ACS APPLIED BIO MATERIALS

Article

Cite This: ACS Appl. Bio Mater. 2019, 2, 518−526

www.acsabm.org

Peptide-Based Polyelectrolyte Promotes Directional and Long Neurite Outgrowth

Chia-Yu Lin,[†] Shyh-Chyang Luo,[†,⊥] Jia-Shing Yu,[‡,⊥] Ta-Ching Chen,[§,∥,⊥] and Wei-Fang Su*[,†,⊥]

[†]Department of Materials Science and Engineering, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 10617, Taiwan

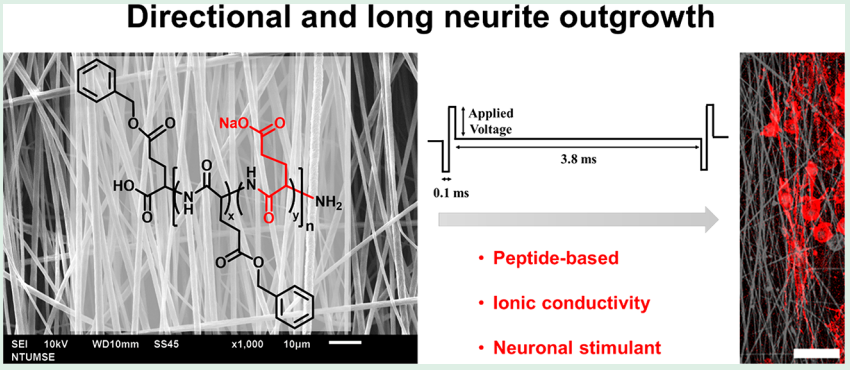
[‡]Department of Chemical Engineering, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 10617, Taiwan

[§]Department of Ophthalmology, College of Medicine, National Taiwan University, Taipei 10002, Taiwan

[∥]Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Tapei 10002, Taiwan

[⊥]Molecular Imaging Center, National Taiwan University, Taipei 10617, Taiwan

Supporting Information



ABSTRACT: Neural tissue engineering has emerged as a promising technology to cure neural damages. Although various synthetic polymers with good biocompatibility and biodegradability have been adopted as candidate materials for scaffolds, most of them require the incorporation of biomolecules or conductive materials to promote the growth of long axons. Herein we demonstrate for the first time a unique peptide-based polyelectrolyte that is ionically conductive and contains a neurotransmitter, glutamic acid. The designed polymer, sodium salt of poly(γ-benzyl-L-glutamate)-*r*-poly(L-glutamic acid) (PBGA20-Na), was synthesized and fabricated into a 3D fibrous scaffold with aligned fibers. Neuron-like rat pheochromocytoma (PC12) cells were cultured on the scaffolds to evaluate cell proliferation and differentiation with or without electrical stimulation. The results show that with both electrical and biochemical cues presented in the polyelectrolyte, PBGA20-Na promotes longer neurite outgrowth compared with the neutral poly(γ-benzyl-L-glutamate) (PBG) and the poly(γ-benzyl-L-glutamate)-*r*-poly(L-glutamic acid) (PBGA20). Furthermore, the neurite length of the cells cultured on PBGA20-Na is more than twice as long compared with the conventional biopolymer, polycaprolactone. In conclusion, PBGA20-Na is a promising biomaterial for neural tissue engineering and drug-screening platforms.

KEYWORDS: *neuron regeneration, tissue engineering, polypeptide, polyelectrolyte, electrical stimulation, glutamic acid*

INTRODUCTION

Millions of people suffer from neurodegenerative diseases arising from the central nervous system, especially in the brain, optic nerve, or retina. For example, the neural damages in the brain lead to Alzheimer's disease, Parkinson's disease, and Huntington's disease.[1,2] The degeneration of retinal ganglion cell leads to glaucoma and irreversible blindness.[3] How to repair the damaged central nervous tissues remains a challenge issue because central nervous tissues have limited intrinsic regenerative capacity after injury.[4] Recently, neural tissue engineering has been considered as a promising technology to transplant functional engineered neural tissues and treat neurodegenerative diseases.[5−7] The key to tissue engineering is to provide a 3D scaffold serving as an artificial extracellular matrix.[8] However, the biocompatibility and biodegradability are crucial properties for an artificial functional scaffold. Furthermore, an architecture that allows neurons to directionally extend axons is also a critical issue for rebuilding neuron communication.

Synthetic scaffolds made of polyesters or oligopeptides have shown successful culture of neurons or stem cells.[7,9,10] However, synthetic polyesters usually lack cell recognizable

Received: November 11, 2018
Accepted: December 13, 2018
Published: December 13, 2018

DOI: 10.1021/acsabm.8b00697
ACS Appl. Bio Mater. 2019, 2, 518−526

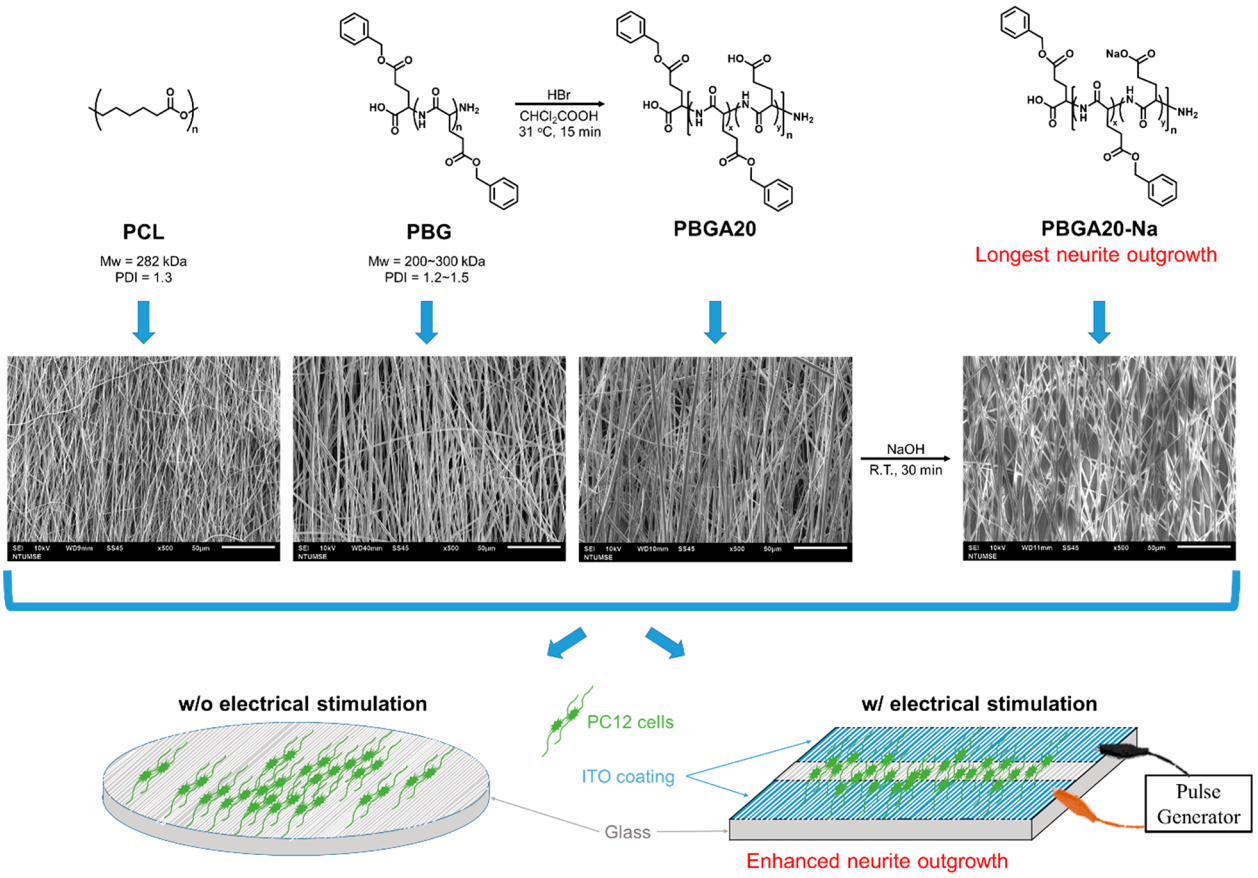


Figure 1. Schematic illustration of synthesizing and fabricating PCL, PBG, PBGA20, and PBGA20-Na scaffolds for culturing PC12 cells without or with electrical stimulation.

ligands and may degrade into acids and alcohols, causing local low-pH and inflammatory reactions.[11−13] Oligopeptides could be synthesized by solid-phase synthesizers or genetic engineering. The process is time-consuming, and the yield is usually low.[14−16] Scaffolds made of conducting polymers combined with external electrical stimulus can stimulate longer axon growth of neurons.[17,18] Nevertheless, conducting polymers are inherently nonbiodegradable,[19] which is not suitable for tissue regeneration purpose. Herein we propose a unique peptide-based polyelectrolyte, sodium salt of poly(γ-benzyl-L-glutamate)-*r*-poly(L-glutamic acid) (PBGA20-Na), which is biodegradable because of the peptide linkages, as shown in Figure 1. It contains glutamic acid, an excitatory neurotransmitter, which can promote synaptic transmission and neurogenesis.[20−22] The ionic conductivity could induce more and faster neurite outgrowth because nerves are electroactive.[19,23] Besides, it can be fabricated into a 3D fibrous scaffold with aligned fibers to guide the directional outgrowth of neurites. In this work, neuron-like rat pheochromocytoma (PC12) cells were cultured on the scaffolds to evaluate cell proliferation and differentiation with or without electrical stimulation. Two structurally resembled polypeptides, poly(γ-benzyl-L-glutamate) (PBG) and poly(γ-benzyl-L-glutamate)-*r*-poly(L-glutamic acid) (PBGA20), were used for comparison. Commercial polycaprolactone (PCL) served as a comparative control in this study.

MATERIALS AND METHODS

Materials. Chemicals for the characterization of cellular behaviors included a Live/Dead viability/cytotoxicity kit (cat. no. L3224, Molecular Probes), Alamar Blue cell cytotoxicity assay (cat. no. BUF102A, AbD Serotec), bovine serum albumin (BSA, cat. no. B4287, Sigma-Aldrich), 4′,6-diamidino-2-phenylindole (DAPI, cat. no. D8417, Sigma-Aldrich), phalloidin-tetramethylrhodamine B isothiocyanate (phalloidin-TRITC, cat. no. P1951, Sigma-Aldrich), Alexa Fluor 488 goat antimouse IgG (H+L) secondary antibody (cat. no. A11001, molecular probes), formaldehyde (37 wt %, cat. no. 50-00-0, ACROS Organics), Triton X-100 (cat. no. X198-07, J.T. Baker), and protease from bovine pancreas (Type I, cat. no. P4630, Sigma). The stock solution of phalloidin was prepared by dissolving 0.1 mg phalloidin-TRITC in 1 mL of DMSO (76.6 μM). The stock solution of DAPI was prepared by dissolving 5 mg DAPI in 5 mL of DI water (1 mg/mL).

Nomenclature. Polycaprolactone and poly(γ-benzyl-L-glutamate) are abbreviated as PCL and PBG, respectively. The random copolymer that contains 20 mol % of poly(α-L-glutamic acid) and 80 mol % of PBG, poly(γ-benzyl-L-glutamate)-*r*-poly(L-glutamic acid), is named PBGA20. The polyelectrolyte derived from PBGA20, sodium salt of poly(γ-benzyl-L-glutamate)-*r*-poly(L-glutamic acid), is named PBGA20-Na. The monomer of PBG, γ-benzyl glutamate-*N*-carboxy anhydride, is abbreviated BGNCA.

Fabrication of Fibrous Scaffold with Aligned Fibers. Homogenous solutions of PCL, PBG, and PBGA20 were prepared by dissolving the polymer powders in a cosolvent of tetrahydrofuran (THF) and *N*,*N*-dimethylacetamide (DMAc) overnight. The polymer solution was placed in a 3 mL syringe, which was mounted in a syringe pump. A high voltage supply was connected to a needle tip. A grounded, metal target was placed horizontally 15 cm away from the needle tip and rotated in a speed of 3200 rpm. Scaffolds were collected on either cover glasses or indium tin oxide (ITO) glasses. The total pump volume was controlled by calculating the product of flow rate and pumping time. Table S1 lists the parameters for the electrospinning process.

DOI: 10.1021/acsabm.8b00697
ACS Appl. Bio Mater. 2019, 2, 518−526

Fabrication of PBGA20-Na Scaffold. The PBGA20 scaffold was immersed in 0.2 M NaOH aqueous solution for 30 min. The scaffold was thoroughly rinsed with distilled water to make sure all NaOH was removed from the scaffold. The scaffold was vacuum-dried at 40−50 °C overnight. The chemical structure of PBGA20-Na scaffold was confirmed by X-ray photoelectron spectroscopy (XPS) (Figure S6).

Biodegradability of Scaffold. Scaffolds prepared for the degradation test were cut into circles with a diameter of 12 mm. For each set, 24−27 circular scaffolds were weighted for their initial total weight (W_0) to reach a minimum value of 15 mg. Scaffolds were put into a six-well culture plate with a density of eight to nine pieces per well. The enzymatic solution was prepared according to the previous study.[24] Protease from bovine pancreas was dissolved in phosphate-buffered saline (PBS) with a concentration of 10 units/mL and adjusted to pH 7.4 before filtration (0.22 μm). The prepared enzymatic solution was added to the six-well culture plate with a volume of 2 to 2.4 mL/well to totally immerse scaffolds. The solution was refreshed every 2 to 3 days to maintain the activity of enzymes. At days 7, 14, 21, 28, 35, and 42, parts of the scaffolds were removed from the enzymatic solution and washed with distilled water six to eight times in an ultrasound bath. The scaffolds were immersed in 95% ethanol for at least 24 h. Finally, the scaffolds were vacuum-dried at 40 °C overnight before the second measurement of the weight (W). The remaining weight (%) was calculated by $100 \times (W/W_0)$.

Electrical Stimulation of PC12 Cells on Scaffolds. Scaffolds on ITO glasses were immersed in antibiotic antimycotic solution (1% v/v in PBS) at 4 °C overnight, exposed to UV light for 20 min, and immersed in fresh culture medium for 20 min until use. PC12 cells were seeded on scaffolds in a density of 8000 cells/cm^2. After 1 day, 100 ng/mL nerve growth factor (NGF) was added to the culture medium. After another 1 day, electrical stimulation started. The processing parameters for the electrical stimulation are based on previous studies.[25,26] The two parts of the ITO served as electrodes to release electrical signals to the cells (Figure S7). A biphasic electrical pulse with the amplitude of 100 mV was released 1 h every day. The culture medium with NGF was renewed every 3 days.

Fluorescence Staining of PC12 Cells on Scaffolds. Scaffolds on cover glasses or ITO glasses were prepared in the same way as previously described. PC12 cells were seeded on scaffolds in a density of 8000 cells/cm^2. After 1 day, 100 ng/mL NGF was added to the culture medium, and the medium with NGF was renewed every 3 days. After 5 or 10 days, cells were fixed by immersing cells in formaldehyde solution (3.7% v/v in PBS) for 15 min. Cells were immersed in Triton-X 100 solution (1% v/v in PBS) for 10 min. Cells were stained for F-actin by phalloidin for 1 h. Cells were stained for the nucleus by DAPI for 5 min and ready for imaging.

Characterization of Neurite Length. The phalloidin labeling was used to measure the neurite length. The neurite length was measured from the cell body to the tip of the neurite using the software ImageJ. Each group was repeated three times. More than 85 neurites were measured for length in each sample. Notably, the measured neurite lengths were not normally distributed. Therefore, the Kruskal−Wallis H test was used instead of the t test to statistically evaluate the differences between the neurite populations.[27]

RESULTS AND DISCUSSION

Figure 1 shows the schematic diagram of the synthesis and fabrication of polypeptide materials used in this study. PBG and PBGA20 were synthesized and characterized in our lab (Supporting Information). The PBG has the weight-average molecular weight in the range of 200−300 kDa, PDI: 1.2 to 1.3 for ease of fiber formation. The PBGA20 was obtained through partial hydrolysis PBG to have a 20% molar ratio of glutamic acid in the peptide. The commercial available biodegradable polymer, PCL, was used as the control throughout the study. It has a similar molecular weight as the polypeptide at 282 kDa, PDI: 1.3. Then, the scaffolds of PCL, PBG, and PBGA20 were prepared using the electrospinning method from their respective polymer solution. The polyelectrolyte, PBGA20-Na scaffold was prepared by treating the PBGA scaffold with sodium hydroxide solution to neutralize the glutamic acid to sodium salt. According to our previous study,[28] the fiber in the diameter of one micron is most suitable for the growth of the cell because its size can accommodate the cell to be adhered on it and its porosity can provide a 3D environment for the communication of cells. The length of neurite outgrowth of PC-12 on the 3D scaffold made from aligned fibers is longer than isotropic fibers due to the directional growth by aligned fibers.[31] Therefore, we used about one micron diameter of aligned fibers throughout this study to rule out the fiber size effect.

Biodegradability of Scaffold. The biodegradability of scaffolds is important because scaffolds are expected to support the growing tissues at the beginning and to be replaced by the extracellular matrix secreted from the newborn tissues at the end.[8] Because the designed polymers in this study are peptide-based biomaterials, PBS solution containing proteases was chosen as a model for the in vivo environment.[24] Figure 2

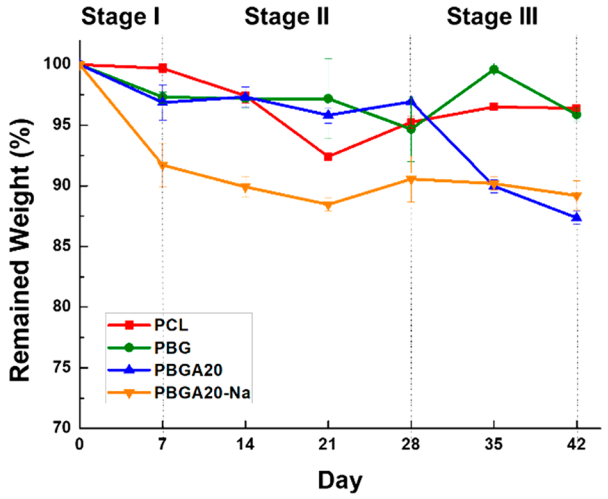


Figure 2. Degradation of scaffolds in protease solution

showed the remaining weights of the scaffolds every 7 days from day 0 to day 42. Three stages of the degradation process can be differentiated. We obtained the average values of data points for certain periods of time in Figure 2 and summarized them in Table 1.

The degradation behaviors of PCL and PBG were similar. They both maintained >99% of their weights on stage I (i.e., day 0∼7), lost 3 to 4% of their weights on stage II (i.e., days 7−28), and kept their weights on stage III (i.e., days 28−42). The remaining weight of the PBGA20-Na scaffold was much

Table 1. % Weight Retention of Scaffolds in Protease Solution for Different Periods of Time

	% weight retention at stage				
	I		II	III	
polymer	day 0	days 0−7	days 7−28	days 28−42	day 42
PCL	100	100 ± 0	96 ± 3	96 ± 1	96 ± 0
PBG	100	99 ± 2	97 ± 1	97 ± 3	96 ± 0
PBGA20	100	98 ± 2	97 ± 1	91 ± 5	87 ± 1
PBGA20-Na	100	96 ± 6	90 ± 1	90 ± 1	89 ± 1

DOI: 10.1021/acsabm.8b00697
ACS Appl. Bio Mater. 2019, 2, 518−526

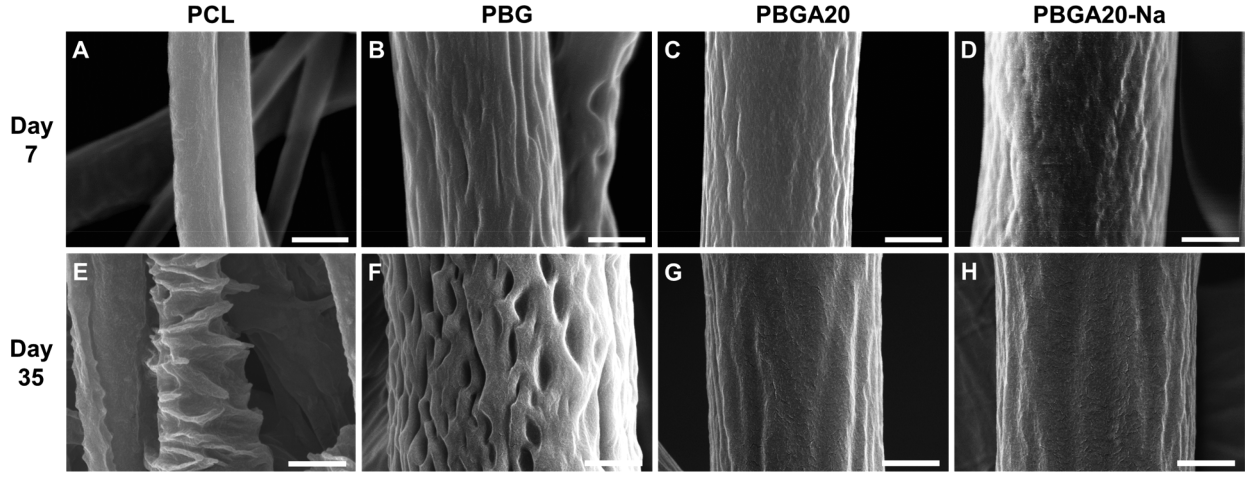


Figure 3. Morphology of fibers after 7 and 35 days of degradation in protease solution. The images of fibers were taken from the scaffolds made of (A,E) PCL, (B,F) PBG, (C,G) PBGA20, and (D,H) PBGA20-Na. Scale bar: 500 nm.

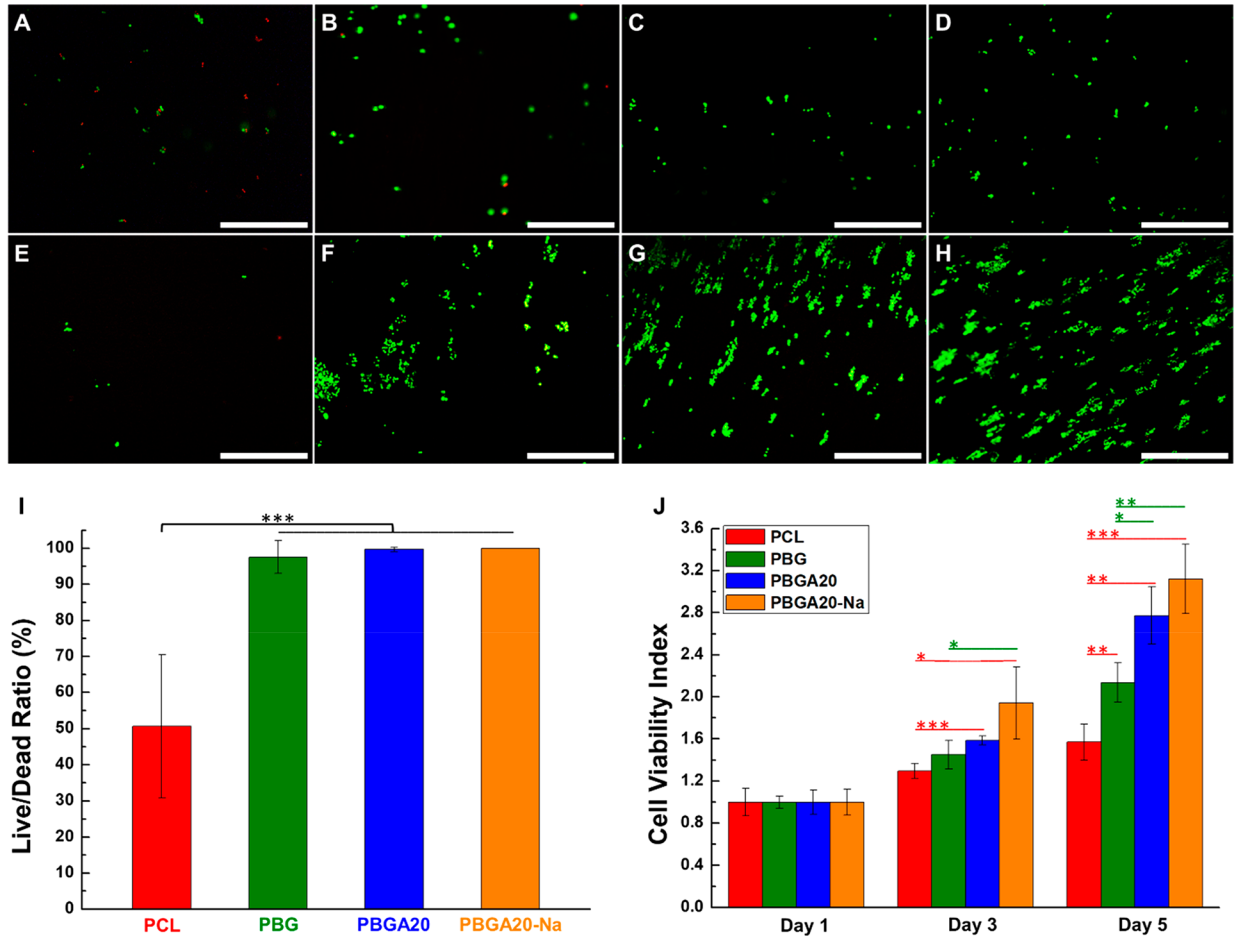


Figure 4. (A–D) Representative Live/Dead fluorescence microscopic images of PC12 cells after 1 and (E–H) 5 days of culture on scaffolds made of: (A,E) PCL, (B,F) PBG, (C,G) PBGA20, and (D,H) PBGA20-Na. Green: live cells. Red: dead cells. Scale bar: 500 μm. (I) Live/Dead ratio of PC12 cells after 1 day of culture. Cell count = 200–550. (J) Cell viability of PC12 cells during 5 days of culture. Error bars represent the standard error of the mean where $n = 3$. t test: $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, and $^{*}P \leq 0.05$. Cell viability was obtained by normalizing the value on day 3 or 5 to the value on day 1.

lower than that of the PCL and PBG scaffolds. The PBGA20-Na scaffold maintained >95% of the weight on stage I, lost 10% of the weight on stage II, and lost the weight on stage III slowly. The remaining weight of the PBGA20 scaffold was similar to that of the PCL and PBG scaffolds in stages I and II and dropped to the same level as PBGA20-Na scaffold in stage III.

The desirable targets for proteases are peptide bonds linked between natural amino acids. PCL contains an ester linkage on the main chain of the polymer. PBG is a peptide of unnatural

DOI: 10.1021/acsabm.8b00697
ACS Appl. Bio Mater. 2019, 2, 518–526

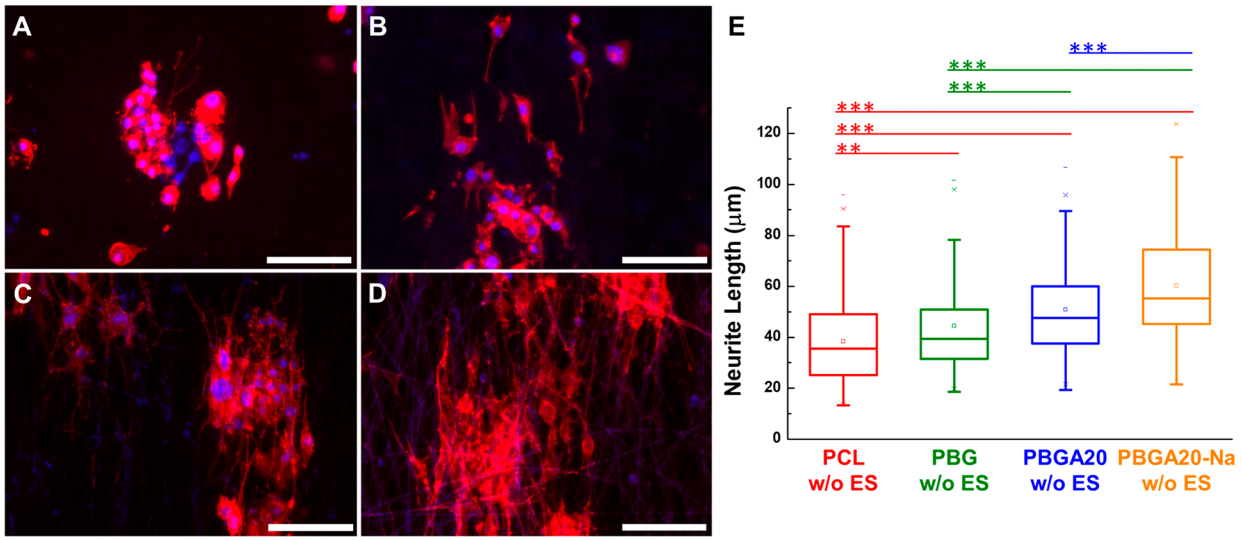


Figure 5. Representative fluorescent microscopic images of PC12 cells after 5 days of differentiation without electrical stimulation (w/o ES) on scaffolds made of: (A) PCL, (B) PBG, (C) PBGA20, and (D) PBGA20-Na. (E) Box chart of PC12 neurite lengths after 5 days of differentiation without electrical stimulation. $n = 3$. H test: $^{***}P \leq 0.001$ and $^{**}P \leq 0.01$. Neurite counts = 85−140 for each group in panel E. Blue: nucleus. Red: F-actin. Scale bar: 100 μm.

amino acids, γ-benzyl-L-glutamaten which contains ester linkage on the side chain of the polymer. PBGA20 and PBGA20-Na contain natural amino acids, glutamic acids. Therefore, the degradation of PCL and PBG in this study might mostly depend on the hydrolytic degradation of the ester bonds, whereas PBGA20 and PBGA20-Na might depend on both enzymatic and hydrolytic degradation.[29]

The diffusivity of water into the matrix, the functional groups in polymers, and the dimensions of the matrix are key factors determining the rate of the hydrolytic degradation.[30] PCL and PBG scaffolds shared the same targeted functional groups (i.e., ester bonds) and the dimension of the matrix (i.e., 12 mm circular area and ~150 μm in thickness). Furthermore, they shared a similar scaffold density (PCL: 17.2 ± 4.4, PBG: 19.7 ± 5.5 μg/mm^3) and water contact angle (PCL: 82.9 ± 5.1°, PBG: 94.1 ± 5.8°). These similarities might contribute to the similar diffusivity of water into the matrix and the similar degradation behaviors of PCL and PBG.

The high hydrophilicity of PBGA20-Na (water contact angle: 43.0 ± 7.6°) helped water diffuse into the matrix and speeded up the hydrolytic degradation of the ester bonds. The water also brought proteases to the scaffold and helped the enzymatic degradation of the peptide bonds. Therefore, the low remained weight of PBGA20-Na is due to the high hydrophilicity and the enzyme-degradable residues, glutamic acids. On the contrary, the relatively high water contact angle of PBGA20 (85.5 ± 6.2°) compared with that of PBGA20-Na is the reason why PBGA20 scaffolds took a longer time to reach the same weight loss as PBGA20-Na scaffolds.

The comparison between the remained weight of PBGA20 in PBS and in protease solution further demonstrates the possibility of peptide bond degradation. Whereas PBGA20 maintained ~94% of the weight on day 42 in PBS, it maintained only ~87% of the weight on day 42 in protease solution.[31] The 7% difference might arise from the degradation of the main-chain peptide linkages.

Figure 3 showed the morphology of fibers after 7 and 35 days of degradation in protease solution. Some of the fibers in PCL scaffolds started to lose their structural integrity on day 35 due to the ester bond cleavage on its main chain. The PBG is the most hydrophobic scaffold among four kinds of samples. A similar polarity cosolvent system of THF and dimethyl acetate was used in the electrospinning of polymers. The radial gyration of PBG in the solution should be smaller than the other three polymers due to the less interaction between polymer and solvent. Thus the polymer surface morphology of the PBG fiber easily contains flaws, for example, pin holes. That is quite different from the other three samples. The flaws become larger after 35 days of study, but the structural integrity of the fiber is remained. The ester side of PBG undergoes hydrolysis during this period, which will not affect the structural integrity of polymer. It is interested to note that PBGA20 and PBGA20-Na also maintained their structural integrity, which indicates that the hydrolysis of peptide chain is slower than the ester linkage, and the hydrolysis might occur primarily on the surface of the fibers. The structural merit of PBGA20 and PBGA20-Na can avoid sudden loss of mechanical strength before a complete degradation.[24,30]

In summary, we successfully fabricated scaffolds that are biodegradable in protease solution. Polymers such as PCL and PBG might hydrolytically degrade their ester bonds. In contrast, PBGA20 and PBGA20-Na might degrade both hydrolytically and enzymatically on their ester bonds and peptide linkages. Furthermore, PBGA20 and PBGA20-Na scaffolds maintained structural integrity during degradation, which is beneficial for a scaffold or medical device that requires consistent mechanical strength before complete degradation.

Biocompatibility of Scaffold. The biocompatibility of scaffolds was evaluated by the Live/Dead assay and the Alamar Blue assay. To evaluate the cell adhesion directly on different scaffolds, the surface of the scaffold was not modified by any adhesion promoter such as polylysine.[31] According to the Live/Dead images on day 1 (Figure 4A−D,I), cells were half-died on the PCL scaffold after 1 day of culture. More than 95% of cells were alive on the PBG and PBGA20 scaffolds. No dead cell was found on PBGA20-Na scaffold. According to the cell viability obtained by the Alamar Blue assay (Figure 4J), cells were significantly less viable on the PCL scaffold than on the

DOI: 10.1021/acsabm.8b00697
ACS Appl. Bio Mater. 2019, 2, 518−526

other three scaffolds on day 5. Cells cultured on PBGA20 and PBGA20-Na scaffolds were more viable than on the PBG scaffold. The live/dead images on day 5 (Figure 4E−H) also echoed the results of Alamar Blue assay. Dead cells were hardly found on day 5 (see enlarged images, Figures S10 and S11 in the Supporting Information), which is possible from the detachment of the dead cells. Barely any live cell was found on the PCL scaffold, which may be caused by the poor adhesion of PC12 on PCL. In contrast, many live cells were found on the PBGA20 and PBGA20-Na scaffolds. All of the results showed that PC12 cells could attach and proliferate well on the PBGA20 and PBGA20-Na scaffolds. However, the PCL scaffolds could not support the survival of PC12 cells.

The high biocompatibility of PBGA20 and PBGA20-Na might be related to the glutamic acid subunits. PCL and PBGA20 shared comparable water contact angles (PCL: 82.9 ± 5.1°, PBGA20: 85.5 ± 6.2°). PCL, PBG, and PBGA20 had similar scaffold densities (PCL: 16.4 ± 3.5, PBG: 19.7 ± 5.5, PBGA20: 21.8 ± 7.6 $\mu g/mm^3$). We can thus speculate that the surface chemistry was the dominating factor in the cell viability in this system. Studies showed that biomaterials immobilized with neurotransmitters can promote neuron cell viability, adhesion, and differentiation.[32,33] As a neurotransmitter, the glutamic acid immobilized on PBGA20 and PBGA20-Na scaffolds might also be attributed to the high biocompatibility.

Neurite Outgrowth without Electrical Stimulation (w/o ES). PC12 cells were triggered by NGF for 5 days on the scaffolds to evaluate the neurite outgrowth (Figure 5). Figure 5E showed that the neurite lengths were in a rank of PCL < PBG ≪ PBGA20 ≪ PBGA20-Na.

The general trend of the cell differentiation was the same as the results we found in the cell viability. Because good neurite outgrowth of PC12 cells requires good cell viability and adhesion, the similarity is reasonable.[34]

The longer neurite lengths on PBGA20 than on PBG scaffolds might arise from the glutamic acid subunits contained in PBGA20. Glutamic acid is a neurotransmitter that is important for cell communication and differentiation and can promote neurogenesis in brain.[20,21,35] The distinct neurite outgrowth happened within 5 days, which was too short for degradation. Hence, cells probably recognized glutamic acid subunits on the polymer chains through direct contact. Studies implied that the immobilized dopamine (a neurotransmitter) can act as a dopamine receptor or transporter and further promote neurite outgrowth.[33] As a neurotransmitter, the glutamic acid immobilized on PBGA20 might also induce neurotransmitter-related cellular behaviors and further enhance the neurite outgrowth.

The longer neurite lengths on PBGA20-Na than on PBGA20 scaffolds might be attributed to the electroactive properties. PBGA20-Na is a polyelectrolyte that transmits electrical signals by dissociating ions into solution. This mechanism is similar to how neurons transport electrical impulses (i.e., action potentials). Action potentials are generated by the depolarization of neuron membranes, which is achieved by the influx of sodium or calcium ions through ion channels.[36,37] Hence, the ion-generated polyelectrolytes might provide an electroactive interface through a naturally mimetic way. Moreover, the sodium ions dissociated from PBGA20-Na might promote the function of ion channels and the initiation of action potentials.

In summary, good cell viability, glutamate signaling by immobilized glutamic acid, and stimulated sodium ion channels were three possible reasons accounting for the results of PC12 neurite outgrowth w/o ES in this study (i.e., PCL < PBG ≪ PBGA20 ≪ PBGA20-Na). To the best of our knowledge, this study is the first one to demonstrate the possibility of polyelectrolytes to induce better neurite outgrowth through stimulated ion channels.

Neurite Outgrowth with Electrical Stimulation (w/ES). Electrical stimulation is considered as one of the strategies to facilitate the regeneration of nervous systems. Exogenous electric fields (EFs) exist in the developing nervous system of vertebrates. EFs can induce more, faster, longer, and directional neurite outgrowth of cultured neurons.[25−27,38] Hence, in this study, we incorporated electrical stimulation to further promote neurite outgrowth. Fibrous scaffolds electrospun on ITO served as the substrates for cell culture and at the same time served as the electrode for electrical stimulation (Figure S7).

The impedance of the scaffolds at the working frequency is listed in Table 2. PCL and PBG showed relatively high impedance. PBGA20 and PBGA20-Na were relatively more electroactive, probably due to the partially or fully dissociable COOH and COONa groups in PBGA20 and PBGA20-Na. The exposed COO^- groups on the surface of the electrode might attract electrolytes in solution and speed up the charge-transfer reaction, leading to lower impedance.

Table 2. Impedance of the Scaffolds and ITO at 250 Hz

material	PCL	PBG	PBGA20	'k	ITO
impedance (ohm)	83.0	78.9	50.8	44.0	18.2

PC12 cells were triggered by NGF with electrical stimulation for 5 days to evaluate the neurite outgrowth (Figure 6). Figure 6E showed that the neurite lengths were significantly longer with electrical stimulation (w/ES) than without electrical stimulation (w/o ES). Neurites on PCL w/ES were significantly shorter than other three materials w/ES ($p \leq 0.001$). The longest neurite (175 μm) was found on the scaffold of PBGA20-Na w/ES.

The shorter neurite lengths on PCL than the other three materials might arise from the poor cell viability on PCL scaffolds. The resistances of PCL (83.0 Ohm) and PBG (78.9 Ohm) were similar, and both of them were low enough for in vitro electrical stimulation.[25,27] Therefore, we postulated that the dominating reason was the poor cell viability that was already shown in Figure 4. It seemed that although electrical stimulation did promote the differentiation of PC12 cells, the overall successful neurite extension needed a minimum cell viability or attachment for cells to anchor and further extend neurites on the scaffolds.

The absence of statistically significant difference between PBG w/ES, PBGA20 w/ES, and PBGA20-Na w/ES might be attributed to the unusual and broad distribution of the neurite lengths arising from the unequal electrical stimulus received by cells (Figure 7B−D). Cells were cultured on fibrous membranes on ITO. The electrical signals were likely to transmit from the ITO electrode, across the fibrous membrane, and finally reach the cells with an aid of electrolytes from the culture medium or from the dissociable scaffolds such as PBGA20 and PBGA20-Na. Hence, the electrical signals received by cells might depend on the spot they adhered to and the corresponding exposure to fibers or medium, which led

DOI: 10.1021/acsabm.8b00697
ACS Appl. Bio Mater. 2019, 2, 518−526

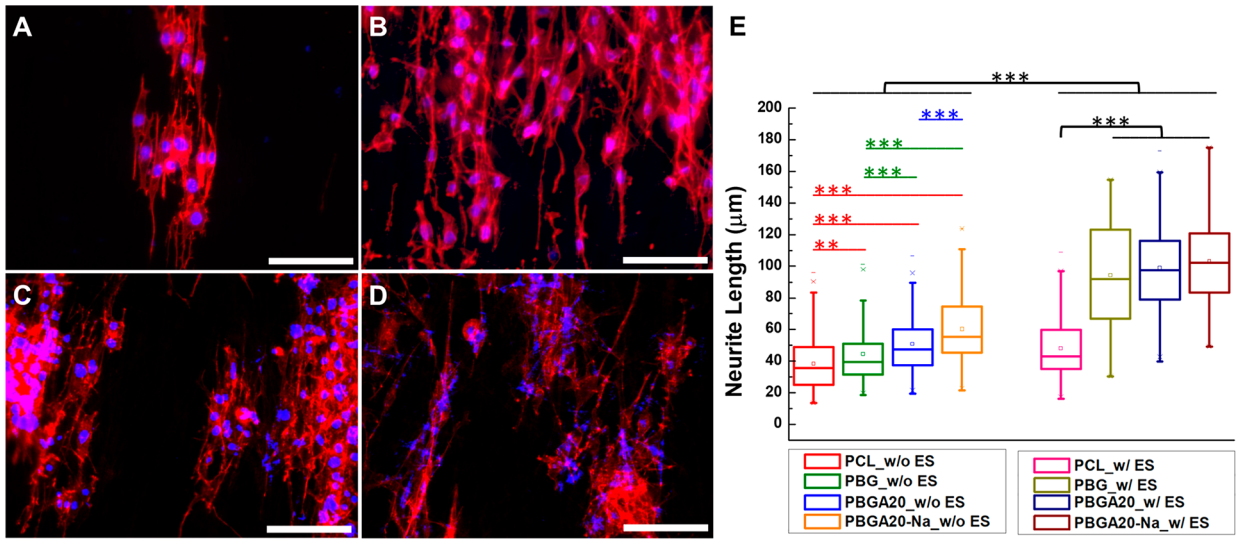


Figure 6. (A–D) Representative fluorescence microscopic images of PC12 cells after 5 days of differentiation with electrical stimulation on scaffolds made of: (A) PCL, (B) PBG, (C) PBGA20, and (D) PBGA20-Na. (E) Box chart of PC12 neurite lengths after 5 days of differentiation without and with electrical stimulation. $n = 3$. H test: $^{***}P \leq 0.001$ and $^{**}P \leq 0.01$. Neurite counts = 85–140 for each group in panel E. Blue: nucleus. Red: F-actin. Scale bar: 100 μm.

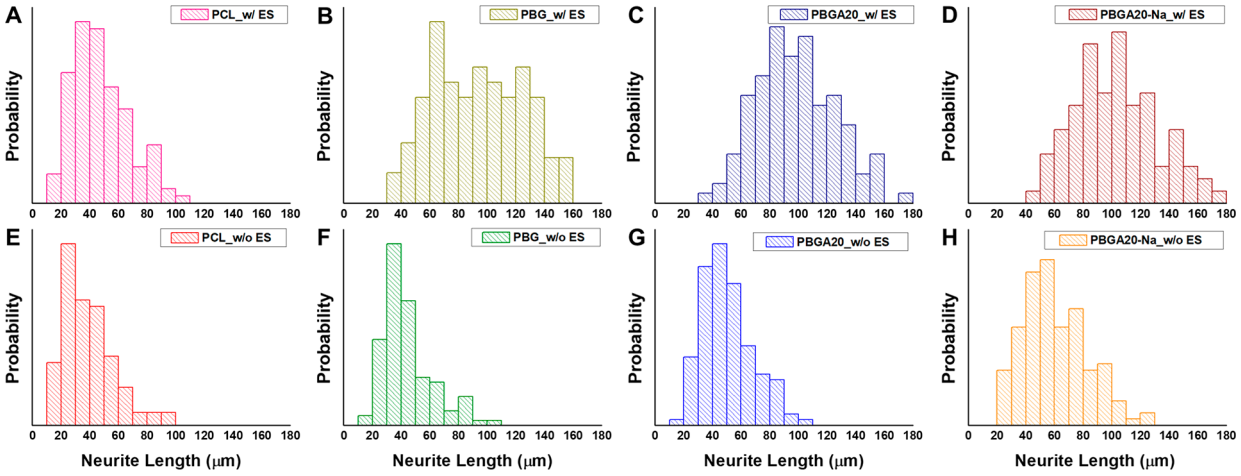


Figure 7. Distributions of PC12 neurite lengths after 5 days of differentiation (A–D) with and (E–H) without electrical stimulation on scaffolds made of: (A,E) PCL, (B,F) PBG, (C,G) PBGA20, and (D,H) PBGA20-Na. $n = 3$. Neurite counts = 85–140 in each group.

to an unequal electrical effects and broad neurite length distribution.

In summary, we successfully demonstrated that electrical stimulation can promote significant longer neurite outgrowth on fibrous scaffolds made by PCL, PBG, PBGA20, and PBGA20-Na. Apart from the impedance of the scaffolds, the cell viability or attachment and the cell exposure to fibers or medium might also influence the ability of cells to receive electrical signals.

Alignment of PC12 Neurites. Directional outgrowth of neurites is important for neurons to transmit signals to the target. The fluorescence microscopic image (Figure 8A) and scanning electron microscope (SEM) image (Figure 8B) demonstrated the extended cell body and the alignment between the neurites and the fibers. We further statistically analyzed the angles of neurites in Figure 8C–H. The aligned fibers guided the directional outgrowth of neurites. In contrast, cells cultured on TCPS (a flat plate made of tissue culture polystyrene) extended their neurites in a random direction. We successfully fabricated fibrous scaffolds with aligned fibers that guided the directional outgrowth of neurites.

CONCLUSIONS

A peptide-based polyelectrolyte containing neuronal stimulant (i.e., glutamic acid), PBGA20-Na, was synthesized and fabricated into 3D aligned fibrous scaffolds for neural tissue engineering. Two other structurally resembled polypeptides, PBG and PBGA20, were used for comparison. Also, another widely used commercial biopolyester, PCL, was used as a comparative control. All materials were used without coating, and cells were fully involved in the scaffolds (Figure S9). Hence, the cellular behaviors were directly related to the scaffolds themselves.

The biocompatibilities of PBG, PBGA20, and PBGA20-Na were significantly better than PCL. Cells extended neurites in alignment with the fibers. Longer neurites were observed on scaffolds with electrical stimulation than without stimulation. Furthermore, the integration of neuronal stimulant into

DOI: 10.1021/acsabm.8b00697
ACS Appl. Bio Mater. 2019, 2, 518–526

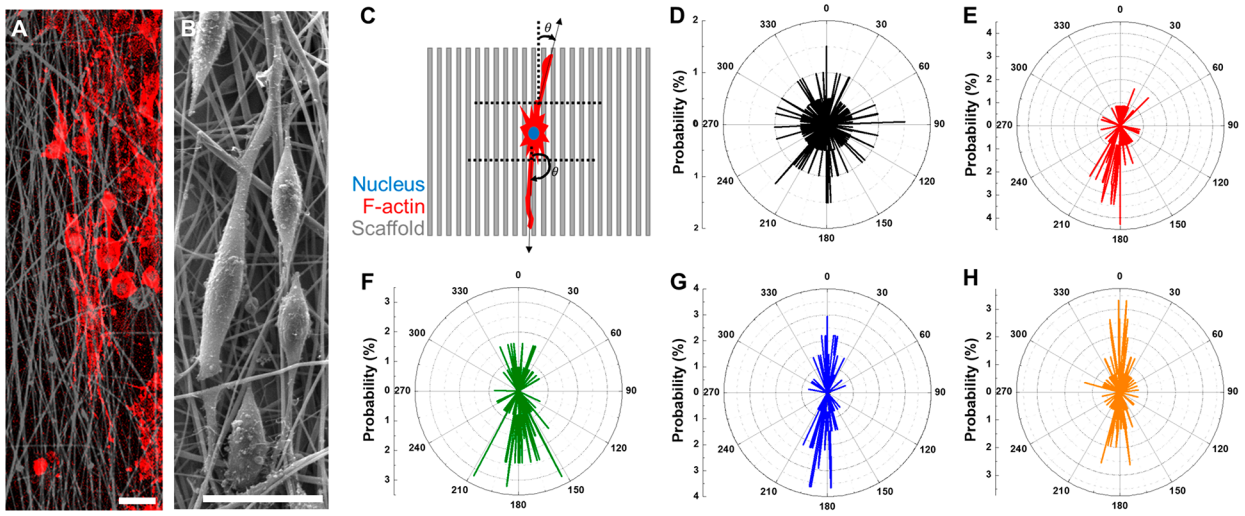


Figure 8. (A) Fluorescence microscopic image (gray: scaffold; red: F-actin) and (B) SEM image showing neurite alignment of 10 day differentiated PC12 cells on PBGA20-Na scaffolds with electrical stimulation. Scale bar: 30 μm. (C–H) Neurite orientation of 5 day differentiated PC12 cells on (D) TCPS and scaffolds made of (E) PCL, (F) PBG, (G) PBGA20, and (H) PBGA20-Na without electrical stimulation. n = 3. Neurite counts = 85–140 in each group.

polypeptides (i.e., PBGA20 and PBGA20-Na) showed an enhancement of cell adhesion, proliferation, and differentiation. Neurite outgrowth on the electroactive polypeptide containing glutamic acid (i.e., PBGA20-Na) was the longest (175 μm) among the four materials. In conclusion, PBGA20-Na is a unique and promising biomaterial for neural tissue engineering and drug-screening platforms.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsabm.8b00697.

Materials and methods; NMR results of BGNCA, PBG, and PBGA20; calculation equation of the degree of hydrolysis in PBGA20; IR spectra of the starting material: L-glutamic acid γ-benzyl ester, the monomer: BGNCA, and the polymer: PBG; GPC result of PBG; parameters for the electrospinning process; XPS of PBGA20 and PBGA20-Na; calculation equation of dye reduction percentage in Alamar Blue assay; the setup for the electrical stimulation; the 3D distribution of 10 day differentiated PC12 cells with electrical stimulation on PCL or PBGA20-Na scaffold; the extra live/dead pictures of PBGA20-Na on day 1; and the magnified live/dead picture of PBGA20-Na on day 5. (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: suwf@ntu.edu.tw.

ORCID

Shyh-Chyang Luo: 0000-0003-3972-1086

Jia-Shing Yu: 0000-0002-0782-2328

Wei-Fang Su: 0000-0002-3375-4664

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the Department of Ophthalmology, Harvard Medical School and Department of Ophthalmology, National Taiwan University Hospital for the ongoing culture of primary retinal ganglion cells (RGCs), retinal explants, and hiPSC-derived RGCs. We acknowledge the financial support from the Ministry of Science and Technology (MOST) and Ministry of Education (MOE) of Taiwan.

■ ABBREVIATIONS

PBGA20-Na, sodium salt of poly(γ-benzyl-L-glutamate)-*r*-poly(L-glutamic acid); PBG, poly(γ-benzyl-L-glutamate); PBGA20, poly(γ-benzyl-L-glutamate)-*r*-poly(L-glutamic acid); PCL, polycaprolactone; PC12 cells, rat pheochromocytoma cells; w/o ES, without electrical stimulation; NGF, nerve growth factor; w/ES, with electrical stimulation; EFs, electric fields; ITO, indium tin oxide; TCPS, tissue culture polystyrene

■ REFERENCES

(1) Poewe, W.; Seppi, K.; Tanner, C. M.; Halliday, G. M.; Brundin, P.; Volkmann, J.; Schrag, A.-E.; Lang, A. E. Parkinson disease. *Nat. Rev. Dis. Primers* **2017**, *3*, 17013.

(2) Kumar, A.; Singh, A.; Ekavali. A review on Alzheimer's disease pathophysiology and its management: an update. *Pharmacol. Rep.* **2015**, *67* (2), 195–203.

(3) Calkins, D. J.; Pekny, M.; Cooper, M. L.; Benowitz, L.; Calkins, D.; Benowitz, L.; Cooper, M.; Crowston, J.; Huberman, A.; Johnson, E.; et al. The challenge of regenerative therapies for the optic nerve in glaucoma. *Exp. Eye Res.* **2017**, *157*, 28–33.

(4) Illis, L. Central nervous system regeneration does not occur. *Spinal Cord* **2012**, *50* (4), 259.

(5) Li, K.; Zhong, X.; Yang, S.; Luo, Z.; Li, K.; Liu, Y.; Cai, S.; Gu, H.; Lu, S.; Zhang, H.; et al. HiPSC-derived retinal ganglion cells grow dendritic arbors and functional axons on a tissue-engineered scaffold. *Acta Biomater.* **2017**, *54*, 117–127.

(6) Hunt, N. C.; Hallam, D.; Karimi, A.; Mellough, C. B.; Chen, J.; Steel, D. H.; Lako, M. 3D culture of human pluripotent stem cells in RGD-alginate hydrogel improves retinal tissue development. *Acta Biomater.* **2017**, *49*, 329–343.

(7) Sun, Y.; Li, W.; Wu, X.; Zhang, N.; Zhang, Y.; Ouyang, S.; Song, X.; Fang, X.; Seeram, R.; Xue, W.; et al. Functional self-assembling peptide nanofiber hydrogels designed for nerve degeneration. *ACS Appl. Mater. Interfaces* **2016**, *8* (3), 2348–2359.

(8) Dvir, T.; Timko, B. P.; Kohane, D. S.; Langer, R. Nanotechnological strategies for engineering complex tissues. *Nat. Nanotechnol.* **2011**, *6* (1), 13.
(9) Zheng, H.; Yoshitomi, T.; Yoshimoto, K. Analysis of Chirality Effects on Stem Cell Fate Using Three-dimensional Fibrous Peptide Hydrogels. *ACS Appl. Bio Mater.* **2018**, *1* (3), 538−543.
(10) Ding, Q.; Qu, Y.; Shi, K.; He, X.; Chen, Z.; Yang, Y.; Wang, X.; Qian, Z. Preparation of Bone Marrow Mesenchymal Stem Cells Combined with Hydroxyapatite/Poly (d, l-lactide) Porous Microspheres for Bone Regeneration in Calvarial Defects. *ACS Appl. Bio Mater.* **2018**, *1* (4), 1084−1093.
(11) Yuan, S.; Xiong, G.; Wang, X.; Zhang, S.; Choong, C. Surface modification of polycaprolactone substrates using collagen-conjugated poly (methacrylic acid) brushes for the regulation of cell proliferation and endothelialisation. *J. Mater. Chem.* **2012**, *22* (26), 13039−13049.
(12) Vacanti, N. M.; Cheng, H.; Hill, P. S.; Guerreiro, J. o. D.; Dang, T. T.; Ma, M.; Watson, S. e.; Hwang, N. S.; Langer, R.; Anderson, D. G. Localized delivery of dexamethasone from electrospun fibers reduces the foreign body response. *Biomacromolecules* **2012**, *13* (10), 3031−3038.
(13) Washington, K. S.; Bashur, C. A. Delivery of Antioxidant and Anti-inflammatory Agents for Tissue Engineered Vascular Grafts. *Front. Pharmacol.* **2017**, *8*, 659.
(14) Holmes, T. C.; de Lacalle, S.; Su, X.; Liu, G.; Rich, A.; Zhang, S. Extensive neurite outgrowth and active synapse formation on self-assembling peptide scaffolds. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97* (12), 6728−6733.
(15) Berns, E. J.; Sur, S.; Pan, L.; Goldberger, J. E.; Suresh, S.; Zhang, S.; Kessler, J. A.; Stupp, S. I. Aligned neurite outgrowth and directed cell migration in self-assembled monodomain gels. *Biomaterials* **2014**, *35* (1), 185−195.
(16) Lim, D. W.; Nettles, D. L.; Setton, L. A.; Chilkoti, A. In situ cross-linking of elastin-like polypeptide block copolymers for tissue repair. *Biomacromolecules* **2008**, *9* (1), 222−230.
(17) Collazos-Castro, J. E.; García-Rama, C.; Alves-Sampaio, A. Glial progenitor cell migration promotes CNS axon growth on functionalized electroconducting microfibers. *Acta Biomater.* **2016**, *35*, 42−56.
(18) Borah, R.; Ingavle, G. C.; Sandeman, S. R.; Kumar, A.; Mikhalovsky, S. V. Amine-Functionalized Electrically Conductive Core−Sheath MEH-PPV: PCL Electrospun Nanofibers for Enhanced Cell−Biomaterial Interactions. *ACS Biomater. Sci. Eng.* **2018**, *4* (9), 3327−3346.
(19) Balint, R.; Cassidy, N. J.; Cartmell, S. H. Conductive polymers: towards a smart biomaterial for tissue engineering. *Acta Biomater.* **2014**, *10* (6), 2341−2353.
(20) Song, M.; Yu, S. P.; Mohamad, O.; Cao, W.; Wei, Z. Z.; Gu, X.; Jiang, M. Q.; Wei, L. Optogenetic stimulation of glutamatergic neuronal activity in the striatum enhances neurogenesis in the subventricular zone of normal and stroke mice. *Neurobiol. Dis.* **2017**, *98*, 9−24.
(21) Brazel, C.; Nunez, J.; Yang, Z.; Levison, S. Glutamate enhances survival and proliferation of neural progenitors derived from the subventricular zone. *Neuroscience* **2005**, *131* (1), 55−65.
(22) Stevens, C. F. Neurotransmitter release at central synapses. *Neuron* **2003**, *40* (2), 381−388.
(23) Guo, B.; Ma, P. X. Conducting polymers for tissue engineering. *Biomacromolecules* **2018**, *19* (6), 1764−1782.
(24) Wang, J.; Boutin, K. G.; Abdulhadi, O.; Personnat, L. D.; Shazly, T.; Langer, R.; Channick, C. L.; Borenstein, J. T. Fully Biodegradable Airway Stents Using Amino Alcohol-Based Poly (ester amide) Elastomers. *Adv. Healthcare Mater.* **2013**, *2* (10), 1329−1336.
(25) Zhu, B.; Luo, S.-C.; Zhao, H.; Lin, H.-A.; Sekine, J.; Nakao, A.; Chen, C.; Yamashita, Y.; Yu, H.-h. Large enhancement in neurite outgrowth on a cell membrane-mimicking conducting polymer. *Nat. Commun.* **2014**, *5*, 4523.
(26) Thrivikraman, G.; Boda, S. K.; Basu, B. Unraveling the mechanistic effects of electric field stimulation towards directing stem cell fate and function: A tissue engineering perspective. *Biomaterials* **2018**, *150*, 60−86.
(27) Schmidt, C. E.; Shastri, V. R.; Vacanti, J. P.; Langer, R. Stimulation of neurite outgrowth using an electrically conducting polymer. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94* (17), 8948−8953.
(28) Su, W.-F.; Ho, C.-C.; Shih, T.-H.; Wang, C.-H.; Yeh, C.-H. Exceptional biocompatibility of 3D fibrous scaffold for cardiac tissue engineering fabricated from biodegradable polyurethane blended with cellulose. *Int. J. Polym. Mater.* **2016**, *65* (14), 703−711.
(29) Nair, L. S.; Laurencin, C. T. Biodegradable polymers as biomaterials. *Prog. Polym. Sci.* **2007**, *32* (8−9), 762−798.
(30) von Burkersroda, F.; Schedl, L.; Göpferich, A. Why degradable polymers undergo surface erosion or bulk erosion. *Biomaterials* **2002**, *23* (21), 4221−4231.
(31) Wang, Z. H.; Chang, Y. Y.; Wu, J. G.; Lin, C. Y.; An, H. L.; Luo, S. C.; Tang, T. K.; Su, W. F. Novel 3D Neuron Regeneration Scaffolds Based on Synthetic Polypeptide Containing Neuron Cue. *Macromol. Biosci.* **2018**, *18* (3), 1700251.
(32) Zhou, Z.; Yu, P.; Geller, H. M.; Ober, C. K. The role of hydrogels with tethered acetylcholine functionality on the adhesion and viability of hippocampal neurons and glial cells. *Biomaterials* **2012**, *33* (8), 2473−2481.
(33) Gao, J.; Kim, Y. M.; Coe, H.; Zern, B.; Sheppard, B.; Wang, Y. A neuroinductive biomaterial based on dopamine. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103* (45), 16681−16686.
(34) Greene, L. A.; Aletta, J. M.; Rukenstein, A.; Green, S. H. [18] PC12 pheochromocytoma cells: culture, nerve growth factor treatment, and experimental exploitation. *Methods Enzymol.* **1987**, *147*, 207−216.
(35) Snyder, S. H. Turning off neurotransmitters. *Cell* **2006**, *125* (1), 13−15.
(36) Kole, M. H.; Ilschner, S. U.; Kampa, B. M.; Williams, S. R.; Ruben, P. C.; Stuart, G. J. Action potential generation requires a high sodium channel density in the axon initial segment. *Nat. Neurosci.* **2008**, *11* (2), 178.
(37) Bender, K. J.; Trussell, L. O. Axon initial segment Ca2+ channels influence action potential generation and timing. *Neuron* **2009**, *61* (2), 259−271.
(38) Haan, N.; Song, B. Therapeutic application of electric fields in the injured nervous system. *Adv. Wound Care* **2014**, *3* (2), 156−165.

DOI: 10.1021/acsabm.8b00697
ACS Appl. Bio Mater. 2019, 2, 518−526