Minireview

Recent Advances in Nanobiotechnology and High-Throughput Molecular Techniques for Systems Biomedicine

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Nanotechnology-based tools are beginning to emerge as promising platforms for quantitative high-throughput analysis of live cells and tissues. Despite unprecedented progress made over the last decade, a challenge still lies in integrating emerging nanotechnology-based tools into macroscopic biomedical apparatuses for practical purposes in biomedical sciences. In this review, we discuss the recent advances and limitations in the analysis and control of mechanical, biochemical, fluidic, and optical interactions in the interface areas of nanotechnology-based materials and living cells in both *in vitro* and *in vivo* settings.

INTRODUCTION

Nanotechnology has gradually evolved towards commercial applications in various fields including advanced materials, computational sciences, bio-analytics and surface sciences. Nanotechnology can potentially make big impacts on biomedicine and ultimately global human society. How can we utilize the recent progress in the forefronts of nanotechnology to make a significant impact on global health? This is tough to answer, since nanotechnology by nature encompasses a large number of interdisciplinary fields from broad areas which need to work closely together. Why are technologies at the nanoscale so important in the biosciences? Living systems and nanoscale objects fundamentally share a common scale at the molecular level (Fig. 1). Building blocks of almost all living systems constitute interacting bio-molecules at the molecular scale. Thus advances in nanotechnologies are bound to provide an interface and have a direct application in the life sciences. In this review, we highlight emerging areas such as quantum dots and nanoparticles, nanoliter fluidic systems, label free detection, single molecule, and single cell analysis (Fig. 2). These rapidly growing areas can also influence our fundamental understand-

ing of biology and medicinal sciences including tissue engineering (Fig. 3).

What advances have been made at the individual bio-molecule level? It is now possible to detect trace amounts of bio-materials inside living organisms. This has opened a new avenue of detection with minimal sample preparation, increased accuracy and shortened the total required experimental time. Such a novel approach further enables us to explore much broader experimental states to look for new solutions. High-throughput measurements in large-scale experiments at both the cellular and bio-molecular level are more readily available than before and this can illuminate what one researcher aptly called “biological dark matter”. Advances in the synthesis of tunable nano-materials which interact with biological matter in novel ways have led to more efficient discoveries of new labeling and therapeutic agents (Miele et al., 2012; Zhang et al., 2013).

SYSTEMS BIOLOGY

How would a nanotechnological approach change new drug development if we could precisely model a biological system as a whole? So far, a reductionist's approach has been mostly used to understand biological processes by taking small pieces of system at a time, and looking at a small number of inter-relations with other entities. An emerging viewpoint in biology is to globally map out a large number of processes and inter-connections in a given biological system (Kirschner, 2005). This knowledge can be used, for example, to evaluate new methods for identifying drug target molecules. Systems biology fundamentally takes a different approach to consider a biological entity as an information processing network with each individual module responding to signals from various nodes (Ideker, 2004). Thus biological complexity, which has plagued our understanding of how living systems behave in response to a given externally or internally generated signal, can now be ad-

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dressed in a more theoretical manner. Such a modeling scheme could enable building hypotheses that are testable in systematic experimental settings.

Two basic paradigm shifts are suggested to make this transition feasible. The first avenue for change is to mine large amounts of data in numerous experimental conditions *via* high-throughput technology. Such experiments rely heavily on automated technologies to enable probing the large numbers of state spaces of stimuli and responses of a biological system. The second avenue would use computational and mathematical tools, which play crucial roles in building these large scale models and enable the testing of various hypotheses in a given system network. A systems biology approach can provide us a new way to understand the interactions among elements uncovering emergent properties in intracellular and intercellular signaling through quantitative measurements, modeling and reconstruction. For example, dynamically changing levels of mRNA and protein affect protein-to-protein interactions and DNA-to-protein interactions. The accurate quantitation of biological information in a high-throughput manner is central to systems biology (Aderem, 2005; Chuang et al., 2010). The four essential features in measurements of biological information are quantitative, global, dynamic, and systematic perturbation of networks.

Several high-throughput technologies are progressively becoming adopted in systems biology areas. Automated DNA sequencers, microarrays for global gene expression profiles, and mass spectrometry and others have been the workhorses for high-throughput generation of inter-relationships in systems biology networks. A comprehensive understanding of detailed biological networks would be necessary to reliably predict biological and phenotypical consequences. However, there are several challenges. For instance, conventionally used experimental tools alone cannot provide enough spatio-temporal resolution in measurements.

Since systems biology requires large amounts of information and data to predict responses to environmental cues, it is common to iteratively refine details in a given model. Maerkl and Quake (2007) recently introduced an integrated microfluidic device for high-throughput measurement of molecular affinities to build inter-relation networks. The technique depends on mechanically induced trapping with microfluidic valves. Attomoles of DNA and transcription factors were used per data point to obtain ~40,000 data points to measure molecular interactions of transcription factors with DNA belonging to helix-loop-helix family. Another technique integrates digital PCR with microfluidic manipulation and microarrays to acquire information from a single bacteria (Ottesen et al., 2006), which is not feasible in traditional cell culture based approaches. This allows the building of gene inventory in a population of mixed bacterial species obtained from a natural environment. Such high-throughput data retrieval on the phylogenetic scale adds information at another level of hierarchy in the biological network.

TECHNOLOGICAL ADVANCES

Multifunctional nanoparticles and nanomaterials

Quantum dots

A quantum dot (QD) is the most basic nanostructure which spatially confines the conduction band, valence band and excitation at a single point. QDs have been applied to *in vitro* live cell imaging, *in vivo* imaging in animals and medical diagnostics (Cheki et al., 2013; Michalet et al., 2005; Stroh et al., 2005).

Cell surface receptors, as small as 50 nm, can be labeled with QDs. Furthermore, the controllable hydrodynamic diameter (HD) and surface charge of nano-crystals are useful in biomedical imaging of tissue and sentinel lymph nodes. *In vivo* imaging of a tumor would be possible by the use of QD515 (HD = 4.4 nm) and QD574 (HD = 8.2 nm) (Jain, 2005). Some challenges include the lack of accurate quantification and the inability to detect multiple proteins simultaneously. The detection of multiple QDs with tunable size in a single imaging setup would provide rich information sets with a single experimental run.

For biological imaging applications, fluorescent nanoparticles and nano-crystals have been already commercialized. Moreover, other simple elements can be used as building blocks. For example, Bruchez et al. (1998) developed nanorods used in DNA conjugation and electrophoresis of Au nano-crystals and DNA tiles organized by nano-crystals. It is further possible to engineer multi-functional nanoparticles. Integrated multifunctional nanoparticles would be useful in targeting agents and diagnostic imaging with remote sensing, triggering, and drug delivery. *In vivo* phage display exploits differences in the tumor endothelium (Ruoslahti, 2002). Nanoparticles functionalized with homing peptides can also target tumors: for example, F3-QDs and LyP-1-QDs target different structures in xenograft tumor (Akerman et al., 2002). Protease-activated QDs, in which the blocker peptide coating on QDs is cleaved by protease, can be used to modulate the cellular uptake of QDs (Zhang et al., 2006b). Matrix metalloprotease-cleavable polyethylene glycol (PEG) nanoparticles can be accumulated in xenograft tumors over non-cleavable controls, and be localized for better tumor targeting (Harris et al., 2008). A programmable drug delivery is also possible (Wagner, 2007). DNA can be heated by the induction of an external electromagnetic field, which first triggers DNA release from nanoparticles, then multistage release, and finally *in vivo* release. This process is reversible and allows triggered self-assembly for sensing application.

Toxicity of quantum dots

One challenge in using nanomaterials such as QDs for *in vivo* studies is its potential long-term toxicity and its effect on *in vivo* fate (Sayes et al., 2006). The toxicity and the potential environmental effects of QDs need to be addressed before these agents can be used for *in vivo* imaging. For example, the renal clearance of QDs depends on its electrostatic property or hydrodynamic radius and this is an important consideration in designing QDs or nanoparticles for *in vivo* use (Choi et al., 2007). Several innovative strategies have been developed to reduce or eliminate toxicity of these nanomaterials. For instance, it was demonstrated that intercellular concentration of QDs could be made nontoxic by coating the surface of the QDs with terminal-functionalized polyethylene glycol (PEG) (Zhang and Monteiro-Riviere, 2009). Since QDs have strong surface coating dependence, PEG coating could improve biocompatibility. The dramatic reduction of the QD uptake into cells at low temperatures (4°C) indicates that the intracellular localization is energy-dependent (Damalakiene et al., 2013). For extracellular use at the cell surface receptor binding, it was shown that a concentration of 5 to 20 nM should be used for minimal toxicity of QDs. It must also be noted that QDs can degrade at the acidic pH of 4.5 to 5 in the lysosome. Thus, cell death was shown to be highly dependent on the environmental exposure concentration, type of QDs and experimental conditions (Hardman, 2006).

Fig. 1. Multiple-scale features of living biological materials and systems.

Use of magnetic nanoparticles and gold nanoparticles in bioimaging

Magnetic nanoparticles and gold nanoparticles are extensively used for bioimaging *in vivo* and *in vitro*, showing a multifunctional potential such as drug delivery and image-guided surgery. Superparamagnetic iron oxide nanoparticles have detected tumors sensitively and have been used as a contrast agent for magnetic resonance imaging (MRI). Most iron oxide-based magnetic nanoparticles are known to be biocompatible (Gupta et al., 2007). Recently magnetic particles have been used to generate heat in the deep tissue in the presence of an external magnetic field oscillating at 0.1 to 1 MHz (Fortin et al., 2007; Lee et al., 2011). When therapeutic agents are attached to magnetic nanoparticles, a well-controlled external magnetic field can localize the drug-nanoparticle complex into the target site. Gold nanoparticles are able to show localized surface plasmon resonance (LSPR) from coherently oscillating electrons under incident light at a specific wavelength (Daniel and Astruc, 2004). LSPR can enhance the signal intensity of fluorescence or surface-enhanced Raman scattering when their reporters are in the vicinity of the gold surface. Moreover, the photothermal property of gold nanoparticles has been utilized for hyperthermia therapy and photoacoustic imaging, since the energy of incident light can be partially converted into heat by gold nanoparticles (Yang et al., 2009).

Nanodevices as extracellular matrix-mimicry

More studies support the importance of nanotopographic control of the extracellular matrix (ECM) for regulating cell motility, proliferation, differentiation, and disease development. Attempts have been made in developing more effective *in vitro* nanoscale devices to recapitulate *in vivo* ECM conditions (Kim et al., 2012b). Polymeric anisotropic nanopatterns promoted the differentiation of cardiac stem cells and myocardial regeneration in the infarcted rat heart (Kim et al., 2010; 2012a). Nanostructured metal, ceramics, polymers, composites enhanced cellular adhesion, proliferation and expression of bone-related proteins and the deposition of calcium complex of bone-forming osteoblast (Lim et al., 2008; Richert et al., 2008; Yang et al., 2013).

For example, nanoscale topographic modification of titanium dental implant leads to superior osteointegration, which is crucial for the long-term stability and efficacy of dental implant therapy (Mendonca et al., 2008). The sustainable release of human growth hormone throughout a nanopored membrane (Kim et al., 2013) is expected to facilitate the tissue regeneration when the delivery scheme for therapeutic proteins is integrated into the implant material.

Although integrating nano-bioimaging technology and therapeutic medical application using multifunctional nanoparticles or nanomaterials poses a significant challenge, this can provide a novel approach in biomedicine such as simultaneous delivery of imaging and therapeutic agents (Gao et al., 2004; 2005; Michalet et al., 2005).

Nano-/microfluidic systems for fluid handling

Recent technological advances provide a means to manipulate sub-nano liter fluids in a programmable fashion. This has contributed to unprecedented progress in the field of microfluidics and the development of tools for performing biologically relevant experiments even with small volume samples at high-throughput levels (Kim et al., 2009).

Various strategies have been developed for automated fluid handling at the microscale. The approaches can either be classified as either single-phase flow with transported fluid in contact with channel walls at all times or multi-phase flow with droplets traveling in an immiscible fluid, where reagents of interest never touch the channel geometry and are not subject to dispersion. Another classification is based on driving forces used to pump fluids at small scales.

Uses of microfluidics in real time qRT-PCR

Currently, large-scale integration allows for fabrication of O (10,000) integrated valves in a microfluidic device (Thorsen et al., 2002). This method usually involves breaking a sample into a very large number of fragments, each addressable compartment being a few hundred picoliters in volume. The integrated microfluidic chip has been utilized for real time quantitative reverse transcription polymerase chain reaction (qRT-PCR) to

Fig. 2. Nanotechnology-based bioimaging and detection of biochemical events. (A) *In vivo* imaging with quantum dots (Stroh et al., 2005). Reproduced by permission of Nature Publishing Group. (B) High-throughput single-molecule DNA sequencing in nanowells in real time (Eid et al., 2009). Adapted by permission of The American Association for the Advancement of Science. (C) Sensitive detection of target molecules on a dendron-coated surface (Kim et al., 2012d). Reproduced by permission of The Royal Society of Chemistry. (D) Label-free in situ monitoring of single DNA nick-sealing events using AFM. Adapted with permission from (Kim et al., 2012c). Copyright (2012) American Chemical Society.

quantitate the expression levels of genes in a single cell (Sanchez-Freire et al., 2012). The transcriptional heterogeneity of monoclonal human colon cancer (Dalerba et al., 2011) and circulating tumor cells (Powell et al., 2012) were revealed by commercially available qRT-PCR equipment in microfluidic platform.

Another common approach involves a series of components which integrate various steps in metering, formulation, mixing, analysis, and storage (Malic et al., 2010; Mark et al., 2010). One module which arises repeatedly in high-throughput analysis is storage. For example, a high-throughput screen for protein crystallization solutions requires a large number of reactants and reaction conditions which need to be stored to allow for crystal formation. Various storage schemes employing two-phase flow (Lau et al., 2007) are now emerging as they allow for serial sample storage of O(1000) in long tubes in a linear fashion. The operation of pneumatic integrated micro-valves can be further automated by using an abstract description language describing experimental protocols (Ananthanarayanan and Thies, 2010). Another approach for control in the multi-phase flow of droplets in microchannels is based on hydrodynamic logic gates (Prakash and Gershenfeld, 2007), thus re-

quiring no external pneumatic control.

Over time, numerous off-chip components have been integrated on microfluidic devices to increase the utility of these devices for a wide array of applications. A recent work on co-fabrication (Siegel et al., 2010) proposes to embed most of these components, microfluidic channels and optical detection schemes, such as lasers and electromagnetic elements, in a single compatible process. This leads to rapid-prototyping of complex designs over a short period of time.

Extracellular matrix-mimicking hydrogels in microfluidic devices

The three-dimensional cell culture on ECM-mimicking hydrogels in microfluidic devices has been applied to *in vitro* high-throughput drug screening. Recently organ-on-a-chip platforms, including lung-on-a-chip, muscle-on-a-chip, and central nervous system-on-a-chip, have been developed to investigate the systematic pharmacokinetics (Tsui et al., 2013). The mechanical stimulus that imitates the breathing conditions were applied to lung cells on a thin and porous polydimethylsiloxane (PDMS) membrane coated with ECM in order to reconstitute the func-

Fig. 3. Nanotechnology-driven tissue engineering, (A) Control of cellular polarization on the nanotopographic pattern (Kim et al., 2012a). Reproduced by permission of The Royal Society of Chemistry. (B) Cardiac cell patch to heal the myocardial infarction (MI) (Kim et al., 2012a). Reproduced by permission of The Royal Society of Chemistry. (C) Controlled release of therapeutic proteins using nanopored polymer membrane (Kim et al., 2013). Reproduced by permission of The Royal Society of Chemistry. (D) Microfluidic lung-on-a-chip to mimic lung tissue and its mechanics (Huh et al., 2010). Reproduced by permission of The American Association for the Advancement of Science.

tional alveolar capillary interface of the human lung (Huh et al., 2010). The mechanically active organ-on-a-chip is expected to provide alternative systems for drug screening and toxicological examination prior to expensive animal experiments or clinical trials.

High-throughput molecular technology in biomedicine

In a given integrated biological analysis platform, usually the throughput bottleneck lies in detection schemes. On line real time detection schemes for a large number of biologically relevant parameters are still uncommon with currently available tools. Fluorescence-activated cell sorting (FACS) still remains one of the most common quantitative methods for cell analysis (Osborne, 2011).

Label-free detection of biomolecules and single cells

Patterned cantilevers binding with cells or biomolecules have been previously used as a detection tool in vacuum. The scheme can be implemented into an ultrasensitive cantilever sensor array (Li et al., 2007), with a 250 nm pitch to allow real-time monitoring of lipid bilayer formation on the cantilevers and fast microorganism detection. Burg and Manalis (2003) introduced label-free detection of bio-molecules and single cells in a micro-channel resonator which did not require the exposure of samples to vacuum, integrating a resonant cantilever and a

sample-carrying channel in the same structure. Binding of various biomolecules or passage of a single cell shifts the resonant frequency of the cantilever to allow fast online detection. An oligo detection method with an ability to fish label-free genes within complete genome, or an un-labeled gene in total cellular RNA was also introduced (Zhang et al., 2006a). Such nanomechanics-based chips can be used for applications in ultrasensitive biosensing and diagnostics. More conventional nanoelements such as nanotube transistors and resonators are used as nano-bio-electronic sensors in micro-nano electronics research. Chemo-absorptive coatings and all electronic ultrasensitive detection provide a direct interface to volatile compounds from the disease-correlated odor source such as skin, sputum, urine, and breath (Oh et al., 2011).

Other major approaches for label-free detection for quantitative bioassays are based on principles of electrochemistry (Sadik et al., 2009) or surface plasmon resonance (Ray et al., 2010). For a multiplexed detection, thousands of discrete DNAs, proteins or other biomolecules can be spotted and immobilized on solid surfaces in a microarray format. The combination of conventional label-free detection methods with micro- or nanofluidic platforms offers a high-throughput detection of target molecules (Zhang et al., 2012). A gold substrate that presents peptide ligands uniformly on the self-assembled layer was utilized to quantitatively evaluate the protein kinase activity in a array

Fig. 4. Application fields of emerging nanotechnology and high-throughput techniques for systems biomedicine.

format on a single substrate using surface plasmon resonance (Houseman et al., 2002).

Observation of a functioning cell, with all its machinery intact, is necessary to make correct predictions about the current state of a cell and to derive meaningful inferences for translation into biomedicine. This approach can be further classified as single cell analysis or the study of controlled cell cultures. Beebe's group introduced a high-throughput microenvironment for studying cell populations (Meyvantsson and Beebe, 2008). Three-dimensional *in vitro* assays generate more controlled microenvironments that can better predict the structure and functions of constituent cells and be more useful in drug screening. Xie's group developed a technique for probing dynamics of a single transcription factor in a living *E. coli* cell (Elf et al., 2007).

DNA sequencing

Contrary to the conventional Sanger DNA sequencing methods which required nearly \$3 billion and 15 years to complete the human genome project, next generation DNA sequencing technologies integrate biochemistry, optics, surface chemistry, microfluidics, and electronics and has revolutionized genome and transcriptome analysis in recent years. This makes it possible to obtain personal genome at around \$50,000 (<http://www.biotechniques.com/news/First-named-female-genome-sequenced/biotechniques-204525.htm>). Nanotechnology aiming to engineer multifunctional nanomaterials and nanodevices may ambitiously open the \$1,000 genome era (Davies, 2010). Rapid and low-cost whole-genome sequencing has been applied to diagnose genetic diseases in neonatal intensive care units (Saunders et al., 2012) and studies are underway to expand the clinical application of next generation DNA sequencing technology in other diseases (Koboldt et al., 2013; Rizzo and Buck, 2012).

Single-cell sequencing as well as deep next generation sequencing have been utilized in progression disease biopsies (Koboldt et al., 2013). Determination of DNA sequences from a single DNA can result in an ultra-high-throughput sequencing of DNA or RNA without any amplification, thus reducing cost and increasing accuracy compared to the conventional Sanger

sequencing techniques. Recently these optical (Eid et al., 2009) or electrochemical (Rothberg et al., 2011) methods for single-molecule DNA sequencing have been commercialized. Furthermore, Zong et al. (2012) identified single-nucleotide and copy-number variations *via* sequencing single cell genome without error-prone exponential amplification. Single-cell genome sequencing will be useful for analyzing rarely available cell samples such as prenatal or forensic specimen.

Furthermore, very recently an additional breakthrough has been made, which can maximize the full power of next generation sequencing and lower PCR errors. A major obstacle to investigating low frequency mutation, especially in normal cells/tissue, is the absence of methods to accurately detect mutations due to the high error rate of DNA sequencing, even in next generation sequencing. A new protocol, termed duplex sequencing offers unprecedented accuracy and sensitivity (Kennedy et al., 2013; Schmitt et al., 2012). Adaptation of duplex sequencing into next generation sequencing can detect ultra-rare mutations, which can account for tumor heterogeneity and accurately compare mutations in normal vs. tumor or diseased states tissues/cells.

Microarray and atomic force microscopy

The optimization of chemical modification or functionalization of solid surface is critical for sensitive detection in microarrays. For example, the control of lateral spacing of capture molecules with nanoscale dendron molecules can lead to more sensitive detection of target biomolecules or enzymatic reactions on the dendron-modified glass surface (Kim et al., 2011a; 2012d). This dendron modification can make it possible to monitor a single nick-sealing event *in situ* by DNA ligase using atomic force microscopy (AFM) (Kim et al., 2012c). The label-free AFM approach can reveal the specific protein-protein interaction (Kim et al., 2011b) and visualize the dynamic translocation of a single myosin molecule along an actin fiber (Kodera et al., 2010).

CONCLUSION

What are the challenges in applying nanomaterials and nanodevices to biomedicine and bio-pharmaceutical industry? Though unprecedented progress has been made on the integration of fluid handling, tools and platforms need to be standardized to meet industrial needs. This is necessary to deliver microfluidic technologies to medical practitioners and biomedical scientist. In the near future, past medical history (PMH), predictive-probabilistic records from DNA sequencing, multi-parameter blood protein measurements, and *in vivo* diagnostic measurements and preventive-design of therapeutic drugs derived from systems biology approaches will lead to a P4 medicine, which is predictive, preventive, personalized, and participatory (Hood, 2013; Hood and Friend, 2011; Sobradillo et al., 2011). Such an approach could transform medical and pharmaceutical health industry by aiding in diagnosis and personalized treatment plan in the near future (Fig. 4).

A challenge lies in integrating medicine, biology, and engineering. It is historically believed that the space mission to the moon brought unprecedented advances in computing technology and infra-structure. If not for the bold move to step on the moon, we could have still been stuck in the old era of computing. A similar notion is slowly emerging in high-throughput biology, where advances in technology will change the fundamental paradigms in biology. Can we provide a catalyst for growth in this field - a 'new prize' like entity that channels the creative technological insights into a coherent vision for biome-

dicine? Technology development for this goal will further advance other branches of high-throughput synthesis and analysis. A better understanding of how biological systems behave will further advance health-care technologies, thus providing therapies currently unheard of.

Recent advances in nanotechnology in biomedicine have also led to the U.S. presidential announcement of brain research through advancing innovative neurotechnologies (BRAIN) initiative (<http://www.nih.gov/science/brain>). Since cell-matrix or cell-device interaction occur at near molecular levels, the nanoscale control at their interaction is essential to mimic physiological conditions. The advance of nanotechnology will generate more innovative high-density nanoscale platforms to record and stimulate excitable cells such as neurons and cardiomyocytes not only for single cells but also for population of cells. The application of nanotechnology in creating biomimetic microsystems and high-throughput molecular techniques will expedite our understanding of complex biological systems and advances in biomedicine.

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