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研究課題名（英文）　Functionalized conducting polymer biointerfaces for cell engineering: specific adhesion of proteins and cells, cell patterning, and dynamic binding/releasing by electrical stimuli.

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研究成果の概要（和文）：

当プロジェクトにおける当初の目標を達成し、さらに導電性高分子の分子設計に基づき細胞の行動と機能をコントロールする革新的な機能性バイオインターフェイスを構築した。インターフェイスは化学的、電気的刺激に対して動的であるので、これら外部刺激が細胞に及ぼす影響も精査した。主な業績として以降のものを含む。20 種以上のチオフェン誘導体によるモノマーライブラリの構築。細胞とマテリアル間の相互作用コントロール。

研究成果の概要（英文）：

During the 3-year of this original project, we have successfully achieved all the original goals and build a new concept to utilize molecularly designed conducting polymer to control cellular behavior on the interface between cells and materials. The interface is also dynamic therefore we can control the interface by both chemical and electrical stimulation. The major achievements include: (1) Successfully build a monomer library consists of more than 20 new thiophene based monomers. (2) Control the cellular interaction with the materials with introduction of the monomers that prohibit proteins and cells binding to the materials. (3) Switch the surface properties from layered polythiophenes assemblies. (4) Cell patterning with top-down fabrication of bottom-up electropolymerization assemblies. (5) Dynamic polythiophene-cell interface with electrical stimulation. We further studied the effect of chemical and electrical stimulation on the cell engineering with these materials. The major results include: (1) Conducting polymer nano-networks for cell engineering. Thiophene monomer with a variety of functional groups would form EDOT nano-networks with the nanofiber dimensions between 20 to 40 nm by controlling the electropolymerization kinetics. (2) Enhanced cell-capturing on the nanostructured conducting polymer with capture agents. More efficient capturing of cancer cells were demonstrated on antibody conjugated polythiophene nanodots.

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2010 年度	8,700,000	2,610,000	11,310,000
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2012 年度	5,400,000	1,620,000	7,020,000
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キーワード：有機化学・導電性高分子・有機導体・バイオテクノロジー・バイオインターフェイス・ナノバイオ・ナノ材料

１．研究開始当初の背景

Biointerfaces between materials and biomolecules or cells represent the key for successful bioengineering applications, which range from drug delivery systems, tissue engineering, and artificial organ implants to sensor devices. Currently, A self-assembled monolayer (SAM) based on thiol-Au interaction is the most popular method for conductive biointerface construction because of its simplicity, chemical availability, and ability to produce thin and uniform films. However, SAM suffered from limited selection of grafted electrode surfaces, extended time for immobilization, and potential-dependent instability of thiol-Au bonding. This is why recent applications of electrically conducting polymers (ECPs) as biointerface have shown promise for nucleic acid detection, neural probe, controlled release of neuron growth factor (NGF), biological voltage-driven actuator and guided cell growth. (*Prog. Polym. Sci.* **2007**, *32*, 876-921). One of the earliest experiments demonstrated that polypyrrole (PPy), one kind of the conducting polymers, control the cell shape and function by the oxidation sate of the polymer. (*Proc. Natl. Acad. Sci. USA* **1994**, *91*, 3201-3204). More recently, several groups have demonstrated the utilization of conducting polymers as the interface on electrodes for controlled cell engineering. The applications ranging from enhancement of axon growth of neuron cell (C. E. Schmidt, USA), composite materials with neuron cells as neuron probes (D. C. Martin, USA), biomimetic calcium pumps (O. Inganäs, Sweden), multi-electrode array for cellular signaling (K. Torimitsu, NTT BRL, Japan), to biocompatible organic implants (G. G. Wallace, Australia). However, most of the research work is intiated and conducted by materail scientists and chemical engineers. Therefore, most of the research use unfunctionalized conducting polymers, including polythiophenes (PT), polypyrroles (PPy), and polyanilines (PAni) and composite materials with unfunctionalized conducting polymer as one component. There is very limited effort toward the possibility of molecular design to introduce functionality through the covalent linkage to the side chain of conducting polymers.

Comparing to traditional polymer biointerface, utilization of conducting polymer coatings for biointerface has the following advantages: (1) thin and smooth films are deposited with < 100 nm in thickness and controlled roughness; (2) interfaces are grafted with a variety of functional groups that are capable of different bioconjugation pathways (3) composition-tunable biointerfaces are prepared under uniform conditions and stable in aqueous buffer and body fluids for long period of time (>1 months); (4) these films are conductive to provide electrical stimuli. (5) Materials are controlled to deposit on selected electrode surfaces; (6) these biointerfaces are synthesized within seconds and amenable to large-scale manufacturing; and (7) they exhibit very low intrinsic cytotoxicity and display no inflammatory response upon implantation.

Overall, development of functionalized conducting polymer biointerfaces for cell engineering will provide a unique tool for bioengineering with the capability to stimulate cells electrically over an extended period of time. Fundamental understanding of the interaction between conducting polymer and cells as well as how to control the interaction is of great importance scientifically. We would like to develop all the scientific knowledge base to promote this research direction.

２．研究の目的

Build versatile thiophene monomer building blocks for controlling specific binding of proteins and cells. Two groups of new monomer building blocks will be synthesized. (1) **material modulation monomers**, particularly hydrophilic thiophene monomers (oligoethylene glycol, zwitterionic). They are used to reduce the entropy-driven nonspecific binding of biomolecules to the conducting polymer. (2) **cell-targeting monomers**. Monomers with ligand to direct specific binding to cell membrane receptors and proteins, include antigen-antibody pairing, carbohydrates, and peptides.

Cell patterning and dynamic binding/releasing by electrical stimuli. A simple and direct methodology to control the spatial relationship between cells on conducting polymers will be developed because the importance of environment to cell growth. We will create a dynamic surface to control the cellular behavior by applying electrical stimuli, a unique toolkit for study and control cell behavior.

３．研究の方法

(1) Synthesize thiophene-based monomer building blocks for specific binding of proteins and cells. In the first stage of this proposal, the

versatility of monomers will be expanded and >20 new monomers will be synthesized in order to confirm the structure to function relationship at the molecular level. These new thiophene-based monomers can be separated into two groups. Material modulation monomers are synthesized to control the surface property of the conducting polymer, particularly reduction of the undesired nonspecific binding in cell culture solution or body fluid. Many thiophene monomers with grafted hydrophilic side-chains will be synthesized and examined, mainly neutral and zwitterionic ones. Oligoethylene glycol chains with different number of repeating units have shown different level of anti-biofouling capabilities in both traditional polymer and self-assembled monolayer. It is necessary to confirm the magic number when conducting polymer biointerface is utilized. Another possible candidate is biomimicking zwitterionic side-chains, including phosphorylcholine and betaine. The degree of non-specific binding is evaluated by quartz crystal microbalance (QCM) under constant flow of protein-containing buffer solutions. The conducting polymer interface is synthesized by electropolymerization of thiophene-monomer containing solutions directly onto gold-plated quartz crystals. Both homo-polymers and copolymers from monomer solution with more than two kinds of monomers are examined and evaluated to determine the boundary condition.

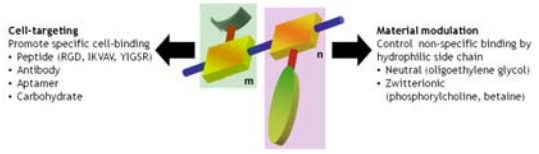

Figure 1. Molecular designs.

Cell-targeting monomers either contains functional groups (-COOH, $-N_3$, $-NH_2$, -SH) suitable for post-polymerization bioconjugation or direct ligand linkage (carbohydrates). Linker length between the ligand and the materials determine the ligand orientation and ability to enter binding domain of proteins and cell surface receptors. Therefore, it is critical to study the linker effect on conducting polymer biointerface as well. Linker with different lengths and functional connections will also be synthesized. Various types of ligand-cell binding domain interactions will be examined, including peptides, carbohydrates, antibodies, and aptamers.

(2) Cell patterning to control the spatial relationship among cells. We first use layer-by-layer electropolymerization to create surface-patterning of conducting polymer biointerfaces. One advantage of conducting polymers is their capability to cover selective conductive surfaces to create patterning. For example, patterns with and without cellular binding ligands can be created by (1) electropolymerization of anti-biofouling conducting polymer biointerface onto the surface electrode, (2) alignment of a mask with the desired areas uncovered, and (3) selective electropolymerize the second layer of cell-binding conducting polymer biointerface on top of the first layer. Utilizing this simple patterning technique, patterns with different shapes (lines, squares, circles, and cross-lines) and spacing will be created as the platform for cell culture. Subsequently, we examine the cell spatial arrangement for controlled cell growth. The controlled cell adhesion and patterning will be performed with several cell lines of interest (PC12 neuron cells, NIH3T3 fibroblast cells, MCF7 cancer cells) With the patterned surface, we have a unique tool to examine the capability of conducting polymer biointerface to control and limit cell-growth onto certain areas of the surface. With small features (<1 μm), we will examine the topological effect on cell growth, e.g. axon out growth on neuron cells (PC12 cells). When large features are constructed, this platform can be applied to study the intracellular connection between cells, e.g. neuron networks, and co-culture of multiple cell lines with each spot decorated with different specific cell-binding ligands.

(3) Dynamic binding/releasing of proteins and cells by electrical stimuli. For cell engineering, it is important not only to statically control the cell binding via ligand-binding domain interactions, but also to dynamically bind/release/trigger cells with external stimuli. These stimuli responsive interfaces are often referred as “smart surfaces”. Conducting polymers are smart materials due their capability to switch between conductive and insulating stages upon applying electricity. These two stages should behave differently in binding proteins and cell growth. Unlike other studies with unsubstituted conducting polymers. Scientific understandings of the binding will be evaluated by the introduction of specific binding side-chains and anti-biofouling. It is important to understand the boundary conditions and the deciding factors to efficiently control the binding and releasing of cells dynamically. Moreover, when cells and tissues perform in the living organism, it is often that they are responsive to a combination of environmental stimuli instead of a single event. With monomer mixtures carefully selected from the thiophene-monomer bank, it is essential to construct conducting polymer biointerfaces with different functional groups on

the surface, e.g. pH-responsive carboxylic acid group, and photo-responsive "masked" cell binding peptides, in order to build the "logic-gates". These logic-gates provide a platform to study the combo-effect of various stimuli. This will lead to a systematic understanding of the external stimuli for cell engineering, eventually resulting in successful tissue reconstruction.

４．研究成果

(1) Target-oriented and efficient synthetic approach for π-conjugated materials. Syntheses and applications of thiophene-based π-conjugated system have been widely investigated. Recently, the attention has been drawn to the efficient synthesis of these organic materials due to the necessity of environmental friendliness and step-economics of the synthetic approach. However, it is often that synthetic chemists do not realize the desired molecular architecture for functional materials and material chemists do not investigate more efficient synthetic approach. In our research, we would like to break the barrier and investigate on the synchronization for facile synthesis of target-oriented functional π-conjugated materials. Among all thiophene derivatives, 3,4-ethylenedioxythiophene (EDOT) is particularly of interest to us due to its small intrinsic band gap in polymers, low onset potential to semiconducting state, and great stability and biocompatibility in aqueous buffer solution. In order to extend the π-conjugation, traditional synthetic approaches require EDOT to be halogenated or metallated in order to proceed with cross coupling reactions. However, these substituents eventually become waste because they are eliminated in the subsequent cross coupling step. Furthermore, these metal halide byproducts usually cause environmental and health concerns. Recent advances in direct C-H arylation provide a more environmental friendly and step-economic approach to obtain π-conjugated materials without the above mentioned disadvantages. After screening the necessary parameters, optimized reaction conditions were obtained with $Pd(OAc)_2$ as catalyst, tri-*m*-tolylphosphine ($P(m\text{-}Tol)_3$) as ligand, and cesium carbonate as base in toluene. Using this recipe, extended π-conjugated molecules which targeted for various applications were synthesized in good yields.

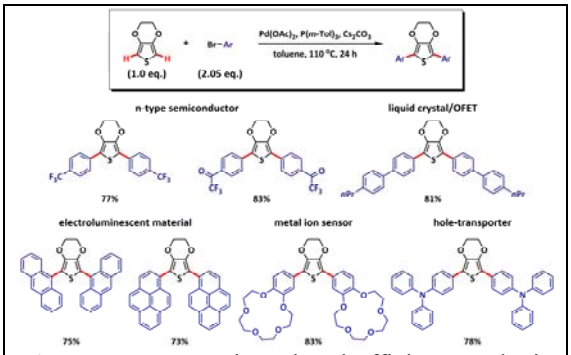

Figure 2. Target-oriented and efficient synthetic approach for functional π-conjugated molecules.

Besides small molecules, investigations on efficient syntheses of π-conjugated polymers were also carried out. There were even fewer selections regarding to the efficient syntheses of thiophene-based conjugated polymers. Oxidative coupling reactions of thiophene monomers are step-growth polymerization and usually yield polymers with broad molecular weight distribution. The advantageous features of direct C-H arylation as mentioned above prompt us to investigate more on their applications toward π-conjugated polymers. As shown in Figure 3, various substituted dioxythiophene-based conjugated polymers were synthesized by palladium catalyzed direct C-H arylation. Depending on the functional group, the molecular weight of the polymers ranged from good (M_n = 6100 ~ 9600 for alkoxy substitution) to moderate (M_n = 2000 ~ 3900 for protected –OH, -COOH, and $–NH_2$) with narrow molecular weight distribution (PDI = 1.1~ 1.2).

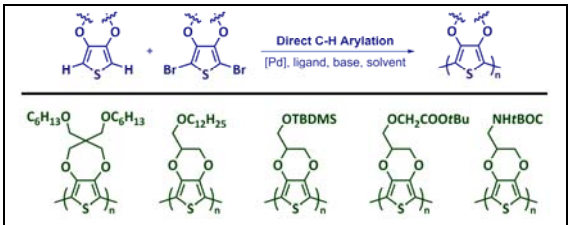

Figure 3. Direct C-H arylation to form functionalized π-conjugated polymers

(2) Understand and control the nano-assembly process of π-conjugated materials. Nanostructures of conjugated materials have become one of the most important research topics in the research because of the nanostructure's influence on the materials performance. Up to date, most research focus on the nanostructures and material properties of unfunctionalized π-conjugated materials and limited studies on nanostructures of functionalized ones. Because of the molecular advantageous features of dioxythiophenes, we are particularly interested to

develop general approaches for their polymeric nanostructures with various functional groups. We first enlarged the dioxythiophene-based monomer library with a variety of molecular building blocks. The monomers can be classified as two groups as shown in Figure 4. Material modulation monomers are synthesized to manipulate the material properties, ranging from highly hydrophobic to highly hydrophilic, and create desired assemblies. The side chains included alkyl groups, perfluorinated groups, oligoethylene glycol groups, and especially zwitterionic phosphorylcholine and betain groups. Our research represents the first example in linking these biomimetic zwitterionic side-chains in conjugated materials to create enzyme/cell resistant conductive surface. The other group of monomers is the ones provide target function. For the biomaterials research, they provide the site for bioconjugation (-COOH, -NH_2, -maleimide, -N_3). The library now consists of >50 monomers with different linkers and functional groups. Mixing the monomers from two groups, we would achieve π-conjugated materials with desired functions and material characteristics. For example, we combined the maleimide- and phophorylcholine-functionalized dioxythiophenes to construct conductive membranes. Upon bioconjugation with neuron-targeting IKVAV ligand, the membrane selective binds PC12 cells with zero binding to the control NIH3T3 cells.

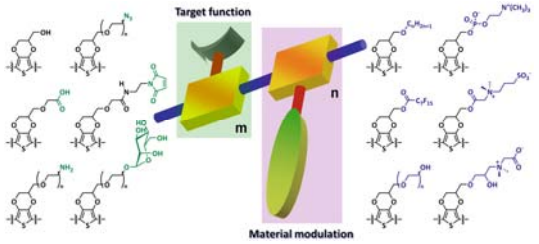

Figure 4. Schematic representation and examples of dioxythiophene monomers.

One advantageous feature of conductive materials is that the surface properties can be switched from hydrophobic to hydrophilic, cell binding to cell resistant by deposition of a new layer of conjugated polymer onto the existing surface. This feature allows us to integrate the material assembly process with "top-down" lithographic process, thereby fabricating patterned surface to constrain the cell's behavior on the electrode. For example, the axon growth of PC12 cells was limited within the cell-binding domain. The proliferation and differentiation of the cells were influenced by the topological restrictions as shown in Figure 5.

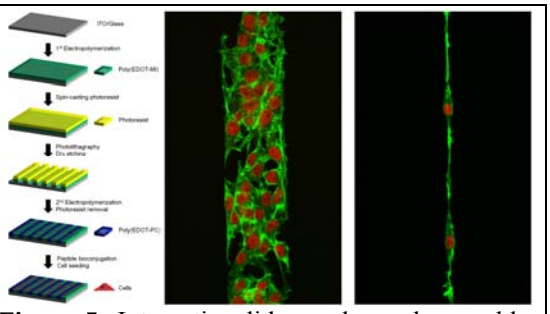

Figure 5. Integration lithography and assembly to create patterns that restrict cell behaviors.

(3) Programmable-controlled dynamic interfaces for electrically stimulating cell growth and release. We successfully demonstrated that cells can be grown on the thiophene film we created and selectively released upon electrical stimulations. The cell growth are enhanced upon the electrical pulse for 5 days and eventually, the cells are released by electrical stimulation.

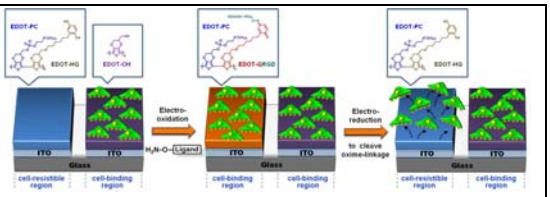

Figure 6. Schematic representation of dynamic cell release.

Overall, we have achieved all the original goal of the proposal and would like to continue exploring these materials for biological applications.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計 15 件）

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