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Highly fluorescent and photostable polymeric nanofibers as scaffolds for cell interfacing and long-term tracking

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In tissue engineering, artificial substrates are developed to mimic the natural *in vivo* micro-environment in which cells reside.^[1] How cells respond to and interact with these substrates provides essential information in stem cell therapies and regenerative medicine.^[2] In order to accurately evaluate the mechanisms behind cell-substrate interactions, fluorescence labeling has been used extensively for visualization and tracking. To date, much effort has focused on cells – e.g. tracking cellular uptake of biomolecules with labeled DNA/RNA^[3] or labeled nanoparticles^[4], or tagging intracellular components^[5]. However, the study of cell-substrate interactions also includes visualization of the substrate on which cells grow. Unfortunately, the development in this aspect remains rarely explored.

Thus far, commercialized fluorescent compounds, such as FITC and Rhodamine, are the most commonly used approaches for labeling and visualizing extracellular materials such as proteins or polysaccharides^[6, 7]. However, the leaching and poor photo-stability of these dyes remain the major obstacles to their wide-spread applications, especially for long-term tracking and imaging purposes. Recently, a semiconducting luminescent polymer was developed for long-term optical non-invasive tracking, but the detailed interaction between cells (especially stem/progenitor cell) and its underlying substrate remains undetectable^[8]. More delicate tracking such as cell-substrate temporal interaction requires new functional polymeric scaffolds with good biocompatibility and fluorescent properties.

We have designed and synthesized a series of highly fluorescent and photostable polycaprolactones (PCLs) covalently linked with fluorescent di(thiophene-2-yl)-diketopyrrolopyrrole (DPP) in the middle of each polymer chain.^[9, 10] The fluorescence properties of these polymers (denoted as PCL-DPP-PCL) in solid states are determined by the average polymer chain length of PCL. In particular, polymers with a number-average degree of polymerization (n) of PCL over 100 show strong yellow fluorescence that is intrinsic to aggregation-free DPP in different states such as solid films and aqueous dispersion of nanoparticles.^[10] Moreover, the highly fluorescent and photostable nanoparticles composed of PCL-DPP-PCL ($n = 185$) can be internalized by tumor cells and multipotent stromal cells for cell imaging.^[9] Nevertheless, the potential of these functional PCL polymers as tissue constructs for tissue engineering has not been explored so far.

Here, we present highly fluorescent and photostable electro-spun polymeric nanofibers that serve as scaffolds for cell interfacing. These fluorescent nanofibers composed of PCL-DPP-PCL ($n = 185$)[9, 10] provide striking contrast for studying cell-substrate interactions and are useful for long-term tracking. As compared to various cellular tracking compounds, the methods of labeling scaffold are still limited to using FITC or Rhodamine, which are always tagged to the end groups of biomolecules that make up the extracellular matrix or scaffold. Unfortunately, in these methods, dyes that are tagged to the matrices often suffer from fast photobleaching because of their intrinsic nature of poor photostability, as they are easily exposed to the environment by being tagged to the ends of biomolecules. This leads to the limitation of their applications in long-term tracking of cell-substrate interactions. As compared to the common sulforhodamine-labeled electrospun fibers in which the dye was physically dispersed in the polymer matrix^[7], our PCL-DPP-PCL fibers show superior photostability and significantly less leaching. Importantly, by using human dermal fibroblasts and primary rat oligodendroglial precursor cells (OPCs) as the model adult somatic cell and progenitor cell respectively, we demonstrate that the cytocompatibility and cell attachment were not compromised as compared to unmodified commercial PCL. In addition, these PCL-DPP-PCL fibers enabled clear visualization of intricate cell-substrate interactions such as oligodendrocyte (OL) myelination.

Figure 1 summarizes the overall experimental approach taken in this study. PCL-DPP-PCL fibers were fabricated from PCL-DPP-PCL and commercial PCL mixture by electrospinning method. The superior photostability of PCL-DPP-PCL fibers offers the opportunity to monitor cell-substrate interaction for long-term tracking. More specifically, as a proof of concept, OPCs were treated with PCL-DPP-PCL fibers to visualize and provide better understanding of OPC myelination, which is related to cell-extracellular environmental interactions.

As shown in Figure 2 (A and B), when processed under the same electrospinning parameters, PCL (Figure 2A, $2.044 \pm 0.367 \mu\text{m}$) and PCL-DPP-PCL fiber scaffolds (Figure 2B, $1.985 \pm 0.62 \mu\text{m}$) possessed similar substrate morphology and average fiber diameters. These images indicated that DPP conjugation did not affect the diameter of electrospun fibers.

We then investigated the photostability of PCL-DPP-PCL in solid states, specifically in fibrillar scaffolds that are ultralong but very thin (sub-micron to nanometers) in diameter.

Another commonly used fluorophore R6G as well as a DPP-based small molecule (DPP-EH) were chosen as controls. As shown in Figure 2 (C-E), only PCL-DPP-PCL nanofibers (Figure 2C) retained relatively strong fluorescence intensity after 60 s of 2W-laser irradiation, while the two small molecular fluorophores, DPP-EH (Figure 2D) and R6G (Figure 2E), showed significant photobleaching phenomenon under the same conditions. These results are consistent with the photostability of PCL-DPP-PCL in colloidal spherical nanoparticles,[9] which further demonstrates that our molecular design of PCL-DPP-PCL is efficient in suppressing fluorescence quenching and improving the photostability of organic fluorophores in both nanoparticles and other forms of solid states (e.g. nanofibers and thin films).[10]

Due to the hydrophilic nature of R6G, obvious leaching (up to nearly 8% after 7 days) of the organic dye was observed, whereas almost no leaching (less than 0.01 %) could be detected from the nanofibers that were composed of either DPP-EH or PCL-DPP-PCL as they are both hydrophobic (Figure 2F). To summarize, both photostability and leaching tests suggested that PCL-DPP-PCL nanofibers were stable and could potentially be used for long-term imaging and monitoring of cell-substrate interactions.

To evaluate the potential of PCL-DPP-PCL fiber scaffolds as constructs for understanding cell-substrate interactions, we cultured human dermal fibroblasts (HDF) as the model human adult somatic cell onto PCL-DPP-PCL scaffolds. After 14 days of culture, cell attachment appeared unaffected by the presence of DPP conjugation. Specifically, HDF attached firmly on both PCL and PCL-DPP-PCL fiber scaffolds (Figure 3A and 3B), as evident by their typical well-spread morphology and the presence of actin stress fibers throughout the cell bodies.

Besides looking at cell attachment, fluorescent fibers may also serve as useful platforms for evaluating more specific cell functionalities that are related to cell-extracellular environmental interactions, such as myelination. As shown in Figure 3C and 3D, OPCs on both PCL and PCL-DPP-PCL fiber scaffolds revealed typical structures with a polygonal soma and a multipolar tree of fine processes.^[11] Furthermore, as shown in Figure 3E, MTT assay indicated similar viability of OPCs on both PCL and PCL-DPP-PCL fibers at each time point. As compared to day 4, OPC viability on day 7 was much higher on every scaffold. On day 15, a slight decrease in cell viability was observed on both PCL and PCL-DPP-PCL fiber scaffolds, which may result from cellular contact inhibition. Altogether, DPP conjugation into

PCL fiber scaffolds did not affect the cell viability and attachment of primary OPCs. Importantly, given the fact that OPCs are very sensitive to their microenvironment, the normal attachment and viability of primary OPCs as observed on our PCL-DPP-PCL scaffolds underscores the cytocompatibility and relevance of our constructs as study platforms for analyzing delicate cell-substrate interactions.

Within the central nervous system (CNS), OPC interacts closely with its microenvironment^[12] and plays critical roles in myelinating nerves. Demyelination leads to devastating outcomes as seen in diseases such as multiple sclerosis (MS). Unfortunately, effective treatments are lacking due in part due to the limited knowledge on OPC myelination^[13]. Therefore, any improvements in experimental strategies and platforms that can facilitate studies and visualization of the myelination process, such as that provided by our PCL-DPP-PCL fiber platform has great potential in advancing the field.

OL-subtract interaction is divided into three stages: contacting, extending and wrapping^[14], according to different modes of interaction^[15]. As shown in Figure 4A and 4B, after 7 days of differentiation, OL contacted and extended along the fiber, but no wrapping was seen. On day 15, obvious wrapping of OL around our fibers was detected (Figure 4C and 4D), although most of the cellular extensions remained at an earlier stage such as extending. The extension of OL clearly wrapped around PCL-DPP-PCL fiber (as shown by the box and arrow in Figure 4D), followed by the formation of myelin membrane which ensheathed the bright red PCL-DPP-PCL fiber (Supplementary video 1). Oligodendroglial-neuronal co-culture has been the traditional strategy for understanding OL myelination process. However, decoupling of indirect (neuronal) effects from direct (oligodendroglial) effects is difficult when using such co-cultures^[7]. Although electrospun fibers have been used to investigate OL-substrate interactions^[16], these fibers were labeled with rhodamine, rendering them unstable and unsuitable for long-term fluorescent imaging and tracking. In contrast, as shown in this study, PCL-DPP-PCL nanofibers retained strong fluorescent signals even after 15 days of prolonged cell culture.

In summary, highly fluorescent PCL-DPP-PCL fibers provide a cytocompatible substrate for primary somatic and precursor cell proliferation and differentiation. Given that fibroblasts attached firmly as seen by stress fiber formation, OPCs proliferated as seen by increased viability, and OPCs differentiated into MBP positive cells that myelinated the fibers, our results demonstrate that PCL-DPP-PCL is cytocompatible and not cytotoxic. More

importantly, the enhanced photo-stability of PCL-DPP-PCL fiber scaffolds renders these constructs as useful platforms for understanding cell-substrate interactions.

Experimental Section

Reagents: DPP-EH was synthesized according to the literature^[17]; the synthesis of PCL-DPP-PCL with number average molecular weight of 38300 was reported in the previous paper^[10]. Rhodamine 6G (R6G), polycaprolactone (PCL, Mw: 80,000), 3, 4- dihydroxy-L-phenylalanine (DOPA), 2, 2, 2-trifluoroethnal (TFE, $\geq 99.0\%$), Tris-EDTA (TE) buffer solution (pH 8.0), triiodothyronine (T3), thiazolyl blue tetrazolium bromide (MTT, 98%), dimethyl sulfoxide (DMSO), 10% formalin, triton X-100 and fluoromount medium, 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich. Laminin (1mg/ml), Alexa-Fluro 488 goat anti-mouse antibody was purchased from Life Technologies. Mouse anti-myelin basic protein (MBP) (MAB387) antibody was purchased from Merck. Basic fibroblast growth factor (bFGF) was purchased from Peprotech

Fabrication of PCL-DPP-PCL fibers: To electrospin fibers for photobleaching and leaching tests, 10% (mol concentration) of R6G, DPP-EH and PCL-DPP-PCL were mixed with PCL (80K) in TFE with 12 wt% of PCL (80K) separately. Each of these three solutions was first treated with ultrasonication, wrapped with aluminium foil and then placed on a multi-axle rotating mixer overnight for thorough mixing and complete polymer dissolution. Thereafter, each solution was dispensed at a flow rate of 1.5 ml/h through a 21G needle for electrospinning. The collector was located 12 cm away. A 16 kV (+12 kV and -4 kV) potential was applied between the syringe needle and the rotating collector (2100 rpm). Finally, the morphology of the fibers was evaluated using scanning electron microscopy (SEM) (JEOL, JSM-6390LA, Japan) after sputter-coating with platinum.

Photobleaching test: All electrospun samples were irradiated with 514 nm light (2W) under the fluorescent microscope for 100 s. A series of fluorescence images of the nanofibers were recorded every 50 ms for the comparison of photostability of the nanofibers with different fluorescence agents.

Leaching test: Each set of electrospun scaffold (n=3, average mass = 40.2 ± 1.4 mg for DPP-EH, 45.6 ± 1.7 mg for PCL-DPP-PCL, 43.0 ± 3.3 mg for Rhodamine 6G) was immersed into 10 ml of $1 \times$ PBS buffer (pH=7.4) in a 15 ml centrifuge tube. The centrifuge

tubes were then placed in the shaking bath at 37 °C and 0.5 mL of solution was collected every 12 h. The leaching percentage of R6G, DPP-EH and PCL-DPP-PCL was determined by fluorescence intensity of each fluorophore that leached into PBS buffer over the total theoretical mass of fluorophore in the scaffolds (i.e. 0.072 ± 0.003 mg for DPP-EH, 4.15 ± 0.15 mg for PCL-DPP-PCL and 0.054 ± 0.004 mg for Rhodamine 6G).

Preparation for poly(DOPA)-laminin coated fibers: To ensure OPC attachment onto fiber scaffolds, coating of PCL and PCL-DPP-PCL fibers was carried out using our published protocol^{[18][19]}. Briefly, after cutting the scaffolds into 2 cm²- discs to fit 24-well plates, all scaffolds were pre-wet with deionized water overnight. Thereafter, the scaffolds were submerged into 0.5 mg/ml of DOPA that was dissolved in poly(DOPA) coating buffer (50 mM NaCl and 10 mM Bicine, pH=8.5) and agitated at 120 rpm on an orbital shaker for 4 h. Following that, all scaffolds were washed with deionized water to remove residual monomer and lyophilized overnight. Next, after UV sterilization, poly(DOPA) coated scaffolds were immersed into 300 µl of laminin solution at 10 µg/cm² for 2 h.

Primary OPC isolation and differentiation: Approved by the Institutional Animal Care and Use Committee (IACUC) at Nanyang Technological University, Singapore, primary OPC was isolated from p1-p2 neonatal rats^[20]. The cortices of neonatal pups were digested with 0.01 % trypsin and 0.01 mg/ml Dnase I for 15 min at 37 °C. After mechanical homogenization, the suspension was passed through a 70 µm cell strainer (BD Biosciences, USA) and seeded in T75 flasks at a density of 10⁶ cells. After 9-10 days of culture, the OPCs were purified by shaking on an orbital shaker for 18-20 h at 37 °C. Thereafter, the cell suspension was passed through a 20 µm sterile screening Nylon pouch (Sefar American, USA) and the OPCs were then seeded onto poly(DOPA)-laminin coated scaffolds at a density of 50, 000 cells in OPC differentiation medium with 10 ng/ml bFGF and 15 nM T3. The medium was changed every other day.

Cell viability assay: At indicated time points, three independent scaffolds were collected and washed with 1× PBS. After immersing into 1 ml of MTT solution (1 mg/ml in 1× PBS), the scaffolds were incubated at 37 °C for 4-6 h. Thereafter, MTT solution was discarded and 500 µl of DMSO was added into each well to react with the purple formazan product on the scaffolds. Finally, absorbance at 570 nm was read using a microplate reader (Tecan®, Infinite 200, Austria).

Cell skeleton staining: At indicated time points, cell-seeded nanofiber scaffolds were collected and fixed in 10 % formalin and permeabilized with 0.3 % Triton-X 100 in PBS for 20 min. Thereafter, the samples were incubated with phalloidin-488 (1 : 500) and DAPI (1 : 1000) at 37 °C for 30 min followed by imaging under a Zeiss LSM 710 confocal microscope.

Immunofluorescent staining: After 15 days of culture, scaffolds were fixed with 10 % formalin and permeabilized with 0.1 % Triton-X 100 in PBS for 20 min. After being blocked with 1.5 % goat serum for 1 h, the samples were incubated with primary anti-MBP antibody (1 : 200) overnight at 4 °C followed by incubation with secondary antibody (1 : 700) for 1 h at room temperature. DAPI was used for nuclei counterstaining. Finally, all samples were mounted and imaged under a confocal microscope (Zeiss LSM 710).

Statistical analysis: Quantitative values are presented as mean $\pm$ standard deviation. After F-test, the one-way ANOVA and Turkey post-hoc tests were used when samples had equal variances. Otherwise, the Kruskal-Wallis and Mann-Whitney *U*-tests were used.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Figure 1. Schematic illustration of PCL-DPP-PCL fibers fabrication and interaction with Oligodendrocyte precursor cell (OPC).

Figure 2. SEM images of electrospun nanofiber scaffolds: (A) PCL, (B) PCL-DPP-PCL fiber scaffolds. Fluorescence images of: (C) PCL-DPP-PCL fibers (20% mol in PCL), (D) DPP-EH fiber (20% mol in PCL), and (E) R6G fiber (20% mol in PCL) in 1 min under 514 nm light irradiation (2W). The scale bars shown in images recorded at 60 s are applicable to images recorded at 0 s, 20 s and 40 s for (C), (D) and (E). Leaching profile (F) of R6G, DPP-EH and PCL-DPP-PCL nanofibers in PBS buffer at 37°C in 7 days.

Figure 3. Actin cytoskeleton structures of HDFs (A and B, 40X, day 14) and OPCs (C and D, day 3) on PCL (A, C) or PCL-DPP-PCL (B, D) fiber scaffolds, as revealed by Phalloidin staining. And (F) quantification of OPC cell viability (mean $\pm$ SD, N = 3). * $p < 0.05$ (ANOVA), ** $p < 0.01$ (ANOVA) and *** $p < 0.001$ (ANOVA) compared with absorbance (570nm) of same group on day 4, ## $p < 0.01$ (ANOVA) compared with absorbance (570nm) of same group on day 7.

Figure 4. OPC-fiber interaction between MBP⁺ (green) OL and PCL-DPP-PCL fiber scaffolds (red) on day 7: (A) (40X) and (B) (100X); day 15 (C) (40X) and (D) (100X). Blue color represents DAPI.

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