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Current Application of Micro/Nano-Interfaces to Stimulate and Analyze Cellular Responses

YOON-KYOUNG CHO,[1,2] HEUNGJOO SHIN,[1,2] SUNG KUK LEE,[1,3] and TAESUNG KIM[1,2]

[1]School of Nano-Biotechnology and Chemical Engineering, Ulsan National Institute of Science and Technology (UNIST), Banyeon-ri 100, Ulsan 689-798, Korea; [2]School of Mechanical and Advanced Materials Engineering, Ulsan National Institute of Science and Technology (UNIST), Banyeon-ri 100, Ulsan 689-798, Korea; and [3]School of Energy Engineering, Ulsan National Institute of Science and Technology (UNIST), Banyeon-ri 100, Ulsan 689-798, Korea

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Abstract—Microfabrication technologies have a high potential for novel approaches to access living cells at a cellular or even at a molecular level. In the course of reviewing and discussing the current application of microinterface systems including nanointerfaces to stimulate and analyze cellular responses with subcellular resolution, this article focuses on interfaces based on microfluidics, nanoparticles, and scanning electrochemical microscopy (SECM). Micro/nanointerface systems provide a novel, attractive means for cell study because they are capable of regulating and monitoring cellular signals simultaneously and repeatedly, leading us to an enhanced understanding and interpretation of cellular responses. Therefore, it is hoped that the integrated micro/nanointerfaces presented in this review will contribute to future developments of cell biology and facilitate advanced biomedical applications.

Keywords—Micro/nanointerfaces, Microfabrication, Microfluidics, Nanoparticles, Scanning electrochemical microscopy, Interfacing biology.

INTRODUCTION

Biological systems are highly complex and differ from electrical systems in the strong effect of crosstalk between signals in the cell or among cells.[48] It is often difficult or almost impossible to understand how biological systems respond to changing physical and chemical conditions. However, wide applications of micro/nanofabrication technologies which offer many advantages over the conventional instrumentations have made it possible to take novel approaches in investigating biological systems that would have been impossible in the past. Applied to building the cell culture environment in a miniaturized chip, namely microenvironment, for instance, these new technologies also have provided exquisite ways to communicate with cells (bacterial as well as mammalian) via various microscale interface systems (microinterfaces including nanointerfaces) that are capable of controlling and monitoring diffusible cell-signaling molecules with subcellular resolution in time and space to stimulate and analyze cellular responses simultaneously and repeatedly.[8,35,40]

Furthermore, current technological advances in microinterface systems have allowed scientists to examine biological systems at unprecedented levels of detail in cell biology, synthetic biology, and bacterial physiology.[8,10,58] Microscale experimental techniques have opened a path to characterize how a population of cells respond to their environment, communicate with each other, or undergo complex processes such as gene expression and gene network dynamics. These technologies have already made significant contributions not only to the study of multiple cells but also to the study of single cells. The capability of investigating individual cells in parallel and within a short period of time has led to single cell-based characterization such as heterogeneous gene and protein expressions.[32] In other words, gene expression levels in response to various stimuli have largely been inferred from observations obtained at the population level in the past. For instance, a plasmid system based on the *araBAD* promoter has been constructed by Guzman *et al.* who showed that the gene expression levels in cultures varied over an approximately 300-fold range in different concentrations of inducer, arabinose, indicating the intermediate levels of gene expression at subsaturating induction of the *araBAD* promoter.[29] One year later, it was found that the intermediate expression levels observed in culture resulted from the proportion

Address correspondence to Taesung Kim, School of Mechanical and Advanced Materials Engineering, Ulsan National Institute of Science and Technology (UNIST), Banyeon-ri 100, Ulsan 689-798, Korea. Electronic mail: tskim@unist.ac.kr

of cells that are fully induced, rather than from the intermediate expression in any individual cell.[69]

The ability to control the extracellular environment is important not only in examining intracellular signaling pathway and dynamics of gene regulation but also in testing mathematical models of gene regulation in cell biology, pathology, clinical medicine, and even in synthetic biology. In particular, microinterface technologies are at the center of a new scientific discipline called synthetic biology[8] that focuses on understanding the existing systems and on constructing and examining novel biological systems from scratch.[10,26]

Many articles have recently reviewed various microenvironments from the viewpoint of microfabrication technologies that are developed to envision *in vivo* cell culture environment by producing and detecting controllable and reproducible cues to cells.[22,57] In this review, however, our interests are focused on three latest technologies: (1) microfluidic interface systems that can impose extracellular, chemical, and mechanical stimuli onto cells; (2) nanoparticle interfaces that not only apply noninvasive, intracellular stimuli by delivering various chemical compounds into cells but also read out the cellular responses; and (3) microfabricated SECM-based interfaces that monitor electrochemical cellular responses at the molecular and cellular level in both invasive and noninvasive manners. Figure 1 shows an illustration of cyclic experiments to reveal cellular responses by using combined micro/nanointerface systems.

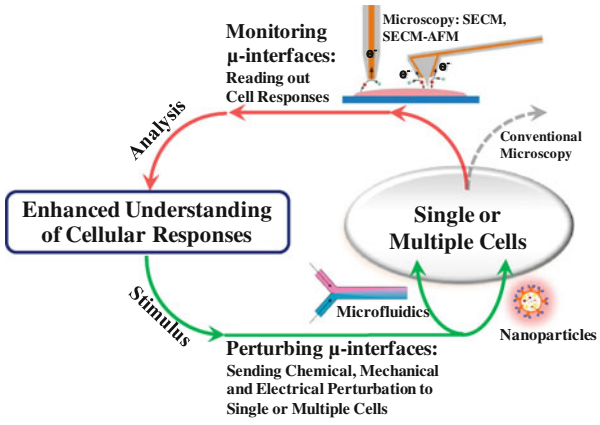

FIGURE 1. Schematic showing the various microinterface systems utilized in studying cellular responses from single and multiple cells. Microfluidics-based interfaces impose extracellular, biochemical, mechanical, and electrical stimuli onto cells; nanoparticle-based interfaces both impose and detect intracellular stimuli into cells; and SECM-based monitoring interfaces read out the responses of cells. Cyclic experiments will help develop biomedical applications.

MICROFLUIDIC BIOCHEMICAL INTERFACES: EXTRACELLULAR STIMULUS

In 2001, a subcellular gradient interface was developed using a laminar flow within a microfluidic device in which a mammalian cell was positioned and multiple laminar flows were used to produce biochemical interfaces of cell-signaling molecules.[72] This subcellular gradient interface made it possible to deliver small molecules to local regions of a single mammalian cell. In a parallel progress, many biochemical microinterfaces were presented which enabled the study of a number of cells and their interactions. One representative microinterface system utilized a Christmas tree-shaped microfluidic channel network along which soluble compounds were mixed uniformly and a linear gradient interface was generated at the downstream.[19,37] Also, linear and nonlinear gradient interfaces of interleukin-8 were produced by using a microfluidic device developed by Jeon *et al.* and used in the study of neutrophil chemotaxis.[36,37] Similar biochemical interfaces are now being used to investigate the growth and differentiation of human neural stem cells (hNSCs) under various growth factors such as epidermal (EGF), fibroblast (FGF), and platelet-derived growth factors (PDGF)[15] which are also used to observe the metastasis of human breast cancer cells.[63]

In addition to biochemical interfaces of a single chemical compound, multiple biochemical interfaces have also been developed, which consist of many chemical compounds (cell-signaling molecules). Atentia *et al.* have developed a new chemical interface/gradient generator called the "microfluidic palette" that can produce multiple spatial chemical interfaces in a chamber by using two glass substrates, a PDMS gasket and a PMMA layer, to access the chamber.[4] The device is capable of generating gradient interfaces of three different compounds simultaneously, and green fluorescence proteins (GFPs) labeled *P. aeruginosa* are observed to migrate toward the highest concentration of glucose, implying a chemotaxis behavior.

In addition to microfluidic chemical interface systems that employ diffusive mixing and convective flow, a class of devices (techniques) have recently emerged that use microscale flows to generate gradient interfaces in one layer but culture cells in another layer across a diffusible membrane so that they eliminate or reduce direct shear stresses on cells significantly.[4,14,41,74] Kim *et al.* have used a two-layered structure to separate the cell culture layer from the chemical interface layer by using polyester membranes.[41] They cultured cells on top of the membrane (upper layer) but produced sharp, movable chemical

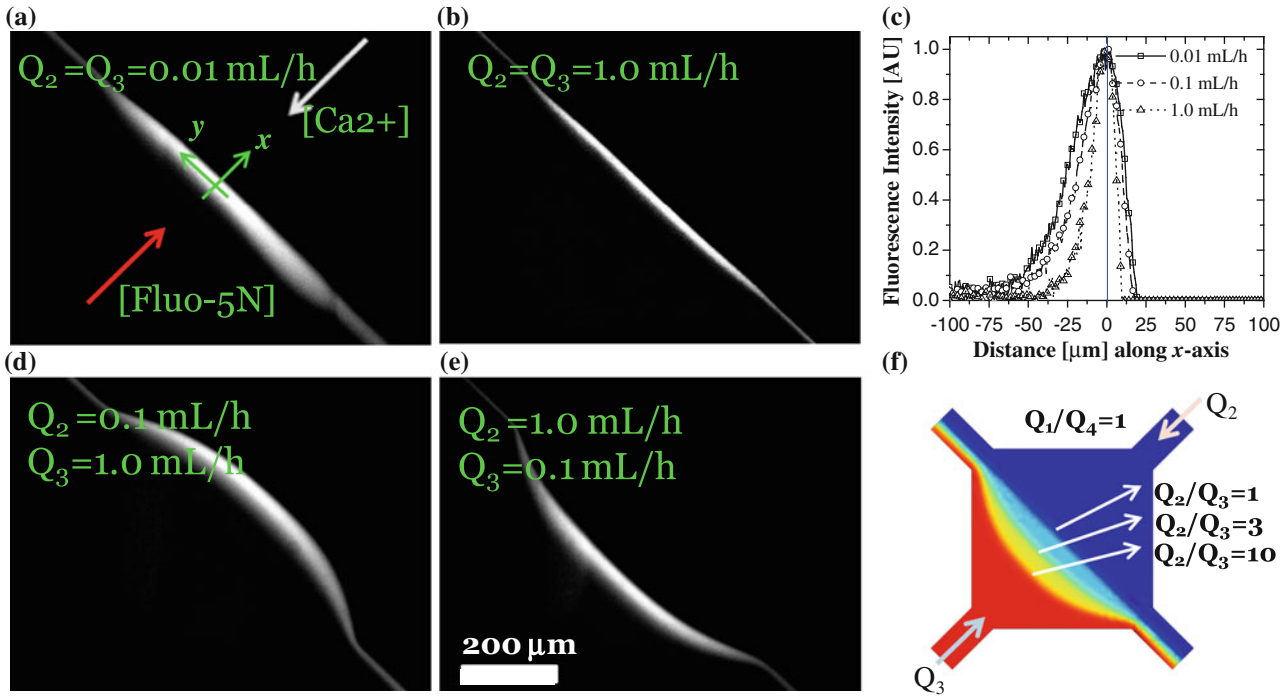

FIGURE 2. Microfluidic chemical interfaces. (a) and (b) Symmetric gradients (thick and thin, respectively) and (c) the normalized fluorescence intensities of three different flow rates. (d) and (e) Movable and asymmetric gradient interface, respectively, and (f) Simulation result of gradient profiles. This figure is reproduced from the Biomedical Microdevices 11:65–73, with due copyright permission from Springer.

interfaces in the bottom chamber by controlling the flow rates differently at two inlets. They also demonstrated that the device can control the thickness of chemical interfaces, relocate the boundary of interfaces, and produced the sharpest chemical interface to date as shown in Fig. 2.

In a similar fashion, many microfluidic biochemical interfaces/generators have been developed to control induction, proliferation, differentiation, and migration of cells.[3,28,46] In particular, Irimia *et al.* have developed a microfluidic device that enables temporal and spatial control of chemical gradients and studied the responses of neutrophil migration to dynamic changes of chemical gradients. Neutrophil migration was found to be governed by the average concentration of Interleukin-8 and the direction of its gradients.

Dynamic chemical interfaces for cell cultures are another important development in applications of microinterfaces. King *et al.* have developed a so-called "microfluidic flow-encoded switching system" that made it possible to deliver many different concentration profiles of soluble molecules onto cells by controlling a single differential pressure (flow ratio).[42] Not only have they provided a way to dynamically control a wide range of durations of exposure and pulse frequency, width and length, but they have also enabled the study of temporal aspects of multiple cellular stimuli in parallel, resulting in a significant decrease of experimental labor and time required in series experiments. It is worth noting that this method can be used to explore the dynamic effects of viscous shear stresses as well as many cell-signaling molecules that have previously been impossible in microenvironments fabricated for perfusion culture experiments.

OXYGEN GRADIENT INTERFACES

Like the soluble biomolecules in liquid, gases are also significantly important for cell-signaling biomolecules in developmental and cell biology. Oxygen, in particular, plays a crucial role in differentiating embryonic and adult stem cells and cell apoptosis.[75] The fate of stem cells, for instance, can be controlled to maintain pluripotency and reduce the possibility of spontaneous differentiation by lowering the concentration of oxygen. Thus, microscale oxygen interfaces can be a novel method of studying mechanisms associated with cell signal regulation. Park *et al.* have developed a microfluidic oxygen interface systems that can produce various profiles of oxygen gradients by using arrays of electrodes transducing current into oxygen via electrolysis.[59] They have demonstrated that C2C12 cells at oxygen-rich areas (>~25%) experience

apoptosis, but those at normal oxygen concentrations (<20%) grow normally. The boundary at which local oxygen concentrations are between 25 and 28% coincides with that of apoptosis. Furthermore, oxygen gradients on substrate can be easily controlled in time and in space because the amount of oxygen doses can be controlled by the current flowing through the electrode arrays.

TEMPERATURE GRADIENT INTERFACES

Microscale temperature gradient interface in a microfluidic device has been characterized and used to perturb a developing *Drosophila* embryo in space and time.[52,53] Using a typical Y-shaped channel,[30] a *Drosophila* embryo was immobilized at the center of the microchannel, and each half of the embryo was exposed to two fluids with warm and cool temperature to control the development of the *Drosophila* embryo because biochemical processes are sensitive to the temperature of *in vivo* and *in vitro* (micro)environments. The microfluidic device is characterized numerically and experimentally to secure a stable temperature step (interface) over the embryo, and the difference of nuclear density in the two halves of the embryo is visualized to be relatively sharp, demonstrating that intracellular cycle regulation corresponds to the extracellular temperature step that is as sharp as it was originally designed and fabricated. Of course, the warm temperature develops faster than the cool temperature, implying that temperature interfaces interfere with the rate of development. Furthermore, time-specific switch of a temperature step is used to investigate the patterning of Even-skipped[24] that normally resolves into seven stripes in a specific order. However, in the presence of temperature step (anterior half 27 °C and posterior half 20 °C) and a brief temperature reversal (anterior half 20 °C and posterior half 27 °C), for example, hunchback expression in embryos was found to be more variable than that in normal development. Hence, the temperature interface system is considered useful for regulating and understanding intracellular mechanisms as well as biochemical cell-signaling networks.

PHYSICAL CONFINEMENT INTERFACES

Miniaturized mechanical confinement is useful in trapping or immobilizing a single cell; consequently, microfluidic technologies have been widely used to build physical confinement interfaces for a single cell culture and assay. For example, a microfluidic device is employed to investigate the persistence of single cells of *E. coli* by growing cells in straight microchannels along which the cells are allowed to proliferate and differentiate.[5] Using these mechanical confinement interfaces, Balaban *et al.* have revealed that phenotypic switching takes place between cells in the absence of antibiotics (with normal growth rates) and those in the presence of antibiotics (with reduced growth rates) so that inherent heterogeneity in bacterial cell populations is related to persistence. Furthermore, microfluidic confinement of single cells is used to study the behavior of quorum sensing and growth because only microfluidic confinement can provide us with a way to control and monitor cell-signaling networks at the single cell level.[9]

Recently, migration of cancer cells constrained in straight microchannel interfaces (25 μm in width and 3 and 12 μm in height) has been investigated by Irimia and Toner.[34] They seeded breast cancer cells at one side of the microchannels and observed their motility including the moving direction and velocity. Most cells move persistently from the seeding side, but many of them reverse their moving direction at intermediate points of the microchannel and move back at the same speed. The velocity of migration increases to certain values of the cross-sectional area, and there seems to be an optimal cross-sectional area in which more cells are in contact with all the four surfaces of the microchannels rather than with two or three channel walls. Moreover, different cell lines showed different velocity along the same channels. These new technologies based on physical confinement interfaces for cell study at the single cell level enabled therapeutic studies of cancer cell metastasis and proved to be very useful tools for inducing and inactivating cell-signaling networks for more biomedical applications.

TRANSCELLULAR NANOPARTICLE INTERFACES

While microfluidic interface systems provide controlled methods to apply extracellular, chemical, and mechanical stimuli to a single or multiple cells, the engineered nanoparticles provide a new methodology to actively interface with cells, apply intracellular stimuli to the cells, and control the functions and the fate of the cells.[38] Recently, nanoparticles have been widely developed as a tool for applying intracellular stimuli and also for probing the cellular responses. Nanoparticles are usually internalized through endocytosis, and well-designed nanoparticles facilitate the transportation of active ingredients such as anticancer drugs or imaging entities which, otherwise, would not be easy to travel across the cell membrane. As schematically illustrated in Fig. 3, the multifunctional

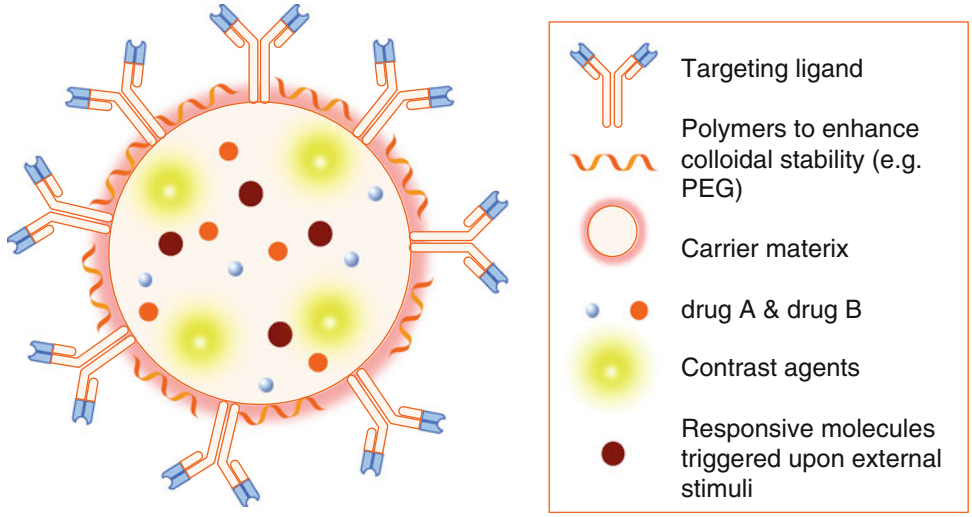

FIGURE 3. Multifunctional nanoparticles that can interface with cells to monitor cellular response *in vivo* applications. The nanoparticles can encapsulate multiple active entities such as therapeutic drugs, image contrast agents, and responsive molecules that can be activated upon external stimuli. The surfaces of nanoparticles are conjugated with active targeting moieties as well as polyethylene glycol (PEG) coatings to avoid nonspecific adsorption and cellular uptake by macrophages.

nanoparticles encapsulate imaging probes and/or anticancer drugs, and their surface can be modified with target specific ligands and coating materials to facilitate active tumor targeting and drug delivery. Since multifunctional nanoparticles and their applications in drug delivery and cell imaging have been reviewed elsewhere,[17,23,47,50] and since the effects of materials, sizes, and shapes of nanoparticles on cellular responses have been studied,[13,31,38] our review will focus on the studies that used nanoparticles as carriers to transport active ingredients and as a means to apply intracellular perturbation and investigate the induced effects on regulating cell functions and behaviors.

NANOPARTICLE-ASSISTED INTRACELLULAR STIMULUS

Cells control their functions through numerous steps of intracellular signaling pathways that are often triggered by the binding of surface receptors and the ligand molecules. Jiang *et al.* have investigated how nanoparticles modified with antibodies can regulate the specific process of membrane receptor internalization.[38] Gold nanoparticles (GNPs) of well-defined sizes ranging from 2 to 100 nm, modified with Herceptin, are employed to study the specific interactions between Herceptin-modified nanoparticles and its receptor ErbB2, a receptor tyrosine kinase overexpressed in various ovarian and breast cancers. Compared to unmodified GNPs, the Herceptin-modified GNPs showed significant enhancement of cellular uptake when incubated with ErbB2 overexpressing human breast cancer cells (SK-BR-3). It has also been observed that the binding with membrane receptors and endocytosis, and the subsequent downregulation of membrane ErbB2 expression strongly depend on the size of nanoparticles. Nanoparticles of 40 and 50 nm showed the highest effects, and almost a twofold enhancement in cell death has been reported as opposed to Herceptin treatment alone. Jiang *et al.* have well demonstrated that the engineered nanoparticles can actively induce selective membrane receptor internalization, downregulate the expression level of the receptor protein, and, therefore, control the subsequent cellular responses.[38]

Like the GNPs, quantum dots (QDs), novel semiconductor nanocrystals, are also an excellent example of nanoparticles interfacing with cells.[73] They are fluorophores with superior optical properties such as high fluorescence efficiency, minimal photobleaching, constant excitation wavelength, and tunable emission spectra.[73] QDs can be conjugated with biological moieties such as proteins, nucleic acids, and antibodies, and they can be used for *in vivo* molecular imaging. One significant advantage of QDs is the possibility of simultaneous imaging of multiple biomarkers. QD probes emitting at different wavelengths can be modified with different tumor markers and can be used for cell or tissue imaging simultaneously. This may potentially improve sensitivity and specificity significantly for cancer detection. However, the cytotoxicity of QDs, mainly attributed to oxidative degradation of heavy metal, remains a problem despite various efforts such as coating of heavy metal core with biocompatible polymers.[62]

Recently, several attempts have been made to develop less cytotoxic nanoparticles. For example, Li *et al*. have reported novel poly(DL-lactide-*co*-glycolide) (PLGA) nanoparticles loaded with conjugated polymers (CPs).[51] CPs are macromolecules with π-conjugated backbones showing high fluorescence signals. CP-loaded PLGA nanoparticles were incubated with MCF-7 breast cancer cells, and the cellular image taken by using the confocal laser scanning microscopy showed low cytotoxicity, as well as high brightness and good photostability.[51] Similarly, Altinoglu *et al*. have utilized calcium phosphate as a carrier system for near-infrared (NIFR) fluorescence imaging of breast cancer tumors.[2] Because the calcium phosphate is a natural material that can be easily found in bones and teeth, it is presumed to be nontoxic. The calcium phosphate nanoparticles (CPNP) are loaded with NIFR dyes, indocyanine green (ICG), with a diameter of 16 nm, which allows real time, noninvasive fluorescence imaging in living animals. Compared to the free fluorophore, ICG and the ICG-doped CPNPs showed a 200% greater quantum efficiency as well as a 500% longer photostability under typical clinical imaging conditions.

Typically, interactions between nanoparticles and cells are usually investigated by using light or fluorescence microscopy or by applying scanning and transmission electron microscopy. However, electrochemical detection techniques (which will be reviewed in detail later) can be also employed to pick up local interactions between nanoparticles and cells. For example, the interactions between Hela cells and silver nanoparticles are studied using a dual mediator system.[12] $Fe(CN)_6^{3-/4-}$ and $IrCl_6^{2-/3-}$ are used for the mediators which have similar hydrophilicity which dominates the permeability. The electrochemical feedback experiment of $Fe(CN)_6^{3-}$ neither showed any permeation of the mediator nor any nanoparticles through the cell membrane. $IrCl_6^{2-}$ is electrochemically reactive to silver nanoparticles on which $IrCl_6^{2-}$ is recycled to $IrCl_6^{3-}$, resulting in an enhanced oxidation current. Therefore, the effect of nanoparticles adsorbing to the cell surface can be exclusively measured using the $IrCl_6^{2-/3-}$ mediator.

NANOPARTICLE-ASSISTED READING OUT OF INTRACELLULAR RESPONSES

Nanoparticles can be utilized both in applying an external stimulus onto cells and in reading out the responses from cells. Kopelman and colleagues have developed a nano-PEBBLE (Photonic Explorer for Biomedical use with Biologically Localized Embedding) technique in which some nanoparticles can be used as sensors to detect intracellular responses or metabolites such as ions and small molecules. For example, polyacrylamide (PAA) nanoparticles have been used as good matrices for ion sensors utilizing their neutral and hydrophilic nature, while nanoparticles with hydrophobic nature are more favored for oxygen sensors.[50]

Nanoparticle sensors typically contain sensing components within chemically inert matrix. When analyte molecules permeate through the matrix and selectively interact with the sensing components (e.g., fluorescent indicator dyes), the sensor signal changes. The nanoparticles sensors containing fluorescent indicator dyes can be used to detect intracellular ions (H^+, Ca^{2+}, Mg^{2+}, K^+), radicals (OH radicals), small molecules (O_2, singlet oxygen, H_2O_2), and cellular electric fields.[50,60]

The Mg^{2+} PEBBLE sensor is a good example of nanoparticle sensors to probe intracellular response.[50,60] The conventional methods of detecting Mg^{2+} have drawbacks of poor detection selectivity between Mg^{2+} and Ca^{2+}. Coumarine 343 is a very sensitive small hydrophilic dye and has superior selectivity for Mg^{2+} over Ca^{2+}, but it cannot penetrate the cell membrane by itself. Therefore, the Mg^{2+} PEBBLE sensor is designed by encapsulating Coumarine 343 and a reference dye, Texas Red, within PAA nanoparticles. It has been demonstrated that the Mg^{2+} PEBBLE sensors reliably probe the intracellular Mg^{2+} concentrations in C6 glioma cells.[50,60] Later, nanoparticle sensors were utilized to investigate the role of ionic environment of phagosomal vacuoles in human macrophases to control phathogen invasion.[56] When the concentration of Mg^{2+} within the *Salmonella* containing vacuole was varied, the ionic concentration within the macrophases was rapidly regulated and did not affect the *phoP* activation. Noninvasive, nanosized probes to detect the ionic concentration within the cells can provide a unique methodology to investigate the underlying mechanism of intracellular activities.[56]

The nanoparticle-assisted, intracellular response-monitoring sensors have also been employed to probe the electric fields distribution within the living cells.[50,77] The technique is composed of a voltage-sensitive fluorescent dye, di-4-ANEPPS, encapsulated within silane-capped (polymerized) micelles. When introduced into astrocyte cells (DITNC) by endocytosis, it is possible to obtain an electric field profile throughout the entire cell including the membranes. These kinds of nanoparticle-assisted, intracellular response-monitoring techniques can provide a unique capability to monitor the intracellular electric fields within the entire cell and, thus, have a great potential to help expand our knowledge of the role of cellular electric field in biological processes within the cell. While the optical

detection method (e.g., confocal microscopy for cellular images) is one of the most popular methods of investigating the cellular responses on external or internal stimuli, the novel detection methods using SECM provide unique capabilities to image local electrochemical responses of cells. In the following sections, we will review a few examples of SECM-based microinterfaces for monitoring cellular responses.

SECM INTERFACES FOR ANALYZING CELLULAR RESPONSES

Ever since its invention in 1984, SECM has attracted vast research interest as a comprehensive analytical tool for living cells because of its capability to image electrochemical behaviors and cellular activities of individual cells in high temporal and spatial resolution.[6] Compared to other analytical methods such as fluorescence microscopy, confocal microscopy, and atomic force microscopy (AFM), SECM is more suitable for providing cellular biochemical information including cell responses to external stimuli, molecular transfer across cell membranes, or cell respiration along with cell morphology as reviewed by several articles reflecting this great amount of attention.[7,33,66,70,78] However, this review study deals exclusively with the recent academic achievement in the field of electrochemical characterization of single cells using SECM.

The SECM scanning modes have been used to investigate the uptake and release of chemicals through cell membranes, cellular responses to chemical stimulation or toxicity, and intracellular chemicals. A brief description is given below for the principle of SECM.

The SECM is enabled by ultramicroelectrodes (UMEs) to collect localized electrochemical data using the minimized double-layer charging effect and IR drop, fast diffusion steady-state rate, and small size (Fig. 4). The electrochemical properties of a substrate are measured by collecting redox current through the electrode surface while the probe is rastering horizontally in the vicinity of the substrate. The redox current is governed by the diffusion of the redox species onto the electrode because of the high chemical reaction rate and negligible effect of convection and migration. As the electrode approaches the substrate, the diffusion of the redox species is hindered (negative feedback mode on insulating substrates) resulting in a reduced redox current (Fig. 4b). The oxidized species at the electrode surface diffuse onto the conductive substrate and reduce back to the original redox species (Fig. 4c). This recycling process enhances the flux of the redox species and increases the redox current as the electrode approaches the conductive substrate (positive feedback mode). In the TG/SC mode, the tip generates an electrochemical reactant which diffuses onto substrate and is detected at a substrate (Fig. 4d). On the other hand, the tip collects the redox current from the redox species produced and released from the substrate such as cells (SG/TC mode, Fig. 4e). If the electrode scans the substrate with constant distance from the substrate, then the redox current indicates the electrochemical activity of the substrate (constant distance mode). With the use of redox species not permeable to and reactive with cell surfaces, the electrode can image cell topography whereas the probe scans only the x–y plane without motion in z direction (constant height mode).

SECM INTERFACES FOR CELLULAR ACTIVITY MONITORING

Gao *et al.* have used the SG/TC mode to quantify enzyme activity within a single cell.[25] The activity of peroxidase (PO) inside human neutrophils is measured by detecting benzoquinone (BQ) produced with the help of PO inside the cell.[25] First, the cell surface is

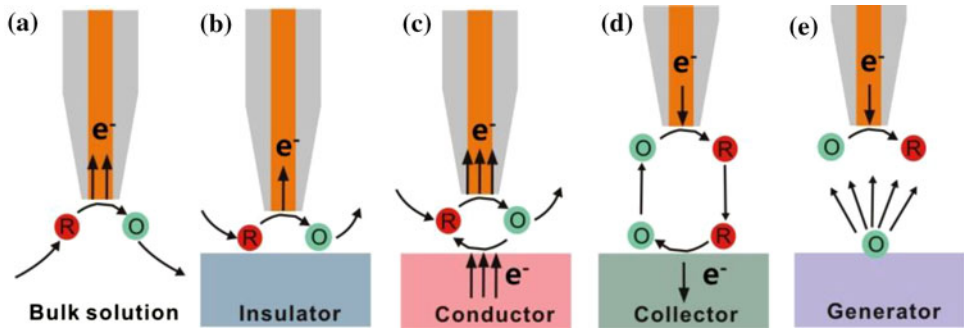

FIGURE 4. Principle of SECM; (a) hemispherical diffusion to UME, (b) negative feedback on insulating substrate, (c) positive feedback on conductive substrate, (d) TG/SC mode collecting current through substrate, (e) SG/TC mode current collection from redox species diffused from substrate.

perforated by digitonin to diffuse hydroquinone (H_2Q) and hydrogen peroxide (H_2O_2) into the cell. Then, the diffused H_2Q is converted into BQ catalyzed by PO inside the cell. BQ diffuses through the predefined micropores onto the electrode surface and is reduced at the electrode. This method introduced a new approach to quantifying intracellular chemical components inside a single cell without cytolysis. Torisawa *et al.* have utilized SECM to electrochemically monitor cellular signal transduction.[76] Secreted alkaline phosphatase (SEAP) is a good reporter protein for monitoring gene expression inside the cells because the protein is secreted into the cell culture medium, and the amount of secreted SEAP is directly proportional to the changes in the intracellular SEAP mRNA. The SEAP expression inside human breast cancer cell lines transfected with pNFκB–SEAP that had been triggered by NFκB was monitored by collecting the oxidation current of *p*-aminophenol converted from *p*-amionphenylphospate monosodium salt in the presence of SEAP secreted from the cell line. The same group used the detection method to develop an electrochemical assay using HeLa cells transfected with various plasmid vectors encoding SEAP.[67] More recently, Takahash *et al.* have measured the expression level of epidermal growth factor receptor (EGFR) at the single cell surface using SECM.[71] They collected redox current from redox species that had been converted to be electrochemically reactive by enzymes attached to the EGFR via antibodies. This method enables the prediction of cell fate by reading out the initial level of EGFR because the complex of EGF–EGFR stimulates signal transduction, resulting in many biochemical changes including gene expression. The initial stage of cell signaling can be investigated by monitoring the level of EGFR at the cell surface because EGF is a growth factor regulating the cell growth, proliferation, and differentiation by binding to EGFR.

SECM INTERFACES FOR RESPIRATORY ACTIVITY MONITORING

The measurement of oxygen consumption and production of cells is a good indicator of the viability of cells as well as their respiratory and photosynthetic activities. The difference between somatic embryo-derived and seedling-derived peanut plants in terms of stomatal physiology, chlorophyll distribution, and photosynthetic activity has been investigated by measuring the oxygen evolution near the cell membrane.[61] With this research, the SECM was proved to be a good analytical tool for investigating plant bioelectrochemistry such as cell fate, differentiation, signal transduction, and developmental biology. Zhu *et al.* have excluded the effect of the cell topography on the electrochemical detection of oxygen consumption of single neutrohphil cells by sequential scanning before/after killing the cell.[80] Under the constant height mode, the SECM collects the oxygen reduction current ($i_p = i_{p1} + i_{p2}$) which is not only governed by cell respiratory activity (i_{p1}) but also by cell topography (i_{p2}). The portion of electrochemical current affected by cell topography is compensated by the successive constant height mode scanning over the cells just after killing the cell. However, this repeated scan method cannot avoid the current variance resulting from continuous cell morphology changes while the first scan proceeds.

The cellular response to toxic molecules can be used for exploring the toxicity of specific chemicals or for developing drugs. Carano *et al.* have studied the antibacterial effect of Ag^+ on *E. coli* by monitoring the cell respiratory activity in the presence of Ag^+.[11] A Pt microelectrode encapsulated by gas-permeable polymer membrane (high density polyethylene, HDPE) inhibiting electrode fouling is used to measure the concentration of oxygen near the cell surface. Ag^+ is known to affect mammalian cells and also to inhibit bacterial and fungal growth. The uptake and efflux of Ag^+ through fibroblast cells is imaged by monitoring the local concentration of Ag^+ at the micromolar level.[79] For toxicity studies using SECM, the produced species at the electrode should not affect or harm the cell activity for high sensitivity which is facilitated by adopting a micropipette-supported ITIES (interface between two immiscible electrolyte solutions) tip as the SECM electrode. Through water/1,2-dichloroethane (DCE), the interface charge is transferred; consequently, additive oxygen reduction does not occur in the water side.

A combination of the fast-scan cyclic voltammetry (FSCV) and SECM scanning method has been used for quantitative and selective detection of oxygen and hydrogen peroxide burst from zymosan-stimulated macrophage cell.[64] As the electrode scans over single live cells, the cyclic voltammograms are collected and analyzed to characterize the kind and concentration of target chemicals at each data acquisition point. FSCV combines the characteristics of amperometry and voltammetry; the former quantifies and the latter distinguishes the chemicals of interest. Another advantage of FSCV is the negligible feedback effects of the electrode to substrate distance on the electrochemical current. By selecting the optimal scan rate and the electrode–substrate distances, the electrochemical current is influenced only by the concentration in the vicinity of the electrode surface because the diffusion layer is very limited to the electrode surface in FSCV.[65]

SECM INTERFACES FOR TRANSCELLULAR CHEMICAL MEASUREMENTS

In a manner similar to measuring the oxygen depletion near the cell surface, other biologically important transcellular chemicals can be detected by SECM. Glucose uptake and lactate production profiles above single cancer cells are measured by integrating the glucose oxidase and lactate oxidase immobilized on the electrode surface using electropolymerization.[16] The target molecules diffuse into the polymer layer where oxygen converts to hydrogen peroxide catalyzed by corresponding enzymes. The electrode collects the oxidation current of the produced hydrogen peroxide. This detection scheme can be applied to biosensing a variety of chemicals which have corresponding enzymes.

In order to understand cellular activities, one of the main properties of interest is the volume changes of the cell. SECM is an excellent tool for imaging the morphology of cells while providing electrochemical data near the cell without a mechanical contact to the cell surface unlike atomic force microscopy. However, to facilitate the dual functions of SECM, the measurement should be conducted in the constant distance mode which needs an extra scanning mode including the constant current mode,[49] shear force mode,[39] or two-electrode mode.[45] All these methods lack the capability of providing an absolute electrode to the substrate distance and need redox species which can affect the electrochemical detection of chemicals of interest. Diakowski and Ding have applied AC–SECM (alternating current—scanning electrochemical microscope) to image the topography of the cell without using redox mediators by measuring the impedance changes as the electrode scans over African green monkey kidney cells.[20] Under AC potential, the collected current and the phase changed in accordance with the cell height changes in the constant height mode. And the impedance also reflects the cellular activity changes in the presence of stimulus (phorbol-1,2-myristate-acetate-3) that stimulate cellular oxidative metabolism and cause respiratory bursts.

APPLICATION OF SECM–AFM INTERFACES TO SINGLE CELLS

Another strategy for enhancing the spatial resolution of scanning is combining SECM and AFM. The main strength of AFM is its high repositioning capability and high spatial resolution with smaller electrode sizes. The SECM–AFM can be classified into two types based on the electrode position at the AFM tip. The first type of SECM–AFM is integrated with UME at the apex of a tip.[1,21,27,55] Conventionally, AFM scans the substrate in contact, thus an electrical short circuit can occur during the conductive sample scanning using this type of SECM–AFM. In order to avoid this short circuit and facilitate a constant distance mode, a sequential surface scanning mode is applied in which the first scan records the substrate topography and the successive scans collect the electrochemical data at a fixed distance.[54] It is difficult to apply this type of SECM–AFM to imaging cell surface because of the temporal variance in cellular morphology. The second type of SECM–AFM images substrate topography using a tip apex while electrochemical data is collected through a ring microelectrode located at a fixed distance from the tip apex.[18,43,68] Kueng *et al.* have shown the feasibility of the SECM–AFM as a scanning tool for detecting glucose emitted from a live cell by immobilizing glucose oxidase onto the ring microelectrode, but real cell scanning data has not been reported yet.[44] However, this type of SECM–AFM is expected to extend the application of SECM to the constant-distance mode monitoring of cellular activity because of the intrinsic fixed electrode–tip distance.

CONCLUSION

This article reviewed various bio-interfaces fabricated by the use of microfabrication technologies to facilitate two-way communications with cells. Cells communicate with each other via mechanical, chemical, and electrical ways such that microscale interfacing systems are highly required in investigating and better understanding cell-signaling mechanisms. We reviewed the microfluidic devices that make it possible to perturb cell signaling by producing chemical, mechanical gradients/interfaces not only over a single cell but also over a population of cells. These devices impose extracellular stimuli onto single or multiple cells. However, nanoparticles-based bio-interfaces not only allow noninvasive delivery of biochemicals into cells and thereby provide intracellular stimuli onto cells, but they also allow us to probe the cellular signals within the cells. These methods show a high potential to provide unique biochemical and physical interfaces with cells and to be used for probing cellular responses with high sensitivity and specificity. Lastly, the SECM-based probing interfaces were reviewed and compared to microfluidics and nanoparticle-based stimuli because they enable the measurement of cell responses by detecting electrochemical signals from both the surface and the inside of cells. All these efforts have been made to precisely stimulate and analyze cellular responses including cell-signaling networks in time and space. In the near future, we would be able to control,

design, and engineer cell functions with the help of multifunctional microinterfaces, spawning many advanced biomedical applications.

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