Ink-jet printed chitosan precise patterning for engineered 2D neuronal networks

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Abstract

The aim of this work was to demonstrate chitosan micropatterning effectiveness to realize spatially guided 2D neuronal networks of primary rat neurons and neurons derived from human induced pluripotent stem cells (h-iNeurons). Morphological characterizations were carried out to validate the formation of a well-defined neuronal networks.

Introduction

The main challenge in neuroscience is to understand how structural organization of a neuronal network affects its activity. Thanks to their accessibility and easy handling, *in vitro* studies remain an important tool for exploring the relationship between structure and function. Controlled spatial organization is crucial during the development of neuronal networks, for physiological and pathological studies and drug screening.[1] Several studies used patterned substrates to control spatial organization, guide the growth of neurites, and obtain well-defined neuronal networks for functional studies Among patterning techniques, ink-jet printing has attracted great interest thanks to the direct-write approach, leading to efficient material deposition, cost-saving and minimized contamination.[2]

In this work, bidimensional patterns with selected micrometric geometry were obtained using Drop-On-Demand (DOD) inkjet technology. A grid geometry was selected in order to obtain an interconnected structure over the sensors layout of the microelectrode array device (MEA60, MultiChannel Systems, Reutlingen, Germany) for electrophysiological characterization. Chitosan (CHI) biopolymer was employed to realize a suitable low viscosity ink. Chitosan is a copolymer of glucosamine and N-acetyl-glucosamine, obtained by the deacetylation of chitin; it is well known for its low-cost, biocompatibility, biodegradability, muco-adhesiveness, antibacterial activity as well as its bioaffinity.[3] Recently, we demonstrated the effectiveness of neat CHI as adhesion factor for 2D neuronal cultures.[4,5]

In this work, we demonstrated the effective deposition of precise micrometric sized patterns of CHI on a glass support. Cell adhesion and growth on patterned substrates was assessed by *in vitro* neuronal cell culture of two different cell populations: primary rat neurons and h-iNeurons. After 15 days in culture, samples were fixed and immunolabeled to characterize the expression of specific neuronal markers.

Materials and Methods

Chitosan-based bio-ink was obtained by dissolving CHI in 1 M glacial acetic acid (0.1-1% w/v) and characterized by viscosity tests using a rheometer (Physica Anton Paar MCR 301). A DOD piezoelectric printer (Dimatix Materials Printer DMP-2800) was used to print the pattern onto glass support with a printing frequency of 1 kHz and a voltage of 40 V. To check the final shape and size of the printed patterns, CHI was labeled by means of Fluorospheres™, and observed by fluorescence microscopy (Olympus BX-51). Primary neuronal cells and h-iPSCs derived neurons were plated onto the patterned surface at a seeding concentration of 250 cell/mm^2 and 300

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Key words: Chitosan, ink-jet printing, patterned neuronal network, microelectrode arrays (MEAs).

Acknowledgments: The D. Di Lisa research activity is co-financed by Programma Operativo Por FSE Regione Liguria 2014-2020, n. RLOF18ASSRIC".

Disclosures: The authors declare no known competing financial interests or personal relationships that could have appeared to influence the work reported in this abstract

Conference presentation: This paper was presented at the Third Centro 3R Annual Meeting - L'era delle 3R: modelli *in silico, in vitro* e *in vivo* per promuovere la ricerca traslazionale - 30 September - 1 October 2021, Evento online organizzato dal Politecnico di Torino.

Received for publication: 9 July 2021.
Accepted for publication: 7 September 2021.

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Biomedical Science and Engineering 2021; 4(s1):148
doi:10.4081/bse.2021.148

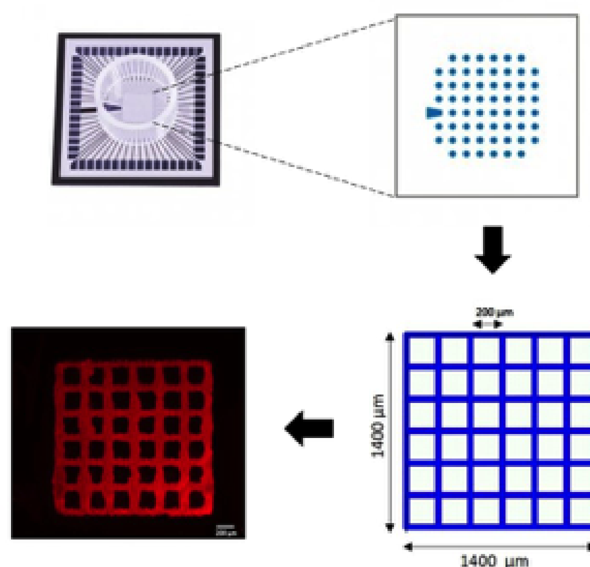

Figure 1. Pattern design, printing and characterization process.

cell/mm², respectively.

To evaluate neuronal network growth, onto the patterns, immunocytochemistry using fluorescence microscopy was employed. Microtubule-associated protein (MAP-2) and Dapi were used to show dendritic arborization and neurons nuclei respectively.

Results

Viscosity tests carried out on CHI solutions showed that a concentration of 0.5% w/v is the best condition to fall within the working range provided by the printer manufacturer. Inkjet patterning trials proved that the bio-ink formulation developed can faithfully reproduce patterns with a resolution of ~50 µm, through the deposition of 10 pl drops (Figure 1). Figure 2 shows the state of the neuronal network on a printed substrate after 15 days *in vitro* (DIV15). Despite an initial homogeneous seeding, neurons were able to adhere and grow exclusively following the pattern printed, confirming the excellent bioadhesive properties of CHI even at low deposited amounts. Furthermore, the prolonged stability of printed patterns in culture environment is demonstrated.

Discussion and Conclusions

In this work, we showed the print of a biocompatible pattern for the guided growth of two-dimensional neuronal cell cultures using CHI bio-ink without any additional treatments. By means of this simple, flexible, and low-cost technique, different and specific architectures can be easily designed and functionally studied. This work provides a useful tool for investigating the structure's influence on the functionality of a neuronal network.

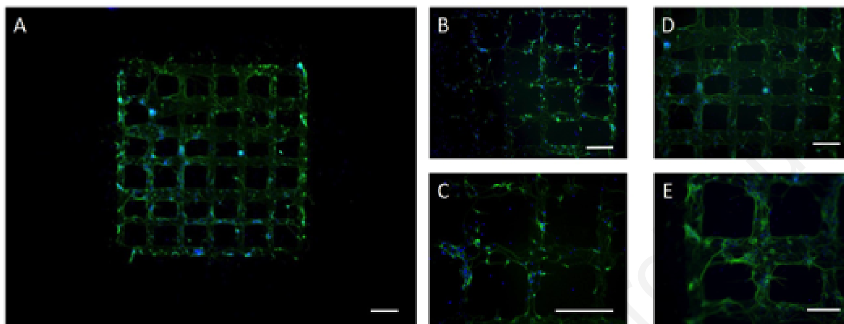

Figure 2. Immunochemistry staining images of primary neuronal network at DIV 7 (A-C) and DIV 15 (D, E), labeled for MAP2 (green) and DAPI (blue). Scale bar: A-D 200µm, E 100 µm.

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