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ORIGINAL PAPER

Novel polypeptide composite fibrous scaffold with internal chemical boundary

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Abstract

Cell migration determines the complete development of mammalian tissue and other pathological phenomena. To investigate the effect of chemical stimuli on such behavior, cells are triggered to translate by the concentration gradient of different molecules on 2D substrates in vitro. But to date unfortunately the polymeric scaffolds for cell migration in 3D environment with chemical stimuli have not been proposed and developed yet. Herein, a novel 3D composite scaffold with an internal chemical boundary is fabricated by electrospinning and mask-assisted electrospray so that the deposition of PBG-N_3 particles is confined at specific area initially. The chemical boundary is subsequently formed after selective surface modification of the particles via click reaction. Using a fluorescent alkyne, the boundary of modified regions is clearly observed by fluorescence microscope. This innovative bio-material has the potential to serve as a promising scaffold for investigating the effect of chemical stimuli on cell migration and growth in 3D environment and further on to the application in tissue engineering.

Keywords Polypeptide · Cell migration · 3D scaffolds · Chemical boundary · Surface modification

Introduction

Cell migration and growth is a vital process in organisms related to wound healing, immune system, and cancer invasion [1–4]. Although investigating migration behavior in vivo provides direct evidence for this process, by the assistance of artificial scaffold in vitro, significant reduction of time and cost are expected as comparing to conduct experiments in living animals. In order to control the migration pathway of target cells in vitro, structural guidance and physical/chemical stimuli are two major factors for promoting the cells to move in the desired direction on 2D substrates. Practically, the guidance is generated by constructing topographical structures for limiting the migration pathway [5, 6]. The physical stimuli can be gradient of stiffness or rigidity of substrates, [7–9] electromagnetic field, [10] and near-infrared radiation, [11] while the chemical stimuli is obtained by surface modification of various molecules or proteins on the substrates, and cells are stimulated and then migrates across the boundary [8, 9, 12]. However, 2D substrates used in these experiments hardly mimic the real environment in organisms [13]. Recently, 3D bio-scaffolds fabricated by electrospinning or 3D printing have been adopted for studying cell migration in 3D environment due to their structural resemblance to extracellular matrix (ECM) [14]. Despite the effect of chemical stimuli on cell migration in 3D environment has been studied using 3D scaffolds by the assistance of microfluidic systems, [15, 16] internal chemical boundaries directly constructed within these type of materials are still undeveloped, since the formation of chemical gradient or boundary in porous bio-scaffolds directly is much more challenging than on 2D substrates. Therefore, it is urgent and essential to design and fabricate 3D scaffolds that combine the concept of chemical boundary for triggering cell migration and growth.

Herein, we demonstrate a novel polypeptide electrospun fibrous scaffold containing an internal chemical boundary with the potential of triggering cell migration

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and growth in 3D environment. The chemical boundary was constructed by partially depositing micro-particles of N_3-terminated polypeptide at selected regions in the scaffold via mask-assisted electrospray. Then the surface modification of the particles can be modified easily by alkyne-azide click reaction with different reagents to chemically guiding the cell growth at specific area or direction. Thus, the formation of boundary in the 3D scaffold can have a potential application in effective cell migration and growth required in tissue engineering.

Experimental section

Materials

All reagents were purchased from commercial suppliers such as Aldrich & Sigma (USA) and were used directly unless otherwise specified. Anhydrous solvent was prepared by removing water through molecular sieves and purging with nitrogen overnight, and anhydrous benzylamine was dried over calcium hydride by distillation.

Characterization

Chemical structures of all synthesized products were confirmed by FT-IR (PerkinElmer, Spectrum 100) and NMR (Bruker, AVIII HD 400 NMR). Molecular weight of polymers was determined by gel permeation chromatography (Waters, 1515 Isocratic HPLC pump). Morphology of scaffolds and particles were characterized by field emission scanning electron microscopy (JEOL, JSM 6510) after platinum coating, and fluorescence image was captured by fluorescence microscopy (Olympus, IX71) with UV excitation at wavelength of 351 nm.

Synthesis of monomer and polymer

Synthesis of BG-NCA

To a refluxed suspension of L-glutamic acid γ-benzyl ester (4.0 g, 16.9 mmol) in anhydrous ethyl acetate (120 ml), triphosgene (2.5 g, 8.4 mmol) was added and the reaction was proceeded for 2 hr in nitrogen. After cooling to room temperature, hexane was added into the resulting solution for recrystallization twice at -20 ℃. The crystal was filtrated out and dried in a vacuum oven at 40 ℃ overnight to afford the title compound (yield: 77%). NMR and FT-IR spectra are available in supporting information (Figs. S1, S2 and S9).

Synthesis of high MW PBG

To a suspension of BG-NCA (2.5 g, 9.5 mmol) in anhydrous benzene (250 ml), 965 µl freshly prepared sodium methoxide solution (obtained by dissolving sodium (75.0 mg) in anhydrous methanol (5.0 ml) and anhydrous benzene (15.0 ml)) was injected. The mixture was allowed to react for 48 hr with stirring in nitrogen. The product was precipitated in methanol, filtrated out, and dried in vacuum oven at 40 ℃ overnight to afford the title polymer (yield: 93%). NMR and FT-IR spectra are available in Supporting Information (Figs. S3, S4 and S9). M_n: 251 kDa, M_w: 254 kDa, PDI: 1.01 (Fig. S10).

Synthesis of low MW PBG

To a solution of BG-NCA (2.5 g, 9.5 mmol) in anhydrous DMF (25 ml), 845 µl freshly prepared initiator solution (anhydrous benzylamine in anhydrous DMF, 50 mM) was injected. The solution was allowed to react for 72 hr with stirring in nitrogen. The purification process, NMR and FT-IR spectra were identical as that of high MW PBG. The product yield is 70% and molecular weight is M_n: 62 kDa, M_w: 80 kDa, PDI: 1.30 (Fig. S10).

Synthesis of low MW PBG-N_3

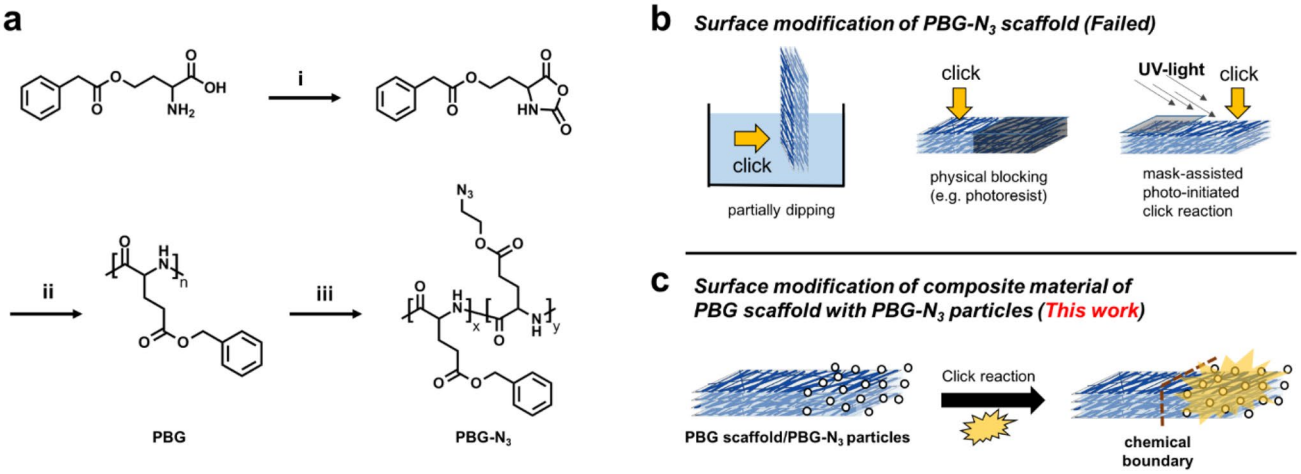
The synthesis was referred to Guo et al. using low MW PBG as starting material, while the alcohol for esterification, 2-azidoethanol, was synthesized according to Norberg et al. [17, 18]. The products of NMR and FT-IR spectra are available in supporting information (Figs. S5-S9). (yield: 93%)

Scaffold fabrication

Aligned fibrous scaffolds and micro-particles were fabricated by electrospinning and electrospray respectively. The composite scaffold, named PBG/PBG-N_3 composite, was fabricated by electrospray of PBG-N_3 particles on selective region of an electrospun PBG scaffold through a mask. In this process, a plastic film was used to mask the scaffold partially such that the particles would specifically deposit on the selective region. Processing parameters are shown in the Supporting Information.

Surface modification of scaffold

PBG/PBG-N_3 composite scaffold (0.5cm*0.5cm) was immersed in DI-water (400 µl) followed by an addition of $CuSO_4 \cdot 5H_2O_{(aq)}$ (10 mM, 5 µl), fluorescent dye (TAMRA



Scheme 1 (**a**) Synthesis of PBG and PBG-N_3: (i) triphosgene, ethyl acetate, 2 hr, reflux; (ii) sodium methoxide, benzene, 48 hr (for high MW PBG); benzylamine, DMF, 72 hr (for low MW PBG); (iii) 2-azidoethanol, benzyl alcohol, p-toluenesulfonic acid, 1,2-dichoroethane, 24 hr, 55 °C. (**b**) Common methods for obtaining chemical boundary via surface modification by click reaction but failed. (**c**) Novel method of incorporating particles with azide functionality into certain region of the scaffold to produce chemical boundary after surface modification by click reaction in this work

alkyne)$_{(aq)}$ (10 mM, 1 µl), and freshly prepared sodium ascorbate$_{(aq)}$ (2.5 mM, 80 µl). The mixture was allowed to react for 2 hr by the assistance of shaker (40 rpm) in the dark. Then, the scaffold was rinsed with DI-water for several times, and immersed in DI-water for 3 days to remove the unreacted dye.

Results and discussion

We hypothesized that if a chemical boundary could be formed in a 3D scaffold consisting of aligned polymeric fibers, cell migration and growth in 3D environment might be observed at specific area direction and area. The new 3D scaffold based on poly(L-γ-benzyl glutamate) (PBG) with N_3 terminated polypeptide particles is developed for potential neural tissue engineering application in this work. We are interested in the development of neural tissue engineering. The poly(L-γ-benzyl glutamate) (PBG) scaffold is biocompatible and contains neuron stimulate glutamate which has been demonstrated in promoting neuron growth [19–21].

A facile scaffold fabrication was attempted to construct chemical boundary on the preformed electrospun 3D fibrous scaffold. More specifically, we can modify the surface of certain region of a scaffold via alkyne-azide click chemistry in aqueous condition to create an area with surface functionality separated from the unmodified area, generating a chemical boundary between the two regions. The chemical boundary can be built by different chemical reagents for desired functionality. By this method, we can obtain a variety of chemical boundaries from lots of polymeric materials just depending on the reagents for click reaction. To ensure both biological and chemical properties are present in the scaffold, the material used here is a copolymer of benzyl glutamate and azido functionality, poly(L-γ-2-azidoethyl glutamate-co-γ-benzyl glutamate) (PBG-N_3), which was synthesized from PBG (Scheme 1a). Calculation of the integral in ^{1}H-NMR spectrum of PBG-N_3 showed the conversion rate of side chains was about 30%. The copolymer was fabricated to fibrous scaffolds by electrospinning, and then the scaffolds were chemically modified by click reaction.

In order to limit click reaction to proceed in certain region of PBG-N_3 scaffold, rather than the whole substrate, to form chemical boundary, we attempted to construct it by several common methods, including partial immersion, physical blocking by photoresist, and mask-assisted photo-initiated reaction (Scheme 1b). However, all of the above strategies unfortunately failed. For partial immersion method, all reactants are absorbed onto the scaffold by capillary effect through the fibers without the formation of chemical boundary.[22] Substance from the physical blocking cannot be completely removed from the scaffolds, not only clear boundary cannot be formed but also the cytotoxicity associated with photoresist is not suitable for biomedical application. Also, radicals generated from photo-initiated reactions degrade many biopolymers including peptide [23].

Therefore, we modified our approach to achieve the goal by combining electrospinning of PBG fibers and mask-assisted electrospray of PBG-N_3 particles to produce a composite scaffold with a clear boundary containing two regions. The morphology, including alignment of fibers and shape of particles, was optimized by tuning the processing parameters

of electrospinning and electrospray using different molecular weight of polymers. In this composite scaffold, only the PBG-N_3 particles can undergo surface modification via click chemistry. Since particles were confined to deposit at certain region of the scaffold, click reaction will particularly proceed at selected area rather than the whole area of the scaffold, and chemical boundary demarcated the modified area from the pristine area was fabricated (Scheme 1c). The obtained scaffolds can serve as a 3D substrate and they have potential for studying the effect of chemical stimuli on cell migration and growth in 3D environment. Simultaneous electrospinning and electrospray also provide composite scaffolds consisting of fibers and particles. However, the required property of aligned fibers in neural tissue engineering cannot obtain through this process. To overcome this issue, we fabricated PBG scaffolds with aligned fibers first, and then deposited PBG-N_3 particles at a selective region of the scaffold by the assistance of a mask.

The morphology of the fibrous scaffold, particles, and the composite material are shown in Fig. 1a-c. The boundary of particles deposition is illustrated by a yellow dash line in Fig. 1c, which demarcates the pristine fibers on the left-hand side and the particle-decorated fibers on the other side. Also, we made sure the direction of boundary be perpendicular to the alignment of fibers so that cell migration will be triggered by chemical stimuli with the assistance of physical guidance provided by the fibers. Figure 1d and e show images with higher magnification focusing on two points beside the boundary in Fig. 1c, demonstrating the clear difference between two regions. Although the particles were deposited from the top of the scaffold, by controlling the size of particles, they were still able to pass through the voids between fibers to deposit into the depth of the scaffold in z-direction as revealed in the circles in Fig. 1e (see also Fig. S11). Garcia Garcia et al. demonstrated the

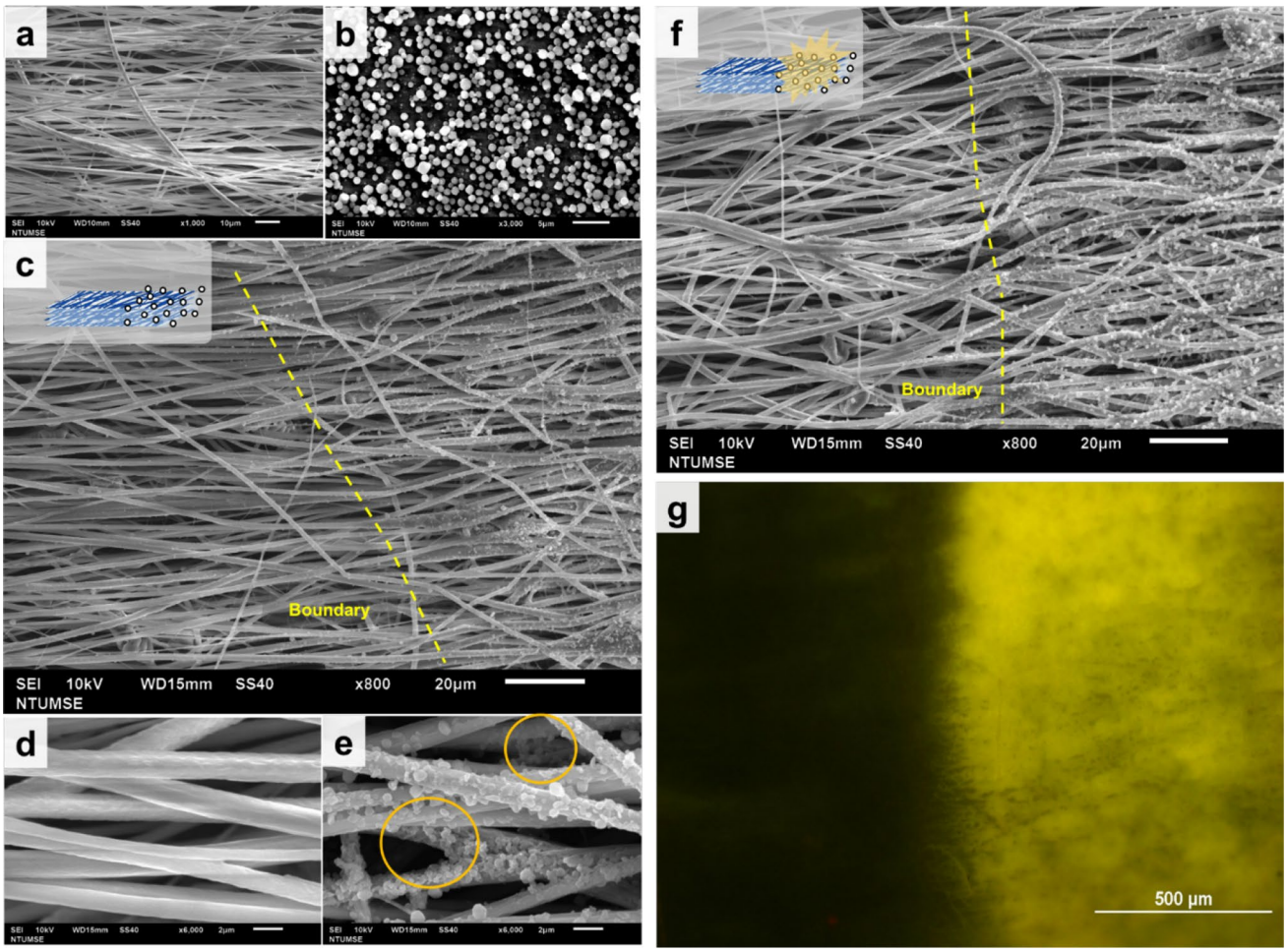


Fig. 1 Morphology of (**a**) PBG electrospun fibrous scaffold (scale bar: 10 μm), (**b**) PBG-N_3 electrosprayed particles (scale bar: 5 μm), and (**c**) PBG/PBG-N_3 composite with boundary from particle deposition (scale bar: 20 μm); (**d**), (**e**) SEM images of two sides of the deposition boundary, showing the pristine and particle-decorated fibers, respectively (scale bar: 2 μm); (**f**) Morphology of PBG/PBG-N_3 composite after click reaction and immersion in water for 3 days, showing the boundary is remained (scale bar: 20 μm); (**g**) Fluorescence microscopy image of PBG/PBG-N_3 composite after click reaction with dye (TAMRA alkyne) (scale bar: 500 μm)

particles could distribute throughout the depth of scaffolds by alternatively electrospinning and electrospray, which is more labor-intensive process than our method [24].

Interestingly, the particles attached firmly on the fibers no matter how many times we rinse the scaffold with water. We speculated that solvent did not evaporate completely as particles arrived at the scaffold during the process of electrospray, and thus fibers and particles were slightly fused together by the residual solvent. This simple strategy provided us a composite scaffold with no need of cross-linking or annealing process to stick the two components together. The residual solvent was removed completely by vacuum evaporation after the substrate was prepared. Actually, the stability of composite scaffold can be fully explained by Flory-Huggins theory. The theory serves as the fundamental basis for understating polymer solution and blend thermodynamics, allowing for quantification of the affinity between a polymer and a plasticizer [25]. Extensive studies have demonstrated that the thermodynamic and morphological properties of polymer blends, block copolymers, and polymer solutions critically depend on the Flory-Huggins interaction parameter, denoted as χ parameter [26]. In our study, we utilized PBG to fabricate fibrous scaffolds through the process of electrospinning and PBG-N_3 particles were placed on the designated area through electrospray to form polypeptide composite fibrous scaffolds. The good compatibility is achieved between PBG and PBG-N_3 because the two polymers contain the same chemical structure except for a small N_3 terminal group (mol.wt.42) at the end of PBG-N_3 (PBG mol.wt, 80,000). The chemical effect of small size N_3 can be totally ignored. The same solvent system (THF/DMAc) was used for both polymers to do the fabrication of the composite scaffold which results in a strong affinity between the two polymer components. Thus, accordingly to the Flory-Huggins theory, the interaction parameter (χ) of two components is very small to have a very stable composite scaffold.

In order to confirm the creation of chemical boundary, we used a fluorescent dye containing alkyne (TAMRA alkyne) for click reaction. Both of the particles and boundary can be remained after click reaction in aqueous condition as shown in Fig. 1f. Since only particles in the composite were able to react, TAMRA alkyne was selectively linked at the region with particle deposition in the scaffold, resulting in a chemical boundary and a partially illuminated fluorescent image as shown in Fig. 1g. Although someone may argue that apart from chemical effect, morphology difference can also induce cell migration. Most literature revealed, stiffness and rigidity of materials are main physical stimuli for cell migration [7–9, 27]. In our composite scaffold, we can assume that its stiffness is similar to the fibrous only scaffold. The particles are discontinuous and smaller than fibers (Figure 1e). When they are on the surface of fibers would not increase stiffness of substrate and would be ignored by cells [28]. We also demonstrated previously the stiff PBG chemically modified into containing glutamic acid can perform much better cell viability and growth than virgin PBG [20]. Thus we reasonably presume that chemical stimuli would be the dominant factor for cell migration in our composite scaffold rather than physical stimuli.

Conclusion

We successfully fabricated a novel 3D fibrous composite scaffold consisting of PBG fibers and PBG-N_3 particles with a clear boundary of particle deposition. The part with particles is able to undergo click reaction for surface modification, and thus chemical boundary of various functional groups can be obtained inside the 3D scaffold. To the best of our knowledge no other research groups have reported this kind of 3D polymeric substrate in literature. The novel scaffold fabricated by combining electrospinning and mask-assisted electrospray is useful for the biologists to investigate cell migration in 3D environment and has a potential application in effective cell migration and growth required in tissue engineering.

Nomenclature The abbreviation of synthesized products are denoted as following: L-γ-Benzyl glutamate N-carboxyanhydride (BG-NCA); poly(L-γ-benzyl glutamate) (PBG); poly(L-γ-2-azidoethyl glutamate-co-γ-benzyl glutamate) (PBG-N3). Polymers with molecular weight exceeding 150 kDa are noted as "high MW", while the others are noted as "low MW".

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10965-023-03695-6.

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Declarations

Conflict of interest None.

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