博士論文（要約）

論文題目　Cytocompatible Phospholipid Polymeric Biomaterials for Real-time Evaluation of Cellular Functions
(細胞応答のリアルタイム計測のための細胞親和性リン脂質ポリマーバイオマテリアル)

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The living organisms consist of different types of cells, which have been proved as the basic structural, functional and biological unit of life. Along with the development of biotechnology, scientists have found that eukaryotic cells have various behaviors which construct and significantly influent normal metabolism of life. These cellular behaviors include direct mitosis, differential grown and adhesion, cell migration, cell matrix swelling and contraction, gap junction and fusion those arise between adjacent cells, and cellular apoptosis, and they are modulated not only by various stimuli from the extracellular environment but also by intrinsic cues encoded by genes, which give rise to the macromolecules that accomplish these processes and behaviors. Accurate tracking of the physical and chemical changes in the cellular environment is essential for understanding dynamic cell behaviors, which can provide insights into basic cell biology and also be applied to regenerative medicine, especially for the early detection and treatment of diseases to alleviate the symptoms of patients. The behavior of a single living cell is affected by surrounded chemical and physical stimuli molecules and parameters, *e.g.*, electrochemical stimulation, growth factor stimulation, external light irradiation, mechanical forces those function on the cell membrane, among others. Conventional investigations those focused on the exploring the mechanism of living cell behavior in a microenvironment can be classified into two types, that is, a) discontinuous evaluations, and b) unidirectional evaluations.

Cytocompatibility of the design biodevices/biomaterials is another important factor that directly influent the evaluation of cell behavior, because the living cells have to interact with those biomaterials without causing any untoward effect. Polymers those bearing zwitterionic groups, such as phosphorylcholine (PC) group, carboxybetaine (CB) group, or sulfobetaine (SB) group, have been widely used in the research of biomaterials, due to their ability to resist non-specific adsorption of proteins caused by electrostatically induced hydration. 2-Methacryloyloxyethyl phosphorylcholine (MPC) polymers contain extremely hydrophilic PC groups and have a plasma membrane-inspired structure that improve the water solubility of MPC-based polymers and inhibit nonspecific interactions with proteins, and the cytocompatibility has been proven in PC group containing polymers with diverse structures and functionalities, indicating that these polymers have significant potential for applications in polymer science, biomaterials science, and biomedical engineering. Therefore, two series of biomaterials containing PC groups were designed and prepared in this thesis to satisfy real-time monitoring of intra- and extracellular reactions, further to precisely regulate the living cell behaviors.

(a) Fabrication of a non-invasive polymer nanoprobe for real-time monitor the intracellular bioreactions/biomolecules

Monitoring the dynamic distribution of mRNA in living cells in real time could aid in the early detection of cell pathogenesis, which could lead to more accurate clinical diagnoses and effective treatments. Standard in vitro assays such as DNA microarrays, reverse transcription polymerase chain reaction (RT-PCR), and Northern blotting can quantify changes in gene expression levels of a cell

population. However, none of these methods can be applied to living cells. Cells must be lysed to extract specific macromolecules for these procedures and thus can only offer a static view of cellular events at a given moment. Therefore, to overcome the limitations of these traditional methods and enable the live imaging of mRNA in cells, we need noninvasive probes to detect mRNA with a high degree of sensitivity as well as high spatial and temporal resolution. Targeting such probes to a specific subcellular location without damaging the cell also poses a considerable challenge.

A water-soluble amphiphilic phospholipid polymer, poly(MPC-*co*-*n*-butyl methacrylate (BMA)) (PMB), was recently shown to penetrate the cell membrane by simple diffusion, and cells were stained within 1 min by PMB labeled with a specific fluorescence dye. PMB can form stable aggregates, which can act as carrier molecules in aqueous solutions at defined concentrations, demonstrating the potential for PMB to serve as a noninvasive nanoprobe in combination with different functional groups or other entities. The extremely hydrophilic phosphorylcholine groups can improve the water solubility of MPC-based polymers and inhibit nonspecific interactions with proteins, which was previously a problem when using molecular beacons (MBs) to monitor mRNA inside the cell. MBs are specially designed DNA hairpin structures that are widely used as fluorescent probes for the detection of complementary intra- and extracellular molecules (i.e., mRNA and proteins, including cytokines) in real time. Highly permeable MPC polymers conjugated with MBs could be used to rapidly label biomolecules and allow the extended monitoring of intracellular events.

The intracellular evaluation describes the synthesis of a cytocompatible, noninvasive hybrid nanoprobe composed of a PMB derivative and MB, that is, poly(MPC-*co*-BMA-*co*-*N*-succinimidyloxycarbonyl tetra(ethylene glycol) methacrylate (PENHS)) (PMBS)-MB. The PMBS, an MPC polymer with an active ester group in the side chain, was synthesized from the monomer by conventional radical polymerization using Perbutyl-ND or AIBN as an initiator. Chemical structures of the polymers were confirmed using ^{1}H NMR. The average molecular weights of the polymers were measured using a gel permeation chromatography (GPC) system in water/methanol (3:7) solution containing 10 mmol/L of lithium bromide. Poly(ethylene oxide) was used as the standard for the calibration curve. A fluorescent molecule, Lucifer Yellow Cadaverine (LYC) was conjugated to the side chain of PMBS polymers through the reaction of the *N*-hydroxysuccinimide (NHS) group of PMBS with the amine group of LYC in a sodium-borate-buffered solution (0.05 mol/L, pH 8.5) at 25 °C. Surface tension measurements indicated that the polymeric nanoprobes had different conformations in aqueous solution, specifically at a concentration of 1.0 mg/mL. The PMBS, containing the large, hydrophobic BMA, formed polymer aggregates. Four kinds of MBs were designed which are named free MBs, activatable MBs, always MBs and random MBs, respectively. Activatable MBs formed a 29-base hairpin structure, with 5' and 3' ends modified by the addition of, respectively, the fluorophore Cy3 and Black Hole Quencher-2 (BHQ-2), and was conjugated to the PMBS polymer chain, form PMBS-MB nanoprobe to specifically targeting human GAPDH mRNA molecules. The carcinoma cells used to test

the probes remained 100% viable after incubation with PMBS-MB probes. Single-stranded DNA-binding proteins (SSBs) were used to check the stability of PMBS-MB probe, followed by the evaluation of hybridization kinetics and sensitivity in the presence of GAPDH mRNA fragment. The polymeric nanoprobes demonstrated not only a high target specificity but also resistance to nonspecific adsorption of proteins compared with unconjugated MBs and were able to penetrate the cytoplasm of the cells, allowing the live imaging of mRNA. Thus, membrane-penetrating, amphiphilic, phospholipid-based polymers can be combined with nano/sub-nano-scale oligonucleotide MBs to generate highly sensitive nanoprobes that can be used to deepen our understanding of basic cellular processes and could also be applied toward the early detection, accurate clinical diagnosis, and effective treatment of diseases in the future.

(b) fabrication of a cytocompatible redox-active extracellular matrix for real-time monitor the extracellular bioreactions (redox reactions)

Bioelectricity stands for the potential and polarity changes of biological organs, tissues and cells in biological processes. It is the performance of normal physiological activity, and an essential feature of biological activity of living organism, that is always accompanied by the changes of cell membrane potential and electron transfer inside the cells. Thus living cell behavior and bioelectricity has very closely and important relationship. Hydrogel, as a three-dimensional (3D) matrix, has been intensively investigated in the biomaterials research fields. Cytocompatible polymeric hydrogels have been synthesized to culture cells and evaluate cell behaviors, such as cell adhesion, detachment, migration, proliferation and differentiation. Therefore, redox-active cellular matrix-like cytocompatible polymer hydrogel will be attractive materials for investigation of living cell responsive behavior to the bioelectrical stimulation, further to promote the fabrication of living cell-based devices, and development of electrochemical therapy.

A water-soluble and amphiphilic phospholipid polymer, poly(MPC-*co*-*n*-butyl methacrylate-*co*-*p*-vinylphenylboronic acid (VPBA)-*co*-vinylferrocene (VFc)) (PMBVF), was chose for incorporation into a hydrogel matrix that promotes encapsulation of living cells and acts as an electron transfer mediator. PMBVF was synthesized using the corresponding monomers by conventional radical polymerization with AIBN as an initiator. Chemical structures of the PMBVF were confirmed using ^{1}H NMR. The average molecular weights of the polymers were measured using a gel permeation chromatography (GPC) system in water/methanol (3:7) solution containing 10 mmol/L of lithium bromide. Poly(ethylene oxide) was used as the standard for the calibration curve. This hydrogel, PMBVF/PVA, formed spontaneously and encapsulated *Shewanella* in 3D structures, after mixing with poly(vinyl alcohol) (PVA) solutions. Visual analysis showed that the encapsulated *Shewanella* maintained viability and metabolic activity even after long-term storage. Cyclic voltammetry (CV) measurement indicated that the PMBVF/PVA hydrogel had stable and high electron transfer efficiency. Amperometric measurement showed that the hydrogel could maintain the electron transfer efficiency

even when *Shewanella* was encapsulated. Similar result on cytocompatibility was obtained while a human cervical cancer cell (HeLa) was encapsulated into this hydrogel. The PMBVF/PVA hydrogel not only provides a mild environment for long-term encapsulated cells survival but also maintains electron transfer efficiency from the cells to the electrode. Also, an electrical stimulation combined with mechanical stimulation of hydrogel to investigate the cell cycle progression (HeLa-Fucci) was performed and cell cycle was affected obviously. All of the information indicates that this PMBVF/PVA hydrogel is a successfully prepared cytocompatible redox-active hydrogel material for regulating cell behavior from extracellular environment.

In conclusion, to evaluate the behavior of living cells, two serious of cytocompatible phospholipid polymer biomaterials, 2-Methacryloyloxyethyl phosphorylcholine (MPC) polymers, were prepared successfully, that is PMBS-MB nanoprobe and PMBVF/PVA hydrogel. The noninvasive PMBS-MB nanoprobe has highly sensitivity, resistance to nonspecific adsorption of protein and cell membrane-permeability, allowing the live imaging of intracellular molecules, mRNA; the cytocompatible redox-active PMBVF/PVA hydrogel can provide mild environment for the encapsulated living cells, further study the behavior of living cells from a three-dimensional electrical stimulation and mechanical stimulations from extracellular environment. Thus, both of these two kinds of biomaterials, their derivatives and an effective combination of them together will help us precisely regulate living cell behaviors.