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Preparation of POMA Nanofibers by Electrospinning and Its Applications in Tissue Engineering

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***Abstract*—**In this manuscript, we produced neat electrospun poly(*o*-methoxyaniline) (POMA) fibers and utilized it for applying the growth of neural stem cells. The transparency and morphology of as-prepared POMA fibers was characterized by UV-visible spectroscopy and scanning electron microscopy, respectively. It was found to have no adverse effects on the long-term proliferation of the neural stem cells (NSCs), retained the ability to self-renew, and exhibit multipotentiality. Results of immunofluorescence staining studies confirmed that POMA electrospun fibers could provide a great environment for NSCs and enhance its differentiation.

***Keywords*—**Electrospun, polyaniline, neural stem cell, differentiation.

I. Introduction

IN the biological field, research on CPs for biomedical applications expanded greatly in the 1980s with the POMA fibers can be a potential substrate for the maturity of discovery that these materials are compatible with many biological molecules such as those used in biosensors. By the mid-1990s, CPs were also shown, via electrical stimulation, to modulate various cellular activities, including cell adhesion, migration, DNA synthesis, and protein secretion [1]-[4].

The exploration of polyaniline (PANI) for tissue engineering applications has progressed more slowly than the development of polypyrrole for a similar application [5]-[7] although recently there has been more evidence that PANI and its variants can support cell growth [8]-[10]. Subsequent studies further confirming this ability of PANI and its variants to enhance cell growth sparked interest towards the biocompatibility of PANI in vivo for tissue engineering applications. Among these is from Lelkes and co-workers who electrospun a novel blend of conductive camphorsulfonic acid-doped emeraldine PANI (CSA-PANI) and gelatin and provided detailed investigations of the ensuing submicron-sized fibers [8]. Kim and co-workers who developed electroactive fibers from blends of PANI and poly[(L-lactide)-*co*-(ε-caprolactone)] (PLCL) for regeneration of muscle tissue [11]. The result presented that PC12 cell viability is significantly higher on CPSA-PAni/PLCL fibers than on PLCL-only fibers, and the electrical conductivity of the fibers affects the differentiation of PC12 cells. Most of those groups investigated the biological properties in the blending fibers, instead of neat polyaniline that have poor solubility in common organic solvents.

NSCs possess the ability to selfrenew and differentiate into neurons for treating neurodegenerative diseases or brain injuries [12]. Knowledge of how cell fat can be mediated by extrinsic and intrinsic factors. Recent exciting work has demonstrated that functional biomaterial substrates can enhance proliferation and differentiation of NSCs [13]. This is important for efficiently enabling cell-based nerve regenerative strategies.

In this study, we attempt to explore novel biomaterials for NSCs, it has further investigate the use of electrospun polyaniline fiber mats. We demonstrate the feasibility of generating a novel type of biocompatible fibers in the form of neat inherently conjugated POMA not blended with other bio- or polymeric macromolecules. This study provides evidence for NSCs for tissue engineering as well as neuroscience research applications.

II. Materials and Methods

A. Synthesis of Poly(O-Methoxyaniline) (POMA)

Emeraldine (EM) salt of POMA was prepared through the oxidative polymerization of o-methoxyaniline (OMA) (Fluka) by ammonium peroxydisulfate (Fluka) in 2.0 *M* $CaCl_2$/HCl solution, which had been cooled in an ice bath (~0°C), under magnetic stirring for 12 hours. An intense blue-green precipitate was collected and washed several times until gray salts were eliminated. Afterwards, it was converted to EM-based POMA by treatment with excess 1.2 *M* NH_4OH, and then dried at 60 °C under vacuum drier for 24 hours. *M*w:69000, *M*n:20750, and the polydispersity of 3.326.

B. Reduction of POMA

The obtained POMA (0.4g) was dispersed into a solution of 4mL hydrazine hydrate in 40mL 1.0 M ammonium hydroxide and stirred for 10h. The reaction mixture was then filtered, washed with distilled water several times, and dried under dynamic vacuum at 40℃ for 24h. Finally, the POMA was reduced to the leucoemeraldine oxidation state (0.35g, 88%).

C. Electrospinning of POMA Fibers

A 5.0mL plastic syringe (TOP®, 5 cc/mL) was filled with the emeraldine base of POMA powder which was dissolved in tetrahydrofuran (THF) / dimethylformamide (DMF) (50:50, v/v) to make a 4 wt % solution and connected to a syringe pump (KD Scientific Model 200) in a horizontal injection. The solution was pushed through a capillary blunt steel needle (23G×1″; R.B.(0.63 × 25 mm) U.T.W.) at a feeding rate (*Q*) of

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0.02 ml·min^{-1}. The steel needle was connected to a high voltage power supply (*V*) (Matsusada, AU-40R0.75). A grounded dish (100×20 (Φ×H mm), Schott Duran®) and cover glass (24mm, Deckgläser) were placed at 12cm distance between the nozzle and collector (*H*). The POMA solution was fed at an applied voltage of 20kV for the collecting time of 0, 1, 2, 3, and 4 minutes, respectively. UV-Visible spectra were collected using a UV-Visible spectrometer (JASCO V-650).

D. Isolation and Culture of Neural Stem Cells

NSCs were isolated from the brains of Sprague–Dawley rat embryos at E14.5. Briefly, these tissues were cut into small pieces and stably reacted in sterile papain solution containing 30 mg/ml papain, 50 mM ethylene diamine tetraacetic acid(EDTA), 2 mg/ml cysteine, and 150 mM $CaCl_2$ (5 babies/1 ml) at 37°C for 15 minutes. They were then reacted with DNase I for 5 minutes and then added into 10% horse serum. After centrifugation and washing by HBSS twice, the dissociated cells were collected in a serum-free medium containing Dulbecco's modified eagle's medium-F12 (DMEM-F12), 10 ng/ml basic fibroblast growth factor (bFGF), 20 ng/ml epidermal growth factor (EGF), and N_2 supplement, 0.5% Penicillin and 1% Streptomycin. The number of live cells was counted by trypan blue exclusion assay in a hemocytometer.

NSCs were cultured in non-coated flasks above culture medium and maintained at 37°C in a humidified atmosphere of 5% CO_2. After 1 ~ 3 days in vitro, cells were undergoing cell division and the proliferating cells formed neurospheres, which were suspended in the medium. Several weeks later, adherent cells were discarded and suspended neurospheres were collected by centrifugation, mechanically dissociated, and subcultured as global cells in a new culture flask with fresh medium containing the same concentration of bFGF. These cells grew into new spheres after 1 ~ 3 days, that is, NSCs proliferated and formed new neurospheres. The process of subculture was repeated again to achieve the purified NSCs and proliferating neurospheres.

E. Immunofluorescence Staining

Cells plated in a six-well plate were washed two times with PBS then fixed for 15 minutes at – 20°C with ice-cold methanol. After being blocked for 30 minutes with PBS containing 3% BSA, the cells were incubated for 1 hour with primary rabbit anti-Tuj 1 antibody (diluted 1:200), rabbit anti-MAP2 (diluted 1:500), rabbit anti-S100 (diluted 1:500) or mouse anti-O4 (diluted 1:500). After washing, Texas red-conjugated second antibody (diluted 1:200), Alexa Fluor®488-conjugated second antibody (diluted 1:500), or Alexa Fluor ®594-conjugated second antibody (diluted 1:500) were added for 1 hour. The cells were then washed for 3 × 15 minutes with PBS. Hoechst 33258 or DAPI were added in the last wash as needed. Fluorescence microscopy was performed using a Nikon ELIPSE TE2000-U microscope (Nikon, Tokyo, Japan). Image acquisition and peripheral device control were automated using Metafluor image software (Universal Imaging Corporation, West Chester, PA). Experiments were repeated three times with similar results. The average percentage of immunoreactive ratio within migrated cells immunoreactive for anti-MAP2, S100 or O4 was calculated from the numbers of phenotypic marker cells divided by the number of DAPI-stained cells. At least 200 cells were counted in each condition. Data were collected from three independent experiments for each condition (n = 3/condition) and expressed as mean ±SD. All statistical analysis was performed using one-way ANOVA followed by the Duncan's test., with $p < 0.05$ considered significant.

F. Scanning Electron Microscopy

The surface morphology and the cross-section of the electrospun POMA fibers were observed using a Hitachi S-4100 field-emission scanning electron microscope at an accelerating voltage of 15 kV. Prior to observation, the specimens were sputter-coated with gold. After 7 days of culturing, NSCs attached to the electrospun POMA nanofibers were washed with PBS twice then fixed in 3.7% formalin solution for 10 minutes. The sample was then post-fixed with a solvent mixture of acetone/95% ethanol (50:50, v/v), dehydrated by ethanol in an increasing concentration gradient, and dried at room temperature. Afterwards, the sample was coated with gold and observed under a SEM.

G. Statistical Analysis

Data are expressed as mean-standard deviation (S.D.). Differences were determined by one-way analysis of variance (ANOVA) with Duncan's test. Statistical significance was displayed as $p<0.05$, 0.01, or 0.005.

III. Results and Discussion

A. Chemical Oxidation of POMA

The leucoemeraldine base (LB) form of the POMA was dissolved in an NMP solution. Subsequently, trace amounts of the oxidant, $(NH_4)_2S_2O_8$, were introduced into the POMA solution gradually for in situ monitoring of the sequential oxidation process of the conjugated components by UV-Visible absorption spectroscopy, as shown in Fig. 1. Initially, only one absorption band was visible at 321 nm, which was associated with the π–π* transition of the conjugated ring system [14]. Upon the addition of trace amounts of the oxidant, slow oxidation of the conjugated segments in POMA was observed. The original absorption band detected by UV-Visible spectroscopy underwent a blue shift from 321 to 307nm.

At the same time, a new absorption peak appeared at 625nm, which was assigned to the exciton-type transition between the HOMO orbital of the benzoid ring and the LUMO orbital of the quinoid ring [15]. After its intensity reached a maximum, λmax of absorption began to undergo a blue shift from 625 to 572nm. One possible interpretation of this result is presented as follows. During the continuous oxidation of POMA in the LB oxidation state the POMA was first converted to the emeraldine oxidation state. After the absorption peak reached maximum intensity, it exhibited a blue shift. This blue shift is indicative of the conversion from the emeraldine base state to the pernigraniline oxidation state.

B. Transparency of Electrospun Poma Substrates

Substrates were electrospun from pure POMA with five different electrospinning times, namely, 0, 1, 2, 3, and 4 minutes. UV-Vis transmittance of POMA fibers electrospun onto cover glasses depended on the various electrospinning duration. As shown in Fig. 2 (a), the cover glass is spotless and transparent that penetrability can reach 90% without the electrospinning process. By increasing the electrospinning time of POMA, the substrates gradually became opaque, *viz.,* T% was reduced from 90% to 10% following the deposition of POMA fibers on the cover glass. This can be observed in Fig. 2 (b) to (e). A qualitative analysis of the appearances from inserted photographs (a) to (e) indicates that independent of electrospinning time at 0, 1, 2, 3, and 4 minutes. It can also be noted that the transmittance of POMA substrates was significantly decreased with respect to electrospinning time. With regard to cell culture, the POMA substrate is thought to contribute to the ease of examination hence POMA electrospun for 3 minutes was considered suitable for our experiments.

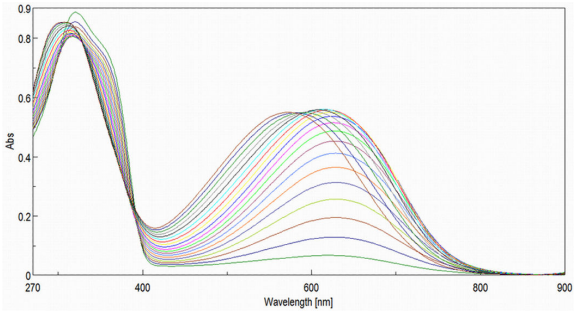

Fig. 1 UV-Vis absorption spectra monitoring the chemical oxidation of POMA in the leucoemeraldine oxidation state in an NMP solution

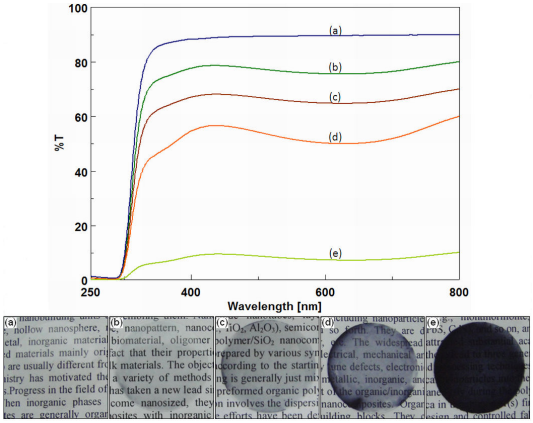

Fig. 2 UV-Vis spectra of POMA fibrous substrates obtained with 4 wt % POMA electrospinning solution at V = 20 kV, H = 12cm and Q = 0.02ml·min–1. Transparent tests for electrospun POMA formed on cover glass with five electrospinning times: (a) 0min, (b) 1min, (c) 2min, (d) 3min, and (e) 4min

C. Electrospun Fibrous Poma Substrates in Neural Stem Cells Culturing

The interconnected fiber structure of electrospun POMA substrates formed with a random distribution of nonwoven web provided the appropriate surface topography for tissue engineering applications, as illustrated in Fig. 3 (a). In addition to visualization by light microscopy, we used scanning electron microscopy for close inspection of the NSCs morphology. As highlighted in Fig. 3 (b), NSCs cultured on electrospun POMA substrate extended neurites (or filopodia) which attached onto the long POMA fibers (red arrowhead). SEM image also showed large neurites (or filopodia) growing along the POMA fiber, suggesting that the POMA fiber could also provide mechanical support and even a positive cue to encourage neurite outgrowth. These results also confirmed that NSCs preferentially grow and spread out on micron-scale three-dimensional structure fibers.

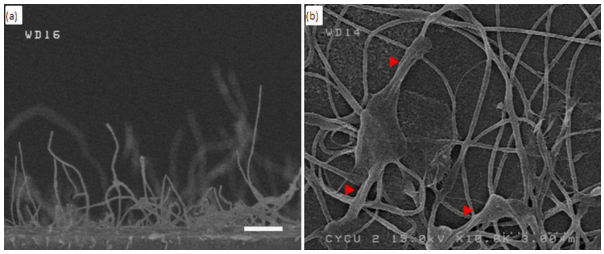

Fig. 3 The SEM image of (a) cross-sectional POMA fibrous substrates obtained with electrospinning time of 3 min and 4 wt % POMA solution at V = 20 kV, H = 12cm and Q = 0.02 ml·min^{-1}, (Magnification of × 3,000 and Scale bar = 5μm) and (b) neural stem cells cultured on fibrous substrate for 7 days. Arrowheads show the neurites (or filopodia) attached onto POMA fibers. Magnificaiton of × 10,000 and scale bar = 3μm

D. Nscs on Electrospun Poma Nanofibers: Maintenance and Differentiation

Photomicrographs of NSCs cultured on PDL-coated and electrospun POMA substrates at an initial density of 5×10^4 cells/ml, in serum-free medium to allow cell attachment, are shown in Fig. 4 (a). It could be seen that as early as 2 days in culture NSCs attached onto the electrospun POMA substrates and self-differentiating cells already extended out from the neurospheres (blue arrow). The number and adhesion of outgrowth from migrated cells within the neurospheres extended their territory steadily over the course of 12 days along the electrospun POMA substrates. On the contrary, cultured NSCs on PDL-coated substrates withdrew their extending processes after 12 days of culture (red arrowhead).

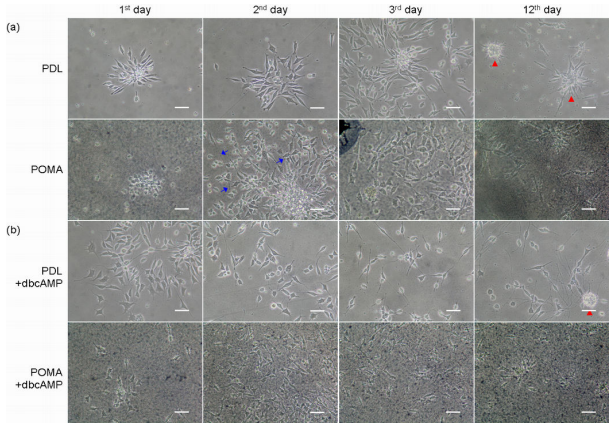

Fig. 4 Photomicrographs illustrate morphology and outgrowth development of NSCs on PDL and POMA substrates. Cells were treated with and without 0.5 mM dbcAMP for 1 day, 2 days, 3 days, and 12 days. Scale bar = 100µm. The blue arrow indicates extending processes, and the red arrowhead indicates neurospheres form

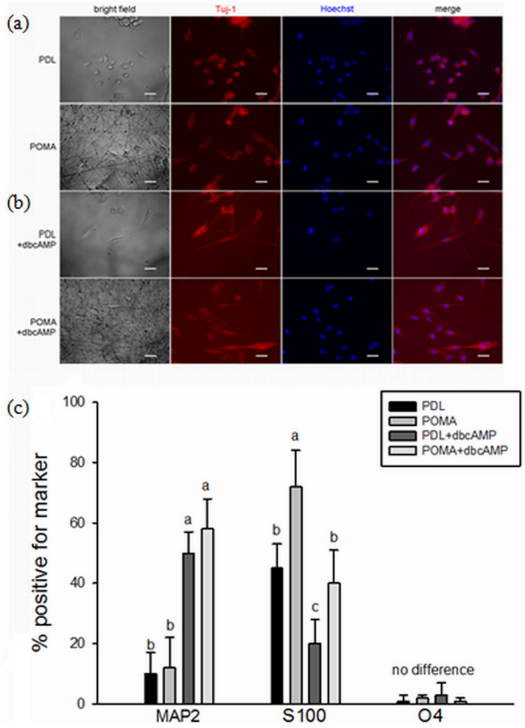

Fig. 5 Neuronal differentiation of NSCs on polystyrene coated with PDL, POMA. The effect of the substrates was evaluated based on the immunofluorescence staining result. Fluorescent photomicrographs represent NSCs phenotypes of (a) cellular activities and (b) differentiation generated from neurospheres on PDL or POMA substrates after 6 days. Tuj-1 (red) expressed in the immature neuron was immunoassay and cell nuclei were counterstained with Hoechst dye (blue). Scale bar = 50µm. (c) Comparison of neuronal and glial commitment of NSCs cultured for 7 days on coated with PDL or POMA. Percentages of cells staining positive for MAP2 (neuron maker), S100 (astrocyte maker) and O4 (oligodendrocyte maker) were evaluated. Data are presented as mean ± SD, n = 3. Values with different superscript letters are significantly at $p<0.05$ analyzed by Duncan's test

N(6),2'-O-dibutyryladenosine 3':5' cyclic monophosphate (dbcAMP) which is a membrane permeable analog of cAMP enhanced neuronal differentiation of neural stem cells [16]-[18]. With dbcAMP, NSC displayed enhanced neurite outgrowth (Fig. 4 (b)).

Since NSC culture represents a heterogeneous population of glial and neuronal cells that cannot be discriminated based on their morphology, we verified the differentiation capacity using immunofluorescence staining. Fig. 5 (a) shows fluorescent images of cells cultured for 6 days on the array and then immunologically stained using TuJ-1 antibody against neuronal β-tubulin (a neuronal differentiation marker) [19]-[21]. The cellular nuclei were labeled with Hoechst dye.

On the other hand, in Fig. 5 (b), NSCs were directly induced to differentiate by dbcAMP on both substrates after 2 days of culture. Differentiated cell neurites sent out long, straight, and slender neurites into surrounding areas. Examination of the immunostaining results with Tuj-1-positive cells revealed similar growth morphology for cells grown on PDL-coated and electrospun POMA substrates, suggesting similar effects of both substrates on the growth of differentiating neurons. For the duration of culture, differentiated neurons began to aggregate on coated PDL substrates but remained quite dispersed on electrospun POMA substrates, as shown in Fig. 4 (b). This indicates that differentiated NSCs are prone to aggregate on PDL-coated substrate (red arrowhead). Clearly, the biomimetic fibrous substrate is superior to the structure provided by a coated substrate.

In the presence of dBcAMP, the neural stem cells proliferated less and more readily extended axon-like protrusions. However, early stages of cell commitment to neuronal or astrocytic fate are present, as evidenced by immunocytochemistry The comparison of differentiation commitment between POMA and PDL revealed a significantly higher amount of S100positive cells on the POMA substrates ($p<0.05$) (Fig 5 (d)). It was not observed the MAP2 expressing cells in the undifferentiated ones. Immunostaining for the neuronal marker revealed higher percentage of cells MAP2 positive in medium containing dbcAMP as compared to dbcAMP free culture, both on POMA and PDL. These results indicate that POMA not only improved NSCs growth, but also enhanced differentiation of NSCs into the neurons and astrocytes.

IV. Conclusion

In this study, we demonstrated for the first time the feasibility of generating a novel fibrous substrate in the form of neat inherently conjugated polyaniline family not blended with any biomacromolecules. The properties of POMA were evaluated with a various analytical techniques such as UV-vis and SEM. The transmission spectra of POMA exhibits lower optical clarity with increasing the electrospinning time. The POMA electrospun for 3 minutes that bead-free fiber was considered suitable for our cell culture studies. Those experimental data demonstrated that the electrospun POMA nanofibers performed better than PDL-coated in supporting attachment, growth and differentiation of NSCs. Therefore, the

present study provides evidence that POMA nanofibers can be a potential substrate for the development of NSCs for neuroscience research applications.

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