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<https://doi.org/10.1021/acs.langmuir.9b01425>

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VERSION

AM (Accepted Manuscript)

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Rebelo, Ana M. Rodrigues, Changqing Liu, Karl-Herbert Schafer, Monika Saumer, Guang Yang, and Yang Liu. 2019. "Poly(4-vinylaniline)/polyaniline Bilayer Functionalized Bacterial Cellulose for Flexible Electrochemical Biosensors". figshare. <https://hdl.handle.net/2134/38368>.

Poly(4-vinylaniline)/polyaniline bilayer functionalized bacterial cellulose for flexible electrochemical biosensors

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Abstract

Bacterial cellulose (BC) nanofibril network is modified with an electrically conductive polyvinylaniline/polyaniline (PVAN/PANI) bilayer for construction of potential electrochemical biosensors. This is accomplished through surface-initiated atom transfer radical polymerization of 4-vinylaniline, followed by *in situ* chemical oxidative polymerization of aniline. A uniform coverage of BC nanofiber with 1D supramolecular PANI nanostructures is confirmed by FTIR, XRD and CHN elemental analysis. Cyclic voltammograms evince the switching in the electrochemical behavior of

BC/PVAN/PANI nanocomposites from the redox peaks at 0.74 V, in the positive scan and at -0.70 V, in the reverse scan, (at 100 mV.s⁻¹ scan rate). From these redox peaks, PANI is the emeraldine form with the maximal electrical performance recorded, showing charge-transfer resistance as low as 21 Ω and capacitance as high as 39 μ F. The voltage-sensible nanocomposites can interact with neural stem cells (NSCs) isolated from subventricular zone (SVZ) of the brain, through stimulation and characterization of differentiated SVZ cells into specialized and mature neurons with long neurites measuring up to 115 $\pm$ 24 μ m length after 7 days of culture without visible signs of cytotoxic effects. The findings pave the path to the new effective nanobiosensor technologies for nerve regenerative medicine, which demands both electroactivity and biocompatibility.

Keywords: bacterial cellulose, polyaniline, poly(4-vinylaniline) interlayer, functionalization, biosensor

INTRODUCTION

Biosensors are a class of biological devices used for numerous applications, and “combines a biological component to detect an analyte and a physicochemical component to produce a signal which is measurable ¹”. Such biocomponents have micro- and even nanoscopic dimensions and thus, smart nanostructured materials with electrically conductive properties are required for the sensitivity and performance detection. Cellulose-based electroconductive paper has recently been explored for a wide range of potential applications such as in tissue engineering scaffolds ², sensors ³, energy storage devices ⁴, electronic displays ⁵ and other electronic devices ^{6,7}. These cellulosic-based devices exhibit many attractive features such as lightweight ⁸, bendable

⁹, ease synthesis ⁷, low cost ⁵ and versatile ¹⁰. There has been an eminent interest in using cellulose as a green and large-scale resource material owing to its high source availability, classified as the most abundant material on earth ^{5,11}. Cellulose is commonly found in plants in the forms of cotton and wood, but it is also present in cell walls of fungi and algae ¹². Certain kinds of bacteria can also produce cellulose – bacterial cellulose (BC) –, showing higher crystallinity and chemical purity than that of cellulose from plants ^{13–15}. With an exceptional porous 3D network and thin ribbon-like microfibrils ¹⁶, BC has much greater tensile strength ¹⁷ and can retain larger amounts of water (about 99% in weight) ¹⁸. Considering such advantages and demonstrated non-cytotoxicity, BC has been approved by US Food and Drug Administration (US FDA) for numerous biomedical applications ¹⁹. BC can be grown on several substrates with desired shapes ²⁰, and can be chemically modified to gain new functionalities to fully exploit its properties. In such instances, it can be treated to acquire a certain range of electrical conductivity either by blending ²¹, grafting or mixing with metal nanoparticles ¹⁸, intrinsically electrically conductive polymers (ICPs) ^{22–25}, ionic solutions ²⁶ and carbon nanotubes ²⁷. Polyaniline (PANI) is one of the most versatile ICPs and has been used in electronic and optic applications ^{28,29}. It has conductivity in the range of 10^{-10} up to 10^5 S.cm^{-1} ³⁰, and can be easily synthesized by chemical and electrochemical methods ³¹. PANI is environmentally stable ³², low-cost ³³, has facile and reversible doping chemistry that allows the control over the conductivity ³⁴, free-volume solubility ³⁵ and optical activity ³⁵.

Substrates like BC with high richness in hydroxyl groups can strongly tether other polymer chains to its surface, in particular PANI ³⁶. However, PANI grown directly on BC nanofibrils tends to form denser clusters covering BC nanofibril network, which can prevent the full conductivity range being achieved for the composites, along with good

mechanical integrity of the PANI coating. To overcome such limitations, PANI can be covalently grafted onto BC nanofibrils with the aids of a poly(4-vinylaniline) (PVAN) interlayer, resulting in an uniform distribution of the nanorod-like PANI on BC, as bespoke grafted structures in our previous published work ³⁷. The optimal fabrication of flexible and electrically conductive cellulose paper sheets with versatile properties has been validated with the presence of PVAN interlayer, that is also expected to contribute to an enhancement of cell adhesion owing to a more homogeneous nanostructured PANI-based coating of BC substrate ^{37–39}.

BC/PVAN/PANI nanocomposites synthesis was initially accomplished via surface-initiated atom transfer radical polymerization (SI-ATRP) followed by *in situ* chemical oxidative polymerization (COP) of aniline. Detailed analysis to the nanocomposites was performed to examine their morphological, chemical and electrochemical characteristics, which was also complemented with viability and differentiation assays with neural stem cells (NSCs). PVAN/PANI bilayer grafted BC membranes can be used in the biosensor technologies for identification and characterization of differentiated NSCs in stem cell-based therapies, for instances as herein demonstrated, to treat a wide range of neurological disorders, including Alzheimer's (AD) and Parkinson's diseases (PD), as well as spinal cord injuries, that demands specific neural cell-types ⁴⁰.

EXPERIMENTAL SECTION

Preparation of BC membranes

BC membranes were produced from fermentation of *Gluconacetobacter hansenii* (ATCC® 53582TM) inoculated at 10% in culture medium containing 6.8 g.L⁻¹ disodium hydrogen phosphate dodecahydrate, 5 g.L⁻¹ peptone, 5 g.L⁻¹ yeast extract, 1.5 g.L⁻¹ citric acid, 20 g.L⁻¹ glucose. After incubating at 30°C for 4 days under static

conditions in 6-well plates, the as-prepared BC nanofiber membranes were purified by soaking in pure water for 2-3 days and boiling in a 4 g.L⁻¹ NaOH solution for 40 min to remove medium and any adsorbed bacteria. The purified BC hydrogels 3.2±0.5 mm thick were oven-dried overnight at 60 °C.

Preparation of BC/PVAN/PANI composites

BC/PVAN/PANI synthesis was completed in three steps as described in our published work ³⁷ and schematically exemplified in Fig. 1. In summary, oven-dried BC membranes were mixed with 4-dimethylaminopyridine (DMAP, Sigma Aldrich), dry-dimethylformamide (dry-DMF, fisher scientific) and triethylamine (TEA, Sigma Aldrich) under stirring, followed by drop-by-drop addition of 2-bromoisoturyl bromide (BiBB, Sigma Aldrich) in an ice-bath. The reaction was carried out at room temperature for 24 hours kept under inert atmosphere with argon, using BiBB:TEA:DMAP=1:2:2 molar ratio in relation to BiBB. The ready-made BC-BiBB membranes were soaked in DMF (Sigma Aldrich) for 2 days as purification step. For grafting PVAN onto BC surface, 4-VAN (97% Sigma Aldrich) was added at BiBB:VAN=1:5 molar ratio to copper (II) chloride (CuCl₂, Sigma Aldrich) and N,N,N',N'',N''-pentamethyldiethylenetriamine (PMDETA, fisher scientific) in DMF with stirring, after which ascorbic acid (ASCA, Sigma Aldrich) was injected dropwise. BC-BiBB membranes were later included into the reaction with vigorous stirring for 24 hours at room temperature under argon atmosphere. The molar ratio used to CuCl₂ for ATRP reaction was CuCl₂:PMDETA:ASCA:VAN=1:2:5:500. BC-grafted-PVAN composites (referred to as BC/PVAN) were thoroughly washed with DMF and water, and left in an excess amount of DMF overnight for removal of any PVAN homopolymer physically adsorbed. The conductive PANI layer on BC/PVAN

was prepared via COP of aniline. Overall, 0.25 M of ammonium persulfate ((NH₄)₂S₂O₈, APS, Sigma Aldrich) was dissolved in 10 mL of 1 M hydrochloric acid (HCl, 32%) with later immersion of BC/PVAN membranes. Then, 0.20 M of aniline (ANI, Sigma Aldrich) was added dropwise and left running for 6 hours. The ready-made BC/PVAN/PANI membranes were thoroughly washed with 100% ethanol (Sigma Aldrich) and soaked in pure water for several days until pH raises up to 7.

Figure 1. Schematic diagram of the synthesis of BC/PVAN/PANI nanocomposite membranes. a) SI-ATRP of VAN onto BC membranes followed by COP of aniline in acidic medium (HCl) with APS as oxidant. b) Optical image of the as-prepared BC/PVAN/PANI nanocomposite membrane. c) Chemical structure of BC, PVAN and PANI.

Characterization

The surface morphology and atomic composition of BC nanofibril composites was analyzed using scanning electron microscopy accoupled with energy-dispersive X-ray spectroscopy (SEM-EDS). The samples were assembled on carbon tape and sputtered with 0.1mm thick gold/palladium for 60 seconds with a rotatory-pumped coating system

(Q150R S, Quorum Technologies, UK) prior to SEM observation (GeminiSEM, Zeiss). Infrared spectra were acquired with a Shimadzu FTIR-8400S spectrophotometer (USA) equipped with an attenuated reflectance attachment (ATR) in the wavenumber range of 4000-600 cm^{-1} . X-ray diffractograms (XRD) were acquired with a bench-top X-ray diffractometer (D2 PHASER, Bruker) using Cu radiation (30 kV, 10 mA, $K\alpha$ radiation ($\lambda = 1.542 \text{ \AA}$)) measured between 2θ of 0° and 40° in steps of 0.02° . The degree of crystallinity of BC was calculated considering the Segal crystalline index (CI) equation, as follows ⁴¹:

$$\%Crystallinity = \frac{I_t - I_a}{I_t} \times 100\%. \quad (1)$$

where I_t is the total intensity of the (2 0 0) peak for cellulose I β at $22.7^\circ 2\theta$ and of the (0 2 0) peak for cellulose II at $21.7^\circ 2\theta$, and I_a is the amorphous intensity at $18^\circ 2\theta$ for cellulose I β and at $16^\circ 2\theta$ for cellulose II.

CHN elemental analysis of the samples was determined using a CE-440 Elemental Analyzer from Exeter Analytical Inc. to estimate the amount of PVAN and PANI grafted on BC through atomic nitrogen (N) quantification. The grafting yield (GY) of PVAN and PANI on BC surface was calculated using the equation^{42,43}:

$$GY = \frac{m_{after} - m_{before}}{m_{before}} \times 100\%, \quad (2)$$

where m_{after} is the mass of the composite and m_{before} is the mass before the grafting.

Electrochemical measurements

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were performed in solid state using a Galvanostat/Potentiostat (Eco Chemie microAutolab type III).

BC/PVAN/PANI membranes were placed between two conductive glasses (Fluorine-doped tin oxide, FTO), acting as working and counter electrodes. The membranes were previously dipped into sulfuric acid solution (1M H₂SO₄) prior to any measurement. CV was recorded for different scan rates (50 and 100 mV) and EIS was obtained upon superimposition of a sinusoidal AC potential of 10 mV with applied DC bias of 0, 0.5, 1, 1.5, -0.5, -1 and -1.5 V, over the frequency range of 9997 Hz to 0.1 Hz.

Cell culture, viability and differentiation

Cell isolation and culture

Neural stem cells (NSCs) from subventricular zone of the brain (SVZ) were isolated from 1–5 day old postnatal BALB/c mice (P1-P5), following German local regulations for animal welfare. After appropriate cell purification and digestion procedures, cells were expanded for 7 days in DMEM-F-12 GlutaMAX medium supplemented with 1% penicillin/streptomycin (10,000 u Penicillin, 10 mg.mL⁻¹ Streptomycin, Pen/Strep), 1% bovine serum albumin (BSA, 35% in PBS), 2% neural cell culture supplement β -27 (without retinoic acid), β -mercaptoethanol (50 mM), 0.2% recombinant human fibroblast growth factor (rh β FGF, 10 μ g.mL⁻¹) and 0.1% recombinant human epidermal growth factor (rhEGF, 10 μ g.mL⁻¹). The culture was kept in the incubator at 37 °C and 5% CO₂ atmosphere with medium replenished every 2 days, followed by dissociation.

Cell viability

Cell viability was assessed in our previous work ³⁷ using live/dead assay. Briefly, SVZ cells were seeded at 2x10⁴ cells.cm⁻² and kept for 7 days in proliferation medium under the atmospheric conditions described above. On the last day of culture, the staining solution (1% calcein acetoxymethyl ester, calcein AM + 1% propidium

iodine, PI, in PBS) was added into each well and incubated for 20 min at 37 °C. The total number of live and dead cells were calculated for each condition based on 4 images taken per well using a fluorescence microscope (Olympus, IX71, Japan), which were used to estimate the cell viability:

$$\% \text{ cell viability} = \frac{\text{number of live cells}}{\text{total number of cells}} \times 100\% \quad (3)$$

More details on the procedure can be found in our most recent study ³⁷.

Cell differentiation

Cell differentiation was performed to assess the ability of SVZ cells to differentiate in the presence of BC composites. Cells were harvested on BC-based membranes for 7 days at cell density of 5×10^4 cells.cm⁻² using differentiation medium composed by DMEM-F-12 GlutaMAX medium supplemented with 1% Pen/Strep, 1% bovine serum albumin (BSA), 2% β -27 with retinoic acid and β -mercaptoethanol. BC composites and glass coverslips were previously coated with extracellular matrix (ECM) gel diluted in DMEM-F-12 at 1:200. ECM-coated coverslips were used as control for differentiation. For cell tracing during the culture period, SVZ cells were labelled with a CellTrace™ CFSE Cell Proliferation Kit (Invitrogen, Thermofisher Scientific) before harvesting, following the protocol described by the supplier. In summary, after cell culture and dissociation, CFSE staining solution (1:1000 dilution in phosphate buffered solution, PBS) was added to cell suspension at 1 mL per each 1 million cells and incubated at 37 °C carefully protected from the light. Complete culture medium was added thereafter and incubated for more 5 min. Cells were resuspended in fresh and pre-warmed differentiation culture medium to induce cell differentiation. SVZ cells were observed at different check-point times (2 hours, 24 hours and 7 days) with fluorescence microscope in the green fluorescence mode. On the 7th day, cells were fixed with 4%

paraformaldehyde solution (PF) and immunostained with green fluorescence protein (GFP) and red fluorescence protein (RFP) for detection of neurons and glial cells, respectively, and cell nuclei were observed with DAPI (blue). Cell membranes were first permeabilized with 0.5% Triton X-100 for 10 min. Unspecific binding was prevented with 1% normal donkey serum (NDS) incubated for 1 hour, followed by the addition of the primary (1:200 anti-rabbit β -tubulin III for neurons + 1:500 anti-mouse α -GFAP for glial cells) and secondary antibody (1:1000 DAM-488 for β -tubulin III + 1:1000 DAR-594 for α -GFAP) solutions which were incubated for 1 hour, one at a time. Each step was followed by sequential washes with phosphate buffered saline with tween 20 (PBST) and/or PBS (except for NDS). Finally, cells were incubated with DAPI for 10 min and washed with pure water. BC substrates and coverslip with ready-stained cells were mounted on glass slides with fluorescence mounting medium for observation with fluorescence microscope. All membranes used were subjected to a dialysis treatment for one week and further steamed sterilized at 121 °C prior to their use for any biological characterization.

Statistical analysis

The percentage of differentiated cells after 7 days of culture was determined based on the average number of cells showing processes in three CFSE cell-labelled images per replica and per condition. Neurite length of 10 neurons was measured from three images captured for each condition after 2 hours, 24 hours and 7 days, using ImageJ software. Neurite length was considered to be the distance from the soma to the end of the longest neurite (if there is more than one neurite) or to the end of the longest branch at each branch point for branched neurites ⁴⁴. The results were expressed in terms of mean $\pm$ standard deviation. Statistical analysis was performed with one-way ANOVA test with p value < 0.05 considered as statistically significant.

RESULTS AND DISCUSSION

Morphological characterization

Fig. 2 shows representative SEM images of BC, BC/PVAN and BC/PVAN/PANI nanocomposites with corresponding fibers' diameter distribution. As seen, BC provides a nanofibrillar support that is suitable for subsequent chemical modification^{45,46}, with approximately 90% of the fibers measuring below 40 nm in diameter (Fig. 2d). It has been found both pristine BC and BC/PVAN consist of smooth nanofibers, which entails a uniform PVAN coating successfully deposited (Fig. 2b). However, after grafting with PVAN, almost 90% of the fibers had their diameters increased up to 80 nm (Fig. 2e).

Figure 2. SEM images of (a) BC, (b) BC/PVAN, and (c) BC/PVAN/PANI and (d-f) respective histogram of fibers diameter with relative and cumulative frequencies.

With PANI functionalization, the smooth BC/PVAN nanofibers turned into an uneven surface with diameters up to 600 nm (Fig. 2c,f) and predominance of nanorod-like arrangement over those typical PANI aggregates (Fig. 3a). The occurrence of these supramolecular structures of PANI is strongly dependent on the reaction conditions, which determines the presence of an elongated polyconjugate system with high conductivity^{47,48}. One-dimensional (1D) nanostructures, like nanorods and nanofibers,

are usually preferred for molecular electronic applications for numerous reasons, but specially for their superior electrical and optical properties ⁴⁹. This is attributed to the reduced charge carrier scattering ⁵⁰, along with a higher charge carrier mobility that facilitates the doping process ⁵¹. In our previous study ³⁷, we showed that PVAN promoted the growth of 1D PANI nanostructures instead of the typical random and irregular 3D PANI agglomerates, which was attributed to the surfactant effect of PVAN. Further, PVAN acts as a soft template with more growing sites for PANI chains on BC nanofibers, which may prevent the entanglement of grafted chains and hence, the aggregation, as schematically illustrated in Fig. 3b.^{49,52}.

Figure 3. a) High-magnification SEM image of BC/PVAN/PANI nanocomposites, showing PANI coating surface with nanorod-like structures (red arrows) and with aggregates in cauliflower-like arrangement (green arrows). b) Schematic representation of the molecular structure of a BC fiber grafted with PVAN/PANI bilayer. c) Representative SEM-EDS image of BC/PVAN/PANI composite membranes showing the atomic distribution on the surface, and respective d) sum map spectrum and e) X-ray maps of C1s, O1s and N1s. Blue circles in c) and e) mark areas where higher concentration of N1s was recorded.

The atomic composition of the surface layered of the composites is shown in the SEM-EDS image of Fig.3c, with the respective sum-map spectrum (Fig. 3d) and the X-ray maps (Fig.3e). As seen, the atomic distribution of carbon (red spots) and oxygen

(green spots) on the surface is quite uniform and intense all over the selected area, showing an apparent slightly higher concentration of nitrogen in certain areas (marked blue area in Fig.3c,e) that interestingly corresponds to more agglomerated and irregular regions. This indicates there are areas where probably PVAN and especially PANI (nitrogen-containing structures) covered BC more effectively. In our previous work ³⁷, TEM-EDS of a cross-sectional area showed a higher concentration of nitrogen on the top layer, which confirmed PVAN and PANI covered efficiently the surface of BC nanofibers. The high intensity of oxygen particularly observed in Fig.3c can be explained by the presence not only of BC (EDS depth resolution up to 2 μm) but also remaining APS used during aniline polymerization and retained in the structure, confirmed with the presence of Sulphur (S) in Fig.3d. Despite the observed, about 80% of the atomic weight of BC/PVAN/PANI surface is composed of carbon, as it would be expected (BC, PVAN and PANI are carbon-based materials), while nitrogen and oxygen correspond to 20% of the total composition (Fig.3d), which is consistent with the molecular structure schematically illustrated in Fig. 3b.

The thickness of each layer may be controlled with adjustments in the respective polymerization parameters, especially through ATRP. It is important to highlight that changes on PVAN interlayer may also affect PANI morphology and then, the conductivity of BC/PVAN/PANI composite. For instances, a higher content of PVAN grafting degree, as a non-conducting polymer, may lower the conductivity of BC/PVAN/PANI composite. Nevertheless, this may also contribute to increase the formation of more conductive PANI supramolecular structures (nanorods) and thus, enhancing the electrical conductivity of the overall composite. However, additional studies are required to reach the best performance of the composite.

Chemical characterization

Fig. 4 shows the FTIR spectra of unmodified BC, initiated BC-BiBB, and functionalized BC/PVAN and BC/PVAN/PANI membranes, indicating the assigned vibrational fingerprint modes of the diverse functional groups present. Pristine BC evinces a strong absorption band at 3600-3100 cm^{-1} due to the OH stretching and OH bending identified at 1641 cm^{-1} . CH stretching and symmetric bending of CH_2 of BC backbone are visible at 2887 cm^{-1} and 1425 cm^{-1} , respectively ⁵³. The successful immobilization of the initiating sites onto BC backbone can be confirmed in the spectrum of BC-BiBB with a new peak that arises at 1721 cm^{-1} , attributed to the O-C=O group of the initiator (BiBB) ⁵⁴. This is accompanied by a slight decrease in the intensity of vibrations ascribed to the OH bending at 1641 cm^{-1} observed in unmodified BC ⁵⁵. A significant lowering of peak intensity at 3343, 2893 and 1641 cm^{-1} is also witnessed after consecutive gratings with PVAN and PANI, indicating the esterification of the hydroxyl groups of BC followed by the growth of PVAN/PANI polymeric chains on the esterified sites ⁵⁶. This confirms the effective attachment of the bilayer onto BC that are covalently bonded. The peak at 1518 cm^{-1} of BC/PVAN membrane spectrum may be attributed to C=C stretching mode characteristic of aromatic rings ^{57,58}. In BC/PVAN/PANI spectrum, C=C stretching vibrations of the quinoid (Q) and benzenoid (B) rings from PANI can be observed at 1557 cm^{-1} (N=Q=N) and 1483 cm^{-1} (N-B-N), respectively ⁵⁹, whereas C-N stretching vibrations (C-N of secondary amines) of the quinoid and benzenoid rings are found at 1290 cm^{-1} and 1249 cm^{-1} , accordingly ³. The intensity of benzenoid and quinoid vibrations is approximately in the same range, which reflects the existence of equal amounts of Q and B moieties ⁶⁰. This indicates that the conductive PANI was efficiently synthesized onto BC-substrate ³⁸. Polaron formation of the quinonoid structure ($\text{Q}=\text{N}^+\text{H}-\text{B}$ or $\text{B}-\text{N}^+\text{H}-\text{B}$) due to doping (at 1118 cm^{-1}) further

corroborates its electronic features ⁶¹. Additionally, the peaks at 1034 and 785 cm⁻¹ can be ascribed to aromatic C-H in-plane and out-of-plane bending vibration of p-disubstituted benzene ring, respectively ^{3,62}. The broad band at 3210 cm⁻¹ is correlated with N-H stretching associated to the “charge-transfer” effect of PANI ⁶³.

Figure 4. FTIR spectra of BC, BC-BiBB, BC/PVAN and BC/PVAN/PANI membranes, with indication of functional groups ascribed to the corresponding vibration modes.

XRD patterns of untreated and treated BC membranes in Fig. 5 show two main diffraction peaks at 14.5° and 22.7° representing the various crystal plane of unit cell structure of I_α and I_β crystalline forms of BC ^{64,65}. The first diffraction peak is associated to 100_{Iα}, 110_{Iβ} and 010_{Iβ} plans and the second, to 110_{Iα} and 200_{Iβ} ⁶⁶. BC

crystallinity was estimated to be as high as 90.5%, similar to what has been found in literature for microbial cellulose ⁶⁵. The grafting of BC with PVAN and PANI layers causes a progressive decrease of the intensity and broadening of these peaks, which indicates lower crystallinity of the material and thus, suggesting successful inclusion of the bilayer onto BC. This effect is particularly obvious in BC/PVAN/PANI membranes. Nevertheless, two detectable diffraction peaks at about 16.9° and 34.9° in BC/PVAN spectrum have been found, which is likely to be associated with PVAN polymer ⁶⁷. The broaden spectrum of BC/PVAN/PANI membranes observed is likely correlated to the amorphous structure of PANI that has largely hindered the crystalline structure of the backbone BC, confirming the presence of a thick polyaniline layer as concluded with the increased diameter of the BC fibers (Fig. 2e,f) ⁶⁸.

Figure 5. XRD patterns of BC, BC/PVAN and BC/PVAN/PANI membranes.

The atomic composition extracted from CHN elemental analysis was performed to ascertain the content in carbon (C), hydrogen (H) and nitrogen (N) of the nanocomposites. Table 1 summarizes the chemical composition measured from the actual BC/PVAN and BC/PVAN/PANI nanocomposite samples in comparison with BC membrane. As N-rich structures, PVAN and PANI incorporation into BC network can be validated from the presence of N in BC/PVAN and BC/PVAN/PANI nanocomposites (Table 1), which is not detected in pristine BC. The successive functionalization steps of BC with PVAN and PANI are reflected in a progressive increase of %N content, as well as in the atomic N/C ratio, which raises from nil (BC) to 0.04 (BC/PVAN) and then to 0.07 (BC/PVAN/PANI). Indeed, PANI molecular structure, with four amines present in its repeating unit, is more abundant in N than PVAN, that has a sole primary amine (see Fig. 3b). This confirms BC was successfully embodied with PVAN and PANI, and strengthens the former results of FTIR and XRD. The grafting yield of both PVAN and PANI on BC estimated by means of weight gain, final states their efficient grafting, which is consistent with other published works ^{39,53}.

Table 1. Elemental atomic composition (%) obtained of pristine BC, and BC/PVAN and BC/PVAN/PANI nanocomposites, showing the corresponding atomic nitrogen/carbon ratios (N/C) and the grafting yield¹.

	C (%)	H (%)	N (%)	N/C	Grafting yield¹ (%)
BC	42.54	6.02	-	-	-
BC/PVAN	41.59	5.35	1.62	0.04	43
BC/PVAN/PANI	46.40	5.40	3.18	0.07	46

¹Calculated by weight gain (section 2.3)

Cyclic voltammetry

Cyclic voltammetry (Fig. 6) was performed on composite membranes to understand the redox processes undergone during potentiodynamic cycling in acidic media. Structural changes due to the oxidation and reduction were detected via current peaks upon doping or dedoping of protons and anions ⁶⁹. Pure BC and BC/PVAN are inherently insulators (grey line in Fig. 6), and hence, their respective cyclic voltammograms appear to be linear when plotted with that of PANI treated BC ⁷⁰. However, the enlarged view of the selected area reveals a trend for a capacitive-like response of BC and BC/PVAN ⁷¹. After incorporation of PANI coating BC/PVAN membranes acquire redox activity, as shown in Fig. 6 (blue and red curves) ³⁰. PANI is usually oxidized in two step-reaction according to its three different oxidation states: leucoemeraldine (LE), emeraldine (EM) and pernigraniline (PG) ⁷². Though, a single redox peak couple is observed at two selected scan rates, as indicated with arrows in Fig. 6. The oxidation potential at 100 mV.s⁻¹ scan rate appears approximately at 0.74 V and -0.70 V in the forward and reversible scans, respectively, corresponding to the conversion of LE into EM form ^{24,73}. During this process, benzoid and quinoid rings (EM) give place mostly to quinoid structures (PG) [Fig. 4]. At lower scan rate (50 mV.s⁻¹), the redox peak couple slightly shifted in the potential axis to 0.57 V and -0.56 V, respectively. This is likely due to the lower flux of charge carriers towards the electrode at slower scan rates (that is proportional to current) ⁷⁴. Other redox peaks associated with the fully oxidized and reduced form of PANI (PG and LE, respectively) were not herein detected, probably related to the type of acid used, the scan rate that was not sufficiently low to enable complete oxidation/reduction of PANI intermediates or because those transitions were not thermodynamically stable under these conditions ^{75,76}. Nevertheless, the oxidation of EM into PG may occur between +0.7 V and +1.5 V

with reduction of EM into LE between -1.5 V and -0.7 V ⁷⁴, that is associated to the release of radical cations compensated by imine ions from the electrolyte and to the formation of quinoid moieties, respectively ²⁴.

Figure 6. Cyclic voltammogram curves of BC, BC/PVAN and BC/PVAN/PANI recorded at 50 and 100 mV.s^{-1} of scan rates, with the respective magnification of BC and BC/PVAN curves, showing the oxidation and reduction processes of PANI. Red and blue arrows point out to the peak currents corresponding to emeraldine (EM) measured at 50 and 100 mV.s^{-1} .

Electrochemical impedance spectroscopy

The electrochemical impedance response of BC/PVAN/PANI was analyzed at different applied voltages that fall into the oxidation/reduction potentials of those corresponding to the oxidation states of PANI described above in CV analysis. Fig. 7b-h shows representative EIS data recorded for BC/PVAN/PANI nanocomposites at different voltage bias which were subsequently curve-fitted into two semi-circles using an equivalent circuit shown in Fig. 7a. The solution resistance (R_s) marks the beginning

of the first semicircle at higher frequencies that comprises a parallel combination of a charge-transfer resistance (R_{ct}) with the respective double layer capacitance described as constant phase element (CPE_1)⁷⁷. R_s accounts for the uncompensated resistance of the electrolyte (H_2SO_4)⁷⁸ and the resistance between the contact electrodes to some extent²⁴. R_{ct} can be ascribed to the electrode/electrolyte resistance concerning the charge-transfer between the electrode and the electrolyte and ionic diffusion, whereas CPE_1 can be assigned to a faradic capacitance (or pseudocapacitance) due to accumulation of charges from PANI redox process^{79,80}. At lower frequencies, the data could be extrapolated into a second semicircle formed by a polarization resistance (R_p) in parallel with a static double layer capacitance (CPE_2) attributed to the separation and accumulation of charges at the respective electrodes⁸¹. The partial absence of the low frequency semicircle has been pointed out as resulting from high ionic conductivity at the electrode/electrolyte interface⁸². CPE accounts for the non-ideal capacitor behaviour (depressed semicircle) of the nanocomposites and the deviation of the diffusion line from the 45° slope (Warburg element) at low frequencies^{80,83}.

Table 2. Data extracted from the Nyquist plots shown in Fig. 7.

V_{bias} (V)	R_s (Ω)	R_{ct} (Ω)	CPE_1 (F)	R_p (Ω)	CPE_2 (F)
0	51.3	21.2	26.7×10^{-6}	1.1×10^{12}	1.5×10^{-6}
0.5	51.3	20.7	39.0×10^{-6}	1.1×10^{12}	6.4×10^{-7}
1	53.3	291	43.3×10^{-6}	1.1×10^{12}	1.5×10^{-6}
1.5	55.7	500	18.0×10^{-6}	1.1×10^{12}	1.6×10^{-6}
-0.5	47.3	35	24.1×10^{-6}	1.1×10^{12}	2.1×10^{-7}
-1	49	532	26.2×10^{-6}	1.1×10^{12}	9.6×10^{-7}
-1.5	49.8	2350	19.5×10^{-6}	1.1×10^{12}	1.3×10^{-7}

R_s , arising mainly from electrolyte resistance, is largely independent on the applied DC bias (V_{DC}), as it would be expected. The parameters herein used to describe the semicircle at low frequencies (R_p , CPE_2) also showed not to be significantly affected by the applied potential, indicating that the diffusion limited electron transport and the charge accumulation were purely of a double layer charging on BC/PVAN/PANI surface ⁸⁴. However, notorious variations on R_{ct} can be observed, which reflects the redox properties of the composite.

According to the cyclic voltammogram of Fig. 6, +0.7 V sets the potential at which LE is transformed into EM, which is the highest conductive form of PANI. At +0.5 V, EM oxidation state is nearly entirely reached and thus, R_{ct} is low. From +0.5 to +1.5V, R_{ct} continuously increases up to 500 Ω owing to the conversion of EM into PG. This confirms that PG oxidation potential should take place above +0.7 V and below +1.5 V, as stated before in the CV analysis. When -0.5 V potential is applied, PANI is converted back to EM salt form as R_{ct} decreases. However, decreasing the potential down to -1.5 V the resistance increases to 2350 Ω , which corresponds to the conversion of EM to the fully reduced and also the least conductive form of PANI (LE). Contrarily, the capacitance CPE_1 increased up to 43.3 μF at +1 V and reached the lowest value of 18 μF at +1.5 V related with the accumulation of charges from the charge transfer process in a faradaic process. These results corroborate those obtained from CV and show that EM is the most conductive form of PANI with also higher capacitive properties. Deviations measured in R_{ct} and CPE_1 for symmetrical potential couple (e.g. -0.5 V *versus* +0.5 V) might be related with the heterogeneities of the membranes and with the slight displacement of the redox peak couple of PANI into its half-oxidized form (EM salt).

Figure 7. a) Randles equivalent circuit used to fit the impedance spectra shown in b-h. b-h) Nyquist plots of representative impedance data of BC/PVAN/PANI nanocomposites collected from different applied DC potentials upon superimposition of a sinusoidal AC potential of 10 mV. The solid lines (—) represent the fitting by the circuit in a).

The electrochemical behavior of BC/PVAN/PANI composites shows identical response of typical PANI coatings⁸⁵ sustained by a 3D BC structure readily for charge transport. The electrochemical activity of PVAN/PANI layer results mainly from a mix of protonic and electronic conduction upon redox reactions induced by the electrolyte and/or the applied potential⁸⁶, but we have recently demonstrated that BC matrix also contributes moderately to the electrochemical response measured⁸⁷, that most likely come almost exclusively from proton conductivity of the electrolyte embed in the structure and electrolyte/BC fibers interaction. This confirms BC confers good support for selective ions transport, whereas PVAN/PANI bilayer acts as a voltage- and

impedance-sensible capacitive coating. Such combined properties can assist in the construction of biological interfaces in biosensor technologies.

SVZ cell viability and neural differentiation

SVZ cell viability of BC membranes was assessed in our previous study ³⁷ using live/dead assay. The results showed that over 80% of cells were viable on BC membranes after 7 days of culture despite an apparent lower cell number on BC/PVAN/PANI compared to other conditions (about 50 % in relation to TCTP), which was attributed to some residual chemicals after COP reaction, such as HCl and aniline monomer, even though purification steps were performed prior to any biological characterization.

SVZ cell differentiation on ECM-coated BC-based scaffolds were checked after 2 hours and 24 hours using a non-cytotoxic green dye (CFSE) as tracking agent (Fig. 8), and were immunostained with GFP, RFP and DAPI in the end of the culture period. On the 7th day about 50% of SVZ cells were differentiated on coverslip and pristine BC, which increased up to 70% on BC/PVAN/PANI membrane (Fig. 8e), showing progressive cell maturation over the culture period. Differentiation was observed as soon as 2 hours after seeding with no significant difference in the following 22 hours, as expected for early differentiation times characteristic of primary NSCs ⁸⁸. An obvious higher cell number can be however witnessed on BC/PVAN, particularly on the seeding day, possibly due to the hydrophilicity of BC/PVAN compared to that of BC/PVAN/PANI ^{89,90}. This indicates that BC/PVAN provides a good substrate for cell adhesion. However, longer cell processes can be noticed on BC/PVAN/PANI than in any other substrate.

Figure 8. SVZ cell differentiation labelled with CFSE after 2 hours and 24 hours on a-b) BC, c-d) BC/PVAN, e-f) BC/PVAN/PANI and g-h) coverslip, respectively.

Immunostaining performed on the last day of culture (Fig. 9a-d) corroborates these findings, revealing highly differentiated cells on PANI-reinforced nanocomposites. Despite the higher cell number seen on BC/PVAN and coverslip, it is an evident conclusion that BC/PVAN/PANI composites promotes statistically higher differentiation degree with well-developed neurons and extended neurites compared to other conditions (Fig. 9e,f). Further, from Fig. 9 it seems that more cells were preferentially differentiated into neurons (GFP) on conducting membranes, whereas in other conditions astrocytes (RFP) were the predominant neuronal lineage. This apparent prevalence of neurons on BC/PVAN/PANI, as electrically-sensible cells⁹¹, leads to the conclusion that the functionalization of BC with PVAN/PANI bilayer may act as an external differentiating factor into highly specialized neural cells, which can be attributed to the electrochemical properties of PANI that stimulates the formation of well-developed neurites.

Figure 9. Representative fluorescence images of differentiated SVZ cells after 7 days of culture on a) BC, b) BC/PVAN, c) BC/PVAN/PANI and d) coverslip, with labelled neurons (GFP), glial cells (RFP) and cell nucleus (DAPI). e) Percentage of differentiated cells after 7 days. f) Evolution of neurite length over time of neuronal cells seeded on scaffolds and coverslip. * refers to statistical significance with 95% of confidence level (p value <0.5).

BC/PVAN/PANI membrane as an electrochemical biosensor

SVZ cells, a class of NSCs, have ability to differentiate into several types of neurons and glial cells under specific (external or internal) inducing agents, including electrochemical stimulus, surface morphology and properties and biochemical cues ^{92,93}. Such nanostructured PVAN/PANI bilayer in a 3D nanoporous BC network have higher surface area to volume ratio in contrast to the typical synthesized PANI agglomerates ⁹⁴, which can facilitate cell adhesion and functions of neural cells ⁹⁵, as well as the charge-transfer and redox reactions in electrochemical processes ⁹⁶. Therefore, increased selectivity, sensitivity and spatial resolution can be achieved with BC/PVAN/PANI nanocomposite to act as a biosensor device in NSCs differentiation.

Different neural cell types exhibit different electrochemical behavior, which can be used as an indicator of certain neural differentiation. Most of the nerve impulses in the nervous system (NS) occur with the release of neurotransmitters (small chemical molecules) from a neuron (presynaptic neuron) that triggers an action potential to a neighboring neuron(s) (postsynaptic neuron(s)). These neurotransmitters induce different functions in the human body and are cell-specific, i.e. one type of neurotransmitter can only be synthesized/received by certain class of neurons ⁹⁷. As schematically illustrated in Fig. 10, the small signaling chemical molecules can undergo oxidation in the extracellular medium with liberation of electrons, which in turn causes the reduction of PANI at the surface of BC/PVAN/PANI membranes. Reversible redox reactions taking place in PANI can be easily detected and measured via EIS and CV, as shown in sections 3.4 and 3.5. Higher concentration of released neurotransmitters would imply an increased frequency of redox reactions, which may cause quicker charge-transfer (lower electrical resistance) and redox switch of PANI oxidation states on BC/PVAN/PANI membrane surface. Each type of neurotransmitter, such as dopamine,

causes also different electrochemical responses that can be easily detected via CV, for instances. The chemical stability of BC/PVAN/PANI composites and thermostability for over 100 °C, as confirmed in our previous studies^{37,98}, ensures product stability and functional feasibility of BC/PVAN/PANI for biosensor applications. At the same time, such nanofibrillar and porous BC substrate can support the multiple focal adhesive contact points of cells on the nanocomposite membrane with efficient attachment, differentiation and nutrients/oxygen circulation⁹⁹. This enhanced electroactivity may be reflected in NSCs differentiated into specialized and mature neurons with well-developed neurites.

Figure 10. Schematic representation of BC/PVAN/PANI nanocomposite membrane performing as a dual mode electrochemical biosensor (impedimetric or amperimetric) for detection of certain types of neurons according to the neurotransmitter released and their concentration. Neuron stem cells are cultured and differentiated into several types of neurons. If dopaminergic neurons are detected, these can be transplanted to the brain to treat several neurological diseases, like Alzheimer and Parkinson. Briefly, dopaminergic neurons release dopamine (neurotransmitter) that is oxidized into dopamine o-quinone. The electrons released from dopamine oxidation process causes polyaniline to undergo redox reactions between the different oxidation states of polyaniline (emeraldine, pernigraniline and leucoemeraldine) that can be detected via CV (amperimetric response).

The dual functionality of BC/PVAN/PANI nanocomposite membranes may be used to assist in stem cell-based therapies with the differentiation and characterization of NSCs *in vitro* into determined neural cells prior to transplantation to the deficient site. Such cell therapies are highly demanding and have shown particular benefits in the treatment of several neurological disorders and spinal cord-injuries that lack of specific

neural cell-types. With advanced design and formulation, the flexible BC/PVAN/PANI nanocomposite membrane developed from this work may facilitate the state-of-the-art applications of specific nanobiosensor.

CONCLUSION

Electrically conductive BC/PVAN/PANI nanocomposite membranes were successfully synthesized in a three-step reaction. SEM images revealed the typical nanofibrillar structure of BC that allows the growth of PVAN/PANI bilayer coating that was strongly and uniformly tethered to BC. PVAN interlayer boosted the formation of rod-like PANI structures which can enhance the electrical conductivity performance. These functional nanocomposites respond to voltage-changes from charge transfer redox reactions taken place on PVAN/PANI surface inside the BC network. It is therefore possible to induce the electrochemical activity through BC/PVAN/PANI composites as a potential biosensor for analysis and detection of several biological analytes or structures. The validation of non-cytotoxicity and neural differentiation potential of SVZ cells confirmed that the functionalized BC nanocomposites may be included in the advanced *in vitro* medical devices that can detect and/or stimulate neuronal differentiation of NSCs for stem cell-based therapies of the Neuron Systems.

ACKNOWLEDGEMENTS

This research was supported by a Marie Curie International Research Staff Exchange Scheme Project of the 7th European Community Framework Program (Grant No. PIRSES-GA-2010-269113), entitled “Micro-Multi-Material Manufacture to Enable Multifunctional Miniaturized Devices (M6)”. The author Ana Rebelo also acknowledge

the PhD studentship awarded through a mini-CDT on “Intelligent, adaptable electronics for healthcare and wellbeing” of Loughborough University, as well as Dr Xiuli Chen for her technical inputs in the initial preparation of polymerization.

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