Neonatal rat ventricular myocytes interfacing conductive polymers and carbon nanotubes

Nuria Alegret · Antonio Dominguez-Alfaro · David Mecerreyes · Maurizio Prato · Luisa Mestroni · Brisa Peña

Abstract Carbon nanotubes (CNTs) have become promising advanced materials and a new tool to specifically interact with electroresponsive cells. Likewise, conductive polymers (CP) appear promising electroactive biomaterial for proliferation of cells. Herein, we have investigated CNT blends with two different conductive polymers, polypyrrole/CNT (PPy/CNT) and PEDOT/CNT to evaluate the growth, survival, and beating behavior of neonatal rat ventricular myocytes (NRVM). The combination of CP/CNT not only shows excellent biocompatibility on NRVM, after 2 weeks of culture, but also exerts functional effects on networks of cardiomyocytes. NRVMs cultured on CNT-based substrates exhibited improved cellular function, i.e., homogeneous, non-arrhythmogenic, and more frequent spontaneous beating; particularly PEDOT/CNT substrates, which yielded to higher beating amplitudes, thus suggesting a more mature cardiac phenotype. Furthermore, cells presented enhanced structure: aligned sarcomeres, organized and abundant Connexin 43 (Cx43). Finally, no signs of induced hypertrophy were observed. In conclusion, the combination of CNT with CP produces high viability and promotes cardiac

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functionality, suggesting great potential to generate scaffolding supports for cardiac tissue engineering.

Keywords Carbon nanotubes · Neonatal cardiomyocytes · Conductive polymers · Cardiac tissue engineering

Introduction

Regeneration of electroactive cells, e.g., neurons and cardiac myocytes, requires electronic conductive environments that can be provided by the interface of substrates. For this reason, developing conducting biomaterials with easy-to-tailor properties is highly valuable. Nanotechnology has gained increased attention to provide novel tools for innovative therapeutic devices and treatment. More specifically, the discovery and manipulation of innovative conductive nanomaterials, such as carbon nanotubes (CNTs), has created the opportunity for biomedical applications, (Place et al. 2009; Dvir et al. 2011) thus, providing tools specifically for the interaction with electroactive cells at the nanoscale (Silva 2006).

In the last decade, CNTs have been largely investigated as substrates for the development of neuronal circuits; (Marchesan et al. 2017) for such use, the development of a hybrid neuronal-nanomaterial network served also as a platform to examine neuronal detection of reactions to environmental physical and chemical features. In this regard, interfacing neurons with CNTs emerged as an effective tool for manipulating neuronal activity at multiple levels of tissue complexities that is at the single neuron, synaptic network, and multilayered tissue levels (Malarkey et al. 2009; Fabbro et al. 2012; Pampaloni et al. 2018). Some of us have previously demonstrated that CNTs boost the electrical activity of neurons and, furthermore, increase the number and length of their neurites (Malarkey et al. 2009). The biological effect is related to changes in the electrical properties of neurons observed while exposed to CNT. The ability of CNT to change the electrical properties of neurons makes them great candidates for novel tissue engineering of other electroactive cells, in particular myocyte cells, such as cardiomyocytes. Neurons and cardiomyocytes (CMs) share several electrical properties and ion channels, and we have demonstrated that changes observed in neurons can be reproduced in CMs (Martinelli et al. 2012; Fabbro et al. 2013; Martinelli et al. 2013; Peña et al. 2016, 2017).

The interest in using nanoscaled-based scaffolds for cardiac tissue engineering has gained large attraction. Preliminary proof-of-concept investigations demonstrated that changes in the electrical properties of neurons observed while exposed to CNT is similar to that observed in CMs culture: neonatal rat ventricular myocytes (NRVM) cultured on CNTs-coated glass coverslips showed improved viability, proliferation, maturation, and electrical properties compared with control substrates (Martinelli et al. 2012). Furthermore, multiple investigations have demonstrated that CNT-based scaffolds promote cardiomyocytes grown, increased expression of gap junctions and, overall, a more mature phenotype, with enhanced electrophysiological behavior and implemented networking and maturation into the functional syncytia (Martinelli et al. 2013; Peña et al. 2017). Moreover, carbon nanotubes appear to exert a protective effect against the pathologic stimulus of phenylephrine. All these properties are unique in the current vexing field of tissue engineering, and offer unprecedented perspectives in the development of innovative therapies for cardiac repair (Peña et al. 2017).

On the other hand, conductive polymers (CP) have emerged as a novel and very promising type of electroactive biomaterial. CP can be synthesized alone, combined into composites, as hydrogels, or as electrospun fibers (Diao et al. 2021; Wang et al. 2021a, b; Cao et al. 2022a, b; Cao et al. 2022a, b). Such versatility offers a wide range of options to modify their chemical, biological, and physical properties, thus becoming very important materials for biosensors, tissue implants, drug delivery devices and tissue engineering scaffolds. Preliminary ex vivo and in vivo studies demonstrated that the conductive nature of CP-based patches had immediate effect on the electrophysiology of the heart and did not induce proarrhythmogenic activities (Mawad et al. 2016). However, the interaction between cardiomyocytes and CP has not yet been fully investigated. Thus, we hypothesize that the development of highly conductive substrates containing CNT might have a superior effect on the cardiac cell cultures. Among CP, the ones most used for biological applications are polypyrrole (PPy) and poly(3,4-ethylenedioxythiophene) (PEDOT). Numerous studies have demonstrated

high biocompatibility of PPy and PEDOT with live cells such as, murine neonatal and adult cardiomyocytes, and iPSC-derived cardiac myocytes (Kim et al. 2018). More specifically, PEDOT:PSS is usually used as coated films for tissue engineering, multi-electrode arrays (MEAs), and sensor devices. Not only cells showed great ability to attach on them with good physiological status, ECM production, and reduction of internal arrythmias, but also in electrophysiological recordings, PEDOT-based electrodes showed high-quality recordings (Roshanbinfar et al. 2018; Garma et al. 2019; Dominguez-Alfaro et al. 2021). Likewise, PPy has been used as coating in fibrous scaffolds and conductive additive in hydrogels for tissue engineering, showing great biocompatibility with limited inflammatory response after implantation but brittle nature and difficult manipulation (Jin et al. 2012; Baei et al. 2020; Liang et al. 2021; Parchehbaf-Kashani et al. 2021; Wang et al. 2021a, b). Furthermore, hydrogels containing PPy have shown improved mechanical and conductive properties which are required for cardiac cells, while the products of degradation showed no cytotoxic effect (Zanjanizadeh Ezazi et al. 2020).

Within the path to develop a synthetic scaffold that would be biocompatible, immunologically inert, conducting, biodegradable, and infection-resistant, we have combined the outstanding effect of CNTs with the highly conductivity of PPy and PEDOT as film substrates for NRVM interface. Even so the usage of these conductive materials separately is not novel, here we propose its original combination. We have previously demonstrated that CNT have a boosting effect on NRVMs culture due to the structure and electrical properties (Martinelli et al. 2012; Martinelli et al. 2013). Herein, the addition of conductive polymers is not only aimed at improving the conductivity of the substrates, but also as matrix to construct pure conductive tridimensional scaffolds composed of CNT, as we previously demonstrated, without the necessity of involving insulating polymers. (Alegret et al. 2018; Dominguez-Alfaro et al. 2020a, b) Our purpose is to understand the interaction and reaction of cardiac cells with CNT and CP and to investigate the benefits that these materials can provide to cardiomyocyte expansion and functional behavior. Knowing this will allow us to move toward the development of 3D scaffolds composed uniquely of CP/CNT for cardiac tissue engineering.

Methodology

Materials

Multiwalled carbon nanotubes (CNTs) were purchased from Nanoamor Inc. (Stock# 1237YJS: > 95%, 20–30 nm diameter, 0.5–2um length). Pyrrole (Py, 98%) and 3,4-Ethylenedioxythiophene (EDOT; 98%) were purchased from Sigma-Aldrich. Iron (III) chloride hexahydrated (FeCl3·6H2O) was acquired from Fisher Scientific Inc. Ethanol (synthesis grade) was purchased from Carlo Erba Reagents SAS. All reagents and solvents were used as received with no further purification.

Preparation of the CP/CNT substrates

Bidimensional films of CP/CNT blends were prepared by airbrushing PEDOT/CNT and PPy/CNT dispersions on a glass coverslip, following 5 min annealing at 80 °C to ensure adherence. Such dispersions were obtained from 3D sponges embedding CNT with PPy or PEDOT as previously reported. (Alegret et al. 2018; Dominguez-Alfaro et al. 2020a, b) Briefly, a sugar-based mold containing CNT and FeCl3 was used as template to polymerize PPy or PEDOT through vapor phase polymerization (VPP). Once the polymerization was completed, self-standing microporous 3D structures were obtained after water-driven dissolution of the sugar, with compositions of ca. 50% of CNT and 50% of the polymer, according to thermogravimetric analyses. X-ray photoelectron spectroscopy (XPS) showed no traces of Fe in the final product. Furthermore, all the materials presented excellent conductive properties ($|Z_{PPy/CNT}| = 7.8$ kΩ for PPy/CNT, and $|Z_{PEDOT/CNT}| = 6$ kΩ for PEDOT/CNT). (Alegret et al. 2018; Dominguez-Alfaro et al. 2020a, b) Afterwards, each 3D scaffold, i.e., PPy/CNT and PEDOT/CNT, was smashed into a fine powder, resuspended with water (10 mg/mL) and airbrushed 10 times onto glass coverslips until a homogeneous film-coated surface was obtained. The films without CNT were prepared by airbrushing PPy/PSS and PEDOT/PSS dispersions. Gelatin-coated coverslips were used as control.

Neonatal rat ventricular myocytes culture (NRVMs)

NRVMs were prepared from six, 1- to 3-day-old pups by enzymatic digestion as previously described with minor modifications (Martinelli et al. 2013; Peña et al. 2017). All animal studies were performed as reported before and according to the guidelines of the University of Colorado Denver Animal Care and Use Committee (protocol #00,235). Briefly, ventricles were separated from the atria using scissors, then dissociated in calcium-free and bicarbonate-free Hanks with Hepes (CBFHH) buffer containing Heparin (10 $U \cdot mL^{-1}$) and digested in a CBFHH solution containing 1.12 $mg \cdot mL^{-1}$ of trypsine (Sigma Aldrich) and 20 $g \cdot mL^{-1}$ of DNAse. Cardiomyocytes were enriched (>90% purity) over non-myocytes cells by two sequential pre-plating steps on 60 mm dishes in MEM, supplemented with 5% bovine calf serum and 2 $g \cdot mL^{-1}$ vitamin B12 and cultured as previously described (Cahill et al. 2013). Unattached cells (predominantly myocytes) were collected, and $3 \cdot 10^5$ cells were cultured on 2D CP/CNT-coated dishes for 3, 7, and 14 days and subsequently analyzed. Gelatin-coated glass substrates were used as control. All experimental conditions were tested in triplicate from at least three independent cell isolations.

Calcium imaging

Intracellular calcium signaling of NRMV after 14 days of culture was recorded to assess the electrical activity of CMs growing on the CP/CNT and controls. Cell-permeant fluo-4 AM was added to each sample and incubated for 15 min, according to manufacture instructions. Samples were then washed three times with warm media and, subsequently, spontaneous calcium transients were recorded for 35 s. All recordings were performed using a Zeiss LSM780 spectral, FLIM, 2P, SHG confocal. Experiments were carried out in quadruplicate from at least three independent experiments and averaged.

Immunocytochemical staining

After 3, 7, and 14 days of culture, immunocytochemistry was performed to distinguish the population of NRVM, using the cardiac-specific α-sarcomeric actinin antibody (1:100, Sigma), from fibroblasts, stained with the cytoskeleton marker vimentin antibody (1:100, Abcam). Goat anti-mouse antibody conjugated to Alexa Fluor 488 (Invitrogen) and goat anti-chicken Cy5 antibody (Abcam) were respectively used as secondary antibodies at 1:200. Anti-Connexin 43 (Cx43) (1:200, Sigma) was used to determine the gap junction distribution between NRVMs after 14 days of culture; as a secondary antibody, goat antirabbit antibody conjugated to Alexa Fluor 594 was used (1:200, Invitrogen). The staining protocol is briefly summarized as follows: cell cultured on coverslips were washed with PBS and fixed with 4% paraformaldehyde (PFA) in PBS for 15 min. Then, cells were permeabilized with 1% Triton X-100 for 1.5 h, blocked in 2% BSA in PBS for 45 min, and incubated with primary antibodies overnight. Secondary antibodies were incubated for 45 min, and, finally, cell nuclei were stained with DAPI (1:1000) for 2 min. Fluorescent images were taken from five regions of each sample ($n = 4$) with a Zeiss AX10 observer Z1. Instrument settings were kept constant between for each experiment.

Images evaluation and statistical analysis

For immune staining analysis, data was collected in quadruplicate from three independent experiments. For calcium signals, data was collected and analyzed from at least 10 fields per sample and averaged. ImageJ was used for data processing as follows: for calcium transients' evaluation "time Series Analyzer V3" plugin was used and for cell counting, "Cell Counting" plugin was used; both analyses were performed in at least 40 fields per sample type 9.

Calcium measurements are reported as the fractional change in fluorescence intensity relative to baseline (F/F0), which was determined as follows. Within a temporal sequence of fluorescence images, a region of interest (ROI) was drawn around a portion of each NRVM to be analyzed. The fluorescence signal from each terminal was calculated as the pixel-averaged intensity within each ROI, yielding the common sinusoidal beating plot. The absolute minimum value of the pixel averaged intensity from each of these ROIs in the whole temporary region was taken as the baseline fluorescence (F0) for that cell. At each time point, the fluorescence value was ratioed against the baseline value to yield F/F0. The amplitudes of Ca^{2+} transients were determined by the maximum values of the beating peaks;

the frequency of the Ca^{2+} transients were calculated by the time differences between adjacent peaks. The precise boundary of each ROI was kept constant between all the analyzed beating cells.

The purity of cardiac cells was calculated according to the following equation:

$$\%\ \text{NRVM} = \frac{\text{Number of NRVM}}{\text{Number of total cells (NRVM + Fb)}} \times 100 \quad (1)$$

The density of NRVMs (#cells/mm^2) was calculated following Eq. 2 and normalized to the value of PEDOT/CNT obtained at day 3 for each set of experiments:

$$\text{Density} = \frac{\text{Number of NRVM}}{\text{Area of the image } (\mu\text{m}^2)} \quad (2)$$

The size of the cardiac cells was calculated from the % area of NRVM-positive fluorescence obtained from the measurement using ImageJ, as shown in Eq. 3:

$$\text{Size NRVM} = \frac{\text{Area of the NRVM } (\mu\text{m}^2)}{\text{Number of NRVM}} \quad (3)$$

Data distribution was evaluated with Shapiro–Wilk normality test (GraphPad Prism 8). Statistical significance between experimental groups for calcium imaging was determined using t-test analysis, while for the cell counting ordinary one-way ANOVA followed by unpaired Tukey's multiple comparisons test was used. In all cases, adjusted *P* values of less than 0.05 were considered statistically significant.

Results and discussion

Even though the field of 3D cell cultures is growing very fast, the usage of synthetic polymers as substrates for primary cells is still a challenge. Initially, we tried to incubate NRVM on the 3D PPy/CNT and PEDOT/CNT scaffolds we have previously developed (Alegret et al. 2018; Dominguez-Alfaro et al. 2020a, b; Dominguez-Alfaro et al. 2020a, b). Unfortunately, although live/dead staining suggested that NRVMs survived, they were not able to expand and grow inside the prepared 3D sponge-like structures. These suggested that cardiac cells are much more delicate and sensitive to the culturing environment than neurons or astrocytes, we previously reported. With the aim of confirming the biocompatibility of our conductive materials with cardiac cells, we decided in this work to evaluate the interaction and maturation of NRVMs cultured on bidimensional films fabricated from airbrushing the 3D PPy/CNT and PEDOT/CNT scaffolds, as shown in Fig. 1 (see Fig. S1 for optical images of the resulting films). We would like to point

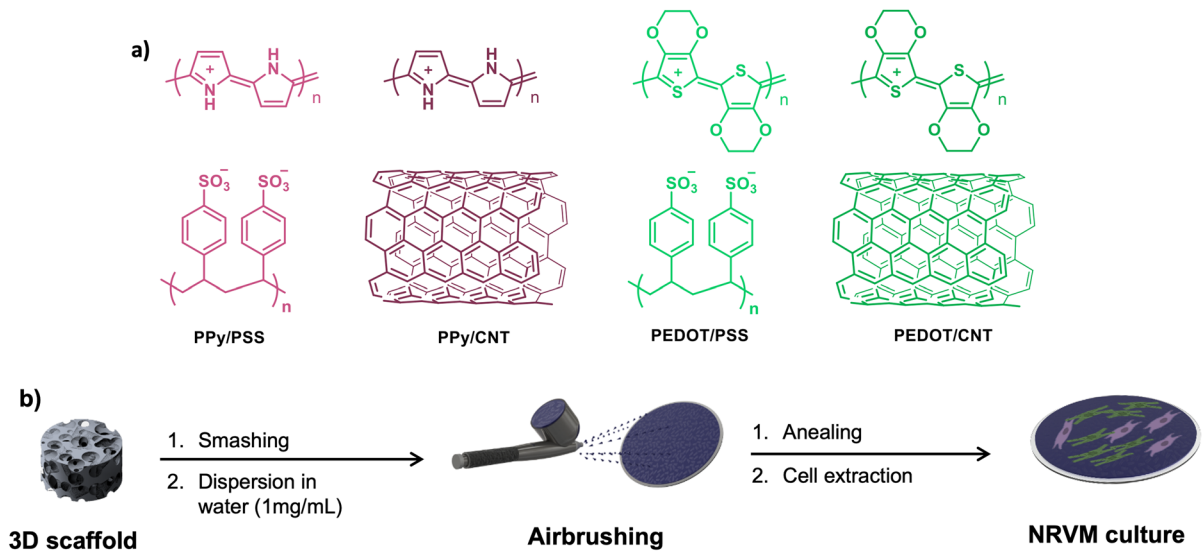

Fig. 1 **a** Chemical structures of PPy:PSS, PPy/CNT, PEDOT:PSS, and PEDOT/CNT used as conductive 2D substrates. **b** Experimental procedure to form the 2D substrates: smashed and dispersed conductive 3D scaffolds that we previously reported, were airbrushed on glass coverslips and annealed before co-cultures of NRVMs and Fibroblast were seeded on top

out that commercial aqueous solution of PEDOT:PSS (Clevios PH1000 from Heraeous) was also included in the initial methodological design; however, it resulted very cytotoxic, and all the cells incubated on this material died within the firsts 3 days (results not shown).

Beating behavior: in vitro NRVM cultured on CP/CNT substrates

Co-cultures of NRVM and fibroblast seeded on the conductive substrates were maintained for up to 14 days; spontaneous beating activity of NRVMs was observed from day 2 and up to day 14. Several facts are worth mentioning: (i) high beating coordination was observed in the NRVMs when cultured on the conductive CNT-based substrates; (ii) the cell-material interaction was very strong, as no material was released during the synchronized beating in all the CP/CNT substrates (see video S1, S2, S3, S4 and S5 in the Supplementary Information); (iii) cells were able to survive for longer periods of times in all the CP/CNT substrates. All these observations are indicatives of the high biocompatibility of these materials, the excellent affinity of cardiac cells to the conductive films, the validation of the coating application technique, and the high functionality that these conductive materials provide to cardiac cells.

To evaluate the effect of CP/CNT substrates on CMs function, spontaneous intracellular calcium oscillations were analyzed and correlated with CMs maturation. In general, a more homogenous and strong calcium transient is equivalent to a better maturation of CMs (Karbassi et al. 2020). Therefore, we assessed CMs function by imaging the spontaneous calcium transients of NRVMs cultured for 14 days in the CP/CNT substrates. Figure 1 shows the effect of the conductive CP/CNT substrates on cardiac function. Interestingly, we found that only the NRVMs cultured on CNT-containing substrates have a more homogeneous spontaneous calcium oscillations, synchronized beating with similar frequencies, while most of the NRVMs cultured on the control substrates and on the substrates without CNT yield to more arrhythmogenic beatings (Fig. 2a and Fig. S2 in the Supplementary Information). This suggests that the presence of CNT enhances the function of cardiomyocytes with a healthier beating even after 14 days of culture (most of the investigations using NRVMs are performed within 7 days on culture). This is in good agreement with our previous experience: we demonstrated that interfacing CNTs with cardiac myocytes results in enhanced maturation and electrophysiological properties, as well as viability, growth, and proliferation. (Martinelli et al. 2012; Martinelli et al. 2013; Peña et al. 2017). We also observed tight interactions between CNTs and NRVM membranes and, hypothesized that such interactions, together with the morphology and conductivity of the CNTs, may

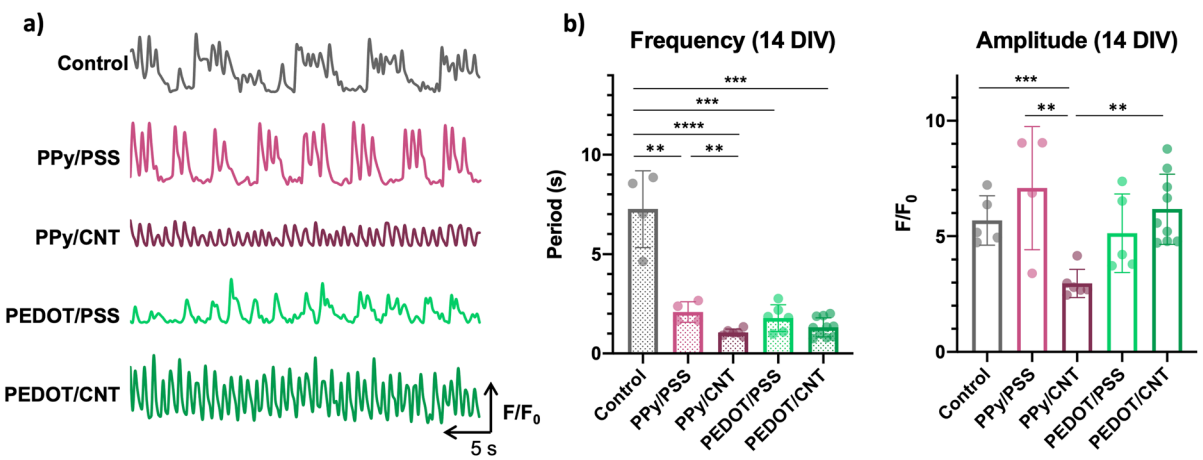

Fig. 2 Effect of conductive substrates on cardiomyocytes function: **a** Calcium imaging plot of the cardiac cell spontaneous beating behavior of cultured NRVMs on glass control and conductive film substrates. **b** Amplitude and frequency calculations from calcium imaging

improve electrical coupling by providing a soft artificial extracellular matrix which may ultimately facilitate cell adhesion, physiological growth, and maturation. However, we have not yet fully understood the precise mechanism for the observed effects of CNTs. We then calculated the amplitude (intensity of the calcium signaling) and the period (seconds between oscillations) of the calcium signaling in order to obtain more detailed information; Fig. 2b shows the results of the spontaneous calcium transients of NRVMs cultured in different substrates. As shown in Fig. 2, NRVMs cultured on conductive materials, i.e., PPy/PSS, PPy/CNT, PEDOT/PSS, and PEDOT/CNT, had more frequent and rhythmic calcium oscillations, i.e., lower beating period, when compared with cells cultured on gelatin controls. This can be attributed to the enhanced electrical conductivity provided by the conductive substrates, which correlates with previous investigations (Roshanbinfar et al. 2018, Baei et al. 2020). Among them, NRVM cultured on substrates containing CNTs showed no arrythmias, while NRVM cultured on substrates without CNTs presented a more arrhythmogenic behavior in around half of the cells analyzed (see Fig. S2 in the Supplementary information for detailed plots). NRVM cultured on PEDOT and PPy substrates present less arrhythmogenic behavior than NRVM cultured on gelatin control substrates. Regarding the beating amplitude, NRVM cultured on the PEDOT/CNT and PPy/PSS substrates showed a trend of higher amplitude when compared with NRVM cultured on the other substrates. On the contrary, the addition of CNT on PPy substrates reduced the amplitude significantly, having the cells cultured on PPy/CNT substrate the lowest beating intensity. Therefore, our findings support the concept that the combination of CNT with conjugated polymers, such as PPy and PEDOT, improves CMs beating behavior and increases their frequency, while PEDOT/CNT is pointed to enhance intercellular function toward a more mature cardiac phenotype, i.e., high beating intensity. Such observation supports our previous and abovementioned hypothesis of the importance of the conductivity on the cardiac maturation: blending conjugated polymers with CNT increases the conductivity of the substrate interface with NRVM, thus boosting even more the maturation and electrophysiological effect of the cardiac cells.

NRVM imaging and phenotype maturation

The organization and phenotype of the NRVMs cultured on the CNT-based substrates after 3, 7, and 14 days were investigated by immunocytochemistry. Our NRVM isolation protocol comprises mainly cardiomyocytes, and ca. 10% cell population of cardiac fibroblasts (Ballerini 2013; Martinelli et al. 2013). Therefore, specific markers for the different cells present in the co-culture were used as follows: α-sarcomeric actinin staining was used as cardiac-specific marker, as it is a protein involved in cardiac muscle contraction; vimentin, a cytoskeletal fibroblast protein, was used to identify cardiac fibroblasts.

As shown in Fig. 3, fluorescence microscopy revealed the typical cross-striated structure of the cardiac sarcomere in NRVMs grown in all the substrates. Although most investigations focus on the study of CMs for cardiac tissue remodeling, fibroblasts play a critical role in maintaining normal cardiac function, as they have important functions in the myocardium including synthesis and deposition of extracellular matrix, cell–cell communication with cardiomyocytes, and they modulate cardiomyocyte electrical and hypertrophic activity (Souders et al. 2009; Pellman et al. 2016). At first glance, as expected, few fibroblasts were observed after 3 days of culture in all the substrates; however, the number of fibroblasts increased over time, due to their highly proliferative nature. However, after 7 and 14 days of culture, the fibroblasts on CNT-based substrates were smaller and presented a thinner shape, suggesting a non-activated nature and, thus, non-pathological phenotype. Overall, we observed that the substrates containing CNT enhance CMs survival with a more controlled proliferation of fibroblasts.

To give quantitative validation of such observations, we counted the number of α-actinin-positive and vimentin-positive cells. As shown in Fig. 4a, during the first 3 days of culture, NRVM are in a proliferative stage and this is enhanced when CNTs are present in the substrates, as we have previously demonstrated (Peña et al. 2017). Indeed, the quantitative analysis show that the amount of CM present in the culture had a greater percentage of α-actinin-positive cells on the conductive coating substrates ($74.5 \pm 13.6\%$ for PPy, $71.9 \pm 12.0\%$ for PPy/CNT, $67.6 \pm 12.3\%$ for PEDOT and $73.6 \pm 9.6\%$ for PEDOT/CNT) when compared with cells cultured

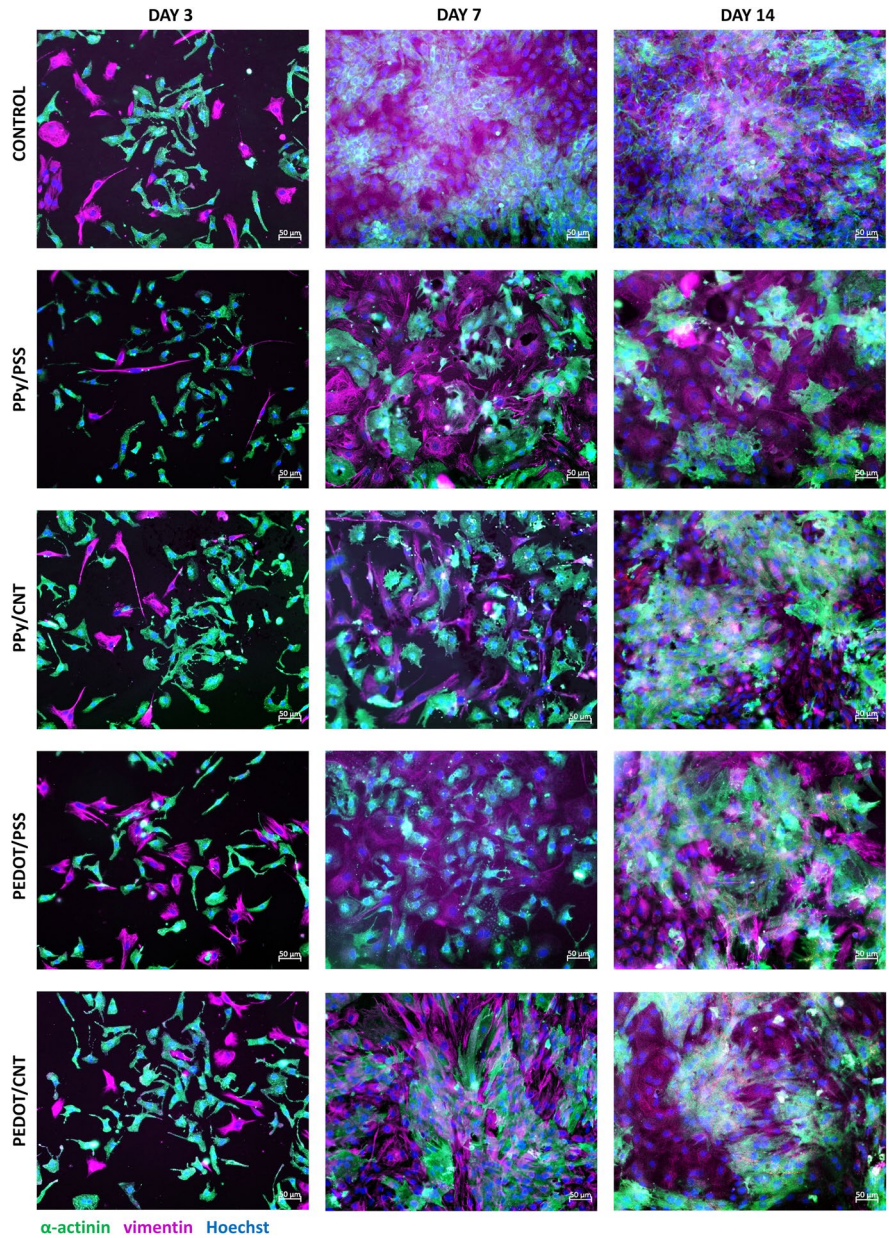

Fig. 3 Immunostaining after 3, 7, and 14 days of co-cultured CMs and fibroblasts on bidimensional films of different substrates: (from top to bottom) gelatin control, PPy, PPy/CNT, PEDOT, and PEDOT/CNT. In green, the CMs; in pink, the fibroblasts

on the 2D gelatin controls (66.0 ± 15.1%). This fact suggests the higher adhesion, expansion, and survival effect of PEDOT, PPy, and CNT on NRMVs. During the following time periods, 7 and 14 days, the % of CM was reduced as the fibroblasts proliferated and reached its maximum percentage: the percentage of cells that were α-actinin-positive was nearly constant after 7 and 14 days of culture in most of the groups (from 55.5 ± 14.2 to 59.1 ± 17.0% for PPy; from 53.0 ± 15.3 to 54.9 ± 23.5% for PPy/CNT; from 59.0 ± 14.7 to 66.4 ± 13.7% for PEDOT; from 62.8 ± 17.7 to 60.6 ± 14.6% for the control) except for PEDOT/CNT, which showed a much higher % of CM at day 7 (43.9 ± 14.9%) than at day 14 (47.9 ± 16.9%). In summary, the percentage of CMs was higher than the percentage of fibroblast on the PPy, PPy/CNT, and PEDOT substrates, i.e., more than 50% of cells were CMs, in all the time points, thus suggesting that

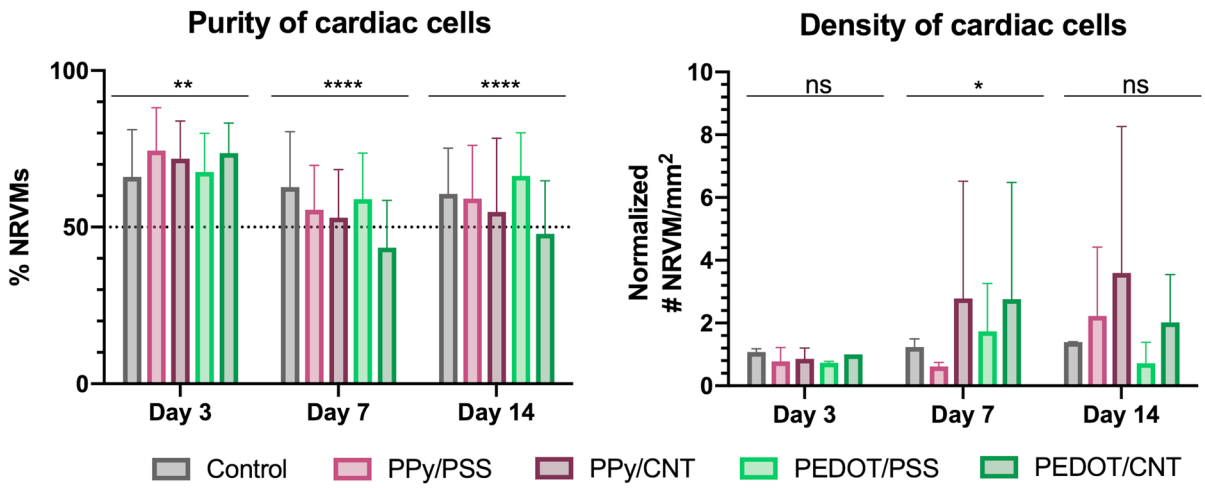

Fig. 4 Cardiomyocyte index plot defined as (left) % of CMs present and (right) area occupied by CMs within the well plate. (ANOVA-Tukey's test within day groups)

these materials are able to reduce fibroblast long-term expansion capacity and enhance CMs survival. In contrast, the number of fibroblasts significantly increased in the PEDOT/CNT systems. Interestingly, from all the conductive systems, the presence of CNT on the substrates presented the lowest percentage of NRVMs after 7 and 14 days of culture, suggesting the enhanced proliferative effect on fibroblast of this nanomaterial.

On the other side, the density of CMs, i.e., number of α-actinin-positive cells present per mm^2 of sample, can give an idea of the amount of CMs covering the surface, and thus the capacity of the materials in promoting tissue formation. For a proper comparison, we have normalized the values calculated of the number of NRVM per mm^2 for each substrate and at each time point to the value obtained for the PEDOT/CNT at day 3 of the same experiment (Fig. 4b). From all the materials, PEDOT/CNT was chosen because in most of the experiments, it was the substrate that had the substantial amount of CMs with a more homogeneous, non-arrhythmogenic, calcium transients when compared with the other materials. First of all, the density of NRVM/mm^2 is similar for all the substrates at day 3, suggesting that the attachment and survival within the firsts days is similar to all materials: 1.08 ± 0.11 for gelatin, 0.78 ± 0.44 for PPy, 0.86 ± 0.35 PPy/CNT, and 0.73 ± 0.04 for PEDOT, normalized to PEDOT/CNT. Nevertheless, it is noteworthy that the amount of NRVMs on CNT-containing substrates increase significantly after 7 days (2.78 ± 3.73 PPy/CNT and 2.76 ± 3.72 for PEDOt/CNT) and it is much higher than the substrates without CNT (0.62 ± 0.12 for PPy and 1.74 ± 1.52 for PEDOT) or the gelatin controls (1.24 ± 0.26). This indicates that CNTs enhance the survival of NRVMs over time, as previously reported (Martinelli et al. 2012; Martinelli et al. 2013; Peña et al. 2017). Interestingly, NRVMs cultured on PPy-based substrates experience a notable increase in the density during the last 7 days of the experiment (2.23 ± 2.18 for PPy and 3.60 ± 4.67 for PPy/CNT). On the contrary, in the case of PEDOT and PEDOT/CNT, such density increase is observed at day 7, but greatly reduced at day 14 (0.72 ± 0.67 for PEDOT and 2.02 ± 1.53 for PEDOT/CNT). Such observation suggests that PPy-based substrates favor cell survival for long periods. Regarding the gelatin controls, the density value of NRMVs is mainly constant, 1.39 ± 0.01 during all the time points, suggesting a large cell expansion. Finally, we calculated the size of NRVMs (m2/cell) on all the substrates (see Fig. S3 in the Supplementary Information). We observed that PPy-based substrates promote NRVM enlargement when compared with controls, while PEDOT-based substrates exhibited no significant differences when compared with controls. These facts suggest that PEDOT and PEDOT/CNT do not induce hypertrophy.

Overall, even though the purity and density of NRVMs on CNT-base substrates has not been the highest among all the tested substrates, calcium imaging indicated that these substrates have induced the more homogeneous, non-arrhythmogenic, and healthy-like calcium transient pattern. Because of this observation, we evaluated the intercellular communicative channels levels and localizations through immunostaining of gap-junction protein Cx43 of the NRVMs cultured on PPy/CNT and PEDOT/CNT after 14 days and compared the results with gelatin coated substrates as controls. The magnified images in Fig. 5 show representative images of NRVMs stained for Cx43 (red), α-actinin (green), and vimentin (pink) after 2 weeks of culture. At first glance, they revealed that the CMs cultured on the PPy/CNT and PEDOT/CNT substrates presented an increased area of Cx43 with a more organized localization when compared with the gelatin-coated glass substrates. Furthermore, multiple studies suggested that the organization of Cx43 is crucial for a healthy cardiac function and impulse propagation, and the alteration of such organization may lead to arrythmias (Kostin et al. 2004; Severs et al. 2004). Thus, we can conclude that both PPy/CNT and PEDOT/CNT systems induce a more organized Cx43 localization which correlate with our calcium imaging analysis results described above and thus, this indicate the great potential of CNT to be

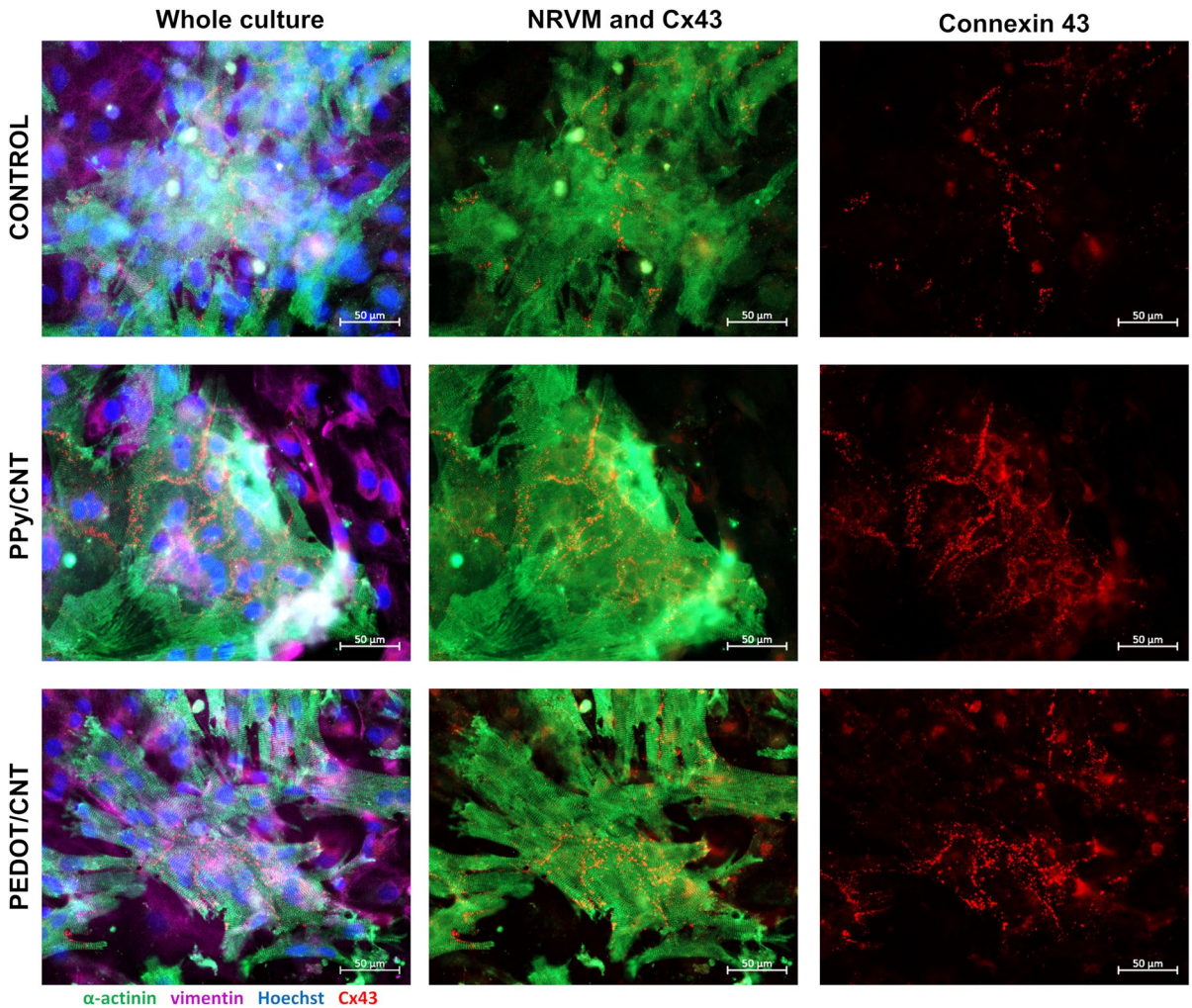

Fig. 5 Fluorescence imaging staining of Cx43 gap junctions (in red) to evaluate the intercellular communication after 14 days of co-cultured CMs (green) and fibroblasts (pink) on gelatin, PPy/CNT, and PEDOT/CNT bidimensional films

used in scaffolds to improve CMs function and better survival in cardiac tissue engineering applications (Ballerini 2013).

Conclusions

In this work, the in vitro interaction and functional effect of CNT combined with conjugated polymers (PPy and PEDOT) was evaluated on primary neonatal rat ventricular myocytes. Topological and electrophysiological cues for native CMs were provided, which demonstrated that the combination of CNT with the highly conductive PPy and PEDOT produces an excellent ensemble with high biocompatibility and unique conductive and interface capabilities, providing improved cell growth without any external stimuli. In particular, CNT-based substrates yielded to improved cellular function, thus more homogeneous spontaneous beating behavior with higher frequency, as shown with robust Ca^{2+} oscillation imaging. Moreover, cells cultured on PEDOT/CNT exhibited the highest beating amplitude, suggesting an enhancement of the intercellular function toward a more mature cardiac phenotype. In contrast, almost half of the cells cultured on substrates without CNT coatings yielded to arrhythmogenic beatings, suggesting the ability of CNT to maintain the cells in a healthier state. In addition, immunocytochemistry demonstrated that the conductive systems yielded to aligned sarcomeres, reduced fibroblast proliferation, and promoted long-term CMs survival, being the CNT-based substrates the ones with these enhanced properties on CMs after 14 days of incubation. Moreover, a more organized localization of Cx43 with increased percentage area was observed in both PPy/CNT and PEDOT/CNT which confirms that this material promotes a more health beating of CMs even after 14 days in culture.

Overall, our work demonstrates that the CP/CNT blend is excellent for interfacing NRVM and may improve the intercellular communication and function toward a more mature cardiac phenotype. Therefore, our next goal is to modulate the mechanical properties of the resulting 3D scaffolds to fit the optimal characteristic to host the cell in a 3D architecture. We would like to highlight that the combination of CP/CNT results in non-biodegradable scaffolds that prevent the release of free CNTs within the body, thus avoiding any possible toxic effects. We anticipate that the scaffolds composed of such an assembly will be interesting for both electrodes for recording or stimulating cell behavior and also for engineering artificial tissue efficiently in a tridimensional environment.

Author contribution N.A. developed the project and the experiments, wrote the main manuscript text, and prepared the figures. A.D. prepared de PEDOT samples and reviewed de manuscript. D.M., M.P., and L.M. reviewed the manuscript and gave final approval. B.P. supervised the experiments, reviewed the manuscript, and gave final approval.

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Ethics approval All animal procedures were performed according to the guidelines of the University of Colorado Denver Animal Care and Use Committee (protocol #00235).

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