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Multiscale engineering of functional organic polymer interfaces for neuronal stimulation and recording

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Development of new materials and technologies for neural interfacing, stimulation and recording has attracted increasing attention. Specifically, the applications of new polymeric materials and technologies for neuron science and engineering could benefit the treatment of neural diseases and recovery of nerve injuries, as well as establishment of brain-machine interfaces that benefit patients suffering from neural diseases or injuries. In the present review, we summarize recent progress on chemistry and processing of organic polymers across a broad range of electrically insulating polymers, semiconducting polymers and conducting polymers for neural interfacing. We particularly focus on cutting-edge techniques for neuronal stimulation, including topographical and/or biochemical guidance, optical/electrical stimulation, and techniques for recording of neuronal responses.

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1 Introduction

Nerve regeneration involves axonal sprouting, growth, and extension, as well as reconstructing synaptic relation with target cells, and recovering nerve-guided physiological activities. One way to control the growth and differentiation of grafted neural stem cells is to use appropriate extracellular matrix materials serving as microenvironments for cell attachment and growth.^{1–6} Functional organic polymer interfaces have become a promising biotic/abiotic platform for regeneration and functional recovery of damaged nerve tissues. The substrate/neuron interfaces play an important role in neural repair by neurostimulation or neuromodulation through biochemical stimulation or implanted neural electrodes to restore the function of an injured central nervous system and maintain its balance.^{7–9} In recent years, many studies have focused on the development of organic polymers and nanostructures. The unique biocompatibility, mechanical properties, variable morphologies and biological signal-transmitting ability of these interfaces have made them indispensable implantable matrices for nerve tissue engineering.^{10,11} In this review, we summarize functional organic polymers and engineering techniques suitable for neural interfacing, including micro/nano-scale grooved surfaces,

micro/nano fibers, surface modification, and optical/electrical stimulation. Techniques that have been used for detection of neuronal responses, which are important to establish the bidirectional feedback loop with neuronal stimulation, are also summarized.

1.1 Research context of neuronal stimulation

The nervous system, including the central nervous system (CNS) and peripheral nerve system (PNS), plays an important role in a human biological process, such as cognition and individual cell function. Neuronal diseases such as stroke in the brain and injuries of the spinal cord may impose tremendous consequences, such as lifelong disability, and its recovery is difficult.¹² For the PNS, peripheral neuropathy can improve the regeneration of nerve axons and neuronal function by using different transplants.^{13–16} However, conventional autograft therapy has some serious disadvantages, such as loss of autograft function and the mismatch between autograft and recipient. And its counterpart allograft faces similar problems with disease transmission and immunogenicity. Thus, these conventional therapies are not competent to completely repair damaged nerves and function recovery due to the complexity of the nervous anatomical system.

To address these existing challenges, neural tissue engineering has rapidly emerged as a new field and has achieved lots of advances.^{7,17–20} This technology applies principles of tissue engineering to neural therapy and focuses on regulating cell behavior and tissue recovery/regeneration through biomimicking implanted materials with good biocompatibility, such as extracellular matrix (ECM) analogs.²¹ The implanted materials should

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provide a number of chemical substances (*e.g.* appropriate biochemical factors and drugs) as well as physical stimulation (such as wettability, porosity, temperature, light, and electric fields) to support the adhesion and growth of neuronal cells to promote neuron differentiation, stimulate neurite growth, enhance neuronal performance and recording, and promote transmission of neuronal signals.²² Such tissue-engineering based nerve regeneration could provide hope for the treatment of Alzheimer's disease,^{23–25} Parkinson's disease,^{26,27} and a series of other degenerative brain disorders.^{28–30}

1.2 Overview of previously developed materials/technologies for neuronal interfacing

Tissue engineering scaffolds, as potential biomaterials for neural regeneration, can be fabricated from either natural or synthetic materials or biotic/abiotic composites.^{2,31–35} Natural biomaterials include proteins and polysaccharides. Both collagen-based scaffolds and silk fibroin nanostructured scaffolds can improve cell proliferation and differentiation performance due to their high biocompatibility and outstanding integration with native issues.^{36–40} Other polysaccharides and their derivatives, such as chitosan, alginate, agarose, hyaluronic acid, dextran, chitin, cellulose and so on, have also exhibited excellent performance in neural regeneration.^{41–51} On the other hand, numerous synthetic materials and engineering strategies have been developed for the repair of nerve lesions in the past decade. To stimulate or record neurological functions, the majority of the neural scaffolds are composed of stable and corrosion-resistant metals (*e.g.* gold, platinum, iridium, platinum-iridium alloy, titanium, and stainless steel),^{52–55} inorganic semiconductors (*e.g.* silicon, germanium, gallium arsenide),^{56–61} or organic polymeric materials (*e.g.* conducting polymers), or carbon-based materials (*e.g.* carbon nanotubes, graphene hydrogels containing protein-based materials), or tissue-engineered constructs containing cells.^{62–67} These synthetic materials have been popular choices for developing next-generation neural interfaces. For instance, some metal electrodes such as Utah- and Michigan-type series have been used for clinical stimulation and recording of neurons. Nevertheless, a major limitation of these metal electrodes is that they are often invasive and that complications such as inflammatory responses could occur at the metal/tissue interfaces, mainly due to the mechanical mismatch.

Carbon nanotubes (CNTs) and carbon nanofibers (CNFs) as synthetic materials have attracted lots of interest in neural tissue engineering.^{68–70} They can contact cell membranes tightly to improve the differentiation of stem cells into favorable cell types. CNTs and CNFs can also direct neuronal growth and neurite extension due to their excellent mechanical and electrical properties, as well as special surface chemical properties. In addition, graphene has emerged as another type of two-dimensional (2D) carbon-based electrically conducting material for neural regeneration.⁷¹ However, there are still challenges in the clinical applications of CNTs, CNFs and graphenes in terms of their long-term degradability and mechanical mismatch with neuronal cells and tissues.

Until now, lots of fabrication techniques have been developed to create biomimetic scaffolds, including micro/nanomachining,^{72,73} photolithography,^{74,75} colloidal lithography,⁷⁶ chemical etching,⁷⁷ electrospinning,⁷⁸ 3D printing,^{79–82} and self-assembly.⁸³ Various interfaces of scaffolds can be precisely modified using these technologies. Synthetic biomaterials including 3D scaffolds and nerve conduits have shown significant promise in strategies for repair and regeneration in the peripheral and central nervous systems.⁸⁴

1.3 Why polymers for neuronal stimulation?

Among various functional materials, organic polymers have been widely used in nerve repair and regeneration. The rational design of organic polymer materials for neural tissue engineering offers several distinct advantages over other materials, such as mechanical flexibility and synthetic variety that enables specific tailoring of mechanical, chemical, optical, electrical, and biological properties.⁸⁵ Various interfaces of scaffolds with precise material geometries, localization of biomolecular cues, and mechanical properties could be controlled and defined. For instance, to further reduce the mechanical mismatch between the polymeric substrates and nerve tissues, the integration of functional polymers and flexible polymers (*e.g.* parylene C,⁸⁶ polydimethylsiloxane (PDMS),⁸⁷ polyimide (PI),⁸⁸ and silk fibroin⁸⁹) is a main solution to achieve enhanced mechanical flexibility for fabricating flexible neural electrodes of different shapes and sizes. For conducting polymers as neural scaffolds, there are various approaches to achieve tunable conductivity, such as secondary doping,⁹⁰ thermal treatment,⁹¹ and addition of organic solvents⁹² or surfactants.⁹³ These advances in both polymer science and engineering have enabled electrical and/or optical stimulation, as well as biochemical cues to improve the function of polymers as scaffolds for neural interfacing, stimulation and recording (Fig. 1).

Polymers as a large family can be divided into three primary groups, insulating polymers, semiconducting polymers and conducting polymers, according to their distinct electrical conductivities. A variety of insulating polymers have shown good biocompatibility, easily tailored topography, and mechanical properties in neural interfacing and engineering. In contrast to insulating polymers, the scope of the semiconducting polymers that have been used for neural interfacing remains limited (Fig. 5). A few semiconducting polymers have been used for photothermal and/or photoconductive stimulation of neurons with good spatiotemporal control. In addition, most of these reported semiconducting polymers are intrinsically hydrophobic, and thus extra interfacial modification is needed to improve the biocompatibility. Conducting polymers are attractive for the field of neuroscience due to their low electrochemical impedance, biocompatibility, and tissue-friendly properties. The conducting polymers reported so far are mainly limited to polyanilines, polythiophenes, polypyrroles, and their derivatives. Both semiconducting polymers and conducting polymers also have unique properties that arise from their nanoscale sizes, such as their large surface area, high electrical conductivity, electrochemical stability, and potential quantum confinement effects, which enables their

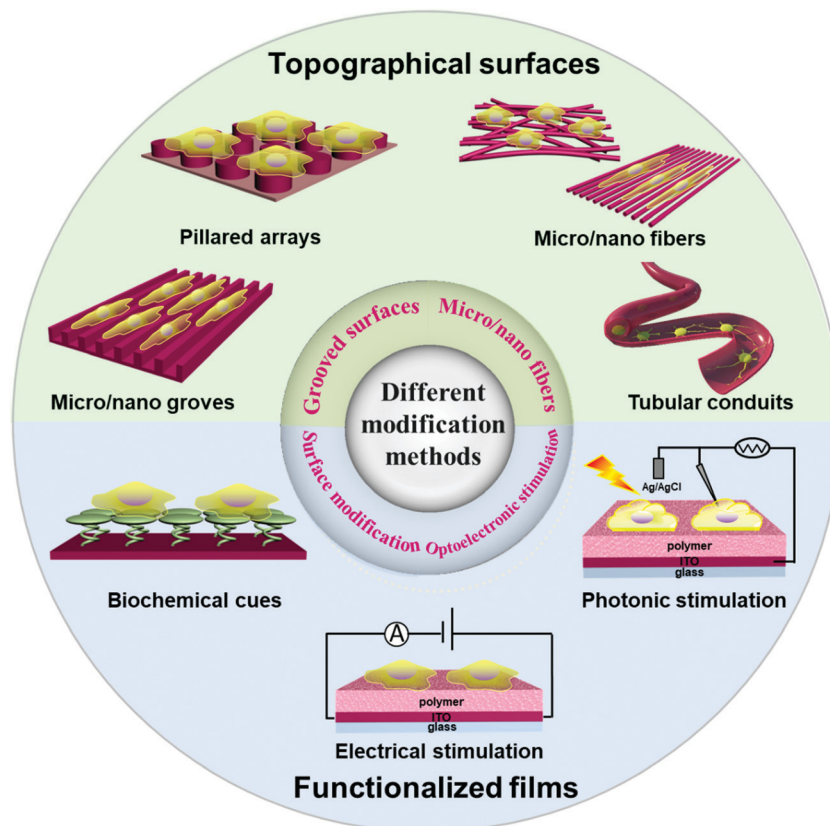


Fig. 1 Schematic illustration of polymer/neuron interfacial engineering for topographical and biochemical guidance of neural differentiation/growth, and functionalized polymer films (e.g. electrically conducting or semiconducting polymers) for spatiotemporal control of neuronal stimulation.

applications in biomedical fields including drug delivery systems, medical devices, and tissue engineering.^{94,95}

In the following sections, we describe recent advances of these three types of polymers for neural interfacing and stimulation, in the order of insulating polymers, semiconducting polymers, and conducting polymers.

2 Polymers for neuronal interfacing and stimulation

2.1 Insulating polymers for topographical and/or biochemical guidance of neurons

This section focuses on electrically insulating polymers for neuronal interfacing and stimulation. Biocompatible insulating polymers as implants are able to establish long-term intimate interaction with host neurons. Instead of giving a comprehensive review of all the polymers that have been used for neural interfacing, here we highlight several representative studies to illustrate the effects of scaffold morphology, biochemical cues and surface wettability of scaffolds on the repair and regeneration of neuronal cells.

2.1.1 Assessment of neuronal response to topographical guidance. In the past decade, various studies were reported on the fabrication of different insulating polymer substrates with topographical surface patterns to induce cellular regeneration.

Many studies confirmed the effects of extracellular scaffolding substrates with micro/nano grooved topographies, including roughness, patterned film, and distribution of pores on the surface, which can influence not only the cell attachment but also the cell morphology, viability, differentiation and proliferation rate, orientation of growth and biological activity.^{100–102} Alignment as a metric of the engineering surface is important to guide neuronal directed growth.^{7,103,104} Neurites have been quantified in many ways to show the assessment of the neuronal response to topography, including somal or nuclear orientation for alignment,¹⁰⁵ circular statistical methods for angular data of nuclei or neurites,¹⁰⁶ a Fourier-transform method for analysis of the overall alignment effect¹⁰⁷ and so on. These methods of assessing cellular and neurite outgrowth provide a quantitative manner for showing how neurons respond to topography.⁷ Many studies discussed in this section evaluate how the topography of insulating polymer substrates affects the alignment of neurons or the differentiation and extension of neurites in a desired orientation.

Electrospinning is a particularly powerful technique to produce oriented fibrous substrates with desired sizes and dimensions.^{108–111} For instance, Mahairaki *et al.* prepared poly-L-ornithine/laminin-coated polycaprolactone fiber matrices of different diameter (nanofibers and microfibers) and orientation (aligned and random) (Fig. 2a and b) by electrospinning and functionalizing fiber matrices with ECM molecules to create an



Fig. 2 (a) Schematic illustration of PCL-DPP-PCL fibers fabrication and interaction with Oligodendrocyte precursor cell (OPC). (b and c) OPC cell and PCL-DPP-PCL fiber interaction on day 3 and day 7.⁹⁷ (d) Schematic illustration of the fabrication of the micropatterned substrates. (e) Fluorescence micrographs of the protein pattern, visualized by adding a fluorescent dye (SR101) to the protein solution. Fluorescence micrographs of 2 DIV neurons on the three patterns stained with an axonal marker (tau-1; red) and a somatodendritic marker (MAP2; green).⁹⁶ (f) Schematic of a DRG seeded fiber-coverslip assembly. (g) Neurite outgrowth is observed both within and along the surface of the fiber scaffolds.⁹⁷

environment biocompatible for regulating the neuronal growth and differentiation. On the basis of their ability to sense the topography of the tissue-specific microenvironment, neural precursors (NPs) derived from human embryonic stem cells showed polarized cell morphology and the best viability with processes extending along the axis of aligned fibrous matrices. In contrast, NPs on plain tissue culture surfaces or random fiber substrates form nonpolarized neurite networks and perform a polygonal shape and grow without particular directionality (Fig. 2c and d). This demonstrates that improved fibrillar matrices of polycaprolactone have advantages in neuronal differentiation by changing migration, adhesion, proliferation, and differentiation of nerve cells.⁹⁶ Meanwhile, Corey *et al.* have proved that neurites on highly aligned fiber bundles could change direction and grow

parallel to the fiber with 16–20% increase of the neurite length.¹¹² The aligned hydrogels fabricated by microfluidic techniques can induce neuronal axons and growth along the direction of the aligned gels.¹¹³ Wang *et al.* fabricated aligned nanofiber yarn/hydrogel core-shell scaffolds for guiding 3D myoblast alignment, elongation and differentiation.¹¹⁴ Thus various aligned insulating polymer scaffolds are suitable for neuronal alignment, elongation, and differentiation.

In tissue engineering, long-term imaging and monitoring the cell-substrate interactions requires visualization and tracking of both cells and the substrate. Compared to various cellular tracking compounds, the fluorophores used for labeling scaffolds are still limited to fluorescein isothiocyanate (FITC) or rhodamine, which are often tagged to the end groups of polymers that form

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. Their easy exposure to the environment (O_2 , water, *etc.*) limits their applications in long-term tracking of cell-substrate interactions. To address these issues, Wang's group and Chew's group have collaborated in the design and synthesis of a series of highly fluorescent and photostable electro-spun polymeric nanofibers that serve as scaffolds for cell interfacing.⁹⁷ PCL-DPP-PCL, which was synthesized by covalently linking di(thiophene-2-yl)-diketopyrrolopyrrole (DPP) as a highly fluorescent chromophore into the middle of polycaprolactone (PCL) chains, was physically blended into the electrospun fibers of commercial PCL homopolymers and significantly improved the fluorescence brightness and photostability (Fig. 2a). After 15 days of differentiation, confocal laser scanning fluorescence images show that the extension of oligodendrocyte precursor cells (OPCs) clearly wrapped around the PCL-DPP-PCL fibers (as shown by the box and arrow in Fig. 2b and c), followed by the formation of myelin membrane which enclosed the fluorescent PCL-DPP-PCL fibers. Such highly fluorescent scaffolds of PCL fibers with robust photostability are important to reveal the mechanisms of the topography-guided neuron/substrate interaction at the interfaces.

Furthermore, there have been several other approaches aiming to control cellular alignment during nerve regeneration, such as hydrogels,^{115–118} copolymer brushes,¹¹⁹ fibers,¹²⁰ channels,¹²¹ groove/ridge patterned films^{122–127} and oriented protein deposition.¹¹⁶ Degradable hydrogels as high-water-content biomimetic materials can be processed into different micropatterns to guide the shape and growth direction of cell regeneration and the directional conduction of nerve signals. Hydrogel surfaces can be patterned with multiple proteins to direct cell growth and attachment.^{116,128} Yamamoto *et al.* reported a study on unidirectional signal propagation in primary neurons micropatterned at a single-cell resolution by a microcontact printing (μ CP) method to pattern a mixture of poly-lysine/extracellular matrix gel on an octadecylsilane-coated coverslip (Fig. 2d and e). Neurons were polarized in the intended orientation of the above three pattern geometries, and thus can be controlled to direct signal propagation.^{98,129}

Poly(PEGMA-*ran*-MAETAC) random copolymer brushes composed of poly(ethylene glycol) methacrylate (PEGMA) and 2-methacryloxyethyl trimethylammonium chloride (MAETAC) through surface-initiated atom transfer radical polymerization (SI-ATRP) reactions prepared by Zhou *et al.* could support multiple dendrites and long axons of hippocampal neurons.¹¹⁹ For the neuronal outgrowth on round and trilobal fibers, Chwalek *et al.* have proved that neurons growing on PLA regular round fibers exhibit improved outgrowth as compared to trilobal fibers.¹²⁰ Hinüber *et al.* developed a hierarchically structured nerve guidance conduit (NCG) by slowly resorbing poly(3-hydroxybutyric acid) (P3HB) and then it successfully supported neuron survival and neurite outgrowth.¹²¹ Wu *et al.* developed a core-shell 3D-hollow microfiber by electrospinning and proved that these hollow microfibers as controllable scaffolds

could not only guide cell adherence and direction growth but also finally form controllable neuronal networks.¹³⁰ In addition, Shahriari *et al.* developed a new technique to produce micro-channels with tunable cross sections and porosity by combining fiber drawing and salt leaching to enhance neural regeneration and nerve repair.¹³¹

In addition, axon growth can be promoted on different micro-patterned groove/ridge biomaterial scaffolds such as typical nano-/micro-grooves,^{122,126,127,132} rectangular gradient patterned grooves with different scales,¹²⁵ biodegradable materials with round contact guidance curves,¹²³ and sinusoidal grooves.¹²⁴ Anikeeva and coworkers produced a wide spectrum of polymer-based nerve guidance channels employing thermal drawing process (TDP) and analyzed the effects of channel size, geometry, and surface topography for potentially promoting nerve regeneration (Fig. 2f). These flexible, biocompatible fibrous polymer scaffolds with a variety of geometries and dimensions led to accelerated Schwann cell migration and neurite growth and alignment (Fig. 2g).⁹⁹ Thus microenvironmental cues, such as stiffness, geometry, roughness, and ligand density of the scaffold can not only affect neuronal growth and elongation of neurites, but also guide synapse formation to control the direction of signal propagation. These polymer scaffolds are promising in cell-interfacing biomedical applications.

2.1.2 Effect of multiple biomolecules on neuronal outgrowth.

In the research of interface engineering for nerve regeneration, the substrate/neuron interface can be modified with specific biochemical irritants, such as proteins and chemical cues, to improve their biocompatibility and facilitate neural cell attachment.^{134–137} The biochemical cues could be combined with topographical cues to create a synergistic condition for direction of neural cell behavior. The chemical cue provides chemical attraction or repulsion to control neuron distribution and outgrowth, while the topographic cue provides a specific interface for neuronal adhesion.^{138,139}

Studies of neural tissue regeneration during the past few decades have been facing the challenge of controlling neuronal differentiation into specific nerve cells. Nerve growth factor (NGF) has been widely employed for chemically immobilizing on the surface to facilitate cell-adhesion and guide the neuron polarity.^{134,140–142} As shown in the previous section, aligned nanofibers have the ability to significantly facilitate the differentiation of MSCs into specific cell types. Aligned NGF-conjugated nanofibrous meshes have been shown to promote the neuronal outgrowth and specific differentiation.^{143,144} Similarly, Horne *et al.* modified brain-derived neurotrophic factor (BDNF) onto 3D electrospun poly(ϵ -caprolactone) (PCL) nanofiber scaffolds by fiber alignment and aminolysation and proved that these functional polymer nanofibers could enhance proliferation of stem cells and direct the cells to differentiate into specific neuronal and oligodendrocytes.¹⁴⁵ Furthermore, Crompton *et al.* immobilized poly-D-lysine (PDL) onto chitosan by azidoaniline photocoupling to improve cell adhesion and neurite outgrowth.¹⁴⁶ Koh *et al.* blended laminin with poly(L-lactic acid) (PLLA) solution for the electrospinning procedure. Such combined chemical and topographical cues of laminin-coupled nanofibers enhance PC12 cell viability and adhesion.¹⁴⁷

Moreover, pendant acetylcholine analog is a neurotransmitter for the enhancement of neuron adhesion and outgrowth. It can promote cell viability and neurite growth in a concentration-dependent manner.¹⁴⁸ Xing *et al.* synthesized a biodegradable poly(ester-carbonate) with a pendant acetylcholine analog on which the cells exhibited the longest neurite length and the highest percentage of neurites.¹⁴⁹ Long-term culture of neuron cells is required in order to develop a platform capable of sustaining neural activity over months and overcome challenging problems such as neuron-based biosensors. Yu *et al.* used initiated chemical vapor deposition (iCVD) and synthesized a polymeric bilayer (PGD) composed of poly(glycidyl methacrylate) (PGMA) and poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) which would perform a function like an acetylcholine quaternary ammonium.¹³³ The epoxide in PGMA would interact with part of the tertiary amine moiety in the PDMAEMA layer upon iCVD, while PGMA with epoxy was deposited on a culture plate or cover glass (Fig. 3a and b). Neurons on PGD3-coated (controlling the PDMAEMA composition by depositing for 6 min) glass substrates, compared to those on PLL-coated glass, showed more dendrites and axonal projection as well as a higher level of MAP2 (a somatodendritic marker) expression. The dendrites of each neuron can extend around, forming evenly distributed neural networks (Fig. 3c–f).

2.1.3 Effect of surface properties on neuronal outgrowth.

Besides the topography and biochemical cues, physicochemical properties such as wettability of the material/neuron interface also have notable effects on cell attachment and proliferation.^{151–157} Specifically, surface wettability was studied to improve nerve regeneration in repairing damaged cells and implanting scaffold grafts. In addition, cell growth or protein fouling on gliding tissue interfaces or the scaffold surface may lead to undesirable outcomes. Stevens and coworkers produced a bifunctional scaffold

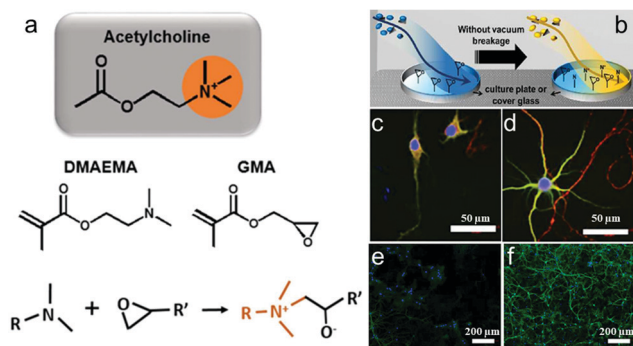


Fig. 3 Polymerization concept of a surface using initiated chemical vapor deposition (iCVD). (a) Chemical structure of acetylcholine, DMAEMA, and GMA and the conjugation reaction between the tertiary amine group in DMAEMA and epoxy group in GMA. (b) Fabricating the polymer-coated surface through sequential introduction of GMA and DMAEMA in the iCVD chamber. (c and d) Confocal microscopy images of neural growth accelerations *in vitro* for 8 days. Neurons immunostained for MAP2 (green) and Tau (red) and costained with the nuclear dye DRAQ5 (blue). (e and f) Immunocytochemical images of neural network morphologies distinguished after 80 days. Cells were immunostained for MAP2 (green) and costained with the nuclear dye DAPI (blue). Neurons grown on PLL (left) and PGD3 (right) surfaces.¹³³

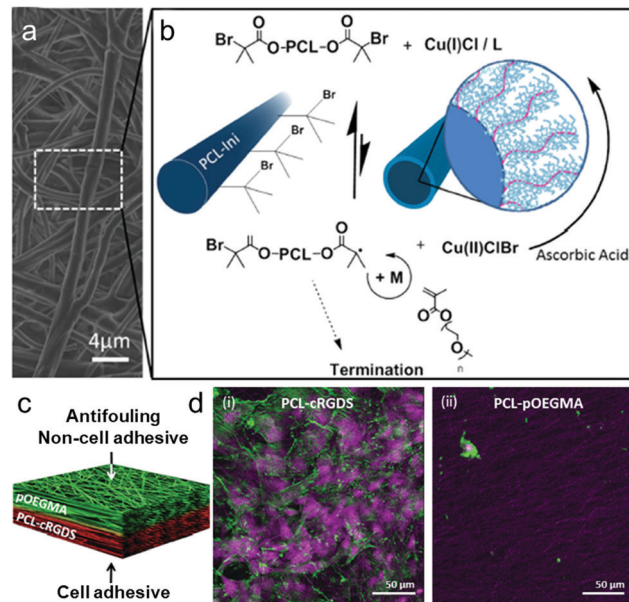


Fig. 4 Grafting of POEGMA bottlebrushes from prefunctionalized electrospun scaffolds to create an antifouling, non-cell-adhesive surface as part of a dual functional scaffold. (a) SEM micrograph of electrospun PCL-POEGMA fibres. (b) ARGET ATRP reaction scheme for polymerization of POEGMA from the PCL-Ini fibers with inset schematic images of PCL-Ini following electrospinning (left) and following polymerization of POEGMA from the fiber surface (right). (c and d) Schematic outlining and cell-adhesive/non-cell-adhesive properties of the bifunctional scaffold structure produced using layered electrospinning with postprocessing polymerization to create an antifouling PCL-POEGMA surface with an opposing cell binding PCL-cRGDS surface. Representative confocal microscopy images of bovine tenocytes cultured for 7 days on electrospun PCL-cRGDS (i) and PCL-POEGMA scaffolds (ii). Cell nuclei stained with draQ5 (purple) and actin with phalloidin (green).¹⁵⁰

structure using layered electrospinning with postprocessing polymerization to create an antifouling PCL-POEGMA surface with an opposing cell-binding PCL-cRGDS (cRGDS: a cell-binding peptide motif of cyclic Arg–Gly–Asp–Ser) surface.¹⁵⁰ Cell adhesion studies were carried out on this bilayered and dual-functionality scaffold with a cell-adhesive surface and an opposing antifouling non-cell-adhesive surface in zonally specific regions (Fig. 4).

Thian *et al.* incorporated nanohydroxyapatite (nHA) particles into the PCL matrix and prepared another typical dual-functional bilayer scaffold with improved mechanical properties *via* a combination of solvent and mechanical blending processes.¹⁵⁸ This PCL/nHA composite film could mimic the composition of tendon–bone interface tissue matrix which exhibited relatively soft tendon tissue and harder bone. These new strategies enable scaffolds that better mimic native tissue and organ structures with tailorable properties for many applications in tissue engineering and regenerative medicine.

Insulating polymeric scaffolds, owing to their superior biocompatibility, mechanical flexibility and processability, have been broadly used to promote nerve regeneration and guide the alignment of neurons or the differentiation of neurites in the desired orientation in neural interfacing. Nevertheless, it remains a challenge to understand the accurate mechanisms

of how nerve cells sense and then respond to different surfaces of insulating polymer scaffolds/substrates. Moreover, much effort needs to be devoted to the development of new techniques for the modification of functional and/or re-orienting units to produce insulating polymer scaffolds with spatiotemporal control and finely tuned functions for a wider range of neural interfacing applications.

2.2 Semiconducting polymers for photothermal or photoconductive stimulation of neurons

Organic semiconducting polymers, due to the presence of delocalized and π -conjugated electrons, are a kind of macromolecular material with optical and electrical properties of semiconductors.^{159–161} In addition, semiconducting polymers could promote biocompatibility by coupling with biomolecules through surface functionalization.^{162–165} Compared to electrically insulating polymers, semiconducting polymers show additional optical properties, photoconductivity and photothermal effect that enable spatiotemporal control in neural interfacing using light irradiation as the stimulus.¹⁶⁶ The past few decades

have witnessed the rapid advances of semiconducting polymers in their synthetic chemistry,^{167,168} processing, device fabrications,^{169–172} and biomedical applications such as fluorescence imaging^{173–175} and photodynamic or photothermal therapy.^{78,176} For instance, Chen *et al.* prepared two types of small photoblinking Pdots using highly fluorescent polymers poly[(9,9-dioctylfluorenyl-2,7-diyl)-co-(1,4-benzo-(2,1,3)-thiadiazole)] (PFBT) and poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-(1-cyanovinylene-1,4-phenylene)] (CN-PPV) to perform super-resolution fluorescence imaging of neuron cells.¹⁷⁷ But only a few semiconducting polymers have been reported so far for neural stimulation (Fig. 5). This section focuses on the recent applications of semiconducting polymers for photothermal or photoconductive activation of neurons.

2.2.1 Semiconducting polymer nanoparticles for photothermal activation of neurons.

Precise controlling of neuronal activity has profound effects on nerve repair. To that end, optogenetics has played an important role in the precise control of neuronal activity.¹⁷⁹ However, the application of current optogenetic methods is largely limited by the need of transgenesis and not responsive to the near-infrared (NIR)

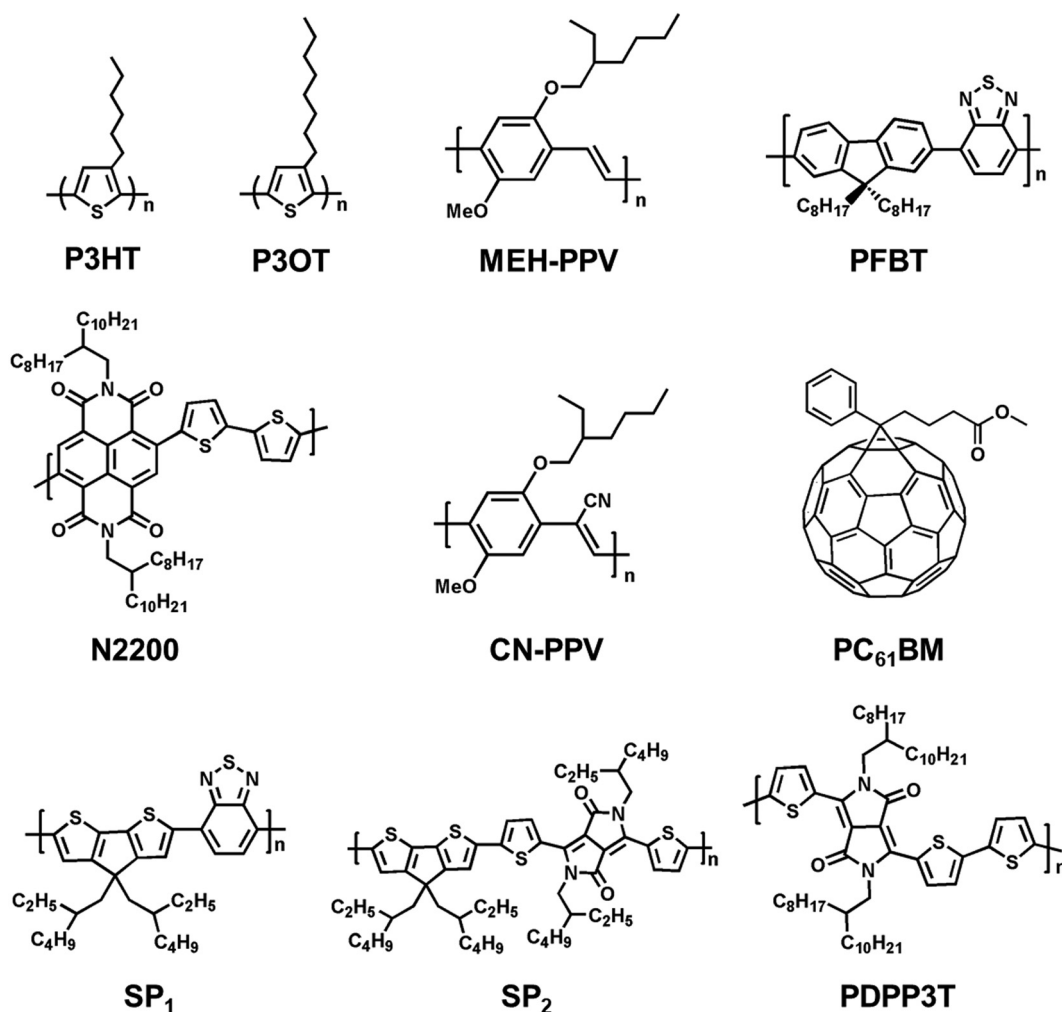


Fig. 5 Molecular structures of representative semiconducting polymers and fullerene derivatives (PC₆₁BM) for neural stimulation via photothermal (SP_{1–2}) or photoconductive effect.

region that enables deep tissue penetration. To address these challenges, for instance, Pu and coworkers reported a nano-bioconjugate of semiconducting polymer as an alternative to optogenetics.¹⁷⁸ Semiconducting polymer nanoparticles (SPNs) were synthesized by conjugating anti-TRPV1 antibody on surfaces of colloidal nanoparticles composed of narrow band gap polymers (SP₁ or SP₂, Fig. 5), which showed good biocompatibility with organisms to specifically activate the neuronal thermosensitive ion channel. The results confirmed that SPN as the photothermal nanomodulator was able to quickly increase the local temperature of TRPV1 ion channels to induce the photothermal activation of TRPV1, leading to specific Ca²⁺ influx of neuronal cells in a reversible and safe manner (Fig. 6). SPNs exhibit targeting, high photothermal conversion efficiency, and fast heating capability to reduce the probability of excessive heat to living cells.

2.2.2 Semiconducting polymer films for neuronal photo-activation. An alternative route to chemical, genetic and thermal approaches for neuron activation is the use of photosensitive extracellular electrodes. Compared to other approaches, this interfacial photoelectric activation could achieve communication between neurons and semiconducting polymers without external electric field and promote better heat dissipation.^{180,181} This facile

tool provides a functional platform for activating neuronal photo-stimulation and cell growth. Photoexcitation of these semiconducting polymers would cause significant depolarization of the neuron as well maintain the specific mechanical, chemical, and biological properties of these materials.^{181–183} Nevertheless, rational selection of semiconducting polymers for light stimulation of neurons is important for maintaining their optoelectronic properties under biological conditions.¹⁸⁴ In particular, oxygen and aqueous solutions may have a detrimental effect, such as oxidation and/or hydrolysis-induced degradation, on the involved polymers. A few examples of photovoltaic polymeric mixtures, poly(3-octylthiophene)/N2200 (P3OT/N2200),^{185,186} poly(3-hexylthiophene)/phenyl-C₆₁-butyric-acid-methyl ester (P3HT/PCBM), poly[2-methoxy-5-(2'-ethylhexyloxy)-*p*-phenylene vinylene]/C₆₁-butyric acid methyl ester (MEH-PPV/PCBM),¹⁸⁷ and near-infrared tandem organic photodiodes¹⁸⁸ have been tested and found to be stable under aqueous solution conditions.¹⁸⁰ In contrast to the poor long-term biostability of silicon-based microphotodiode arrays incubated in saline solution,¹⁸⁹ most of these semiconducting polymers (except polyfluorene derivatives such as poly[9,9-dioctylfluorenyl-2,7-diyl] (PFO)) and their composites with PCBM have shown remarkable biostability during incubation of the thin films in aqueous

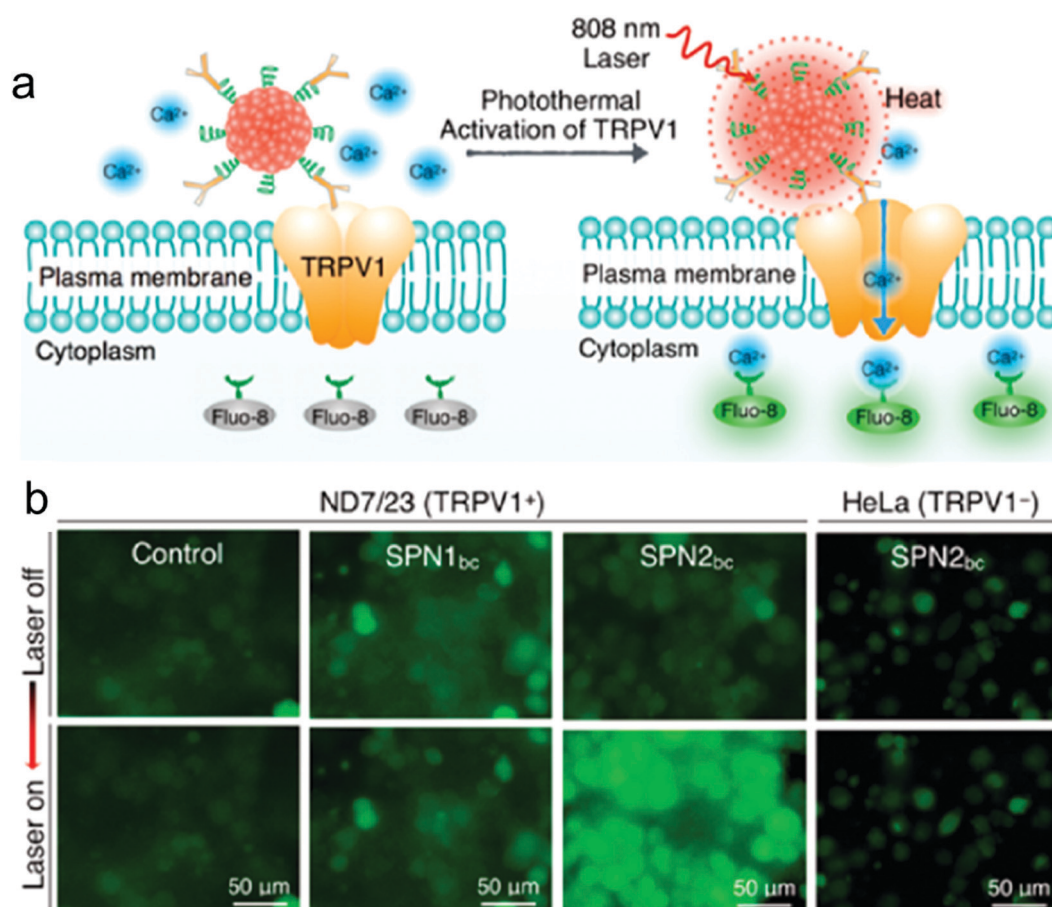


Fig. 6 NIR photothermal activation of the TRPV1 ion channels in mouse neuroblastoma/DRG neuron hybrid ND7/23 cells. (a) Schematic illustration of SPN controlled photothermal activation of Ca²⁺ channels in neurons. The intracellular concentration of Ca²⁺ was monitored in real-time by using Fluo-8 as the indicator, which turned on its fluorescence upon binding with Ca²⁺. (b) Fluorescence images of ND7/23 or HeLa treated with SPN1bc or SPN2bc before and after laser irradiation at 808 nm (104 μW μm⁻²) for 2 s.¹⁷⁸

solutions or cell-culturing media.^{190,191} Such biostability combined with mechanical flexibility and optoelectronic properties makes semiconducting polymers attractive to bioelectronics including neural interfacing.

Ghezzi *et al.* demonstrated photostimulation of neurons by a polymeric mixture-based scheme (P3HT/PCBM), as illustrated in Fig. 7. In this bulk heterojunction (BHJ) structure,^{170,192} P3HT is the electron donor, whereas PCBM is the electron acceptor. To achieve the photoactive neuronal interface, an ITO-coated glass substrate was spin-coated with the photo-voltaic BHJ layer, followed by a poly-L-lysine coating to improve cell adhesion. The patch-clamp technique was used to record the electrical activity of primary rat embryonic hippocampal neurons, cultured on the polymer coated surface. In addition, neuronal network activity was characterized by extracellular recording of primary rat embryonic hippocampal neurons, cultured on a polymer coated ITO microelectrode array.¹⁸⁰

Recently, Wu *et al.* studied photoconductive stimulation of neurons using multiscale-structured P3HT based biointerfaces (Fig. 8a). The micro/nano scale structures of P3HT used for neuronal interfacing and stimulation include self-assembled nanofibers with an average diameter of 100 nm, electrospun microfibers with an average diameter of about 1 μm , and the

lithographically patterned grooves with a width of 3, 25 and 50 μm , respectively. Compared with the P3HT homogeneous film surface, self-assembled P3HT nanofibers promoted neuronal branches and new axon growth, while neurons cultured on P3HT microfibers showed longer and thinner neurites (Fig. 8b–f).^{78,193} These results suggest that two-dimensional patterned semiconducting polymer films can be a potential candidate for high-performance and stable optoelectronic synaptic devices for the photoconductive stimulation of neurons exhibiting functionally mature neuronal phenotypes and repairing injured peripheral nerves. In addition, the scale of the extracellular two-dimensional patterned semiconducting polymer matrix also influences cellular behaviors, including migration, growth, and differentiation of the neuronal cells. But further understanding of the mechanism at the biotic/abiotic interfaces requires development of new techniques for *in situ* monitoring of the neuronal response on the patterned films of semiconducting polymers under light irradiation.

Semiconducting polymers have made certain achievements due to their good biocompatibility, excellent light-harvesting properties and the capability of converting light to thermo/electrical signals in neural interfacing. However, most of the recently reported semiconducting polymers used in neural

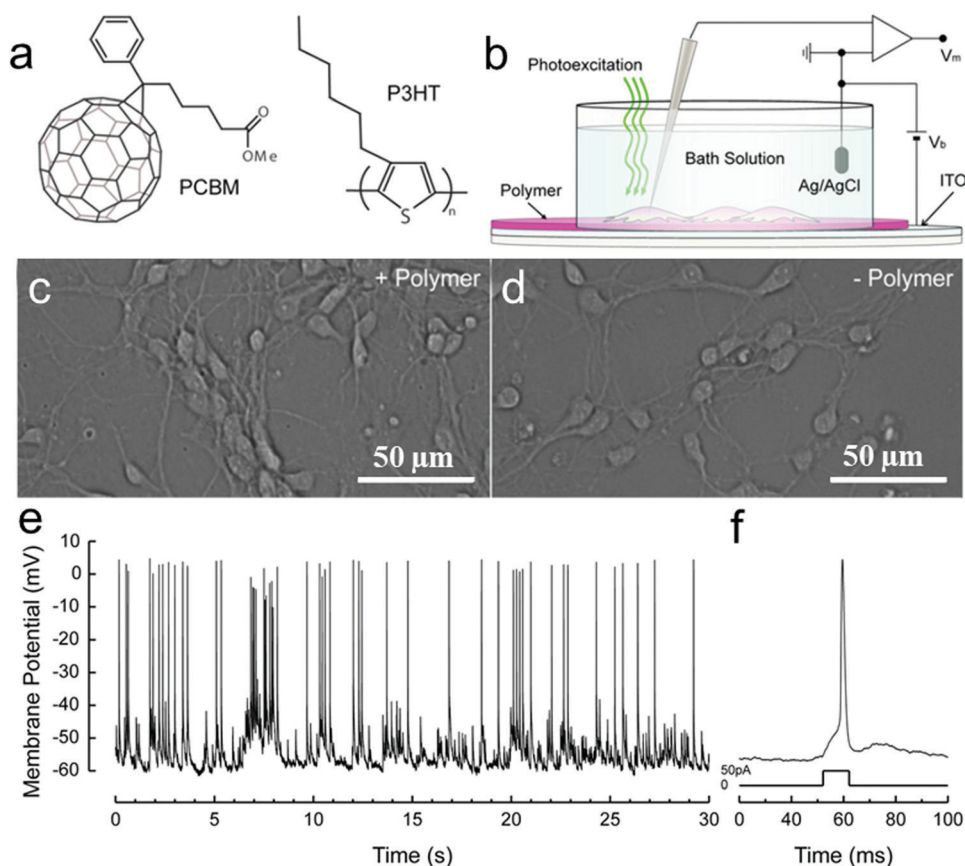


Fig. 7 Solid-liquid organic photodetector: the operation principle. (a) Chemical structures of rr-P3HT and PCBM. (b) Scheme of the photo-sensing interface, with the neuronal network grown on top of the polymer active layer during patch-clamp recordings. Hippocampal neurons cultured after 12 DIV on either poly-L-lysine treated ITO/rr-P3HT:PCBM devices (c) or control glass substrates covered only with ITO and poly-L-lysine (d). By adopting a standard patch-clamp technique, we recorded spontaneous activity (e) and action potentials induced by current injection into cultured neurons (f).¹⁸⁰

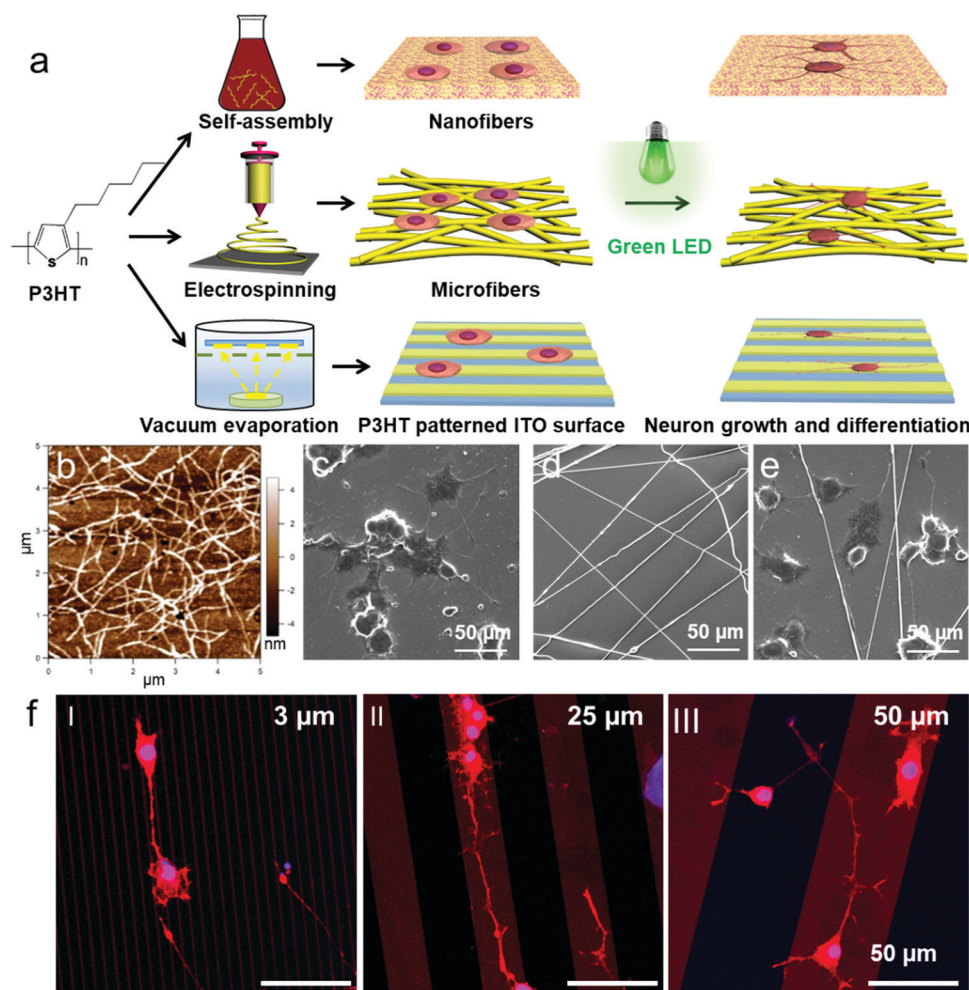


Fig. 8 (a) Schematic illustration of the preparation of P3HT micro/nanofibers and the P3HT patterned surface for PC12 cell culture. (b) AFM images of P3HT nanofibers. (c–e) SEM images of P3HT e-spun microfibers (d) and PC12 cells cultured onto the P3HT nanofiber substrate (c) and (e) P3HT microfiber substrate under LED irradiation. (f) Laser scanning confocal fluorescence microscopy images of PC12 cells cultured on ITO-coated glass surfaces patterned with 3, 25, and 50 μm wide stripes of P3HT with LED irradiation.⁷⁸

interfacing are intrinsically hydrophobic and require further surface modification to enhance the hydrophilicity and biocompatibility for interfacing with cells, tissues, and organs. The development of flexible and implantable semiconducting polymers for neural interfacing and electrophysiological application is a field that needs to be further explored. Furthermore, there is still much potential for exploring 3D semiconducting polymer scaffolds for cell culture, which remains rarely reported in neural interfacing.

2.3 Conducting polymers for electrical stimulation of neurons

The electrical stimulation on nerve cells plays an integral role in recent tissue engineering approaches, because bioelectricity in the human body is important to maintain normal biological functions such as wound healing and signaling of the nervous system.^{12,194,195} The properties of electrically conductive polymers include not only the physical and chemical properties of organic polymers but also the electrical conductivity, stable nano-topographical features, and high mechanical stability.^{196–198}

These properties of conducting polymers have been used to promote the local transmission of electrical stimulus, the growth of cells and tissue repair.^{199–205}

The early work of Langer and coworkers has proved that electrically conducting polypyrrole (PPy) could control surface charge density due to reversible electrochemistry to stimulate neurite outgrowth.²⁰⁶ Since then, a variety of other conducting polymers such as polyaniline (PANI), polythiophene (PT), poly(3,4-ethylenedioxythiophene) (PEDOT), and their derivatives and composites have been reported as substrates for cell adhesion, outgrowth, and proliferation at the polymer–tissue interface through electrical stimulation.^{72,207–212}

In this section, we review the preparation, mechanism, and neural application of conducting polymers in three groups: one dimensional (1D), two-dimensional (2D), and three-dimensional (3D) micro-/nano-structured materials. We particularly focus on the strategies used for the biocompatibility improvement of these conducting polymers and also the integrated novel technologies for fabrication of electrically

conducting scaffolds, such as electrospinning, electrochemical polymerization, and *in situ* vapor phase polymerization.

2.3.1 Micro/nanoscale fibers and tubes of conducting polymers for neural stimulation. 1D conducting polymer materials such as polymer nanofibers or nanotubes were used in neural interfacing.²¹⁵ Among them, polymeric fibers with sizes in the nanometer and micron ranges have recently become very popular materials for tissue engineering applications.²¹⁶ Specifically, 1D nanofibrous scaffolds based on conductive polymer PANI and its derivatives have shown the effect of electrical stimulation on nerve stem cells (NSCs).²¹⁷ Nanofibers of PANI blended with spinnable polymers poly(ϵ -caprolactone)/gelatin (PG) solution (PANI/PG) and prepared by electrospinning facilitated the growth of nerve cells.²¹³ The formation of the polymeric blend system is uniform and there are no beads on the nanofibers. Culturing NSCs on PANI/PG nanofibrous scaffolds with electrical stimulation led to longer neurite length and higher cell proliferation.

PEDOT as the derivative of polythiophene and another promising conducting polymer exhibits particularly low electrochemical impedance and tissue-friendly characteristics *in vivo* for implantable neural electrodes. For instance, Abidian *et al.* reported that PEDOT nanotubes promoted neurite outgrowth and dorsal root ganglion (DRG) cells had better attachment, less

branches, and longer neurites on PEDOT nanotubes.²¹⁸ However, PEDOT produced by those previous approaches could not contact with the carrier PLGA template well to obtain aligned PEDOT fibers. Thus, it is difficult to obtain completely discrete fibers. Martin *et al.* reported a facile, robust method for the fabrication of aligned PEDOT nanotubes through electrospinning and oxidative chemical polymerization (Fig. 9a).²¹⁴ This emerging approach chooses PLGA polymers loaded with EDOT monomer as reactants and FeCl_3 as an oxidative catalyst using electrospinning technology to polymerize PEDOT–PLGA fibers consisting of a PEDOT coated shell and PLGA fiber core. Next, the PLGA fiber core and residual EDOT were removed by immersing the PEDOT–PLGA fibers in chloroform, resulting in hollow PEDOT tubes. The confocal image of DRG cultured on PEDOT tubes shows that DRG sensory neurons attach to PEDOT fiber bundles and grow neurites along the long axes of the tubes (Fig. 9b). In addition, spindle-shaped Schwann cells grown out of the body of the DRGs precisely follow the alignment direction of PEDOT tubes (Fig. 9c).

Xia and coworkers prepared uniaxially aligned conductive PCL–PPy or PLA–PPy core–sheath nanofibers by electrospinning and aqueous polymerization to promote adhesion of DRG cells on core–sheath nanofibers and enhance outgrowth of neurites.^{219–221} Furthermore, the surface functionalization strategy for electrically conducting PEDOT microfibers may



Fig. 9 (a) Schematic presentation of the synthesis of PEDOT microtubes using aligned electrospun PEDOT shell/PLGA core microfibers as templates. (b and c) Confocal fluorescence images of DRG neurites cultured on the aligned electrospun PEDOT microfibers. Beta-tubulin (green), a structural component of neurons, indicates that the DRG neurites followed the direction of fiber alignment (horizontal). Schwann cells are identified by co-localization of neural marker s100 (red) and DAPI (blue).²¹³

provide a powerful tool for controlling neuronal growth and regeneration. Collazos-Castro *et al.* investigated PEDOT:PSS-co-MA-coated electroconducting microfibers which could promote proliferation and migration of CNS glial progenitor cells with electrical stimulation.²²² PEDOT is not only made into 1D microfibers in neural tissue engineering.⁶⁷ In the previous work of Lu and coworkers, it also forms an interpenetrating PEDOT/PSS-PVA/PAA network. This conductive 2D polymer hydrogel interpenetrating polymer network (IPN) film has the advantage of manufacturing a low impedance and biostable interface for implantable optical fiber-electrode arrays.²²³ Liu *et al.* synthesized electrically conductive aligned-nanofibers (PPy-G/PLGA) for optical regeneration of retinal ganglion nerve cells (RGCs).²²⁴ Using the aligned nanofibers as electrical electrodes, the stimulated RGC cell outgrowth was well guided and electrical stimulation led to 137% improvement in cell length. These results indicate that polypyrrole functionalized graphene enhanced electrical conductivity of the fiber, which significantly accelerated RGCs proliferation, differentiation, and enhanced antiaging effect of RGCs under electrical stimulation.

Further *in vivo* studies of these electrically conductive polymeric 1D micro/nanoscale fibers and tubes in animal experiments are needed in the future, in order to demonstrate the

real potential of these materials and structures for clinical applications in guided nerve repair.

2.3.2 2D films of electrically conducting polymers for neural stimulation. In neural tissue engineering, traditional bioelectronic devices such as metal-based needles would initiate adverse reactions such as implant-induced inflammation and immunogenic scarring to the human body, and these disadvantages would cause difficult interfacial communication between bioelectronic materials and neural cells.⁶² 1D fiber-like or tubular structures of conducting polymers have been effective to alleviate the above problems. However, compared to 1D micro/nanoscale fibers and tubes, 2D films of conducting polymers could provide better adhesion and wider growth space for neurons. In addition, 2D films have the advantage of mimicking the cell membrane with improved biocompatibility.²²⁶

Inspired by the characteristics of phospholipid bilayers and specific binding of cell membranes, Zhu *et al.* designed and synthesized 2D biomimetic PEDOT films composed of two different new EDOT monomers,²²⁵ zwitterionic phosphorylcholine-functionalized EDOT (EDOT-PC) which resists non-specific binding to proteins and cells, and maleimide-functionalized EDOT (EDOT-MI) for covalent binding of peptide sequences recognized by integrin (Fig. 10a). A home-designed device



Fig. 10 Electrically stimulated cell behavior on biomimetic PEDOTs. (a) Schematic representation of the approach leading to the design of the target conducting polymer, mimicking the interactions between the cell membrane and the ECM. (b) Schematic representation of the device used for cell growth with applied electrical stimulation. (c) Microscopy images of differentiated PC12 cells cultured in NGF-supplemented medium for 5 days on the biomimetic PEDOT film with applied pulsed electrical stimulation at an amplitude of 60 mV. (d) Median neurite length of PC12 cells on PEDOT and biomimetic PEDOT in the absence and presence of applied pulsed electrical stimulation at amplitudes of 20, 40 and 60 mV.²²⁵

consisting of ITO-coated glass substrate and the electrodeposited biomimetic PEDOT films was used to observe the electrically stimulated cell behavior (Fig. 10b).²²⁷ Differentiated PC12 cells in NGF-supplemented medium for 5 days showed improved adhesion and differentiation on the biomimetic PEDOT films, under applied pulsed electrical stimulation at an amplitude of 60 mV, and more than 99% of PC12 cells survived (Fig. 10c). Meanwhile, the median neurite length of PC12 cells increased obviously in this case (Fig. 10d).

The above biomimicking PEDOT films fabricated by a pair of monomers of EDOT-PC and EDOT-MI are expected to achieve higher conductivities, and efficient and long-term electrical communication at the interface with neuronal cells. The most important feature of these biomimetic 2D films is that they can resist nonspecific binding of proteins and cells to diminish immunogenic scar formation and inflammation, thereby extending the lifetime of the device. Using these materials, electrical stimulation and differentiation of neuronal cells can be enhanced efficiently, thus proving them suitable substrates for neuronal cells/tissue regeneration and recovering of damaged biofunctions.

Some other 2D PEDOT films have also been utilized to overcome neuron-functional deficits associated with neurological disease in human beings.²²⁸ For instance, Lu *et al.* utilized striped microelectrode arrays composed of alternating PEDOT and reduced graphene oxide (rGO) to fabricate two dimensional organic bioelectronic devices *via* lithography technology and the organic bioelectronic devices have been demonstrated to spatially and temporally manipulate the location and proliferation of the neuron-like PC12 cells.²²⁹ PEDOT doped with poly(styrene sulphonate) (PSS) can also be used as an organic electronic ion pump to mediate electronic control of the ion homeostasis in neurons²³⁰ and culture them to form neural networks.^{231–233} PEDOT, PPy and PANI have also been utilized as a bioelectronic interface electrode separately to provide electrical stimulation enhancing PC12 cell differentiation and neurite outgrowth.^{234–237} PEDOT has also been applied to form polymers around living neural cells to facilitate the establishment of long-term, bi-directional communication between host cells and implanted microelectrode devices.²³⁸ Povlich *et al.* synthesized carboxylic acid-functionalized EDOT (EDOTacid) monomer to be copolymerized with EDOT monomer and then bound with peptide, forming peptide functionalized PEDOT (PEDOT-PEDOTacid-peptide) films which induced 3–9 times increased adhesion of primary rat motor neurons.²³⁹

2.3.3 3D scaffolds of electrically conducting polymers for neural stimulation. Although many intricate methods have been developed to generate complex two-dimensional (2D) patterns and gradients of biochemical and mechanical cues, 2D culture conditions may not be appropriate for certain types of cells. Therefore, there is a need to create synthetic materials and 3D structures that resemble the extracellular matrix (ECM) to support three-dimensional (3D) cell culturing and tissue regeneration.^{240–243} Among them, hydrogel-based substrates or scaffolds have attracted growing interest in neuroscience, as hydrogels exhibit biocompatibility, and the mechanical

buffering ability to mitigate the inflammation at the biotic/abiotic interface caused by mechanical mismatch.^{244,245}

Soft hydrogels that can provide microenvironments akin to natural ECM have been shown to support neurite extension for cell proliferation, differentiation and axon extension.^{247,248} Although these soft hydrogels show advantages such as a similar environment for the extracellular matrix for cell regeneration under electrical stimulation, their limited mechanical strength, which may result in collapse under stress, needs to be addressed for certain biomedical applications.

3D conductive materials showed better biocompatibility, supporting ability, dynamic mechanical properties and biochemical functionalities in neural interfacing. To a certain extent, these 3D electrically conductive nanoporous materials could overcome the shortcomings above to make them promising materials for application in nerve repair. 3D functional polymeric scaffolds of novel biomaterials have been proved to strongly support cell culture and tissue regeneration. During implantation over the course of nerve regeneration, 3D nerve guidance scaffolds have a better advantage in tear-resistance, biodegradability, sterilizability, and shape retention.¹²

As a consequence, 3D cell-culturing models better resembling biological microstructures, dynamic mechanical properties, and biochemical functionalities have recently garnered lots of attention to promote cell differentiation and tissue organization.²⁴⁹ Several attempts focusing on biomacromolecules such as nanoporous cellulose gels (NCG) as a template for *in situ* polymerization are beneficial for facile fabrication and remarkable mechanical strength of conducting polymers. For instance, Shi *et al.* presented 3D electrically conductive nanoporous materials synthesized *in situ* from pyrrole vapor in an NCG matrix through oxidative initiation.²⁴⁶ With FeCl₃ oxidants, the supplied pyrrole vapors polymerized into PPy nanoparticles (Fig. 11b). The *in situ* vapor phase polymerization resulted in the NCG/PPy composite aerogels through four processes (Fig. 11a) to obtain NCG/PPy composite alcogel with different PPy contents. PPy adhered to the cellulose nanofiber surface as small particles could increase the conductivity of cellulose nanofibers and enhance cell adhesion on the PPy-coated cellulose nanofibers.²⁵⁰ Under different conditions, the composite hydrogels can still maintain superior mechanical strength (Fig. 11c–e). Xu *et al.* reported the differentiation of neural stem cells (NSCs) to neural and glial progenitors on high-strength hydrogels.²⁵¹ Comparatively, Kim *et al.* demonstrated that PEG–PEDOT:PSS treated with H₂SO₄ optimized conductive hydrogel could support cell adhesion and proliferation without compromising the electrical and physicochemical properties of the hydrogels.²⁵²

In addition, as nanostructured conducting polymers, NCG/PPy composite aerogel can also provide a suitable environment for damaged nerve cells to adhere, regenerate and recover. However, they also face the same problem of implant-induced inflammation and scar formation. When culturing on NCG/PPy composite aerogel with the surfactant dodecyl benzenesulfonic acid (DBSA) dopant, PC12 cells exhibit better attachment, more regeneration and longer neurites.²⁴⁶

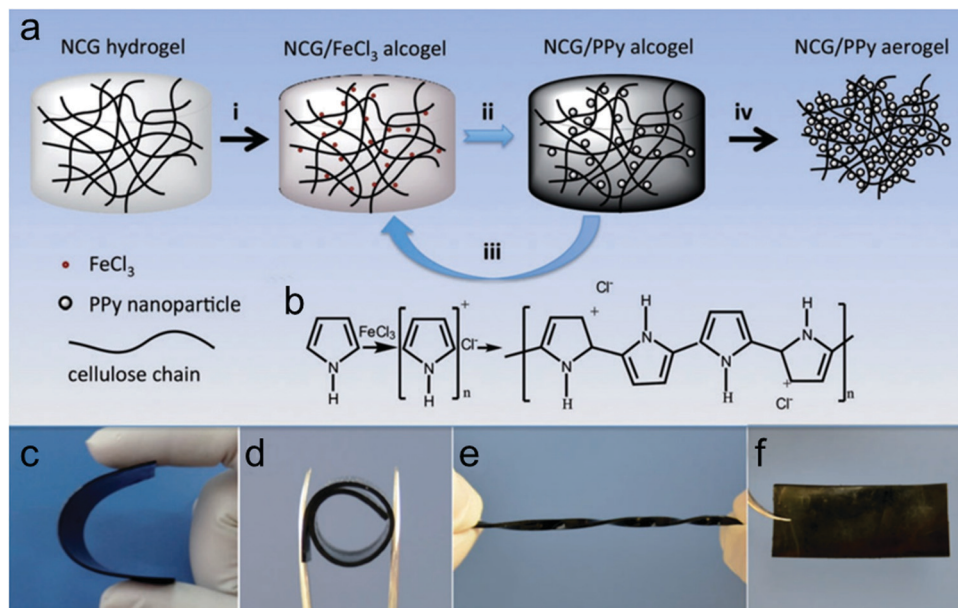


Fig. 11 (a) Preparation of NCG/PPy composite aerogels. (i) The NCG hydrogel with an interconnected nanofibrillar network is solvent exchanged with ethanol, and then impregnated with a solution of FeCl₃ in ethanol to form the NCG/FeCl₃ alcogel. (ii) PPy nanoparticles are formed by *in situ* vapor-phase polymerization of pyrrole monomers supplied as vapor, giving the NCG/PPy-1 composite alcogel. (iii) Repeating the second step two or three times gives NCG/PPy-2 and NCG/PPy-3 composite alcogels, respectively. (iv) Drying with supercritical CO₂ gives the NCG/PPy composite aerogel. (b) Schematic representation of pyrrole polymerization with FeCl₃. Macroscopic views of NCG/PPy-3 composite hydrogels under (c) bending (63 mm × 28 mm × 0.9 mm), (d) rolling (internal diameter of 10 mm), and (e) torsional loading (63 mm × 5 mm × 0.9 mm), and (f) the NCG/PPy-3 composite aerogel (63 mm × 28 mm × 0.9 mm).²⁴⁶

In summary, electrically conducting polymer scaffolds with appropriate space structures for neuronal tissue engineering can deliver local electrical signals to cells, enabling us to achieve efficient tissue regeneration and control over cell behavior. Nevertheless, the poor biocompatibility and biodegradability of pure conducting polymer scaffolds still restrict their clinical application *in vivo*. Thus, in the future design of conducting neural engineering scaffolds, bioactive materials can be introduced into the development of degradable biopolymer blends and composites for the regeneration of clinically relevant electrical-sensitive tissues. Other limitations of the electrically conducting polymers and scaffolds for neuronal stimulation, including the requirement of external power sources and often complicated and invasive wiring, remain to be addressed in future research.

3 Detection of neuronal response

How to detect neuronal response to electrical or optical stimulation or topographical feature of substrates is an important topic on nerve repair at interfaces with functional organic polymers.³¹ In the following sections, we review methods and techniques regarding detection of neuronal responses, including fluorescence microscopy to observe single neuronal cells, use of voltage-sensitive dyes to observe membrane voltage change, microelectrode arrays (MEAs), semiconducting field-effect transistors (FETs) and patch clamp chips (Fig. 12).

3.1 Fluorescence microscopy of single neurons upon stimulation

Fluorescent reporters composed of an archaerhodopsin-derived voltage sensor with fused fluorescent proteins have been widely reported for characterization of dysregulated membrane voltage and neuronal action potentials. Voltage-dependent nonradiative quenching of the fluorescent protein depends on the voltage-induced shifts in the acid-base equilibrium of the Schiff base, which means that the characteristic fluorescence could be regulated by change of the membrane voltage. Zou *et al.* presented genetically encoded fluorescent reporters with bright multicolor to detect neural function.²⁵³ Genetically encoded voltage indicators (GEVIs) of multicolor fluorescence can robustly report action potentials (APs) in cultured neurons and distinguish the activity of excitatory and inhibitory neurons through multiplex voltage imaging. Voltage can not only modulate the fluorescent brightness but also the efficiency of Förster resonance energy transfer (FRET). Although it is difficult to directly measure the voltage-dependent absorption spectra of an Arch mutant in a single cell, the fluorescence could be modulated by a voltage-dependent shift in the absorption spectrum of the rhodopsin (Fig. 13a). Citrine, mOrange2, mRuby2, ArcLight, ASAP1 and QuasAr2 eFRET GEVIs all have efficient trafficking to the plasma membrane. The fast and transient fluorescence response of all those reporters in human embryonic kidney (HEK) cells is presented in Fig. 13b. In addition, far-red fluorescence reporters can enable simultaneous, bright multicolor imaging of cell APs. For instance, QuasAr2-based eFRET

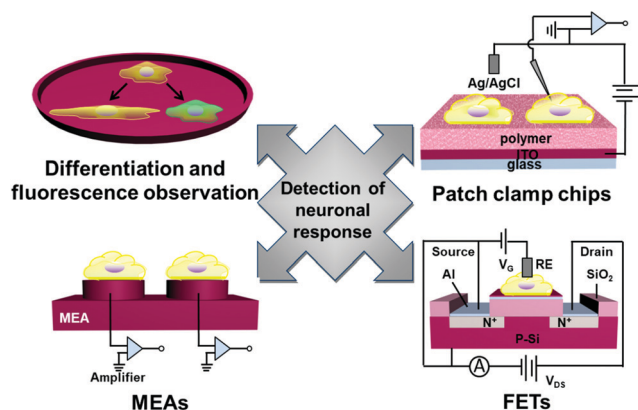


Fig. 12 Schematic summary of four kinds of detection methods for neuronal response.

GEVIs have an advantage in sensitivity, speed, and photostability at the far-red end of the spectra. However, the QuasAr2-based eFRET GEVIs have a limitation that they need intense laser illumination to efficiently detect neural function. Hochbaum *et al.* developed two archaerhodopsin-based sensitive, fast and spectrally orthogonal GEVIs which showed enhanced brightness and voltage sensitivity for genetically targeted simultaneous optical perturbation and measurement of membrane voltage.²⁵⁵

Moreover, Kralj *et al.* used a microbial rhodopsin protein, Archaeorhodopsin 3 (Arch), to detect electrically triggered APs.²⁵⁴ These microbial-based voltage indicators could achieve reliable optical detection of single APs in mammalian neurons. When modulating one cell membrane potential by whole-cell voltage clamp, it could be seen a clear image of the target cellular structure (Fig. 13c). In order to improve imaging capability of neural response activity, Chen *et al.* developed a new class of GEVIs, GCaMP6, which can detect single action potentials and orientation-tuned synaptic calcium transients in individual dendritic spines of the mouse visual cortex.²⁵⁶

Two series of fluorescence indicators above facilitate detecting single trial APs and imaging of single cells distinguished from its neighbors. These fluorescence indicators and progressive optogenetic techniques may find widespread applications in brain-related research. Nevertheless, there still remain some key challenges and limitations of all fluorescence indicators, including efficient trafficking to the membrane, sensitivity, difficulty for application *in vivo* and so on.

3.2 MEAs for detection of neural responses

Microelectrode arrays (MEAs) are widely used to record and stimulate extracellular neuronal activity and they can analyze

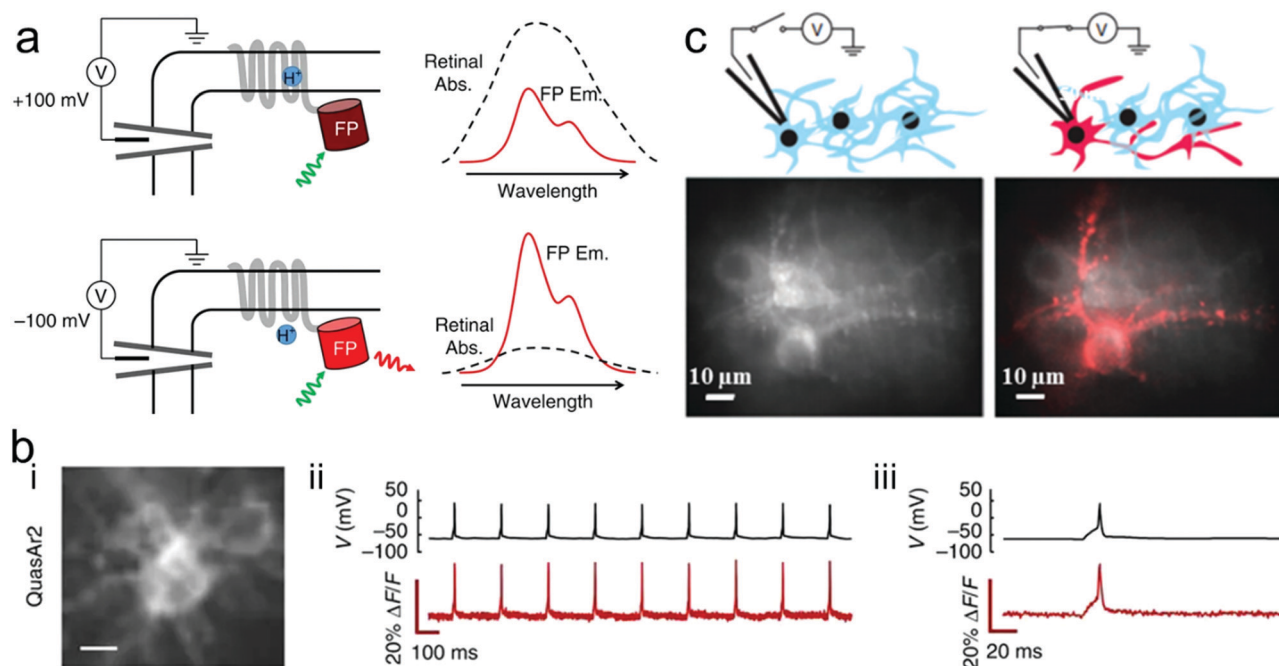


Fig. 13 (a) Proposed mechanism of voltage-dependent fluorescence in eFRET GEVIs. Voltage controls the protonation, and thereby the absorption spectrum, of the Schiff base joining the retinal to the protein scaffold. At a depolarizing (positive) voltage, the microbial rhodopsin has a protonated retinal Schiff base, which absorbs strongly with a peak near 600 nm. The retinal absorption quenches the fluorescence of the appended fluorescent protein (FP). At hyperpolarizing (negative) voltages, the fluorescence is not quenched. Spectra shown are cartoons to illustrate the mechanism. (b) Single-trial recording of neuronal APs with GEVIs. The GEVI construct was expressed in primary rat hippocampal neurons under a CaMKIIa promoter. APs were induced by current injections through a patch pipette. (i) Images of neurons expressing the indicated GEVI. (ii) Simultaneous patch clamp and fluorescence recordings of AP waveforms. Recording was acquired with laser illumination (200 W cm^{-2} for QuasAr2) at an exposure time of 1 ms. Fluorescence traces are presented without temporal filtering nor correction for photobleaching. (iii) Close-up showing single trial, unfiltered, electrically and optically recorded AP waveforms.²⁵³ (c) Application of a voltage to a single neuron caused an increase in fluorescence that distinguished a neuron from its neighbors (top). Time-average Arch fluorescence of multiple transfected neurons (left). Same field of view after membrane potential was modulated by whole-cell voltage clamp (right). Responsive pixels were identified via cross-correlation of the pixel intensity and applied voltage (V , red).²⁵⁴

information from electrogenic cells in different regions of the same tissue over a long period of time.^{258,259} This technology has been utilized to measure external field potentials generated by clumps of beating cardiomyocytes derived from human embryonic stem cells (hESCs).²⁶⁰

For instance, Dipalo *et al.* integrated 3D plasmonic nanoantennas with MEA to combine nanoscale molecular sensing with electrical neural activity recordings.²⁶¹ O'Shaughnessy *et al.* presented a portable, renewable neuron-based biosensor by culturing passaged neural stem and progenitor cells on MEAs to produce functional neuronal networks.²⁵⁷ They replaced the mammalian neurons with neural stem and progenitor cells to extend the life of neuronal networks on microelectrode arrays (MEAs).²⁵⁷ Neuroepithelial cells cultured in serum-free medium containing bFGF rapidly proliferated, differentiated into neurons and astrocytes and began to form multi-cellular clusters on MEAs (Fig. 14a and b). These results showed that a neuronal network could express spontaneous action potentials. Further tests for synaptic activity of neural progenitor-derived neuronal networks were treated with bicuculline, the NMDA (*N*-methyl-D-aspartic acid) glutamate inhibitor (APV) and the non-NMDA glutamate antagonist (CNQX), respectively. The response of neuronal networks to bicuculline increased by about 96%, while it decreased to APV and CNQX and the response to CNQX was almost zero. And the combination of bFGF and BDNF was proved to promote the generation of networks with spontaneous action potentials and more active synaptic activity (Fig. 14c). There is no doubt that neural stem and progenitor cells are suitable and renewable sources for portable biosensors, though a higher number of active channels in the neuronal networks on MEAs needs to be solved to improve biosensors.



Fig. 14 Basic activities of neuronal networks on an MEA: (a) neuroepithelial cells isolated from the embryonic cortex expand and differentiate into neurons and astrocytes on the MEA, (b) cells differentiate into neurons and astrocytes, identified by double immunostaining, and (c) typical spike trains on an electrode of the network after several treatments, *i.e.*, bicuculline, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and 2-amino-5-phosphonopentanoic acid (APV). Reprinted with permission from ref. 257.

In short, electrophysiological signals from neurons can be detected by recording electrical activity with innovative MEA bio-devices.²⁶¹ To improve the ability of MEAs of recording neuronal activity with increased sensitivity, exploring portability tools with better signal-to-noise ratio, more parallel readouts, higher spatial resolution and biocompatibility to reduce active channels in the neuronal networks remains a key issue for neuronal stimulation and recording.

3.3 FET-based neuron sensors

The rapid development of semiconductor micro- and nanotechnologies has stimulated the creation of new sensors and ion-sensitive field-effect transistors that detect both chemical and biological recognition processes, which can be widely applied in different fields including medicine, food technology, chemistry, biotechnology and so on.^{263,264} Organic field-effect transistors (OFETs) with specific surface chemistry, mechanical flexibility and high sensitivity to electrostatic potential are attractive for recording and stimulating neuronal network activity *in vitro*.²⁶² Although organic semiconductors have lower charge mobility compared with inorganic counterparts, they can also exhibit fast and stable detection when operated in direct contact with aqueous solution. Therefore, OFETs have shown some advantages compared to inorganic transistors in detecting neuronal network activity *in vitro*.^{265–267}

Cramer *et al.* presented an original water-gated organic field effect transistor (WGOFET) with pentacene as the semiconducting layer in the liquid-gated device architecture.²⁶⁸ The working principle and electrical connections with adhering cells of the liquid-gated pentacene transistor are presented in Fig. 15a. These devices, consisting of silicon oxide layer and deposition of pentacene films by high vacuum sublimation, show superior characteristics including a well-controlled morphology, biocompatibility and low-voltage operations. In the aqueous environment, they can promote adhesion and differentiation of stem cells into densely interconnected neuronal networks (Fig. 15b). Electrical stimulation verified that WGOFETs can stimulate and record extracellular signals of a population of mature neurons and the transistor is only sensitive to the



Fig. 15 Water-gated organic field effect transistors (WGOFET) neuronal transducer. (a) Schematic model of the device and interactions with adhering cells. (b) Drain current time traces recorded from a differentiated network after stimulation with various intensities. (c) Fluorescent microscopy image which indicates the formation of a functional neuronal network derived from murine stem cells on the transistor surface.²⁶²

stronger signals elicited by mature and differentiated neuronal stem cells (Fig. 15c). These WGOFETs enable stable operations in direct contact with water to stimulate, record and distinguish the differential conditions of neuronal stem cells. They are promising in monitoring the concentration of target molecules and nerve regeneration in nerve tissue engineering. Despite the advances in WGOFETs, long-term biocompatibility experiments *in vivo* have not been investigated and remain an open issue.

Electrophysiological recording of neurons and brain networks *in vivo* is critical to understand information transfer processes in neurons and the brain. However, it is difficult to extract information from noise because of the small electric potentials generated from neuronal circuits.^{270,271} While FET-based sensors could locally amplify and integrate SNR to measure signals from cell cultures, their poorly biocompatible oxide layer makes them limited to *in vitro* signal recording. The organic electrochemical transistor (OECT) was developed to directly combine the channel with electrolyte which permits ion transport between two layers.²⁷² OECTs represent a type of attractive sensor for measuring electrophysiological signals and have better cytocompatibility and integration in neural interfacing.^{273–275} Malliaras and coworkers reported that OECTs composed of the PEDOT:PSS electrodes and stereo electroencephalography and electrocorticography (ECoG) probes could record electrophysiological signals of the epileptiform brain surface.²⁶⁹ The working principle and structure of ECoG probes is presented in Fig. 16a and b. ECoG probes are composed of a thick parylene film substrate, Au and PEDOT:PSS films that are photolithographically patterned into a plate. The devices show superior characteristics including high conformability, biocompatibility and low-operation voltage. The OECT-based ECoGs have been used in the *in vivo* experiment of epileptiform activity in rats, and can promote local

amplification of signals and the recording demonstrates superior SNR of the ECoGs (Fig. 16d–g).

The use of OECTs is likely to detect and record small, local electrophysiological activities with superior SNR *in vivo*. Because of their biocompatibility and highly conformable nature, they are particularly suitable for epilepsy diagnosis, cortical mapping and so on.^{276,277}

3.4 Patch clamp chips for recording of neural responses

In recent years, electrical stimulation has been used widely for many implantable devices to help restore motor activity, treat drug-resistant disease and restore sensory perception of nerve cells. After many efforts dedicated to overcoming the challenges between electrodes and neuronal tissues, another innovative approach, neuronal photostimulation, has shown much potential in photo-physiological neural engineering. It has been shown that photoconductive-stimulation could extracellularly excite any neuron in a network and activity-dependent cellular processes can be dynamically monitored when photoconductive stimulation was used with fluorescence imaging of various molecular probes.^{31,278} To this end, the patch-clamp technique has been used to detect the effect of organic films to neurons. Whole-cell patch-clamp techniques could evoke whole-cell currents and record current amplitude, while single-cell patch-clamp techniques could accurately characterize the effect of photostimulation on membrane potential.¹⁸² The transparent organic cell-stimulating/sensing transistors (O-CST)/patch-clamp has been demonstrated to allow both extracellular current recording and optical read-out of membrane potential modulation.²⁷⁹

Action potentials of astrocyte membrane and primary neurons have been recorded in real time through patch-clamp experiments on thin films of poly(3-hexylthiophene)



Fig. 16 Working principle and structure of OECT-based ECoG probes. (a) Optical micrograph of the probe conforming onto a curvilinear surface (left). Optical micrograph of the channel of a transistor and a surface electrode (right). (b) Layout of the surface electrode. (c) Layout of the transistor channel. (d) Optical micrograph of the ECoG probe placed over the somatosensory cortex, with the craniotomy surrounded by dashed lines. (e) Wiring layout of the transistor, with the blue box indicating the brain of the animal. (f) Recording of a bicuculline-induced epileptiform spike from a transistor (pink), a PEDOT:PSS surface electrode (blue) and 12 of the 16 Ir-penetrating electrodes (black). (g) Current source density map of a bicuculline-induced epileptiform spike.²⁶⁹

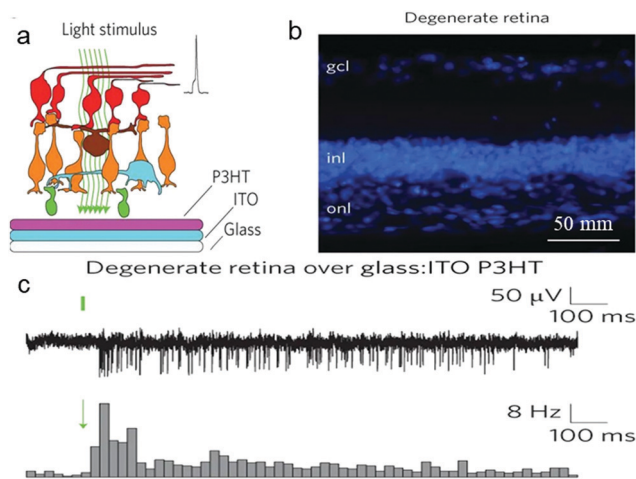


Fig. 17 Photovoltaic excitation of neurons mediated by a P3HT active layer. (a) Schematic illustration of the stimulation/recording interface for degenerate retinas. (b) Confocal images of latero-dorsal degenerate retinal sections labelled with the nuclear stain bisbenzimidazole (gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer). (c) Top row: MUAs recorded with light stimulation (10 ms, 4 mW mm^{-2}) of a degenerate retina over a P3HT-coated glass/ITO substrate. Bottom row: normalized post-stimulus time histograms (PSTHs; bin, 25 ms) computed based on all sweeps recorded in single retinas (10 ms, 4 mW mm^{-2}) for the experimental conditions. Green bars/arrows represent the light stimulus.¹⁸¹

and phenyl- C_{61} -butyric-acid-methyl ester (P3HT:PCBM)^{98,182,227} bulk heterojunctions under light irradiation. Ghezzi *et al.* recorded an active film only composed of the donor component, P3HT, to trigger neuronal firing upon illumination.^{181,280} Degenerated retinas were cultured on P3HT-coated glass/ITO under light irradiation (Fig. 17a and b). The output response of the retina to light showed that photostimulation cannot induce spiking activity in degenerated retinas on glass/ITO, but elicited intense activity in control retina on glass/ITO and degenerated retina on glass/ITO/P3HT. Meanwhile, the light-induced spiking activity of the latter two was similar to each other (Fig. 17c). These data showed that the donor component poly(3-hexylthiophene) (P3HT) under photostimulation has the potential to restore the light sensitivity of degenerated retina.

4 Perspectives and challenges

Neural tissue engineering as an emerging research field integrates neuronal cells, biomaterial scaffolds and bioactive factors into a biocompatible construct. A wide range of innovative functional organic polymers, advanced techniques and detection methods are being utilized to stimulate neural repair and regeneration. Various polymers provide an exciting option to fabricate customizable neural scaffolds with biocompatible architectures. In the future, it can be expected that more innovative polymer materials will be developed further for nanoscale manipulation of neuron behavior, and in turn create more biomimetic, biocompatible and stimuli-responsive (or adaptable) scaffolds for neural regeneration. For example, the development of conductive, ultraflexible and biodegradable polymer materials could be

utilized as the injectable electronics for minimally invasive implantation and show high performance in chronic recording, neural monitoring, and single-neuron-level brain mapping. Moreover, tailor-made organic polymer scaffolds will enable strategies to develop multifunctional composites integrating biosensors, neural prostheses, tissue engineering and drug delivery. In addition, modern polymer-based microrobots with tunable power and speed, precise navigation and operation in biological media could provide new opportunities in neural repair and regeneration.^{281,282} These emerging capabilities of polymer materials and engineering techniques offer diverse biomedical opportunities for neural engineering in the future.²⁸³

Despite advances in both polymeric materials and techniques in neural tissue engineering, many challenges still exist. The complexity of the neural system makes successful clinical neural repair and regeneration still challenging. The main issues include the design of biomimetic scaffolds that can exhibit good biocompatibility, suitable conductivity and mechanical property, excellent host-implant integration and desirable biodegradability. To achieve the goal of stable neuronal stimulation and recording, special attention needs to be paid to robust and biocompatible functional organic polymers and interface-engineering techniques to manipulate the signal transduction at the biotic/abiotic interfaces. The new development in polymer/neuron interfacial engineering plays an increasingly important role in neurological rehabilitation and brain-machine interfaces that have shown promise for the restoration of limb mobility in paralyzed patients.²⁸⁴

5 Summary

The present review summarizes the recent advances of functional polymers for neural tissue engineering. We explored from how functional organic polymers guide cell behaviors and regeneration to the development of neural-response detectors for detecting neurophysiological activities, and highlighted several emerging applications of a polymer scaffold design. The method of fabrication plays a crucial role in determining polymer/neuron interface characteristics such as surface topography or chemical cues, macro/nanostructure, and other properties such as electrical conductivity or mechanical actuation that direct specific cell responses and induce formation of natural tissue. Based on the advantages of good biocompatibility, tunable conductivity, facile synthesis, and simple modification, polymer scaffolds demonstrate their potential in neuro regenerative medicine. Thanks to the rapid development of polymer design and fabrication, novel functional polymer/neuron interfaces have achieved improved tissue responses. Further investigation on the biocompatibility and long-term effects of new polymeric scaffolds would contribute to the development of safe neural prostheses for advanced applications such as brain-machine interfacing.

Conflicts of interest

There are no conflicts to declare.

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