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Carbonization of polymer precursors substrates to direct human iPSC-derived neurons differentiation and maturation

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Abstract

In this work, precursor polymers were processed by electrospinning and photolithography. Polyacrilonitrile (PAN) fibers were obtained by electrospinning, while SU-8 high-resolution micro-patterns on silicon wafers were produced by photolithography. The substrates were subjected to thermal stabilization and pyrolysis to obtain carbon structures. iPSCs-derived Neural Stem Cells (NSCs) were cultured on the carbonized materials to analyze their adhesion and differentiation into neurons on the designed scaffolds. Results show that NSCs recognized the substrates and differentiated into neurons on both scaffold typologies. In order to evaluate differences among substrates used, neuronal marker expression analysis was performed.

Keywords: Photolythography, pyrolysis, carbonization, patterning, neural stem cells.

1. Introduction

Tissue engineered substrates has been highly demonstrated to provide the optimal platforms for the manipulation of cells in vitro. The combination of materials, geometry and structure of the scaffolds is crucial especially for regenerative medicine and disease modelling applications [1]. Recently, the possibility to reprogram patient-derived somatic cells to induced Pluripotent Stem Cells (iPSCs) has provided a renewable source of expandable patient-specific cells that can be differentiated selectively towards Neural Stem Cells (NSCs) and subsequently into neurons [2,3].

The production of supports with micro-environments that, in addition to chemical growth factors, act with mechanical and morphological cues, offers more suitable *in vitro* disease models. In particular, the surface characteristics of the engineered substrates can guide and control adhesion, spreading and most of all, stem cells differentiation by modulating gene expression and intracellular signal transduction. In fact, the cytoskeleton rearrangements deriving from the applied mechanical tension on the cells, induce gene expression levels affecting stem cells function [4].

Different biomanufacturing processes have been developed to obtain substrates with controlled configurations to guide cells orientation and differentiation *in vitro* [5,6]. In particular, over the several scaffolds produced to support and promote lineage differentiation, the optimal substrates are characterized by specific surface features that can modulate the long-term maintenance of a certain phenotype. Both fibrous networks characterized by a high surface to volume ratio and patterns with micro dimensions that can be compared to the selected cells body, have been demonstrated to guide the cells morphology according to the features of the substrate's surface [7,8.]. The unique properties of graphitic carbons have gained widespread attention towards their development and application [9,10]. Carbon materials can be

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synthesized by thermal decomposition and, more specifically, carbon pyrolysis. Different deposition methods of the precursor polymers can influence the graphitic content of the resulting carbon, changing the electrochemical properties of the final material.

Among several possible approaches for such micro-fabrication, photolithography and electrospinning techniques have been used as widespread strategies for producing micro patterns and nanofibers over a large area [11,12]. NSCs differentiation can be promoted on micro channels that can induce cells alignment [13]. On the other hand, electrospinning is renowned as a promising technique for the fabrication of substrates that can mimic the extra-cellular matrix enhancing the adhesion and colonization of stem cells. Furthermore, conductive scaffolds have been demonstrated optimal solutions to influence neurite outgrowth and differentiation of neural stem cells by the application of an electrical stimulation [14]. Finally, the promising biological properties of carbon-based materials make carbonized polymeric scaffolds excellent solutions for neural tissue engineering applications.

In this paper, the manufacturing process of carbonized substrates for tissue engineering applications is reported. The negative photoresist SU-8 polymer was used to produce conductive patterns by photolithography on silicon wafers. Moreover, Polyacrylonitrile (PAN) was used to produce nano-fibers by electrospinning. The pyrolysis treatment was used to obtain conductive nanofibers and micropatterns by the precursor polymers. Therefore, the microstructures and electrospun fibers were analyzed by Scanning Electron Microscopy (SEM). Through an application-based approach, the types of substrates were used to investigate the ability of their configuration to modulate human iPSCs-derived NSCs cells adhesion and differentiation into neurons.

2. Carbonization of polymer precursors

2.1. SU-8 wafers production

Silicon wafers and SU-8 3050 negative photoresist were used for the photolithography. The geometric configuration designed for the photolithography masks that allow a selective cross-link of the polymer is illustrated in Fig. 1. The pattern configuration is characterized by lines having a width (w_0) of 25 μm, and a distance between the lines equal to 25 μm (Fig. 1). The whole pattern has a 5x5 mm^2 area.

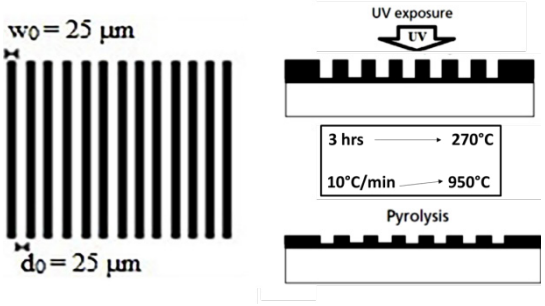

Fig. 1. Photolithography mask and process chain.

Figure 1 reports the process chain that starts with the photolithography of the SU-8 and ends with the pyrolysis of the precursor. A spin-coating speed of 3000rpm was used to obtain a SU-8 layers thickness of 50 μm. An exposure time of sec was chosen to expose the substrates to the UVs. A ramp of 2°C/min was used for the soft bake and the post-exposure bake to reach 95°C and the wafers were cooled down to room temperature to ensure complete cross-linking and avoid stiction forces. To avoid a thermal shock, the wafer was heat-treated at 270°C for 3 hours and subjected to pyrolysis by applying a ramp of 10°C /min till 950 °C in a furnace with inert atmosphere. The two-step heating process allows the releasing of cross-linking stress on the carbon features of the pattern.

2.2. PAN carbon fibers production

Three solutions of 8 wt% of PAN (molecular weight 150,000 g/mol) were prepared in dimethylformamide (DMF). The solution was stirred for 24 h at 40°C. The electrospinning was performed at room temperature and relative humidity of 40%, by applying a flow rate of 0.8 ml/h towards a 21G needle. The voltage was set at 12 kV and the distance between the needle tip and the collector was 200 mm. A single square flat collector was used to collect the electrospun fibers. Figure 2 reports the process steps followed for the obtainment of the carbon fibers.

The fibers were then thermally stabilized at 280°C for 6h prior to the carbonization to control the evolution rate of the PAN volatile components. The samples stabilized are then submitted to pyrolysis in a nitrogen athmosphere to obtain fibers made by pristine carbon. The temperature and the time of exposure in this step are as fundamental as for the stabilization.

The temperature, starting from room temperature, is increased for 60 minutes to reach 300°C. Then the temperature is kept for 60 minutes at 300°C to start the pre-carbonization. Afterwards, the temperature is increased again for 300 minutes to achieve 1050°C. During this step, the final stages of the carbonization are carried out. The entire procedure is shown in Figure 2.

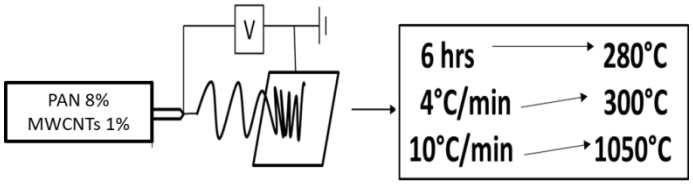

Fig. 2. Electrospinning and pyrolysis process chain.

3. Substrates characterization

3.1. SU-8 patterns

The silicon wafers were observed under optical and SE microscopes to investigate the effects of the carbonization on the imposed configuration after the crosslinking of the polymers. The final morphology is shown in Figure 3 and 4.

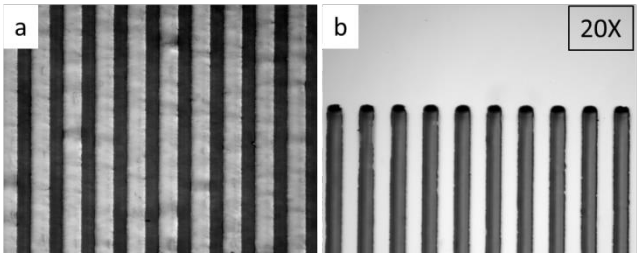

Fig. 3. Optical microscope image 20X of the photolytography configuration.

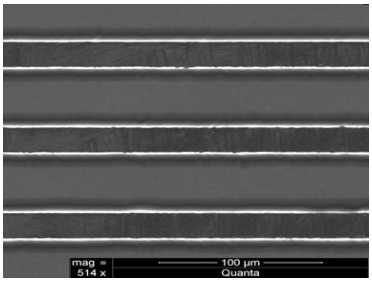

Fig. 4. SEM image of the photolytography configuration.

The ImageJ [15] software was used to properly process the images and measure the pattern dimensions. Table 1 reports the designed pattern configurations of the wafers and resulting dimensions of the pattern after pyrolysis.

Table 1. Imposed pitch by photolithography and pyrolysis of the wafers and resulting dimensions of the pitches.

Nominal feature (µm)	Photolithograpy (µm)	Pyrolysis (µm)
25 (w0)	25.3±1.2	19.2±1.2
25 (d0)	25.6±1.1	30.3±0.9
50 (thickness)	50.8 ± 1.5	14.5±0.1

The reported data highlight the differences between the imposed design and the obtained structures. The exposure process during photolithography enhanced the adhesion of the polymer to the substrate allowing the replication of the nominal features imposed by the design of the mask. In particular, the variation percentage in the geometry of the pattern after photolithography is within a range of ±1µm.
The typical effect of the pyrolysis is the shrinkage effect that causes a reduction of the nominal width of high aspect ratio structures. However, the observed differences between the dimensions of the structures after pyrolysis are acceptable considering the percentage of shrinkage occurring in the same conditions [16].

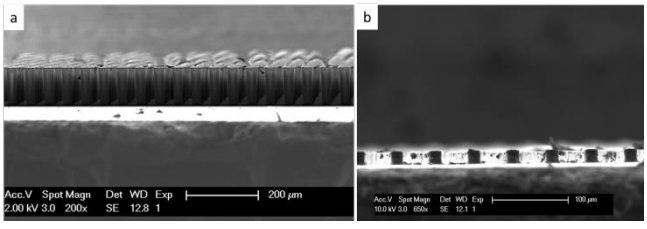

Fig. 5. SEM image of configuration A (detail of the geometric shape of the obtained structures)

The SEM images of the cross-section of the samples were also observed to analyse the profile of the structures after photolithography and pyrolysis (Fig.5). In particular, the exposure and post-exposure baking time during photolithography have been optimized to achieve straight sidewalls through the thickness of the photoresist and the shrinkage effect of the pyrolysis on the thickness of the structures was calculated to be 71%, under the typical shrinkage percentages reported for the same process conditions (86,57%) in Nitrogen atmosphere [16].

3.2. PAN fibers

The morphology of the PAN fibers is reported in the SEM images of Figure 6. The polymer fibers differ from the carbonized ones in relation to the dimensions and distribution of the fibers on the collector. In particular, the diameter of the fibers is reduced by the pyrolysis process, as a consequence of the typical shrinkage of the precursor when carbonized.

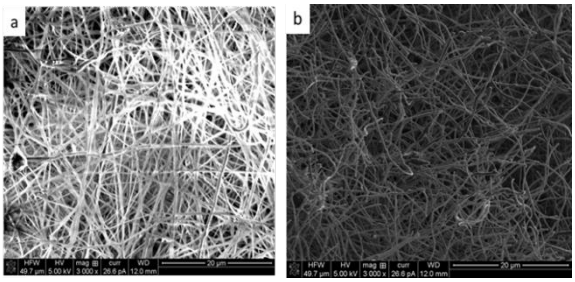

Fig. 6. Electrospun PAN fibers: before (a) and after (b) pyrolysis.

Measures were taken considering three replicas of the electrospinning tests before and after pyrolysis (Table 3).

Table 2. Average diameter of the PAN electrospun fibers before and after pyrolysis.

PAN fibers (µm)	PAN Pyrolyzed fibers (µm)
0.46±0.1	0.38±0.1

Figure 7 shows that the pyrolyzed PAN fibers have no porosity or other defects, except for a small surface roughness. The sample has a homogeneous microstructure with randomly oriented small crystallites.

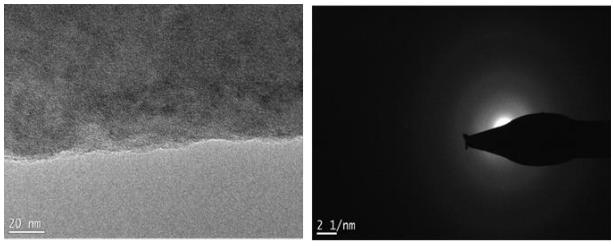

Fig. 7. HRTEM image and bright field image of the pyrolyzed PAN fibers.

The typical Raman spectrum collected from the electrospun PAN carbon fibers is shown in Figure 8.

The G band, centred on 1560–1610 cm-1, is the first order Raman mode induced by in-plane vibration of carbon atoms in sp2 hybridized graphene sheets. As a result, the intensity

and sharpness of G band is signifying the presence of crystalline graphitic phase in the synthesized material. The D band at 1300–1400 cm-1 is the defect originated second order Raman band which indicates the level of disorder in graphitic sp2 structures. The nature of this band is related to one-phonon elastic scattering and it is interpreted as a measure of the quantity of sp3 or dangling sp2 bonds that are causing structural disorders. In this case, both spectra are present and have similar intensities. This is confirmed by the intensity ratio (I_D/I_G) visible in fig. that is used to evaluate the structural ordering of the carbon phases.

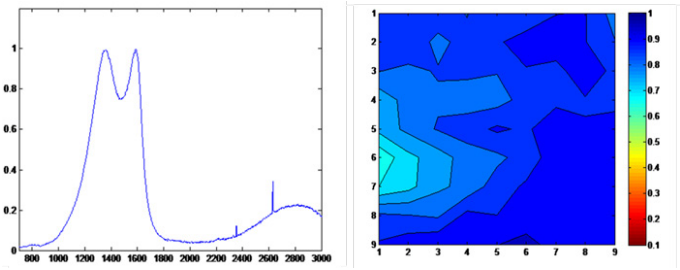

Fig. 8. Raman spectrum and D/G ratio mapping of the pyrolyzed PAN fibers.

4. Cells attachment and orientation

Neural Stem Cells (NSCs) are multipotent cells able to self-renew, proliferate, migrate *in vitro* in supportive culture systems and further differentiate into downstream lineages as neurons or glial cells (astrocytes and oligodendrocytes). We used human iPSC lines reprogrammed and characterized in our laboratory to generate human NSCs (hNSCs) required for analysis of biocompatibility and neuronal differentiation on carbonized substrates.

A protocol based on PSC Neural Induction Medium, (Thermo Fisher Scientific) has been utilized to generate iPSCs-derived NSCs. Human iPSCs, cultured in feeder-free conditions, were split as cell clumps into Matrigel-coated 6-well plates at a 15-20% of confluence. Approximately 24 hours after splitting, culture medium was switched to PSC Neural Induction Medium that was changed every other day for 7 days. Then, passage 0 NSCs were dissociated with StemPro Accutase and plated on Matrigel-coated dish at a density of 1 x 10^5 cells per cm^2. NSC expansion medium (50% PSC Neural Induction Medium, 50% Advanced DMEM/F12) was changed every other day until NSCs reached confluence. The new generated cell lines have been maintained in culture for expansion, characterization, and cryopreservation.

Neural induction was assessed by NSCs-related markers NESTIN, SOX1, SOX2, and PAX6 expression, through immunofluorescence and quantitative PCR (qPCR) analysis.

Morphology of the iPSCs-derived NSCs on both scaffolds has been observed by immunofluorescence to verify the substrates biocompatibility.

Before cell seeding, the substrates autoclave sterilized, were inserted in a 24 well plate and a Matrigel coating was applied.

A concentrated cell suspension (5 x 10^4 cells) was placed onto 1cm^2 area of each support and incubated for 20 minutes before filling the 24-well plate with a proper volume of culture media.

After 5 days in culture, cells on substrates were fixed and permeabilized using Fix&Perm-Reagent kit (SIC), then, blocked for 45minutes with iBindTM Buffer solution (Invitrogen), and stained with Phalloidin that is specific for the cytoskeletal component of the cells. Cell nuclei were counterstained with Hoechst 33342 to highlight living cells. Samples were mounted onto glass slides, observed with an inverted fluorescence microscope (Olympus IX70), and images were analysed with the Image-Pro Plus software v7.0 (Media Cybernetics).

As we previously reported [13] SU-8 high-resolution micro-patterns on silicon wafers is biocompatible with the hNSCs culture. Fluorescence microscopy images shows that also the PAN fibers substrates support both cell adhesion and survival, confirming the biocompatibility of the material (Fig. 9).

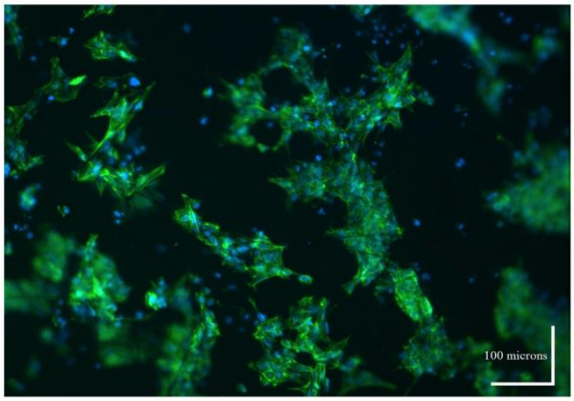

Fig. 9. Immunofluorescence staining of iPSCs-derived NSCs stained for Phalloidin in green, and Hoechst for nuclei in blue, seeded onto the PAN fibers substrate.

5. Neuronal differentiation on carbonized substrates

iPSCs-derived NSCs expanded and characterized after stabilization (passage 8) were used to perform neuronal differentiation.

Before cell seeding, substrates were washed with PBS, autoclave sterilized, and inserted in a 24 well plate. Then a poly-L-ornithine (Sigma-Aldrich) and Laminin (Thermo-Fisher Scientific) coating was applied for 1 and 2 hours respectively at 37°C.

Accutase-dissociated NSCs were passed through a 100-µm strainer (Fisher Scientific) in order to obtain single cell suspension, then collected and centrifuged at 1300 rpm for 4 minutes. The supernatant was removed and human iPSCs-derived NSCs were plated at 3.5 x 10^4 cells/cm^2. A concentrated cell suspension was deposited onto 1cm^2 area of each support and incubated for 20 minutes before filling the 24-well plate with the StemPro NSC SFM (Thermo Fisher Scientific) appropriate volume. This medium promote neuronal precursors proliferation. After two days, the medium was switched to Neurobasal Medium supplemented with B-27 and GlutaMAX-I Supplement (Thermo Fisher Scientific), in order to obtain neurons mixed population. Half culture

medium was changed every 3 days, for 20 days. Neurons generated were collected for RNA analysis and immunostaining assays.

Morphology of hNSCs-derived neurons differentiated on carbon substrates was observed by immunostaining with two specific neuronal markers: Beta tubulin III (TUBB3) and Microtubule-associated protein 2 (MAP2).

Results reported in Fig. 10 show hNSCs-derived neurons after 22 days of differentiation on SU-8 high-resolution micro-patterns on silicon wafers (A) and on PAN fibers (B). Both scaffolds sustained neuronal differentiation, and lead to the generation of TUBB3 and MAP2 positive neurons. In particular neurons orientation on configuration A is strongly influenced by the presence of the conduits: cells seem to align along lines sidewalls and to communicate through them, forming long cytoskeletal protrusions. Meanwhile, on configuration B, hNSCs-derived neurons don't follow a specific direction, but spread on the available surface, creating a packed and complex neural network.

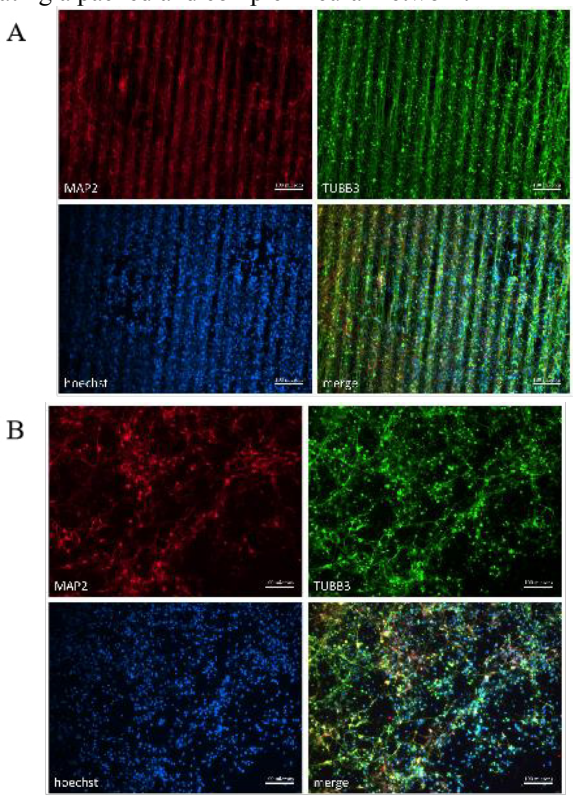

Fig. 10. Fluorescent microscopy images of hNSCs-derived neurons seeded onto the SU-8 high-resolution micro-patterns on silicon wafers (A) and on PAN fibers (B) double stained for MAP2 in red, and TUBB3 in green. Cell nuclei are counterstained with Hoechst in blue.

In addition to immunofluorescence, a qPCR assays based on SYBR-Green have been performed for the neuronal markers Doublecortin (DCX), and MAP2 to test gene expression differences between the substrates. Moreover, in order to assess which kind of neurons was predominant in the cellular culture obtained, a qPCR with specific primers for genes of several neuronal typologies was carried out. Genes tested included: glutamic acid decarboxylase (*GAD*) marker for GABAergic neurons, Choline Acetyltransferase (*CHAT*) for cholinergic neuron, and Tyrosine hydroxylase (*TH*) for dopaminergic neurons. Finally, to estimate the neuronal culture purity from glial cells contamination, expression of an astrocytes specific gene (Glial fibrillary acidic protein - *GFAP*) was evaluated.

Total RNA was extracted from hNSCs-derived neurons after 22 days of differentiation on both scaffolds, and on 24 well plate, used as neurons differentiation internal control. RNAs were collected using NucleoSpin® RNA II kit (Macherey-Nagel) and quantified by a Spectrofluorometer. RNAs were retro-transcribed by ImPromII™ Reverse Transcription System (Promega). Assays were performed on CFX96 C1000 Touch™ Real-Time PCR Detection System, and analyzed with CFX manager software v.3.1 (BioRad).
qPCR reactions were normalized using βACTIN as housekeeping gene. Target markers gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method relative to expression levels in NSCs-derived neurons differentiated on 24 well-coated plate, but with no scaffolds.

Results in figure 11 show that neurons obtained on both scaffolds exhibit a *DCX* and *MAP2* gene expression similar to the neurons differentiated on 24-well plate. Moreover, both substrates sustained the growing and maturation of hNSCs-derived dopaminergic neurons, as suggested by increased *TH* gene expression.
In contrast only SU-8 high resolution micro pattern on silicon wafers seem to promote the gabaergic and cholinergic neurons differentiation, since neurons grown on PAN fibers show a reduced *GAD* and *CHAT* gene expression. Finally in both substrates neurons population, we demonstrated a higher contamination of glia cells expressing *GFAP* gene, compared to the culture on the plates.

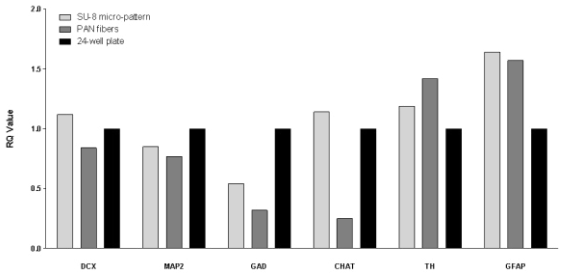

Fig. 11. qPCR analysis for DCX, MAP, GAD, CHAT, TH and GFAP expression in hNSCs-derived neurons. Data were normalized on βACTIN, and calculated in relation to expression levels in neurons differentiated on 24 well-coated plate with no scaffolds (black bars).

6. Conclusion

We have processed precursor polymers by electrospinning and photolithography. In particular, Polyacrilonitrile (PAN) fibers were obtained by electrospinning, while SU-8 high-resolution micro-patterns on silicon wafers were produced by photolithography. The fabricated substrates were subjected to thermal stabilization followed by pyrolysis to obtain carbon structures. iPSCs-derived Neural Stem Cells were cultured on the produced carbonized materials to analyze their adhesion, proliferation and differentiation into neurons on the novel designed scaffolds. The cellular culture results show that both carbonized substrates sustain cell adhesion and survival,

confirming the biocompatibility of the material, and support the neurons differentiation.

In this regard, hNSCs-derived neurons recognized the surface texturing and started to arrange their cytoskeleton according to the morphology of the scaffolds.

Fluorescence microscopy images show that in case of the patterned silicon wafer, the hNSCs-derived neurons are encouraged to organize themselves according to the conduits and communicate through the sidewalls. On PAN fibers the cells are free to colonize the fibrous network with their cytoskeletal protrusions spreading and forming connection in all directions.

Thus, both types of substrate influence the disposition of neurons and the consequent direction of communication. In particular, the first configuration allows the cells to be arranged in an organized manner along the conduits, allowing communication between the walls only in one direction. Instead, on the second substrate, the inter-cellular communication is encouraged at 360° at the expense of a one-way stimulus. Of particular interest will be to evaluate the electrical functionality of the cells on the two kinds of supports to identify the best platform in order to increase the electrical conductivity yield. Furthermore, gene expression analysis revealed that carbonized substrates seem to promote or affect the neuronal differentiation in a given cellular specialization, compared to the general neuronal population obtained on plate culture.

SU-8 high resolution micro pattern on silicon wafers seems in fact to promote more the gabaergic and cholinergic neurons differentiation as compared to the PAN fibers where their maturation is reduced. Both the carbonized scaffolds encouraged the dopaminergic maturation. It's known in literature that carbon material, such graphene oxide, promotes the dopaminergic neuronal differentiation of mouse embryonic stem cells [17], but little is still known about the human neurons specialization when cultured on different materials.

Human cells *in vitro* is still an open field of exploration. The combination of special culture media and growth on substrates composed by several materials can help to direct neuronal maturation towards a single typology.

According to the achieved results, future researches will be focused on the application of an electrical stimulus on hNSCs-derived neurons on the conductive substrates in absence and in presence of a specific surface geometry to identify the best platform to explain their biological function. Then, the contribute of carbonized substrates on hNSCs-derived neurons differentiation in terms of maturation and specialization will be deeply analyzed.

Acknowledgements

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