Chapter 1

Introduction

1.1 Micro- and nanotechnologies in biology

Microtechnology or nanotechnology is no longer a scientific jargon used solely in science or engineering society. Now we can easily find examples that utilize such up-to-date technologies everywhere in daily life, and biology is one of the fields actively adapting such a paradigm change originated by the size.[1, 2] Size, in itself, is an important characteristic factor for us to use to define and describe an object. Viruses, for example, are the smallest living organism. They are so small that they are able to live inside other cells undetected. Thus 'small' can be one of the first characteristic descriptions of virus. However, if we think about the 'small' in depth, we can easily encounter a lot of interesting questions: How do they move? What would be the resistance (or drag force) that they feel when they move? How about their energy utilization (metabolism)? Is that related to their size? Size indeed relates to functions. The new physical and chemical property changes introduced by accessing the extremely small scale can open up a new angle on things that is related to function.

When it comes to biology or medicine, scaling down of tools introduces advantages in terms of small sample amount, fast reaction, multi-parameter analysis, and integration. Early on, microfluidics brought miniaturization and integration together and such efforts

gave birth to micro devices for PCR,[3] capillary electrophoresis (CE),[4] cell counting/sorting,[4] protein crystallization,[5] and integrated devices of multiple functionalities.[6, 7] CE especially shows the power of scaling by accelerating the human genome project.[8] Recently, microfluidics has been actively used in sequencing, which is expected to enable personalized medicine at a reasonable price and timescale.[9] Nanotechnology also shows its potential in multiple applications. Nanovectors such as liposomes[10] or nanoparticles[11] for drug-delivery, nanoparticle-based identification labeling like 'bio-barcode'[12] or 'chemical nose'[13], and silicon nanowire (SiNW)-based biosensors[14] are good examples of nanotechnology applied to biology.

Scaling is an important as well as interesting topic in physics. It is impressive how much progress we've made in understanding new physics at nanoscale since Richard Feynman introduced the concept of nanotechnology in his famous lecture, 'There's Plenty of Room at the Bottom' in 1959. However, it will be more exciting to see how the deep understanding of scale and the wide application of the understandings change the world. Biology and medicine are actively adapting this trend and we will see the outcomes soon in a better quality of life.

1.2 How to study biology: Top-down vs. bottom-up

About two decades ago, there was a huge debate introduced by Eric Drexler in a book entitled *Nanosystems*[15–17] about whether the molecular nanotechnology (MNT) driven by an 'assembler' he proposed can be realized or not. This debate paralleled the discussion on the two major approaches in nanotechnology, top-down or bottom-up (because the concept

of MNT is based on a bottom-up approach). It is still an on-going debate. However, the true meaning of this debate, at least I think, is that people started to think about the way they study and look at things. They started to think about whether they could build up and control at the molecular level. Thus the scope of people's interests has changed. We can find a similar concept in biology as well. Single-cell-based studies are a good example of this. Instead of looking at tissue samples consisting of millions of cells, people become interested in a single cell and its heterogeneous characteristics. Systems biology is also closely related to this paradigm change because it approaches biology with a systemic view, and cells or genes are basic components of the system.[18, 19] The focus of biology moves toward smaller and smaller components, while maintaining its interest in conventional, bulk targets. Now we can categorize the methodologies for biological study into either top-down or bottom-up approaches. Most clinical studies can be categorized as top-down, while rather recent researches–such as on tumor microenvironment or single cells–can be recognized as bottom-up. Categorizing them might be meaningless in itself. However, if it reflects that people start to adopt new way of thinking and analysis, it becomes very important.

We are already seeing the success of the single-cell-based bottom-up approach in biology and medicine.[20–23] Lahav et al. reported that p53 shows pulsed responses to radiation damage at the single-cell level, but not in population measurements.[21] Cohen and coworkers studied the heterogeneous response of human cancer cells to chemotherapy drug by monitoring the levels and locations of ~ 1000 endogenously tagged proteins.[22] Tay et al. also showed that the activation of TNF-α-induced NF-κB signaling is heterogeneous and has a digital response at the single-cell level, which is different from population-level

studies with bulk assays.[20] The most interesting common fact in those studies is that the single-cell-based approaches, which we can call the biological version of the bottom-up approach, illuminate hidden, heterogeneous characteristics of cells that are in contrast to what we have seen in conventional bulk assays. This new set of information will broaden our understanding in biology and will guide us in our fight against diseases in novel and creative ways.

As we have seen from the micro- and nanotechnology fields, there is no reason that either the top-down or the bottom-up approach should lead the studies.[24–26] Combined together, both approaches will open up new feasibility in biology and medicine through a series of breakthroughs, and the micro- and nanotechnologies will play an important role in that. In this thesis, some of those examples will be presented by introducing new technology, device platforms, and analysis schemes.

1.3 Complexity of biology and multi-parameter analysis

Biological systems are complex.[27–29] Even a cell, one of the most basic units of life, can be seen to have extremely complex components under microscopy. It is amazing how all the components are packed into a ~ 10-μm-length scale. And those components are functional: moving, binding, pulling, and replicating. Cancer is another good example of the complexity of biology. Difficulties in cancer treatment arise from the complexity of cancer pathophysiology. The concept of cancer is changing from that of a homogeneous disease to that of stratified heterogeneous diseases, each with its own biological

characteristics, each requiring a specified therapeutic approach. Thus, we need a more effective solution to deal with biology, especially cancer diagnosis and treatment.

Multi-parameter analysis is an effective solution for such needs, and systems biology can be a powerful approach towards predictive, preventative, and personalized medical care.[18, 30] The importance of this approach can be found in many examples. Current clinical treatments are based on monitoring only a few biomarkers, such as prostate-specific antigen (PSA) for prostate cancer, CA125 for ovarian cancer, and HER2 for breast cancer. However, these biomarker tests frequently fail to identify early stages of cancer and allow the tumor to transform to a malignant phenotype before a proper treatment can be instituted. For example, mutation in the KIT receptor tyrosine kinase is the major cause of most gastrointestinal stromal tumors (GISTs). That is why Gleevec, a KIT inhibitor, works well in most patients with GISTs. However Fletcher et al. reported that mutations of KIT or platelet-derived growth factor receptor (PDGFRA) are mutually exclusive oncogenic mechanisms in GISTs and that these mutations induce similar downstream signaling pathways of tumor progression. Actually, about 35% of GISTs have intragenic activation mutations in the PDGFRA, even though they don't have KIT mutations.[31] The global profiling of the molecular signature at a genomic level[32, 33] or proteomic level[34, 35] shows its potential in a number of studies reported in the literature as well. Multi-parameter analysis not only allows a more accurate diagnosis, but also enables early-stage cancer detection. An increased number of biomarkers will lead to a more informative diagnosis, which raises the possibility for the right clinical decision. Earlier disease detection makes proper treatment more likely and improves the survival rate.[36]

However, there are still challenges before multi-parameter analysis will be practical. The first challenge is non-specific binding. Most multi-parameter analysis for biological applications utilizes fluorescence, absorbance, or electrical signals, and for accurate, sensitive measurement the signal-to-noise ratio should be maximized. Looking at multiple targets means a higher chance of non-specific adsorption of target biomolecules on the surface of sensing component, which is known as biofouling. It makes it difficult to deconvolute the real signal from noise. In making the analysis quantitative, this issue becomes more significant.

Another limiting factor is the need to detect small quantities of biomarkers in a small volume, which requires extremely sensitive and fast sensors.[37] This factor has attracted significant interest in rapid measurement of a panel of plasma proteins from quantities of whole blood as small as those obtained by a finger prick.[38–40]

The third challenge is the capture agent. Currently, the antibody is the most commonly used capture agent.[41] High-quality antibodies show good specificity and affinity for the target protein. However, they are expensive and unstable under various experimental conditions such as pH, dehydration, and temperature.[42] This makes it difficult to incorporate them into common fabrication steps for microfluidics or micro-/nanotechnologies. Thus, it is necessary to refine our approach to finding biomolecule capture agents that exhibit a high level of chemical and biochemical stability. [43–46]

While there are still challenges to address, the multi-parameter analysis approach shows its potential in new platforms and is creating a new paradigm. The multi-parameter analysis will be one of the major topics discussed throughout this thesis.

1.4 Thesis overview

This thesis presents the development of chip-based test platforms that utilize nano- and microtechnologies and their biological applications. The scope of the technologies introduced here is broad. It covers the fabrication of nano/microscale devices, efforts to perform multi-parameter analysis experimentally, analyzing data in a quantitative manner, and possible new applications of those technologies. In Chapter 2, I will begin with the nanotechnology-based platform silicon nanowire (SiNW) field-effect transistor (FET). In addition to showing the advantages of label-free, real-time, and electrical measurement, the quantitative detection of single-stranded oligonucleotides with SiNWs in physiologically relevant electrolyte solution is demonstrated. The efforts are further extended to protein sensing as well. Debye screening is one of the major bottlenecks of electrical measurement in solution. To circumvent this problem we utilized electrostatically adsorbed primary DNA on an amine-terminated NW surface for DNA detection, and synthetic peptide as a capture agent for protein sensing. The surface state is important when it comes to smaller, nanostructures, and SiNW is not an exception. In order to look into the surface-state effect on the electrical measurement, two surface functionalization chemistries are compared: an amine-terminated siloxane monolayer on the native SiO_2 surface of the SiNW, and an amine-terminated alkyl monolayer grown directly on a hydrogen-terminated SiNW surface. The SiNWs without the native oxide exhibit improved solution-gated field-effect transistor characteristics and a significantly enhanced sensitivity to single-stranded DNA detection, with an accompanying two orders of magnitude improvement in the dynamic range of sensing. A model for the detection of analyte by SiNW sensors is developed and utilized to

extract DNA binding kinetic parameters. Those values are directly compared with values obtained by the standard method of surface plasmon resonance (SPR), and demonstrated to be similar. The nanowires, however, are characterized by higher detection sensitivity. The implication is that SiNWs can be utilized to quantitate the solution phase concentration of biomolecules at low concentrations. This work also demonstrates the importance of surface chemistry for optimizing biomolecular sensing with silicon nanowires. (Chapter 2 has been taken in part from *Journal of the American Chemical Society* **2006**, 128, 16323–16331).

Chapter 3 to 5 introduce a microfluidics-based platform for performing a single-cell-based protein analysis. A SiNW-based biosensor has some advantages, but is complicated to fabricate and difficult to apply to multi-parameter analysis. Thus, these three chapters present a more simple and practical strategy to study biology in a multi-parameter manner. The technologies introduced in these chapters are relatively simple but highly optimized, and still present new biological findings because they enable analysis of multiple proteins at the isolated, single-cell level, which is difficult to achieve with conventional, bulk analysis. As a starting point of this effort, Chapter 3 presents a method to make high-quality DNA micro-barcodes. To detect proteins, we utilize an approach called DNA-Encoded Antibody Libraries (DEAL) developed in our lab several years ago.[39, 47] DEAL technique is based on orthogonal ssDNAs conjugated to an antibody library where every antibody- specificity is uniquely encoded with a distinct ssDNA sequence. We then can use a more robust biomolecule, as a handle to convert a DNA microarray to a protein microarray. It is a simple but powerful technique, since we can perform multi-parameter protein analysis only if we can find orthogonal DNA pairs (which can be done computationally) and pattern complimentary DNAs on a substrate. We described this in

Chapter 3. We have identified a protocol for generating high-quality, high-density DNA barcode patterns by comparing three microfluidics-based patterning schemes. We find, through both experiment and theory, that the electrostatic attractions between the positively charged PLL substrates and the negatively charged DNA backbone induces significant non-uniformity in the patterning process, but that those electrostatic interactions may be mediated by adding DMSO to the solution, resulting in uniform and highly reproducible barcodes patterned using ~ 55-cm-long channels that template barcodes across an entire 2.5-cm-wide glass slide. Dendrimer-based covalent immobilization also yields good ultimate uniformity, but is hampered by a relatively unstable chemistry that limits run-to-run reproducibility. The potential of this approach has been further demonstrated by assaying cytoplasm proteins from single and lysed U87 model cancer cells. Successful detection of a panel of such proteins represents the potential of our platform to be applied to various biological and, perhaps, clinical applications. (Chapter 3 has been taken in part from *ChemPhysChem* **2010**, 11(14), 3063–3069).

Chapter 4 extends and develops the single cell-based protein detection with DNA micro-barcodes and DEAL technique. We take an approach that integrates microfluidic cell handling and *in situ* protein secretion profiling to assess the functional heterogeneity of single cells, with extensions to small cell colonies. We measured a dozen proteins secreted from cells for the most aggressive type of primary brain tumor, glioblastoma multiforme (GBM). We observed functional phenotypes in terms of secreted proteins with profound cellular heterogeneity but still in a statistically meaningful manner. The unique features that we confirmed from single-cell analysis can present additional useful information to the conventional bulk analysis. Combining physical status of the system (such as cell–cell

distance) and the protein secretion profiles enables study of the tumor microenvironment. We further demonstrate the potential clinical application of this platform by analyzing solid tumor cells derived from a GBM patient. This platform is inexpensive, requires minute amounts of cells and yields a large volume of molecular information, showing great potential for clinical assessment of cellular characteristics in human disease lesions, such as a tumor microenvironment.

One of the challenges that we have noticed from the approach introduced in Chapter 4 is how to analyze the sheer amount of information. By utilizing a microfluidic device with ~ 1000 isolated chambers, we can collect ~ 1000 data sets with a single experiment, and each data set represents highly heterogeneous cellular activity. Thus, we should come up with a good strategy to identify the meaningful information by looking at the heterogeneous data set as a whole. In Chapter 5, I will present an approach that integrates microfluidic cell handling, *in situ* protein secretion profiling, and information theory to determine the extracellular protein-signaling network and the role of perturbations. Protein-signaling networks among cells in a disease lesion play critical roles in a host of pathophysiological processes, from inflammation to tumorigenesis. We assayed 12 proteins secreted from human macrophages that were subjected to lipopolysaccharide challenge, which activates the Toll-like receptor-4 signaling pathway. This process emulates the macrophage-based innate immune responses against Gram-negative bacteria. We characterize the fluctuations in protein secretion of single cells, and of small cell colonies (n = 2, 3,⋯), as a function of colony size. Measuring the fluctuations permits a validation of the conditions required for the application of a quantitative version of the Le Chatelier's principle, as derived using information theory. This principle provides a quantitative

prediction of the role of perturbations and allows a characterization of a protein–protein interaction network.

In Chapter 6, another application of microfluidics will be demonstrated in studying interfacial chemistry for a lung surfactant system. The motivation for this study is to understand the interfacial structure and chemistry of a surfactant layer system when subjected to oxidative stress. In order to achieve that, we combined experimental observations based on field-induced droplet ionization mass spectrometry (FIDI-MS) with computational analysis. FIDI-MS comprises a soft ionization method to sample ions from the surface of microliter droplets. A pulsed electric field stretches neutral droplets until they develop dual Taylor cones, emitting streams of positively and negatively charged submicron droplets in opposite directions, with the desired polarity being directed into a mass spectrometer for analysis. This methodology is employed to study the heterogeneous ozonolysis of 1-palmitoyl-2-oleoyl-*sn*-phosphatidylglycerol (POPG) at the air-liquid interface in negative ion mode using FIDI mass spectrometry. Our results demonstrate unique characteristics of the heterogeneous reactions at the air-liquid interface. We observe the hydroxyhydroperoxide and the secondary ozonide as major products of POPG ozonolysis in the FIDI-MS spectra. These products are metastable and difficult to observe in the bulk phase using standard electrospray ionization (ESI) for mass spectrometric analysis. We also present studies of the heterogeneous ozonolysis of a mixture of saturated and unsaturated phospholipids at the air-liquid interface. A mixture of the saturated phospholipid 1,2-dipalmitoyl-*sn*-phosphatidylglycerol (DPPG) and unsaturated POPG is investigated in negative ion mode using FIDI-MS, while a mixture of 1,2-dipalmitoyl-*sn*-phosphatidylcholine (DPPC) and 1-stearoyl-2-oleoyl-*sn*-phosphatidylcholine (SOPC)

surfactant is studied in positive ion mode. In both cases FIDI-MS shows the saturated and unsaturated pulmonary surfactants form a mixed interfacial layer. Only the unsaturated phospholipid reacts with ozone, forming products that are more hydrophilic than the saturated phospholipid. With extensive ozonolysis only the saturated phospholipid remains at the droplet surface. Later we confirm this finding with a microfluidics-based bubble generator with a model pulmonary surfactant composed of two major phospholipids: DPPC and POPG. With fluorescence imaging, we observe the ozone-induced chemical modification of the unsaturated lipid component of the lipid mixture, POPG. This chemical change due to the oxidative stress was further utilized to study the physical characteristics of the interface through the bubble formation process. The physical property change was evaluated through the oscillatory behavior of the monolayer as well as the bubble size and formation time. Results presented demonstrate the potential of this platform to study interfacial physics of a lung surfactant system under various environmental challenges both qualitatively and quantitatively. (Chapter 6 has been taken in part from *Journal of Physical Chemistry C* **2010**, 114, (29), 9496–9503).

1.5 References

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