This lack of adhesion following the printed solution may be due to the small amount of material, since neurons need a significant amount of PLL. To counteract this, in the last culture, all the patterns were printed with 10 layers of the collagen-PLL solution instead of 1.

FIG. 7 (substrates IV, V and VI) shows that the neurons follow a similar behavior to the other cultures. So any pattern can be distinguished.

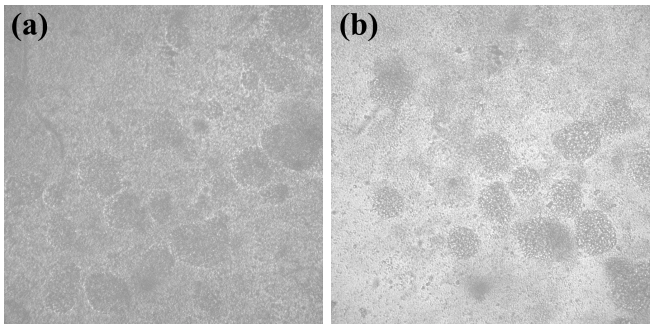

FIG. 7: Image of the last neuronal culture above glass coverslips: (a) sample with only coating (20X) (b) sample with coating and PLL (10 layers) (20X).

A solution to that fact could be printing even more layers of the collagen-PLL solution having more material to the neurons to attach. Another one could be increasing the concentration of PLL in the mixture solution.

IV. CONCLUSIONS

A repulsive background based on PEG coating has been obtained. It has been analyzed with ATR-IR, proving that all the molecules of each stage were well deposited. An adhesive molecule ink has been developed. Moreover, superposed patterns of this ink have been successfully inkjet-printed, after proving that the 100% aqueous solution of the 25:1 mixture of collagen and PLL was the one that best suited the printer's requirements.

Biocompatibility between neurons and the manufactured substrate has been demonstrated since neuronal impulses have been observed through fluorescence emissions. Nonetheless, neuronal spatial distribution has not been achieved. Different patterns with several layers of the collagen-PLL solution have been deposited on the repulsive background, expecting to get a uniform neural network. Instead of that we have obtained neuronal clusters in all the cultures. No remarkable difference was detected between the substrates with only PEG coating and the ones with PEG coating and the collagen-PLL solution. We suspect that it is due to the lack of enough PLL. So in further studies, more layers should be printed or the concentration of PLL in the mixture should be increased.

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