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Fine neurite patterns from photocrosslinking of cell-repellent benzophenone copolymer

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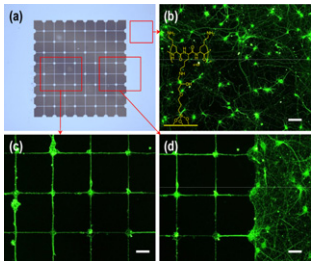
HIGHLIGHTS

- Synthesis of benzophenone copolymer and photopattering of cell-repellent patterns.
- Fine neurite, presumably axon, patterns with excellent pattern fidelity were obtained.
- Benzophenone unit was UV-crosslinked without photo-oxidative damage to poly-D-lysine.

GRAPHICAL ABSTRACT



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ABSTRACT

We have synthesized photocrosslinkable benzophenone copolymer, Poly(St-*co*-MBz), and fabricated cell-repellent patterns of Poly(St-*co*-MBz) on covalently bound poly-D-lysine (PDL) layer via the photocrosslinking. We have successfully obtained fine grid line pattern with line width of 3 μm and fine neurite, presumably axon, patterns with excellent pattern fidelity. We found that benzophenone unit can be crosslinked under the exposure of UV (with the intensity of ~77 mW/cm^2 at 280 nm and ~60 mW/cm^2 at 365 nm) without photo-oxidative damage to PDL, poly-L-lysine, and polyethyleneimine.

1. Introduction

The well-defined neuronal networks in vitro have been emerged as a useful platform for gaining inspiration on artificial neuronal networks that can be adapted to mimic the neuronal networks found in the brain. Patterned neuronal networks had been achieved on the multi-electrode arrays (MEAs) (Chang et al., 2006; Pan et al., 2011; Boehler et al., 2012) and semiconductor devices (Rhee et al., 2005; Patolsky et al., 2006) in order to examine the bi-directional communication between the neuronal networks and the electronic devices. The major technical issues in achieving the desired neuronal networks are the patterning of cell-adhesive materials (CAMs) to guide neuronal growth and the long-term adhesion of neuronal cells. All these technical issues are important in that neuronal networks can be stimulated and their electrical response can be durably recorded at any desired electrode sites with a wide variety of electrode layout.

Cationic polymers like poly-D-lysine (PDL) (Harnett et al., 2007) and polyethyleneimine (PEI) (Liu et al., 2008) as well as extracellular matrix proteins, such as fibronectin and grafted arginine–glycine–aspartic acid peptides (Perlin et al., 2008; Lai et al., 2010), are widely used as a CAM in achieving the patterned neuronal networks on MEAs (James et al., 2004; Jungblut et al., 2009; Boehler et al., 2012), neurochips (Maher et al., 1999), and

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biosensors (Liu et al., 2006). Despite the unique advantages of CAMs, their non-covalent binding nature to the cell-culture substrate frequently limits long-term growth of neuronal cells via dissolution of CAMs from the substrate. To address this limitation, we had covalently bound PDL and modified-PEI onto indium-tin oxide (ITO) and silicon dioxide (SiO_2) substrates using the amine-terminated self-assembled monolayer (SAM) and demonstrated the superior long-term survival of primarily cultured neuronal cells on the covalently bound PDL and modified-PEI than on the physically bound ones (Kim et al., 2011a,b; Baek et al., 2011a,b). The covalently bound PDL and modified-PEI have also promoted the neurite outgrowth.

Many researchers have reported the fabrication of micropatterns of CAMs on the substrates by means of various patterning techniques such as standard photolithography (Sorribas et al., 2002), micro-contact printing (Branch et al., 2000; Lauer et al., 2001; Hwang et al., 2009), and deep UV lithography technique (Lom et al., 1993). Although these techniques can provide micron-scale patterns of CAMs, it is still hard to achieve clear single-cell-level patterned neuronal networks solely from the CAM patterns. Frequently neurites deviate from the patterns or form neuronal bridges between patterns, possibly due to dissolution of physically bound CAMs and interaction between cells and base substrate. Recently, to circumvent the dissolution of physically bound CAMs, we had bound PDL and modified-PEI covalently onto the surface of silicon dioxide and indium-tin oxide (ITO) and found that covalent binding is very effective in enhancing adhesion of neuronal cells to the substrate, resulted in prolonged culture periods (Kim et al., 2011a,b; Baek et al., 2011a,b). However, even the covalent binding was not enough to routinely obtain patterned network of neuronal cells without neuronal bridges crossing space area between patterns. Recently, to enhance the neuronal pattern fidelity, we had tried to cover the space area with bromine-functionalized polyfluorene (BFP) which is hydrophobic and photocrosslinkable (Baek et al., 2011a,b). It had already been confirmed that cells do not adhere to hydrophobic surfaces (James et al., 2004; Chang and Sretavan, 2008; Leclair et al., 2011; Kim et al., 2011a,b). The photocrosslinking capability of BFP enabled the fabrication of patterns with standard photolithography and long-term maintenance of cell-repellent characteristics. In combination with covalently bound cell-adhesive modified-PEI we had demonstrated patterns of cell-adhesive grid patterns with the resolution less than 10 μm surrounded by the cell-repellent BFP, and achieved the clear single cell level patterned neuronal networks.

Despite excellent cell-repellent ability, BFP has some drawbacks in that multi-step procedures are needed in synthesis and the yield is low (~30%). To simplify the reaction we have adopted free radical polymerization instead of Suzuki cross coupling reaction. This synthetic protocol involves fast and efficient two-step procedures and enables precise adjustment of molecular composition and the mass production. Considering hydrophobicity, biocompatibility, and photopatterning capability we have selected styrene and methacryloxybenzophenone as a monomer system for radical co-polymerization. Poystyrene is not only hydrophobic but also biocompatible so that the surface-modified polystyrene (PS) is now commercially available as a substrate for cell culture. Among the various photocrosslinkable moieties such as alkyl bromide (Baek et al., 2011a,b), sulfonyl azide (Schuh et al., 2008), and cinnamic moiety (Yammine et al., 2005), benzophenone derivative was selected as a photocrosslinking agent because of the well-established photochemistry and proven biocompatibility (Dorman and Prestwich, 1994; Prucker et al., 1999; Dhende et al., 2011). Benzophenone is photoactive so that upon UV exposure it undergoes rapid intersystem crossing (the order of $\sim 10^{11}\ s^{-1}$) to yield triplet exited state with almost unit quantum efficiency. At its triplet excited state benzophenone abstracts a hydrogen atom from any neighboring aliphatic C—H bond, generating a radical. The two generated radicals combine together and form new C—C bond, causing crosslinking between close neighboring chains.

The principal features of our method used in the study are as follows: (1) easy chemistry and photopatterning, (2) tight attachment of CAMs and cell-repellants to the substrate via covalent binding and photocrosslinking, (3) enhanced pattern fidelity of neuronal cells beyond single cell level, and (4) pattern compatibility with conventional MEAs. Herein, we demonstrate the photocrosslinking of benzophenone moiety containing polystyrene copolymer as a facile and precise method to get cell-repellent patterns. We also demonstrate axon-level patterned neuronal network by using the cell-repellent polystyrene copolymer pattern in combination with covalently bound PDL layer.

2. Experimental

2.1. Materials and instruments

Metharyloyl chloride, 4-hydroxybenzophenone, styrene, azobisisobutyronitrile (AIBN), 3-glycidoxypropyltrimethoxysilane (GPTMS), PDL hydrobromide (Mw 70–150 K), poly-L-lysine (PLL), and PEI were purchased from Aldrich Co., and used without further purification. Tetrahydrofuran (THF) was distilled over sodium benzophenone ketyl under nitrogen prior to use. ^{1}H NMR and ^{13}C NMR spectra were recorded using a Varian Oxford 300 MHz spectrometer; chemical shifts were reported in ppm unit with tetramethylsilane as an internal standard. The photopatterning of cell-repellent polymer was performed using ultraviolet source (OSRAM HBO 350W/S mercury lamp, spectral intensities of ~77 mW/cm^2 at 280 nm and ~60 mW/cm^2 at 365 nm) equipped in a mask aligner.

The attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectrum was taken with a Nicolet iN10 spectrometer with Ge crystal. X-ray photoelectron spectroscopy (XPS) measurements were performed in an ESCALAB 200R instrument (VG scientific) using monochromized Al-Kα operated at 1486.6 eV, 12.5 kV and 250 W X-ray radiation. The base pressure of the system was 5.0×10^{-10} Torr. XPS spectra were acquired at 90° takeoff angle relative to the surface. The molecular weight and polydispersity were analyzed by a Waters 1515 gel permeation chromatograph (GPC) with a refractive index detector at room temperature (THF as the eluent). Steady-state absorption spectra were recorded on a Shimadzu UV-2401PC spectrophotometer. Contact angles were measured using a contact angle meter (CAM 100, KSV Instrument).

2.2. Synthesis of photopatternable polystyrene copolymer

Synthesis of 4-(methacryloxy)benzophenone. 4-hydroxybenzophenone (7.3 g, 36.85 mmol) was dissolved in 40 mL of CH_2Cl_2 and 5 mL of NEt_3 at room temperature under N_2 atmosphere. Methacryloyl chloride (3.21 g, 30.71 mmol) was slowly injected into a solution and stirred overnight at room temperature. The organic layer was extracted with CH_2Cl_2. The combined extracts were concentrated in vacuo and then dried over $MgSO_4$. The residue was purified by flash chromatography on a silica gel eluting with hexane and dichloromethane (1:5). (7.5 g, 92%). ^{1}H NMR ($CDCl_3$, 300 MHz, ppm): δ 7.89 (d, 2H), 7.82 (d, 2H), 7.59 (t, 1H), 7.47 (t, 2H), 7.27 (d, 2H), 6.39 (t, 1H), 5.81 (m, 1H), 2.08 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz, ppm): δ 195.30, 165.10, 153.99, 137.29, 135.32, 134.77, 132.31, 131.52, 129.79, 128.18, 127.82, 121.44, 18.35.

Synthesis of poly(styrene-*co*-methacryloxybenzophenone) (Poly(St-*co*-MBz)) (Beines et al., 2007). To mixure of styrene (1.55 g, 14.88 mmol) and 4-methacryloxy benzophenone (1.19 g,