Fabrication and Characterization of Nano-FET Biosensors for Studying Osteocyte Mechanotransduction

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Applied Science

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Abstract

Nano-FET biosensors are an emerging nanoelectronic technology capable of real-time and label-free quantification of soluble biological molecules. This technology promises to enable novel in vitro experimental approaches for investigating complex biological systems. In this study, we first explored osteocyte mechanosensitivity under different mechanical stimuli and found that osteocytes are exquisitely sensitive to different oscillatory fluid flow conditions. We therefore aimed to characterize protein-mediated intercellular communication between mechanically-stimulated osteocytes and other bone cell populations in vitro to elucidate the underlying mechanisms of load-induced bone remodeling. To this end, we devised a novel nano-manipulation based fabrication method for manufacturing nano-FET biosensors with precisely controlled device parameters, and further investigated the effect of these parameters on sensor performance.
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Chapter 1
Introduction

1.1 Motivation

The skeleton’s ability to adapt to its loading environment has been recognized for over a century. As such, load-induced bone remodeling offers a unique approach for investigating bone metabolism and bone metabolism-related diseases such as osteoporosis. Increasingly, therapeutic treatments that leverage the native mechanosensitivity of bone for the prevention and treatment of bone fragility (e.g., daily regimen of weight-bearing exercise) are becoming more popular. For these reasons, it is important to establish a thorough understanding of the bone remodeling process at the cellular level.

One approach involves studying the interaction and activity of bone cells under mechanical stimulation in vitro. This approach enables systematic dissection of the different roles fulfilled by various bone cell populations and further allows the systematic observation of this cellular system under a well-controlled environment. With huge advances in molecular biology and a number of enabling biomolecular quantification technologies, this approach has led researchers to make numerous discoveries about the molecular basis of bone remodeling and cellular mechanotransduction, including the mechanosensory and mechanotransductive role the osteocyte, a major bone cell type previously thought to play a minimal role in bone remodeling.

However, still more questions remain to be explored. Technological advances in the fields of micro- and nano-science, molecular biology and biomedical engineering will enable researchers to study those questions which presently cannot be answered. Two promising technologies include the recent development of microfluidic systems for cell culture and handling, and nanoscale biosensors for label-free assaying biomolecular targets. The
combination of these two technologies promises to enable observation of real-time biochemical response of bone cells subjected to mechanical stimulation, and the accompanied intercellular communication between osteocytes and other bone cell populations. These types of dynamic studies, which are not practical with today’s technologies due to performance limitations in molecular detection assays and low-throughput cellular experimentation methods, provide a novel approach for exploring the molecular mechanisms responsible for load-induced bone remodeling and cellular mechanotransduction.

1.2 Objectives

The overarching objective is develop a nanoscale biosensor capable of real-time measurement of soluble signaling molecules released by mechanically-stimulated osteocytes. The specific objectives of this study are:

1. To investigate the ability of osteocytes, subjected to oscillating fluid flow stimulus, to release soluble factors that modulate the activity of osteoblasts and osteoclasts.

2. To design and develop a label-free nanoscale biosensor capable of measuring released soluble signaling molecules in real-time.

1.3 Thesis Organization

This first chapter serves to introduce the reader to the motivation and objectives of this thesis. Chapter 2 summarizes the fundamentals of bone biology and nano-scale FET biosensors relevant to the current study, which emphasis on bone cell mechanotransduction, nano-FET biosensor design, and sensor fabrication. Chapter 3, written in the form of a manuscript, examines the mechanosensitivity of osteocytes to different fluid flow conditions. Chapter 4, also prepared as a manuscript, presents results related to performance of nano-FET biosensors as a function of different nanowire properties. A method for fabricating nano-FET biosensor arrays
with carefully controlled device parameters is also presented. Finally, Chapter 5 summarizes the novel findings of this study and provides recommendations for future research.
Chapter 2
Background

2.1 Bone

2.1.1 Function and structure

Bone serves several distinct structural and metabolic functions in the body. Most notably, the skeleton provides structural support and further facilitates locomotion. The skeleton further provides physical protection for the organs and serves as a store of minerals essential for cellular activities, including calcium and phosphorous. It is further implicated in regulating calcium homeostasis in the body and houses two stem cell populations within the internal compartment of long bones.

As in other connective tissues, bone is composed of cells embedded within an extracellular matrix. The composition of this matrix is different from that of other connective tissues in that it is a composite that consists of both organic and inorganic material. The organic phase is composed mostly of type I collagen, which provides tensile strength and flexibility to the tissue, along with several other constituents including proteoglycans and non-collagenous bone matrix proteins such as osteonecting and bone sialoprotein. The organic phase is stiffened by the inorganic phase, which consists primarily of crystalline hydroxyapatite. The incorporation of the inorganic phase serves to provide rigidity to the bone tissue, thus allowing it to fulfill its structural role in the body.

A second aspect of bone tissue that contributes to its high mechanical strength is its geometry and microstructure (Figure 2-1). Two types of bone microstructure exist which are categorized primarily by their porosity: cortical bone and trabecular bone, with cortical bone being of a much greater density as compared to trabecular bone. Trabecular bone is arranged
in a 3-dimensional open-porous network of interconnecting struts called trabeculae. Cortical bone is made up of mostly concentric lamellae surrounding a network of channels known as Haversian canals. A blood vessel found within each of these canals serves the source of nutrients for the cells that reside within the bone. The concentric lamellae surrounding the Haversian canal further exhibit an even smaller porous network of interconnecting channels and chambers known as the lacunar-canalicular system. In long bones, the primary weight-bearing bones in the body, the interior volume of this tissue is made up of trabecular bone which serves as a weight-efficient load-bearing structure and provides sufficient porosity within the bone to house bone marrow, a source of both mesenchymal and hematopoietic stem cells. The trabecular bone volume is surrounded by a dense layer of cortical bone, which serves to provide resistance to bending and is the primary load-bearing feature in long bones.
2.1.2 Bone Remodeling and Functional Adaptation

Considering bone’s structural and mechanical functions, in which the tissue is constantly subjected to both static and dynamic loading, it is intuitive to understand the importance of bone remodeling as a strategy for self-repair against cracks, fractures, and other loading-induced defects. Bone remodeling is the process of removing both damaged and old bone followed by the formation of new bone. This action is accomplished through different bone cell types. Specifically, osteoclasts are recruited to the site of bone resorption to degrade and resorb the bone matrix while osteoblasts are subsequently recruited to lay down new bone matrix which then becomes mineralized. In adults, this process of bone resorption and bone formation must be carefully balanced as too much or too little bone resorption will lead to bone diseases such as osteoporosis and osteopenia. Besides the ability to self-repair, bone must also be able to adapt its structure to better accommodate the mechanical forces placed upon it. This functional adaptation of bone is convincingly documented in subjects who experience strenuous physical activity (e.g., athletes) and in individuals who experience long durations of mechanical disuse (e.g., astronauts in a weightless environment, patients after long durations of bed rest) and was first demonstrated by Wolff et al. The

2.1.3 Bone cell Mechanotransduction

Load-bearing bones within the body experience dynamic tissue-level strains during functional loading (e.g., walking, running). Deformation of the tissue is transformed into
cellular-level mechanical stimuli in the form of interstitial fluid flow within the bone microstructure due to the formation of pressure gradients.\textsuperscript{5, 6} As the tissue loading profile during physical activity (e.g., walking or running) is cyclic in nature, the fluid flow profile at the cellular level is predicted to be sinusoidal and oscillating (i.e., no net fluid displacement).\textsuperscript{7}

Mechanotransduction is the mechanism by which cells convert a mechanical stimulus into a biochemical response. In bone, the phenomenon of load-induced bone remodeling signifies the existence of at least one type of mechanosensory cell within bone that is capable of responding the fluid flow within the bone porosity and converting this signal into a biochemical response capable of regulating osteoblast and osteoclast activity. Much about this process remains to be determined, however recent evidence suggests that osteoblast and osteoclast activity is regulated, at least in part, through soluble signaling molecules released by mechanosensory cells.\textsuperscript{7-10} A number of these soluble factors have been indentified including prostaglandin E\textsubscript{2} (PGE\textsubscript{2}), sclerostin, receptor activator of nuclear factor kappa B (NF-\textkappa B) ligand (RANKL), and osteoprotegerin (OPG).\textsuperscript{1-3, 11} Briefly, PGE\textsubscript{2} acts on osteoblasts in an autocrine fashion to promote increased bone formation while sclerostin acts as an antagonist to osteoblastic bone formation. RANKL, through complexation with RANK receptors located on the surface of osteoclast precursors, stimulates pre-osteoclast commitment to the osteoclastic phenotype such that total bone resorption in enhanced. OPG, a third molecule secreted by mechanically stimulated osteocytes, acts as a decoy receptor that competes with RANK for the binding of RANKL such that the relative abundance of RANKL to OPG (RANKL/OPG) is indicative of the amount of bone resorption.

Additionally, although it is clear that bone cells are able to sense their mechanical environment, it is not completely certain which of the cells are the key sensory elements in
mechanotransduction. All cells of the osteoblastic lineage, including the undifferentiated mesenchymal precursor, the mature osteoblast, and the terminally differentiated osteocyte have been documented to be responsive to mechanical loading in vitro. However, for reasons detailed below and in the Introductions of Chapter 3, osteocytes are thought to fulfill this role.

2.1.4 Osteocytes

Osteocytes are terminally differentiated from mature osteoblasts, and compose of 90% of the cells found in bone.\(^2\) In addition to their abundance, they are defined by their stellite morphology and unique anatomical location within the bone matrix.\(^1\)\(^2\) Osteocytes inhabit a fluid-filled micro-porous network comprising of cavities within the mineralized bone matrix called lacunae. Extending from the cell bone are long, slender cytoplasmic processes that pass through the bone matrix via small canals termed canaliculi. The processes allow osteocytes to connect to neighboring osteocytes, to the cells lining the bone surface, as well as to the bone marrow compartment. The cell processes and membranes express connexins and gap junctions, thereby allowing electrical coupling as well as intracellular and extracellular transport of signaling molecules through the depths of the tissue. Tissue-level loading of bone induces fluid flow within the lacuno-canalicular network and, due to the tight confines of this porosity, large shear forces are expected to act on the osteocyte cell membrane.\(^5\),\(^8\) Therefore, osteocytes appear to be suitably placed for detecting mechanical stimuli in the bone environment and orchestrating the cellular activities of other bone cells, such as osteoblasts and osteoclasts, via transport of signaling molecules.
2.2 Nanowire FET Biosensors

Nanostructure Field Effect Transistor (nano-FET) biosensors are nano-scale electronic devices used to detect and measure the presence and concentration of specific biological molecules within a given sample solution. These sensors utilize a quasi-one-dimensional semiconducting nanostructure as the active sensing element to detect the presence of local electric fields produced by charged biological molecules.

2.2.1 Biomolecular Detection Technologies

Methods for detecting and measuring the presence, abundance, and activity of biological molecules, such as proteins and nucleic acids enable the investigation of basic biological function, molecular diagnosis of diseases, and development of therapeutic treatments. The combination of poly-acrylamide gel based electrophoresis separation (and related blotting techniques) and absorption-based chromogenic dye staining (e.g., Coomassie brilliant blue or silver staining) has traditionally formed the core technologies for protein and nucleic acid detection. Subsequent development of fluorescence and luminescence-based labeling stains offered the opportunity for multicolor labeling, making multiplexed analysis possible. However these methods were limited to the detection of biological molecules on gels and blots. Immuno-labeling of fluorescent and luminescent probes allowed specific protein detection and facilitated the emergence of immunoassays able to detect and quantify the abundance of a target protein molecule in solution. These optical detection methods suffer from large sample volume consumption, high reagent costs and long assay times which, when combined with the drawback of using labeling molecules, preclude the application of these technologies to many laboratory and clinical applications (e.g., real-time protein quantification and \textit{in vitro} point-of-care clinical diagnostics).
A number of novel label-free protein-detection strategies have since been developed in an effort to overcome some of the aforementioned limitations. Many of these strategies leverage non-optical transduction modalities to circumvent the limitations imposed optical probes. Examples include micro/nano-surface plasmon resonance sensors\textsuperscript{14}, micro- and nano-cantilevers that translate biomolecular interactions into mechanical deformations\textsuperscript{15}, nano-structured field effect transistors (FET) that measure intrinsic biomolecular charge\textsuperscript{13}, and electrochemical sensors that translate biomolecular adsorption to changes in redox current.\textsuperscript{16} Of these technologies, nano-FET biosensors offer unique device characteristics including ultrasensitive, real-time, and label-free measurement capability, compact physical form, CMOS compatible and simple on-chip integration with circuitry and microfluidic systems, direct electrical readout, and multiplexed detection capability.

2.2.2 Nano-FET Biosensor Structure

Nano-FET biosensors are composed of a semiconducting quasi-one dimensional nanostructure (e.g., nanowire or nanotube) bridging the source and drain electrodes (Figure 2-2). The nanostructure serves as the transistors’ ‘channel’ and functions as the sensing element of the device.\textsuperscript{13} The biosensor structure deviates slightly from that of the transistor in the placement and structure of the gating electrode. While the gate electrode in a nano-FET is typically located directly above the channel, separated by a thin dielectric layer, the nano-FET biosensor uses a ‘solution gate electrode’ that is typically composed of platinum or Ag/AgCl and is immersed within the aqueous solution being measured.\textsuperscript{17, 18} Furthermore, the nanostructure surface is functionalized with a layer of analyte specific receptors to impart a degree of specificity to the sensor.\textsuperscript{19} This may be accomplished via chemical bonding using bi-functional linker molecules, or through physical interaction due to adsorption, Van der Waals forces, and weak electrostatic forces.
While the specific nanostructure employed may vary in shape, size and composition, it is important that these structures be quasi-one dimensional and exhibit semi-conductive material properties. Typical nanostructures used in the construction of nano-FETs include single and multi-walled carbon nanotubes,\textsuperscript{20, 21} graphene\textsuperscript{16} and silicon nanowires.\textsuperscript{13, 22, 23} Devices constructed from other semi-conductive materials such as metal-oxides and nanostructures of various shapes including nanobelts,\textsuperscript{24} nanoribbons\textsuperscript{25} and nanobars\textsuperscript{26} have also been reported.

Figure 2-2  (a) Schematic of a single nano-FET biosensor. (b) Schematic of a nano-FET biosensor chip with integrated microfluidic sample delivery system. The nano-FET biosensor array is housed within the microfluidic system. (Adapted by permission from Macmillan Publishers Ltd:\textsuperscript{27}, copyright 2006)

2.2.3 Physics

Nano-FET biosensors utilize semi-conductive nanostructures to detect the presence of charged molecules in the immediate vicinity around the nanostructure surface.\textsuperscript{28} The sensing mechanism is based on the observation that an externally applied electric potential is capable of modulating the number of free charge carriers within a semi-conductive material, thus leading to
a measurable change in electrical conductance. For a cylindrical semiconducting nano-structure with radius $R$ and length $L$, the charge carrier concentration may be described as a function of the applied potential:

$$n_0 = \frac{C(V_G - V_t)}{q\pi R^2 L}$$

where $C$ is the gate capacitance, $q$ is the elementary charge, $V_G$ is the gate voltage and $V_t$ is the threshold voltage. As the conductance of the semiconducting nanowire is described by

$$G = nq\mu \left( \frac{\pi R^2}{L} \right),$$

the current passing through the nanowire ($I_D$) for a given source-drain voltage ($V_{DS}$) is therefore

$$I_D = \frac{\pi R^2}{L} n_0 q \mu V_{DS}$$

where $\mu$ is the carrier mobility. Charged functional groups present on the surface of bound biological molecules such as proteins and nucleic acids provide the necessary electric potential required to induce a conductance change in the semi-conductive nanostructure, thus enabling the direct electrical detection of such molecules using nano-FET devices (Figure 2-3). The strength of a molecule’s surface charge is a function of electrolyte pH and is characterized by the molecules isoelectric point.
Figure 2-3  Real-time nanowire FET sensing results. (a) Conductance versus time data recorded following alternate delivery of prostate-specific antigen (PSA) and pure buffer solution (1 μM phosphate (potassium salt) containing 2 μM KCl, ph 7.4). Subsequent PSA concentrations were 5 ng ml$^{-1}$, 0.9 ng ml$^{-1}$, 9 pg ml$^{-1}$, 0.9 pg ml$^{-1}$ and 90 fg ml$^{-1}$, respectively. (b) Complementary sensing of PSA using p-type (NW1) and n-type (NW2) nanowire FET devices. Vertical lines correspond to addition of PSA solutions of (1,2) 0.9 ng ml$^{-1}$, (3) 9 pg ml$^{-1}$, (4) 0.9 pg ml$^{-1}$ and (5) 5 ng ml$^{-1}$. Arrows on the bottom represent the injections of sensing buffer solution. (Adapted by permission from Macmillan Publishers Ltd: 27, copyright 2006)

The simplified analysis qualitatively illustrates the physical sensing mechanism behind nano-FET biosensors. However, this treatment does not accurately describe the complexities of the nano-FET biosensor system. Equations (1)-(3) describe the electrical behavior of a cylindrical semiconducting crystal subjected to a uniform charge density over the entire nano-structure surface. This assumption fails to take into consideration the non-uniform nano-structure surface charge distribution resulting from discrete biomolecular binding events. The discrete gating mechanism by individual molecules in solution introduces several additional phenomena that significantly influence the sensor’s response including depletion of analyte from solution,
analyte transport mechanisms to the nano-structure surface, and analyte-receptor binding and unbinding kinetics. These effects become particularly important in the detection of rare analytes.

Equations (3) also neglects the influence of electrostatic screening – the spatial arrangement of mobile ions around a charged molecule to effectively neutralize that charge. Importantly, target analytes must be sufficiently close to the sensor surface in order to elicit a conductance change due to electrostatic screening by mobile charges around the analyte molecule (e.g., ions in solution, charge carriers in the semiconducting crystal). The Debye screening length, the distance away from a charged molecule at which the electrostatic potential is diminished to zero due to screening, defines the required proximity.

Debye length (aqueous electrolyte):

\[
k^{-1} = \frac{1}{\sqrt{8\pi \lambda_B N_A I}}
\]

where \( I \) is the ionic strength of the electrolyte (mole/m\(^3\)), \( N_A \) is the Avogadro number, and \( \lambda_B \) is the Bjerrum length of the medium (0.7 nm for water).

Debye length (silicon):

\[
L_D = \sqrt{\frac{\varepsilon_0 \varepsilon_r k_B T}{q^2 N_D}}
\]

where \( \varepsilon_r \) is the dielectric constant of the semiconducting material, \( k_B \) is the Boltzmann’s constant, \( T \) is the absolute temperature in Kelvin, \( q \) is the elementary charge, and \( N_D \) is the density of donors in a substrate. The implications of charge screening and a finite Debye length make several important predictions on nano-FET biosensing performance. Namely, these relations
suggest a conductance dependence on charge carrier concentrations (ionic strength of an electrolyte solution and doping density of a semiconductor), size of the semiconducting crystal, strength of the molecule’s net surface charge and the nanostructure-analyte separation distance. Inclusion of these phenomena into a unified analytical model that accurately describes experimental nano-FET biosensor response is an active area of research.

2.2.4 Performance Parameters

1) **Nano-FET sensitivity** is the induced change in device conductance ($\Delta G_{sd}$), upon exposure to a certain biomolecular stimulus for a constant source-drain voltage ($V_{ds}$). Sensitivity is defined as

$$\text{Sensitivity} = \frac{\Delta G_{sd}}{G_0}$$

where $\Delta G_{sd}$ is the direct conductance change observed in sensing experiments and $G_0$ is the initial device conductance. Because $\Delta G_{sd}$ depends on the specific nanowire parameters (e.g., diameter, mobility, etc.) this value does not reflect the intrinsic sensitivity. Instead, it is more meaningful to characterize the sensitivity using a dimensionless parameter (equation 6). Normalization enables comparison across devices with different physical dimensions.

Sensitivity is closely related to three additional parameters: *detection limit* (the smallest analyte concentration that can be measured, which is dictated by the system signal-to-noise ratio); *measurement resolution* (the smallest detectable change in analyte concentration, which is dictated by noise levels); and *measurement range* (the measurable analyte concentrations, with the upper and lower bounds defined by nanostructure saturation and the detection limit, respectively). A higher sensitivity directly translates into lower detection limit, finer measurement resolution, and a broader measurement range. Typical protein and DNA detection
limits reported in the literature are in the pM (picomolar) to fM (femtomolar) range.\textsuperscript{23, 26} Single virus detection has also been achieved.\textsuperscript{22}

2) **Specificity** is a measure of a biosensor’s relative responsiveness to the target analyte as compared to all other molecules present within the sample solution.\textsuperscript{35} Very specific sensors should possess high sensitivity towards the target analyte and low sensitivity towards all other biomolecules. This attribute is imparted onto a nano-FET biosensor by functionalizing the nanowire surface (either covalently or non-covalently) with a confluent monolayer of analyte-specific receptors/ligands that bind specifically to the molecule of interest.

3) **Response time** is the time it takes for the biosensor system to obtain a stable biomolecular concentration measurement upon introduction of the sample solution to the sensor surface. As with any dynamic system, this characteristic is captured in the step response of the system by the *settling time*. For a nano-FET biosensor, this parameter is influenced by the analyte-receptor binding and unbinding kinetics, transport of analyte molecules to the sensor surface, concentration and degree of mixing of the analyte solution, and the electrical response behavior of the measurement system.\textsuperscript{26, 36} The typical response time of nano-FET biosensors is on the order of minutes.

### 2.3 Optimization of Sensor Performance

Active areas of research in the field of nano-FET biosensing include: (1) optimization of biosensor performance based on the systematic examination of how fundamental device parameters affect sensitivity, specificity, and response time; (2) development of robust large-scale fabrication strategies for the manufacturing of biosensor arrays; and (3) development of an
analytical model capable of accurately describing and predicting nano-FET biosensor performance. In this section, we explore several novel strategies pursued by other research groups to optimize or enhance nano-FET biosensor performance. In the next section, we summarize several large-scale fabrication methods for manufacturing arrays of nano-FET biosensors.

2.3.1 Minimizing the Effects of Charge Screening

As the effect of charge screening acts to minimize the total nanostructure volume gated by surface charges, thus reducing device sensitivity, strategies that increase screening length are expected to improve device sensitivity. The maximum device sensitivity may be obtained in a situation where the effective screening length is much larger than the radius of the nanowire. Improved device sensitivity can thus be obtained through careful design and optimization of several device parameters that influence the effective screening length:

2.3.1.1 Diameter

Reduction of the nanostructure diameter will dramatically increase the surface-to-volume ratio, thus improving device sensitivity. This is the primary advantage of decreasing the channel size and explains why nano-structure FET sensors exhibit significantly higher performance as compared to traditional planar FET devices.

2.3.1.2 Dielectric Thickness

Silicon nanowires form a native oxide layer (~1-2 nm) on their surface that serves as a dielectric layer reducing the electric field strength acting on the nanowire itself. This layer also increases the separation distance between surface-bound molecules and the semi-conductive core
of the nanowire. Thus, a thinner oxide layer is expected to improve sensitivity. Complete removal of the native oxide layer surrounding silicon nanowires has been shown to increase device sensitivity\textsuperscript{37,38}.

2.3.1.3 Functionalization Scheme and Receptor Size

Decreasing the separation distance between the receptor-bound analyte molecule and the nanostructure surface minimizes the effect of electrolyte screening. While antibodies are commonly used to selectively bind analyte molecules to the biosensor surface, the use of smaller capture probes such as antibody fragments or aptamers in place of antibodies can increase device sensitivity without the loss of selectivity.\textsuperscript{19,39} Similarly, utilizing shorter bi-functional linker molecules to attach the capture probes to the nanostructure surface may also increase device sensitivity. The choice of functionalization strategy may also dictate the capture probe density and coverage on the nanostructure surface. A poor capture probe density can result in the non-specific adsorption of molecules on to unmodified areas of the nanowire surface and may also limit the upper detection limit of the sensor due to capture probe saturation.

2.3.1.4 Electrolyte Ion Concentration and pH

The ionic strength of the electrolyte solution influences the efficiency of charge screening as the density of free ions is directly related to size of the electric double layer formed around a charged molecule. Higher ionic strength electrolytes are thus able to screen a charged molecule over a shorter distance. It is therefore advantageous to perform biosensing experiments in low-ion conditions to maximize sensitivity\textsuperscript{30}. However, this is not always feasible as many sensing applications are performed with physiological samples (e.g., serum, whole blood, cell culture media, etc).
The pH of a solution also influences device sensitivity by changing the effective electrical charge on a biological molecule. Biological molecules such as proteins and nucleic acids contain both acidic and basic functional groups that may be positively or negatively charged depending on the availability of protons (H\(^+\)) present in solution. The pH for which the negative and positive charges on the molecule are balanced, resulting in zero net charge, is defined as the isoelectric point (pI). The net charge on a molecule is therefore a function of the electrolyte pH and can become increasingly more positively or negatively charged as the term |pI-pH| increases. While tuning this parameter may be useful in specific applications, the pH of a sample is usually fixed at physiological levels (pH = 7.4).

### 2.3.1.5 Carrier Concentration

The screening length within a semi-conductive nanowire dictates the depth of penetration into the nanostructure for which surface-bound charged molecules are able to gate. For example, the screening length in a silicon nanowire operating with a typical carrier concentration of \(10^{18}-10^{19} \text{ cm}^{-3}\) is ~1–2 nm, suggesting that only a small portion of the total nanowire volume (a ~1–2 nm thick layer at the nanowire surface) is affected by surface charges. Decreasing the charge carrier concentration will therefore increase device sensitivity by increasing the screening length and the total gated volume. This can be achieved by varying either of two device parameters: the nanostructure doping density, or the bias voltage applied through a gate electrode on the nano-FET biosensor. In terms of semiconductors, lower doping densities result in longer screening lengths and therefore produce higher sensitivity devices.\(^{31}\) It is instructive to note that the optimum biosensor sensitivity is obtained when the device is operating in the sub-threshold regime, as under these conditions the trans-conductance is at a maximum.\(^{18, 34, 40}\)
2.3.2 Optimization of Analyte Delivery Efficiency

Biomolecular detection using nano-FET sensors requires the delivery of analyte to the sensor surface. The efficiency of this process can therefore limit the performance of nano-FET biosensors. Several phenomena that influence this process include diffusive and convective molecular transport mechanisms of analyte to the biosensor surface, depletion of free analyte from solution, and non-specific analyte adsorption and binding.

2.3.2.1 Molecular Transport

As with many surface-based biosensor systems, the transport of analyte in solution to the sensor surface plays a crucial role in governing binding kinetics and ultimately sensor performance. This is especially true when analyzing samples with dilute concentrations of target molecules and/or employing microfluidic systems for efficient and automated handling of small sample solution volumes. A number of competing physical processes influence target transport to the sensor surface. Analyte molecules suspended in solution may diffuse randomly within the solution, be convected along with flowing fluid, bind to adjacent surface-bound receptors, or subsequently un-bind to re-enter solution. For diffusion-based transport, the binding (or collection) of biomolecules onto the sensor surface simultaneously depletes the surrounding solution to form a so-called depletion region around the sensor. As the depletion region grows in size, the analyte diffusive flux decreases and thus the collection rate becomes slower. Through finite element analysis, Sheehan and Whitman\textsuperscript{36} determined that the size and shape of the sensor profoundly affects the total analyte flux to the sensor surface and further argues that without directed transport of biomolecules, individual nanoscale sensors would be limit to pico-molar order sensitivity for practical time scales (hours to days).\textsuperscript{36}
While the diffusive depletion zone grows indefinitely, albeit at an ever-decreasing rate, introduction of convective flow (assumed to be laminar) halts this growth resulting in a steady-state depletion zone with a length scale defined by the balance between the convective analyte flux delivered to the depletion zone boundary and the diffusive flux out of the depletion zone. By adjusting the convective flow rate through the channel, it is possible to tune the size of the depletion layer and thus control the rate at which molecules are delivered to the sensor surface. However, as Squires et al. pointed out, the total mass transport varies only weakly with the flow rate as flow rates must be increased 1,000-fold to enhance flux by a factor of 10. The treatment thus far has assumed instantaneous analyte-receptor coupling upon transport of target molecules to the sensor surface. This represents one of two sensing regimes that can be described as being ‘transport-limited’ as the availability of analyte adjacent to the sensor surface limits sensor response.

A second biosensing regime arises when considering the kinetics of analyte-receptor association and dissociation (assumed to be first-order Langmuir kinetics). If delivery of target molecules to the sensor surface occurs at a faster rate than the net analyte-receptor association rate, then the transport is said to be ‘reaction limited’. In general, the reaction kinetics is determined by the fidelity of the immobilized reagents. While each of the two regimes imposes an independent maximum target collection rate, the ‘reaction limited’ condition is most desirable for nano-FET operation as, for a given device, biomolecular sensing is purely a function of analyte concentration. The sensor size and flow rate should thus be adjusted to ensure nano-FET operation within this regime. Alternatively, introduction of turbulent flow or fluid mixing through innovative microfluidic design may also be employed to overcome the limitations imposed by convective and diffusive molecular transport.
2.3.2.2 Depletion of Free Analyte

As silicon and metal-oxide nanostructures share similar surface chemistries with several popular microfabrication substrates (e.g., silicon wafers, glass, quartz), surface modification techniques used to attach receptor molecules to the nanostructure surface can also functionalize the device substrate. In this situation, the total device surface area exposed to the sample solution during biomolecular detection experiments is proportional to the number of receptors available for reaction. Analyte binding events with substrate-bound receptors do not induce a change in nanostructure conductance. Rather, these reactions serve only to deplete free analyte molecules from solution, thus reducing the number of analyte molecules available for reaction with nanostructure-bound receptors. Minimizing the total surface area exposed to sample solutions is therefore, important for maximizing device sensitivity. This effect may be circumvented using nanostructure-specific surface modification chemistries.

2.3.2.3 Non-Specific Adsorption

Random adsorption of biological molecules onto the nano-FET sensor produces a false-positive measurement signal. This effect may be minimized by grafting a dense layer of long non-reactive polymer chains (e.g., poly-ethylene glycol) onto the nanostructure surface alongside receptor molecules. The polymer chains prevent molecular adsorption directly onto the nanostructure surface by acting as a spacer while the co-functionalized receptors are able to bring analyte molecules into intimate contact with the nanostructure through molecular binding. The length of the polymer chains used for this application should exceed the screening length of the system.
Non-specific adsorption of random molecules is especially problematic when working with samples containing numerous types of biological molecules such as whole blood. One strategy that enables detection of a specific analyte within a whole blood sample involves the use of a sample pre-purification procedure before nano-FET measurement. In this method, the whole blood is introduced into a chamber in which analyte-specific receptors have been immobilized on to the chamber walls. Upon binding of free analytes to the surface-bound receptors, the whole blood solution is flushed out of the chamber and discarded. The receptors are then detached from the chamber surfaces and transported to the nano-FET biosensor for analysis.

2.4 Methods for Large-Scale Nano-FET Fabrication

Reliable large-scale fabrication of nano-FET biosensors remains to be a significant technological challenge, impeding the adoption of this technology in medical diagnostic and biomolecular detection applications. Unlike traditional microfabrication methods for producing micrometer-level structures, the difficulties encountered in nano-FET biosensor fabrication arise from the process of establishing electrical contact with nano-sized structures in a high-throughput and reproducible manner.

Early methods for manufacturing nano-FETs involve (1) the growth of individual nanostructures using a chemical vapor deposition process, (2) transfer of as-grown nanostructures from the growth substrate on to a device substrate (a process which results in the random placement and orientation of the deposited nanostructures), and (3) identification of a suitable nanostructure on the device substrate followed by the patterning of metallic electrodes.
onto the ends of that nanostructure using a mask-less nano-lithographic tool (e.g., electron beam lithography or focused ion beam lithography), which has a low throughput and is only suitable for proof-of-principle use. Recently devised batch-fabrication strategies for the wafer-scale production of nano-FET arrays can be broadly categorized into ‘bottom-up’ or ‘top-down’ fabrication methods.

2.4.1 Bottom-Up Fabrication Methods

One strategy for bottom-up fabrication is to make use of pre-synthesized free-standing nanostructures using epitaxial growth methods such as chemical vapor deposition. This strategy affords several advantages over top-down and other bottom-up approaches including (1) strict control over nanostructure dimensions and electrical properties, (2) the ability to assemble nano-FET devices on to flexible and transparent substrates, and (3) the ability to incorporate nanostructures with unique chemical compositions and architecture into nano-FET devices. Successful incorporation of these nanostructures into nano-FET devices relies primarily on the ability to transfer, align and position the as-grown nanostructures onto a device substrate. Contacting electrodes may then be patterned using conventional photolithography to form individual sensors or sensor arrays.

2.4.1.1 Flow Alignment

Flow Alignment is a method that makes use of flowing fluid to align suspended nanostructures along a single orientation along the direction of flow. By passing a nanostructure suspension through a microfluidic structure formed between a poly(dimethylsiloxane) mold and the device substrate, free-flowing nanostructures assemble onto
the device substrate due to surface forces and remain relatively aligned parallel to the direction of flow. The resulting degree of alignment is a function of the flow rate as increased flow velocities result in a narrower angular distribution amongst deposited nanostructures. Furthermore, varying the total flow duration dictates the nanostructure deposition density, with longer flow durations favoring higher nanostructure densities. This method is useful in controlling the degree of nanostructure alignment and the deposition density, thus allowing subsequent electrode formation. Nano-FET fabrication can therefore be achieved by patterning a pair of electrodes parallel to the flow direction using conventional microfabrication processes. For this physical arrangement, minimizing the electrode pair separation distance increases the likelihood of a bridging nanostructure between the electrode pair; however the exact number of bridging nanostructures resulting from this process is probabilistic.

2.4.1.2 Nanostructure Contact Printing

Nanostructure Contact Printing (Figure 2-4) is a method that provides similar results to the flow alignment strategy. This method, however, relies on mechanical shear forces to detach free-standing nanostructures from the growth substrate, and friction to align the deposited the nanowires onto the device substrate. In this method, the growth substrate is inverted and placed on top of the device substrate such that the nanostructures are sandwiched in between. Weights are added on top of the growth substrate to control the applied pressure between the two substrates in order to optimize the deposited nanostructure density. The growth substrate is then pulled over the device substrate at a continuous velocity in order to deposit and align the nanostructures onto the device substrate. The shear velocity and the amount of friction between the two substrates influence the resulting degree of nanostructure alignment. The latter may be adjusted with the use of a lubricant.
Figure 2-4  Contact printing of nanowires. (a) Schematic of the contact printing process for producing well-aligned nanowire arrays. (b) Dark-field optical and (c) SEM images of 30 nm nanowires printed on a Si/SiO2 substrate showing highly dense and aligned monolayer of nanowires. The self-limiting process limits the transfer of nanowires to a single layer, without significant nanowire bundling. (Adapted with permission from 43. Copyright 2008 American Chemical Society.)

2.4.1.3 Thin-Film Nanowire Suspension

Thin-Film Nanowire Suspension (Figure 2-5) is a method that makes use of the observation nanostructures suspended within a liquid thin film will align along a single direction when that film is elongated. Therefore, this method involves the formation of a large balloon from a nanostructure suspension.44 The aligned nanostructures within the thin film can then be transferred to a substrate by placing the substrate in contact with the balloon. Nano-FET fabrication would subsequently proceed as in the case of flow alignment.
Figure 2-5  Blown bubble film approach. Nanostructures are dispersed in a polymer solution. A volume of solution is expanded as a bubble using a die to produce well aligned nanostructures suspended within a thin film. The film is then contacted with a substrate to deposit well-aligned nanostructures onto the substrate. (Reproduced by permission of The Royal Society of Chemistry)

2.4.1.4 Directed Self-Assembly

Directed Self-Assembly (Figure 2-6) relies on the minimization of surface energy to assemble suspended nanostructures into predefined locations on a substrate. In this method, surface modification strategies are used to render certain portions of the device substrate either hydrophobic or hydrophilic. Upon immersion of the substrate into a nanostructure suspension, individual nanostructures will self-assemble onto the hydrophobic portions of the substrate. This strategy can be used to deposit individual nanostructures at specific locations over a large area on a substrate. A similar strategy using analyte-ligand interactions has also been employed in which suspended nanostructures are functionalized with biotin molecules. Upon immersion of a substrate with bound streptavidin molecules at predefined locations, biotin-streptavidin conjugation forces the nanostructures to assemble onto the device substrate at pre-defined locations.
Figure 2-6  Linker-free directed self-assembly. (a) The patterning process of octadecyltrichlorosilane self-assembled monolayer on a solid substrate via photolithography. (b) Self assembly suspended nanostructures onto the bare surface regions on the substrate. Subsequent photolithography and metallization can be used to pattern electrodes onto the ends of the deposited nanostructures. (Adapted by permission from Macmillan Publishers Ltd. Nature Nanotechnology 45: copyright 2006)

2.4.1.5 Dielectrophoresis

Dielectrophoresis (DEP) utilizes electrical fields to manipulate nanostructures. In this method, metallic contact electrodes are patterned on the device substrate prior to the assembly of nanostructures onto the device. By applying a biased alternating voltage across the electrodes, a local non-uniform electric field is produced, exerting a force on suspended semi-conducting nanostructures. This force causes the nanostructures to assemble across the two electrodes. This process is easily amenable to the large-scale production of nano-FET arrays and can be used to fabricate single nanostructure FET devices, however typical DEP trapping processes yield
networked multi-nanostructure FET devices. This process is highly susceptible to the experimental conditions including the size, shape, and properties of the nanostructure to be manipulated, and parameters of the electrical signals, as well as the electrical properties of the surrounding medium.

2.4.1.6 In-situ Growth of Nanostructures

In-situ Growth of Nanostructures is a method that utilizes patterned catalysts to selectively synthesize nanostructures at certain regions on the device substrate. These catalysts (e.g., iron or gold particles) are required to initiate and/or propagate nanostructure growth. In some cases, the direction of nanostructure growth can be influenced with the application of an electric field, therefore enabling control of the resulting nanostructure orientation. This method usually produces numerous nanostructures and is therefore, useful in fabricating nano-FET sensors that utilize a network of nanostructures.

2.4.2 Top-Down Fabrication Method

Top-down methods are typically based on the anisotropic lateral wet etching of nanometer-thin SOI (silicon-on-insulator) wafers to produce well-defined nanostructures from the device layer (Figure 2-7). Micron-sized etch masks are patterned via conventional photolithography and anisotropically time-etched to produce nanometer-wide nanostructures. Source and drain electrodes are degenerately doped, rendering them conductive and unaffected by the etchant. This approach enables wafer-scale formation of semiconducting nanostructures at precise locations on a wafer-scale, making subsequent electrode fabrication relatively straightforward using conventional photolithography. As this method relies on time-controlled anisotropic etching for the removal of material, it is highly susceptible to small changes in
processing conditions such as etch time, processing temperature and mixing, and device-layer doping density and crystal orientation.

Figure 2-7  (a) Schematic of Nano-FET device after anisotropic etching. The silicon-on-insulator active channel (yellow, width w and thickness t) is undercut etched, whereas degenerate leads (red) are etch-resistant. The source (S), drain (D), and underlying backgate (G) are labeled. (b) Scanning electron micrograph of a complete device. (Adapted by permission from Macmillan Publishers Ltd: Nature 45, copyright 2007)

2.5 Nano-FET Biosensors: Challenges and Research Directions

While the promise of highly sensitive, label-free electrical sensors with real-time measurement capability is attractive for numerous medical and basic science research applications, at this point in time, the development of nano-FET biosensors is still in the research phase. Great progress over the past decade has been made in nano-FET fabrication and detection. However, current large-scale fabrication techniques are unable to precisely position individual nanostructures at predefined locations on a substrate in a reliable manner. As such, current
manufacturing processes, which are highly susceptible to processing conditions and probabilistic positioning of nanostructures, result in nano-FET devices with significant variability and device-to-device inconsistency. Thus, continued research in along this direction will be essential in the commercialization of this technology. Further efforts in the area of nano-FET biosensor integration with microfluidic and lab-on-chip devices will also be required to facilitate point-of-care diagnostic applications and real-time closed-loop drug delivery systems. Similarly, these applications will likely require improved sensor specificity and sensitivity in order to handle human serum or whole blood samples. By overcoming these limitations, multiplexed nano-FET biosensor arrays may be applied to a number of applications in the area of combinatorial chemical and biological research, high-throughput screening systems for drug development, point-of-care diagnostics and novel in vitro biology experimentation.
2.6 References


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Chapter 3
Effect of Oscillating Fluid Flow Stimulation on Osteocyte Activity

3.1 Introduction

Bone is a dynamic tissue that can respond to mechanical loading by adapting its mass and structure through the process of bone remodeling. While the concept of functional adaptation in bone is well accepted, the underlying mechanisms by which this is accomplished remains poorly understood. Mounting evidence suggest that a) tissue-level strains are translated to cellular-level mechanical stimuli in the form of interstitial fluid flow within the lacunar-canalicular system, a fluid-filled network of interconnected pores permeating throughout the bone tissue;1-6 b) bone remodeling involves the tightly regulated interaction of multiple bone cell types acting through multiple signaling pathways;7-10 and c) osteocytes, terminally differentiated osteoblastic cells that reside within the lacunar-canalicular system, are able to sense and respond to mechanical stimulation in a coordinated and cohesive manner.11 This ability of osteocytes to sense and respond to fluid flow stimulus has been widely demonstrated in vitro.7,11 Due to their abundance and distribution throughout the bone matrix, osteocytes rather than osteoblasts are thought to be the primary mechanosensors in bone.12,13 This hypothesis is supported by in vivo evidence demonstrating that targeted ablation of osteocytes in mice induces the onset of osteoporosis and results in defective mechanotransduction suggesting that osteocytes play a critical role in this process,14 as well as in vitro evidence suggesting that osteocytes and osteoblasts respond differently to fluid flow stimulation, suggesting that they play different roles in the bone remodeling process.15,16
In vitro mechanical stimulation of osteocytes has been demonstrated to regulate the production of several soluble signaling molecules known to influence osteoblast and osteoclast activity including prostaglandin E2 (PGE2), receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL), and osteoprotegerin (OPG). Briefly, PGE2 acts on osteoblasts in an autocrine fashion to promote increased bone formation while RANKL, through complexation with RANK receptors located on the surface of osteoclast precursors, stimulates pre-osteoclast commitment to the osteoclastic phenotype such that total bone resorption is enhanced. OPG, a third molecule secreted by mechanically stimulated osteocytes, acts as a decoy receptor that competes with RANK for the binding of RANKL such that the relative abundance of RANKL to OPG (RANKL/OPG) is indicative of the amount of bone resorption. At the gene transcription level, physical stimulation of osteocytes has been shown to decrease the RANKL/OPG mRNA ratio while at the same time increase cyclooxygenase-2 (COX-2) mRNA expression. COX-2 is an essential enzyme in the synthesis of prostaglandin E2 (PGE2) and, since PGE2 is not stored by the cell but rather synthesized and released as needed, the expression levels of COX-2 can be directly correlated to PGE2 release. Taken together, osteocytes exposed to physiological level of mechanical stimuli produce and secrete soluble signaling molecules that regulate osteoblast and osteoclast activity in an autocrine fashion with the net effect of increasing bone formation.

The degree of cellular response elicited by osteocytes subjected to fluid flow stimulation remains largely unexplored and has been suggested to be a function of various fluid flow parameters including the applied shear stress magnitude, dynamic flow frequency, flow duration, and the number of loading cycles. Of these four parameters, the first three listed here are independent parameters, while the number of loading cycles is a dependent parameter on both
the total flow duration and the dynamic flow frequency. This relationship between these flow parameters and osteocyte activity is further complicated by the observation that osteocytes are additionally sensitive to the fluid flow stimulus profile (e.g., steady, pulsating, and oscillating fluid flow). These observations illustrate that osteocytes are exquisitely sensitive to different loading conditions and loading profiles. A thorough understanding of loading-induced bone adaptation and the etiology of some bone diseases, such as osteoporosis, therefore requires the systematic characterization of osteocyte biochemical response under different fluid flow stimulus conditions. To date, however, there have been no systematic investigations examining the effect of different oscillatory fluid flow conditions on osteocyte activity. Herein we study the effect of oscillating flow-induced shear stress magnitude, load number, oscillating frequency, and total flow duration on osteocyte mRNA expression levels. We limit our study to the oscillating fluid flow profile because this flow profile is considered to be the most relevant in describing the in vivo loading environment experienced by osteocytes. Specifically, we investigate the effects of these conditions on the mRNA expression of RANKL, OPG, and COX-2, as the expression levels of these genes has been previously reported to be indicative of load-induced bone remodeling. Therefore, we hypothesize that osteocyte are sensitive to different oscillating fluid flow parameters including shear stress amplitudes, oscillating frequencies, and flow durations.
3.2 Methods

3.2.1 Cell culture

MLO-Y4 osteocyte-like cells (gift from Dr. Lynda Bonewald, University of Missouri-Kansas City, Kansas City, MO, USA) were cultured in α-Modified Eagle’s Medium (α-MEM, GIBCO™) supplemented with 2.5% fetal bovine serum (FBS, Hyclone), 2.5% calf serum (CS, Hycone), and 1% penicillin/streptomycin (P/S. GIBCO™). They were subcultured onto collagen-coated polystyrene dishes (type I rat tail collagen, BD Biosciences) and grown in a 37°C, 5% CO₂ atmosphere to 70% confluence. In preparation for fluid flow stimulation, MLO-Y4 cells were subcultured onto collagen-coated glass slides (75 mm x 38 mm x 1 mm), grown to 70% confluence and starved for 12 hours in α-Modified Eagle’s Medium (α-MEM, GIBCO™) supplemented with 0.2% FBS and 1% P/S.

3.2.2 Fluid flow stimulation

Collagen-coated glass slides seeded with MLO-Y4 cells were assembled into parallel-plate flow chambers for administration of oscillating fluid flow stimulus. Each chamber consisted of a polycarbonate manifold which houses a glass slide, a rubber gasket and a polycarbonate cover. The resulting rectangular fluid volume measured 75 mm x 38 mm x 0.28 mm. Oscillating (sinusoidal profile) laminar fluid flow was delivered to the assembled flow chamber through a polyethylene tube that connected the chamber inlet to a glass syringe, which was driven by a custom-built linear actuator. The chamber outlet was exposed to the atmosphere. The desired fluid shear stress magnitude within the flow chamber was determined by varying the volumetric flow rate $Q$ according to the closed form equation
\[ \tau = \frac{\mu Q}{bh^2} \]

where \( \mu \) is the viscosity, \( b \) is the flow channel width, and \( h \) is the flow channel height. The volumetric flow rate and oscillating flow frequencies were controlled by varying the diameter and stroke length of the glass syringe as well as the stroke speed.

Oscillating fluid flow stimulus parameters examined in this study are listed in table 1. The peak shear stress levels experienced by the osteocytes were 0.5, 1.0, 2.0, and 5.0 Pa. These shear levels were selected to encompass the predicted in vivo physiological shear stress ranges experienced by osteocytes within in the lacuna-canalicular system.\(^1\),\(^28\) The oscillating fluid flow frequencies were 0.5, 1.0, and 2.0 Hz. These values were selected to reflect normal walking and running frequencies, and thus mimicking the loading frequency of long bones during these activities. The total flow durations tested were 1, 2 and 4 hours. Experimental controls (no-flow experiments) consisted of collagen-coated glass slides seeded with MLO-Y4 cells (70-80% confluent and starved for 12 hours) that were placed into parallel-plate flow chambers but not subject to fluid flow stimulation for the corresponding amount of time. All flow and control experiments were performed at 37°C in a 5% CO\(_2\) atmosphere.

### 3.2.3 mRNA quantification

After the end of flow, MLO-Y4 cells were removed from the flow chambers and rinsed with phosphate buffered saline (PBS, pH = 7.4). The total RNA was extracted from the MLO-Y4 cells (n = 4) using an RNeasy Mini Kit (Qiagen, USA). The extracted RNA was treated with DNase I (Fermentas) and reverse-transcribed using SuperScript™ III RT (Invitrogen, USA) to synthesize
cDNA. Quantitative PCR (qPCR) was used to amplify and quantify the amount of cDNA in each sample using gene-specific primers (table 2) and SYBR Green I (Roche, USA) in a Mastercyler ep Realplex machine (Eppendorf, USA). For each gene, the copy number for each experimental group was normalized to the 18S (housekeeping gene) and their respective controls such that comparison between the different experimental conditions can be made.

3.2.4 Statistics

Student t-tests (performed with SPSS software) were used to determine significant differences between OFF groups and no-flow groups (control). ANOVA was used to compare means of more than three groups, followed by Tukey posthoc test. Statistical significance was defined as P<0.05 (two tailed).

3.3 Results

Osteocytes subjected to oscillating fluid flow stimulus displayed transcriptional sensitivity towards each of the three fluid flow parameters varied in this study. Figure 3-1 shows the normalized COX-2 and RANKL/OPG mRNA levels for four different shear stress amplitudes (0.5, 1, 2 and 5 Pa) while all other fluid flow parameters are held constant. In generally, osteocytes subjected to oscillatory fluid flow stimulus exhibited elevated COX-2 mRNA levels (Figure. 3-1a) and decreased RANKL/OPG mRNA levels (Figure. 3-1b) as compared to no-flow controls, which have a normalized value of 1. The strength of osteocyte response (degree of increase in COX-2 mRNA levels and decrease in RANKL/OPG) was found to be a function of the peak shear stress amplitude, implying that osteocyte response was dose-dependent. Notably,
COX-2 mRNA expression was not significantly different (p>0.05) from their respective no-flow control groups at lower peak shear stress amplitudes (typically 0.5 Pa flow conditions and occasionally 1 Pa flow conditions depending on the other parameters), but became significantly different from controls (p<0.05) at higher shear stress amplitude flow conditions (Figure 3-1a). Similarly, statistically significant differences (p<0.05) for RANKL/OPG mRNA levels, as compared to no-flow controls, were more readily observed for flow conditions with higher shear stress amplitudes (Figure 3-1b). Osteocytes that experienced the greatest shear stress magnitudes (5 Pa) exhibited the highest COX-2 mRNA levels. This trend was maintained across all experimental groups with different flow frequencies and flow durations (Figure 3-1a). A decreasing dose-dependent response was observed for RANKL/OPG mRNA levels, however the RANKL/OPG ratio continued to decrease only until a minimum threshold value was reached (Figure 3-1b). This is evidenced by the fact that observed RANKL/OPG mRNA levels decreased consistently from a value of 1 (the experimental control value) as shear stress magnitudes increased from 0.5 Pa to 2 Pa for the 2 Hz-1 hr 0.5 Hz-2 hr and 1Hz-2hr experimental conditions. However, this continued decrease was not observed for the 5 Pa shear stress amplitudes in these groups, rather observed RANKL/OPG ratios for 5 Pa were similar to the 2 Pa fluid flow condition. For the 2Hz/2hr and 0.5Hz/4hr experimental conditions, this minimum RANKL/OPG value was reached at the lowest shear stress magnitude tested (0.5 Pa), and further remained at this level for increased shear stress magnitudes (1 Pa, 2 Pa and 5Pa).

The effect of varying the oscillating fluid flow frequency (and thus the number of loading cycles) on osteocytes, for a constant shear stress amplitude and fixed fluid flow duration, is presented in figure 3-2. These results, re-plotted from figure 3-1 in different groupings, demonstrate that osteocytes subjected to different fluid flow frequencies (for the same shear
stress amplitude and flow duration) did not exhibit a significant difference (P>0.05) in COX-2 mRNA expression levels, suggesting that osteocytes are independent of both the frequency of the applied loading and the loading cycle number in the physiological range (Figure. 3-2a). In contrast, RANKL/OPG mRNA levels declined with a faster oscillating fluid flow frequency for shear stress levels of 0.5 Pa and 1 Pa, demonstrating that RANKL/OPG transcription is influenced by either loading frequency, the number of loading cycles, or both (Figure. 3-2b). Again, we note an apparent minimum RANKL/OPG ratio for the 2 Pa and 5 Pa experimental conditions.

We next examined the effect of fluid flow duration on osteocyte COX-2 and RANKL/OPG mRNA expression (Figure. 3-3). COX-2 mRNA levels were found to be consistently elevated in osteocytes subjected to longer flow durations (1 vs. 2 hours, and 2 vs. 4 hours; Figure. 3-3a). This trend was consistent amongst experimental conditions with four different shear stress amplitudes and two different oscillating frequencies tested. RANKL/OPG ratio was found to decrease for longer flow durations for 0.5 and 1 Pa shear stress conditions (Figure. 3-3b). At elevated shear stress flow conditions of 2 and 5 Pa, however, increased flow duration did not appear to induce a step-wise response for osteocyte RANKL/OPG mRNA expression.

### 3.4 Discussion

To our knowledge, this is the first systematic study to examine the separate effects of three independent oscillatory fluid flow parameters on osteocyte response in vitro. Based on previous findings that suggest osteocytes are highly sensitive to their loading environment, we hypothesized that osteocyte COX-2 and RANKL/OPG mRNA expression levels would be
regulated by each of the three oscillating fluid flow parameters studied here: fluid flow-induced peak shear stress amplitude, oscillating fluid flow frequency and total fluid flow duration. To decouple the separate effects of each flow parameter, we subjected MLO-Y4 osteocyte-like cells to oscillating fluid flow stimulation with different combinations of these parameters (table 2). The effect of a single parameter was determined by varying that parameter while holding the remaining two parameters constant, and observing the resulting changes in osteocyte COX-2 and RANKL/OPG mRNA levels. The major findings from this study are (1) osteocytes exhibit distinctly different responses to each of the three independent oscillating fluid flow parameters: Peak shear stress magnitude, oscillating frequency and total flow duration, (2) different mechano-transduction mechanisms likely exist is osteocytes for regulating COX-2 and RANKL/OPG mRNA expression, (3) the effects of each oscillating fluid flow parameter appear to work together in a cumulative manner in regulating osteocyte activity, and (4) physiological loading conditions with higher peak shear stress amplitudes, higher oscillating frequency, and longer loading durations provide the best stimulus for promoting bone formation.

Our results demonstrate that osteocytes subjected to different oscillatory fluid flow conditions exhibited several interesting behaviors. In general, the application of oscillatory fluid flow stimulation up-regulated COX-2 mRNA expression and at the same time down-regulated RANKL/OPG mRNA levels. Given that increased COX-2 mRNA expression promotes bone formation by osteoblasts via the release of PGE$_2$, and a higher ratio of RANKL to OPG favors increased bone resorption through osteoclastogenesis, our findings suggest that oscillating fluid flow stimulation of osteocytes tips the balance between osteoblast and osteoclast activity in favor of increased bone formation.
More notable, however, is the observation that osteocytes responded to changes in all three oscillating fluid flow parameters. This reinforces the idea that osteocytes are exquisitely sensitive to mechanical stimulation. Specifically, COX-2 mRNA expression increased in a dose-dependent manner with progressively higher peak shear stress amplitudes (Figure. 3-1a) and flow durations (Figure. 3-3a). Similarly, RANKL/OPG mRNA levels generally decreased in a dose-dependent manner to higher peak shear stress magnitudes (Figure. 3-1b), oscillating fluid flow frequencies (Figure. 3-2b), and longer flow durations (Figure. 3-3b). This dose-dependent response on shear stress amplitude, loading frequency and flow duration has also been observed by other group studying osteoblast morphological response to increasing fluid shear stresses, nitric oxide production by osteoblasts subjected to different shear stress rates, osteocyte displacement in the presence of different fluid flow frequencies, and flow-induced calcium oscillations in osteocytes, and RANKL and OPG production in osteocytes and osteoblasts. It should be noted that osteocytes subjected to fluid flow conditions with lower parameter values (e.g., 0.5 Pa or 1 Pa peak shear stress amplitudes, 0.5 Hz oscillating frequency, and/or 1 hr flow duration) generally did not exhibit statistical significant differences in COX-2 or RANKL/OPG mRNA levels as compared to their respective no-flow controls due to insufficient stimulation provided by these flow conditions. This is an intuitive and reasonable observation given the dose-responsive behavior of osteocyte towards the various fluid flow stimulus parameters. While the above-mentioned trends are generally well supported by our findings, careful examination of our results reveals several interesting exceptions that allow us to speculate on the mechanisms involved in osteocyte mechanotransduction.

Interestingly, COX-2 and RANKL/OPG mRNA levels were found to respond differently (qualitatively) to the same fluid flow parameters (i.e., not considering quantitative response
differences). This is clearly exemplified by the fact that while RANKL/OPG ratio changed as a result of varying fluid flow frequency, COX-2 mRNA expression showed no significant sensitivity towards this parameter (p>0.05; Figure. 3-2). A more subtle demonstration of this point can be seen when comparing the dose-response regulation of COX-2 and RANKL/OPG mRNA levels to increasing shear stress magnitudes in which the RANKL/OPG mRNA levels did not continue to decrease when subjected to higher shear stresses beyond 2Pa, while COX-2 mRNA levels continued to change even for 5 Pa peak shear stress (Figure. 3-1). This would seem to suggest that loading-induced COX-2 and RANKL/OPG gene transcription are regulated via two different transduction mechanisms. The COX-2 regulation mechanism would appear to be insensitive to loading frequencies while the RANKL/OPG mechanism appears to impose a lower limit for this ratio.

Another interesting observation is that the influence of the different fluid flow parameters on osteocyte COX-2 and RANKL/OPG mRNA expression appear to be additive. For instance, while both higher shear stress magnitudes and longer flow durations independently increased COX-2 mRNA levels, the combination of both elevated shear stress magnitude and longer flow durations result in a substantially higher COX-2 mRNA level than either of these parameters acting alone (Figure. 3-1a). Therefore, the highest measured COX-2 mRNA levels coincide with the fluid flow parameter with the highest shear stress (5 Pa) and longest flow duration (4 hours). A similar situation can be observed for RANKL/OPG in which both shear stress magnitude and cycle number (via either frequency or duration) separately act to decrease this ratio. Yet when combined, the decrease in RANKL/OPG mRNA levels down to the apparent lower limit occurs at lower shear stress amplitudes (2 Pa for 0.5 Hz, 2 hr as compared to 0.5 Pa for either the 2 Hz,
2 hr flow condition or the 0.5 Hz, 4 hr flow condition). This observation suggests that osteocytes respond to each of the three fluid flow parameters in a synergistic manner.

For our experimental flow conditions, higher peak shear stress amplitudes and longer flow durations maximized COX-2 mRNA levels, indicating that these conditions would result in increased PGE₂ production and osteoblast activity. Higher peak shear stress amplitudes, oscillating frequencies, and longer flow durations generally decreased RANKL/OPG mRNA levels which, also by extension, would suggest decreased RANKL/OPG protein ratio and thus decreased osteoclastogenesis. As our experimental conditions were selected to encompass the estimated physiological stimuli experienced by osteocytes in vivo, our findings indicate that physiological loading conditions with higher peak shear stress amplitudes, higher oscillating frequency, and longer loading durations provide the best stimulus for promoting bone formation in vivo.

The experimental methods employed in this study present several inherent limitations which should be carefully considered when interpreting our results. First, the use of MLO-Y4 cells in our study precluded Sost mRNA expression. The Sost gene encodes for sclerostin, a soluble signaling molecule that antagonizes bone formation through binding with LRP5/6 receptors and inhibiting the Wnt signaling pathway. While MLO-Y4 cells behave in many ways similar to primary osteocytes, observations made in our lab, along with private communication with Dr. Lynda Bonewald (University of Missouri-Kansas City, Kansas City, MO, USA) has led us to believe that these cells do not express any appreciable level of SOST. Investigation into the effects of different oscillatory fluid flow parameters on SOST expression would provide a more complete understanding of osteocyte mechanotransduction, and therefore primary osteocytes
would serve as a better cell model for our future studies. Our oscillatory fluid delivery system also limited our study to shear stress amplitudes of 5 Pa and below, and oscillating frequencies between 0.5 and 2 Hz. While we believe that this range of stimulation is adequate for a preliminary investigation into the effects of different oscillating fluid flow parameters on osteocyte activity, observation of osteocyte response to higher loading frequencies and shear stresses would be valuable in elucidating the mechanisms responsible for osteocyte mechanotransduction. Experimental conditions with longer flow durations were not pursued in this study due to concern regarding osteocyte viability due to poor nutrient exchange and oxygen supply levels within the flow chambers for durations over 4 hours. For the purposes of this study, however, the differences in nutrient supply and oxygen levels for different flow durations were accounted for by subjecting both flow and no-flow control groups to the same conditions (i.e., both groups of cells were loaded into parallel plate flow chambers). We recognize that mRNA levels within a cell, while generally indicative of final protein levels, do not provide a direct correlation as post-translational modifications may also contribute significantly to the final protein levels. Therefore, future work currently being pursued in our lab include the characterization of soluble protein products secreted by from mechanically stimulated osteocytes (PGE$_2$, RANKL and OPG) and their direct effect on osteoblasts and osteoclasts. Correlation of these results with our current findings presented in this study would be of great interest in understanding the role of intracellular signaling via soluble molecules in mechanically stimulated bone remodeling.

In conclusion, we subjected osteocytes to different oscillating fluid flow conditions in vitro and examined the effects of three fluid flow parameters (Peak shear stress magnitude, oscillating frequency and total flow duration) on COX-2 and RANKL/OPG mRNA expression. COX-2
mRNA levels were sensitive to the shear stress amplitude and total flow duration, while RANKL/OPG mRNA levels were sensitive to variation in all three fluid flow parameters, however a minimum limit appeared to exist for this ratio. Our findings demonstrate a distinct difference in osteocytes response to the three independent oscillating fluid flow parameters indicating that osteocytes are exquisitely sensitive to different fluid flow stimulus conditions. Our results further suggest that multiple transduction mechanism exist for regulating osteocyte activity and that the effects of each individual fluid flow parameter operate in a cumulative manner. Taken together, our findings suggest that physiological loading conditions with higher peak shear stress amplitudes, higher oscillating frequency, and longer loading durations provide the best stimulus for promoting bone formation.
Figure 3-1  (a) Cox-2 mRNA levels and (b) RANKL/OPG mRNA levels in osteocytes subjected to oscillatory fluid flow stimulus. Experimental groups are grouped with increasing peak shear stress amplitudes for constant oscillating frequency and flow duration. Significant difference of individual flow experiments as compared to their respective no-flow control groups are indicated with a *. (n=4, * p<0.05)
Figure 3-2  (a) Cox-2 mRNA levels and (b) RANKL/OPG mRNA levels in osteocytes subjected to oscillatory fluid flow stimulus. Experimental groups are grouped with increasing oscillating frequency for constant shear stress magnitude and flow duration. Significant difference of individual flow experiments as compared to their respective no-flow control groups are indicated with a *. (n=4; * p<0.05)
Figure 3-3  (a) Cox-2 mRNA levels and (b) RANKL/OPG mRNA levels in osteocytes subjected to oscillatory fluid flow stimulus. Experimental groups are grouped with increasing flow duration for constant peak shear stress amplitude and flow duration. Significant difference of individual flow experiments as compared to their respective no-flow control groups are indicated with a *. (n=4; * p<0.05)
Table 1: *Experimental Oscillating Fluid Flow Conditions.*

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<th>Oscillating Frequency</th>
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<tr>
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<tr>
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<td>2, 4 hr</td>
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<tr>
<td>5.0 Pa</td>
<td>2, 4 hr</td>
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Table 2: *RT-PCR Primer Sequence*

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<th>5’-Reverse-3’</th>
<th>Product size (bp)</th>
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3.5 References


Chapter 4
Effect of Nanowire Number, Diameter, and Doping Density on Nano-FET Biosensor Sensitivity

4.1 Introduction

Nanowire field-effect transistors (nano-FETs) enable label-free detection of molecules with higher sensitivity and shorter detection times compared to conventional bioassays. Research efforts over the past decade have produced significant advances in nano-FET biosensor technology and resulted in highly sensitive proof-of-concept devices capable of detecting exceedingly low concentrations of proteins,1-3 nucleic acids,4,5 and viruses6 in solution. In order to achieve high performance and consistency across devices, understanding sensing mechanisms and the effect of important parameters is important. A number of experimental studies have been reported, which sought to elucidate the sensing mechanism and the effect of various device parameters on nano-FET sensitivity including electrode material,7 nanowire composition,8,9 functionalization method, receptor size,10,11 gate bias,12-14 electrolyte ion concentration,15,16 and analyte delivery methods.17-19 However, the influence of nanowire number, doping density, and diameter on nano-FET biosensor sensitivity remain to be experimentally quantified.

Previous studies explored the influence of nanowire number, doping density, and diameter on device sensitivity in the context of nanowire electrical transport studies,21-23 gas-phase chemical sensing,8,24 aqueous sensing of pH and ionic species,3,25 and nanoribbon FETs.26,27 However, fundamental differences between these sensor systems and nanowire-FET biosensors require that the effect of these parameters be experimentally examined under biomolecule sensing conditions to obtain quantitatively meaningful relationships. Several numerical models were also
established for qualitatively predicting nano-FET biosensor sensitivity dependence on nanowire diameter, doping concentration, and number on nano-FET biosensor performance.28,29

Herein we experimentally determine the influence of nanowire number (the number of bridging nanowires incorporated into each device), nanowire doping density, and nanowire diameter on the sensitivity of silicon-nanowire FET protein sensors using human immunoglobulin G (hIgG) as a model analyte. As existing large-scale nano-FET construction methods such as directed self-assembly,30-32 contact printing,33-35 flow alignment,36 and dielectroporesis37,38 of pre-synthesized nanostructures are incapable of precisely controlling the diameter and/or number of nanowires incorporated into each device, we further present a unique, relatively inexpensive fabrication method to achieve reliable nanowire number and diameter control through a combined use of existing large-scale nanowire integration methods and post processing using nanomanipulation inside a scanning electron microscope (SEM). Using this technique, we are able to fabricate arrays of nano-FET biosensors with carefully controlled nanowire properties for subsequent investigation of their effects on device sensitivity.

4.2 Materials and Methods

4.2.1 Nanowire Synthesis

Phosphorous-doped (n-type) silicon nanowires (SiNWs) were synthesized on a silicon wafer growth substrate using a chemical vapor deposition (CVD) process via the gold-catalyzed vapor-liquid-solid nanowire growth mechanism (550°C, 10-50 Torr, 10% SiH4/H2 silicon source, PH3 dopant source; Illuminex corp., USA). Nanowires synthesized under these conditions had a
distribution of diameters between 60 nm to 120 nm. Two batches of nanowires with different
doping concentrations (10^{17} and 10^{19} atoms/cm^3) were synthesized.

### 4.2.2 Microfabrication of Nanowire Transistors

Wafer-scale transfer of the as-grown nanowires onto a device substrate (degenerately doped
silicon wafer with a 2000 nm thick thermal oxide layer) using the previously reported contact
printing method\textsuperscript{33} resulted in the high-density deposition of well-aligned nanowires. Briefly, the
growth substrate was inverted and placed on top of the device substrate such that the as-grown
nanowires were sandwiched between the two silicon wafers. A weight was then placed above of
the growth substrate to provide a normal force of 20 g·cm\(^{-2}\). Lateral displacement of the growth
substrate over the device substrate at a constant velocity of 20 mm·min\(^{-1}\) resulted in the transfer
of a high-density monolayer of well-aligned nanowires on to the device substrate. Standard
photolithography, metallization, and lift-off wafer-level microfabrication processes were
subsequently used to form 200 nm thick Al electrodes over the aligned nanowires producing
arrays of nanowire transistors (Figure 4-1a). Devices fabricated in this manner consisted of
numerous nanowires bridging the source-drain electrode pair. Nanowires were orientated parallel
to each another along the printing direction, having an identical effective length of \(\sim 5\ \mu\text{m}\) as
dictated by the electrode pair gap. The Al electrodes act to firmly pin the nanowires to the
substrate to permit subsequent nanowire removal via nanomanipulation.

The number of nanowires per device, and their respective diameters were controlled using a
piezoelectric nanomanipulator inside an SEM (Figure 4-1b). The multi-nanowire transistor
arrays were loaded into the SEM equipped with a custom-made nanomanipulation system with
nanometer motion resolutions. The system determines diameters of each bridging nanowire via imaging processing and also identifies nanowire(s) of interest (those to retain and remove). A tungsten nano-probe was then brought into close proximity of the nano-FET devices and placed into contact with the substrate. Unwanted bridging nanowire(s) were physically severed and removed by running the nano-probe along the electrode edges across the nanowire(s) (Figure 4-1c,d,e). Pinning of the nanowires beneath the contact electrodes caused the nanowire to sever precisely at the electrode edges. Electrical characterization was subsequently performed to obtain device I-V characteristics, and verify Ohmic nanowire-electrode contact, the intactness of the thermal oxide layer, and proper n-type FET electrical characteristics (Figure 4-1f).

4.2.3 Incorporation of Sensors into a Microfluidic Device

Nano-FET arrays were encapsulated into a simple microfluidic channel to facilitate easy solution handling during surface functionalization and subsequent biosensing experimentation. Incorporation of the microfluidic channel also served to restrict the total device surface area exposed to the electrolyte solution. The microfluidic channels (5mm×1mm×0.4mm) were fabricated by molding poly(dimethylsiloxane) (PDMS; Microchem Corp., Newton MA, USA) onto an SU-8 50 (Microchem Corp., USA) mold master using standard soft lithography. The channels were then peeled from the mold master and irreversibly bonded onto the nano-FET arrays.
4.2.4 Functionalization

Unless otherwise specified, all chemicals and reagents used for functionalization and biosensing experiments were purchased and used without further purification from Sigma-Aldrich (Oakville, Canada). Surface modification was performed using 2% (3-Aminopropyl)triethoxysilane (APTES) in ethanol (30 min.), 3% glutaraldehyde in phosphate buffered saline (0.01M PBS, pH 7.4, 30 min) and anti-human IgG (Invitrogen, USA) in PBS. Unreacted terminal aldehyde groups were passivated with a 100mM ethanolamine solution in PBS (pH 8.4) and washed with a solution of 0.5% tween-20 in PBS. Successful and repeatable functionalization of silicon oxide surfaces was verified using x-ray photon spectroscopy (XPS) and fluorescence microscopy analysis. Successful conjugation of APTES was verified by the presence of nitrogen atoms on the sample surface using XPS analysis (Figure 4-2). Immobilization of fluorescently tagged anti-hlgG (Alexa Fluor-488 anti-hlgG, Invitrogen) was verified using fluorescence microscopy.

4.2.5 Protein Sensing Experiments

All biosensing experiments were carried out at room temperature using various concentrations of hlgG in 0.1X PBS as a model analyte for immuno-detection applications (Figure 4-3). The low ionic concentration of 0.1X was used to reduce the effect of charge screening by mobile ions in solution. Increasing concentrations of hlgG (from 10 fg·ml⁻¹ up to 10 μg·ml⁻¹) were introduced to the sensor surface, and sufficient time was given to ensure that signal equilibrium was reached before the addition of subsequent solutions. Electrical measurements were performed using a Keithley 2602 Sourcemeter (for all biosensing experiments: V_sd = 10 mV, V_g = -1.0 V, I_sd was
measured and recorded). The gate voltage was applied using an Ag/AgCl solution gate electrode immersed within the electrolyte.

Up to 18 devices were characterized simultaneously throughout experimentation using a LabVIEW-controlled data acquisition system and a multiplexer (National Instruments, USA). For each concentration of hIgG, the steady-state normalized device current \((I/I_0)\) was measured and plotted against the protein concentration. Sensitivity, defined as \((I-I_0)/I_0\), was then determined and plotted against the test parameter. Statistical significance was determined using unpaired student t-test assuming unequal variances \((p<0.05)\).

4.3 Results and Discussion

4.3.1 Device Fabrication

Wafer-scale fabrication of nano-FET biosensors was made possible by several previously reported large-scale nanowire-positioning techniques such as flow alignment\(^{36}\) and contact printing\(^{33}\). Additional post processing is useful for controlling parameters such as nanowire number and diameter. We first explored the use of focused ion beam (FIB) milling for selective nanowire removal. However, as previously reported Hu et al.,\(^{40}\) EDX (energy-dispersive X-ray spectroscopy) analysis of FIB-milled samples revealed substantial deposition and/or implantation of gallium atoms around the milled region (Figure 4-4). Due to the concern of gallium contamination on silicon nanowires, which might alter the nanowires’ charge transport properties, we chose physical nanomanipulation for this nano-FET characterization study.

Physical nanomanipulation, despite being slower compared to the large-scale methods, promises specificity, precision, and programmed motion, and thus, enable the manipulation and characterization of individual nanowires. We use nanomanipulation to select nanowires and
remove unwanted nanowires to form nano-FET biosensors with well-controlled number of nanowires and nanowire diameters. Positioning of nanoprobes with nanometer resolution enabled the removal of a nanowire without disturbing adjacent nanowires. Automation of this process facilitated the manufacturing of single or multiple-nanowire transistors in a reproducible manner (>95% success rate) manner at a speed of ~1 minute per device. Testing confirmed that nanoprobe contact with the substrate during nanowire removal did not damage the oxide layer.

4.3.2 Effect of Nanowire Number

We first examined the effect of nanowire number on nano-FET biosensing sensitivity. Previous studies suggested that the number of bridging nanowires incorporated into a device may be an important parameter in determining sensitivity.\textsuperscript{24,41} Zhang et al.\textsuperscript{24} demonstrated that multiple In\textsubscript{2}O\textsubscript{3} nanowire FET devices were more sensitive than single nanowire devices for gaseous chemical sensing. While the underlying mechanism was not determined, the authors speculated that this observation might be attributed to the formation of nanowire-nanowire junctions between overlapping nanowires. Gruner et al.\textsuperscript{41} further suggested that individual carbon nanotube (CNT) FET biosensors, as compared to CNT network devices, exhibited higher sensitivity. As these sensing systems differ from nano-FET biosensors in terms of sensing environment, molecule of interest and/or nanostructure composition and type, herein we investigate the dependence of nanowire number in nanowire-based biological sensing. Twelve devices with exactly 1, 4, or 7 bridging nanowires (80-100 nm diameter; 4 devices each) were fabricated and characterized in response to increasing concentrations of hIgG (Figure 4-5a,b). All sensors exhibited linear relationships between source-drain current and log[hIgG].\textsuperscript{29} Device sensitivity was found to be maximal for single nanowire devices (0.0477 per decade; n=4) and
decreased for increasing number of nanowires (0.0313 per decade for 4 nanowires and 0.0086 per decade for 7 nanowires, which represents a ~34.4% and ~81.0% decrease in sensitivity, respectively; n=4 for each case), suggesting that single nanowire devices can yield the highest sensitivity.

As no nanowire-nanowire junctions were present in our devices, we attribute this phenomenon to the depletion of hIgG molecules from solution around the nanowires. For a single nanowire device, which experiences an initial source-drain current of $I_{0(1nw)}$, introducing analyte solution results in the binding of $x_{1nw}$ molecules onto the nanowire surface to produce a change in device current $(I-I_0)_{1nw}$, and the sensitivity is $(I-I_0)_{1nw}/I_{0(1nw)}$. Similarly, for a multi-nanowire device ($n$ nanowires connected in parallel) with an initial source-drain current of $I_{0(n nanowires)} = n \times I_{0(1nw)}$, if we assume that there is no appreciable depletion of analyte molecules from solution, then a total current change of $(I-I_0)_{(n nanowires)} = n \times (I-I_0)_{1nw}$ would be expected. Under this assumption, the sensitivity of a nano-FET sensor with $n$ parallel nanowires is then $(I-I_0)_{(n nanowires)}/I_{0(n nanowires)} = n(I-I_0)_{1nw}/nI_{0(1nw)} = (I-I_0)_{1nw}/I_{0(1nw)}$, which is equivalent to that of a single nanowire device. However, for low concentrations of analyte, a finite number of analyte molecules are located in the immediate vicinity around the nano-FET device. Therefore the effect of analyte depletion from the surrounding solution becomes significant and effectively decreases the number of nanowire-analyte binding events experienced per nanowire. Thus, for a multi-nanowire FET device in which multiple closely spaced nanowires compete for the binding of free analyte molecules, the number of nanowire-analyte interactions for each nanowire is expected to decrease for increasing numbers of nanowires such that $x_{(n nanowires)} < x_{1nw}$ resulting in a smaller current change for each nanowire. Therefore, a lower sensitivity is expected for nano-FET devices with multiple nanowires (i.e., $(I-I_0)_{(n nanowires)}/I_{0(n nanowires)} < (I-I_0)_{1nw}/I_{0(1nw)}$). While further
testing is required to conclude that analyte depletion is indeed the cause for the experimental observations, the results indicate that precise control over the number of bridging nanowires incorporated into a nano-FET device is important to optimizing device sensitivity, and that the control of nanowire spacing in multi-nanowire sensors may have practical implications.

4.3.3 Effect of Nanowire Doping Concentration

We next examined the effect of nanowire doping concentration on nano-FET sensitivity. Single nanowire devices, with diameters between 80 and 100 nm, were constructed using nanowires of two different doping concentrations ($10^{17}$ and $10^{19}$ atoms/cm$^3$, n=6 and n=4 respectively). Source-Drain current was measured as increasing concentrations of hIgG solution (1 fg/ml – 10 μg/ml) were introduced to the sensor surface (Figure 4-6). Nano-FET sensitivity was extracted as a function of protein concentration and correlated with the nanowire doping concentration. Figure 4-6 shows that a two-order of magnitude change in doping concentration (from $10^{19}$ to $10^{17}$ cm$^{-3}$) resulted in a ~3.3-fold increase in nano-FET sensitivity from 0.0477 per decade to 0.158 per decade. The sensitivity increase was also accompanied by a significantly lower sensor detection limit (~10 fg/ml vs. ~10 pg/ml) for hIgG.

These results reveal that nano-FET sensitivity is strongly dependent on nanowire doping concentration, with lower doping densities resulting in higher device sensitivity. These findings are in qualitative agreement with computational modeling predictions. However, direct quantitative comparisons cannot be made due to differences in nanowire dimensions used in the present study and previous studies. As previously reported in the literature, this observation may
be attributed to the reduced effect of charge screening by mobile charge carriers in nanowires with lower doping densities.\textsuperscript{28}

\textbf{4.3.4 Effect of Nanowire Diameter}

We finally investigated the effect of nanowire diameter on nano-FET sensitivity. While several nano-FET gas\textsuperscript{8} and pH sensors\textsuperscript{3,25} have demonstrated a sensitivity dependence on nanowire diameter, few experimental studies have examined this dependence for biosensing applications. Numerical modeling results of nanowire biosensors presented by Nair et al.\textsuperscript{28} predicted a negative correlation between nanowire diameter and device sensitivity. However, the quantitative extent of this dependence has yet to be determined experimentally. We therefore, explored the influence of nanowire diameter by fabricating single-nanowire devices with nanowire diameters ranging between 60 nm and 120 nm.

As diameter variations exist among pre-synthesized nanowires, we categorized our devices into three experimental groups based on their nanowire diameters: 60-80 nm, 81-100 nm, and 101-120 nm (n=6 for each case). The sensors were then characterized against increasing concentrations of hIgG to determine device sensitivity. As shown in Figure 4-7, a negative relationship between nanowire diameter and device sensitivity was observed for single-nanowire devices with diameters ranging from 60 to 120 nm (0.116 per decade to 0.192 per decade, \( \sim 65.5\% \) increase in sensitivity; n=6), demonstrating that nano-FET devices with thinner nanowires exhibit a higher sensitivity. This trend is in agreement with the numerical modeling predictions reported by Nair et al.\textsuperscript{28}, in agreement with experimental pH measurements made by
Stern et al.\textsuperscript{42}, and in agreement with nanoribbon-based FET (45-100 nm thick, 1 \(\mu\)m wide, 2 \(\mu\)m long) biosensing results observed by Elfstrom et al.\textsuperscript{26}

### 4.3.5 Conclusion

Our results provide experimental evidence demonstrating the importance of control over nanowire properties in determining nano-FET biosensor sensitivity and further quantify the effect of three nano-FET biosensor parameters (nanowire number, doping density, and diameter) on device sensitivity. We are aware that when the effects of nanowire number and doping density were studied, there were slight variations in nanowire diameter (80nm-100nm), which might introduce a parameter-coupling effect into the measurements. However, the potential coupling effect was captured and reflected itself in the variance of each measurement (standard deviation). The observed sensitivity change resulting from the intentional alteration of a single parameter was still statistically significant (p<0.05) in all experiments.

In summary, we have demonstrated a fabrication method that combines large-scale fabrication and SEM nanomanipulation post processing for constructing nano-FET biosensors relatively inexpensively with well-controlled nanowire number and diameters of nanowires. Using this method, we constructed single-nanowire FET biosensors to experimentally study the effect of nanowire doping density and nanowire diameter on device sensitivity. We experimentally observed that nanowires with a lower doping density produce significantly more sensitive nano-FET sensors and that smaller nanowire diameters also improve device performance. Furthermore, we also experimentally demonstrated the effect of nanowire number on nano-FET biosensor sensitivity and have attributed the decreased sensitivity in multi-
nanowire devices to competitive binding and depletion of analyte from the surrounding solution. This phenomenon further stipulates that separation distance between nanowires in a multiple-nanowire biosensor must be controlled in order to achieve device sensitivity comparable to single-nanowire devices. These findings may serve as a design reference for the optimization of nano-FET biosensor performance, both in terms of improving device sensitivity and minimizing variability across devices.
Figure 4-1  Nanowire FET construction. (a) Batch fabricated device arrays using contact printing and standard photolithography, metallization, and lift-off. Devices have multiple nanowires of various diameters bridging microelectrodes. The microelectrodes are on top of nanowires and pin the nanowires down on the substrate. (b) A batch fabricated nano-FET array mounted on an SEM stage for nanomanipulation (nanowire selection and nanowire removal). (c)(d) Before and after nanowire removal by nanomanipulation. (arrow: retained single nanowire). (e) An array of 6 single-nanowire devices (arrows: remained nanowire) (f) Current-voltage characteristics of a post-processed nanowire FET device ($V_{sd}$ = 0 to -20V, $V_{backgate}$ = -25 to +25 V, increasing $V_{backgate}$ indicated by arrow).
Figure 4-2  Post-functionalization device. (a) Schematic of a single-nanowire FET biosensor after biofunctionalization. (b) XPS analysis verifies successful conjugation of APTES molecules, as indicated by the presence of N atoms.

Figure 4-3  A typical current-time response for 7 Si-nanowire FET biosensors exposed to increasing concentrations of hIgG in 0.1x PBS buffer (from 10 fg/ml to 10 μg/ml).
Figure 4-4   EDX analysis of a device with nanowires removed with focused ion beam (FIB) milling. Ga atoms are present in the proximity of the milled region. Inset: EDX analysis of a region away from the milled region with no presence of Ga atoms.
Figure 4-5  Effect of nanowire number on device sensitivity. (a) Normalized current as a function of protein concentration for devices with 1, 4 and 7 nanowires (n=4 for each group; nanowire diameter = 81-100 nm; nanowire doping density = 10^{19} \text{ atoms cm}^{-3}). (b) Sensitivity as a function of the number of bridging nanowires (n=4 for each group). Decreasing sensitivity was observed for higher numbers of nanowires. (n=4 for each group; *p<0.05).
Figure 4-6   Effect of nanowire doping concentration on device sensitivity. (a) Normalized current as a function of protein concentration for single-nanowire devices with a nanowire doping density of either $10^{19}$ or $10^{17}$ atoms·cm$^{-3}$. All devices (n=4 and n=6 for experimental groups with doping densities of $10^{19}$ or $10^{17}$ atoms·cm$^{-3}$, respectively) contained only a single nanowire (diameter=81-100 nm). A lower nanowire doping density resulted in devices of higher sensitivity and also with a significantly improved detection limit (~10 fg/ml vs. ~10 pg/ml). (b) A ~3.3-fold increase in sensitivity was observed corresponding to the two-order of magnitude decrease in nanowire doping density. (n≥4 for each group; *p<0.05).
Figure 4-7  Effect of nanowire diameter on device sensitivity. Single-nanowire devices with diameters ranging between 60 nm and 100 nm were characterized. Nanowire diameters were grouped into three categories: 60-80 nm, 81-100 nm, and 101-120 nm. (a) Normalized current as a function of protein concentration for single-nanowire devices with different nanowire diameters (n=6 for each group). (b) Smaller nanowire diameters produced more sensitive nanowire FET biosensors. (n=6 for each group; *p<0.05).
4.4 References


Chapter 5

Conclusion and Future Work

5.1 Conclusion

The first objective was to investigate the mechanosensitivity of osteocytes to respond to different fluid flow conditions and measured the mRNA levels of markers influential on bone turnover. We found that osteocytes were able to distinguish between three different oscillating fluid flow parameters including the peak shear stress amplitude, the oscillating fluid flow frequency, and the total flow duration. We further noted differences in COX-2 and RANKL/OPG response to each of the three parameters suggesting that the expression of genes, and by extension the effects of osteocyte signaling to osteoblasts and osteoclasts, are regulated through different signal transduction mechanisms. Finally, we concluded that the effects of each of these parameters operate in a synergistic manner in determining osteocyte activity.

The second objective was to develop a label-free nanoscale biosensor system capable of measuring released soluble factors in real-time. We therefore designed and fabricated an electrical-based nanowire FET sensor system to fulfill this objective. In doing so, we developed a novel nanomanipulation-based fabrication method that allowed careful control over several device parameters, including the number of nanowires incorporated into each device, the nanowire diameter, and the nanowire doping density. This technique was then applied in the fabrication of nanosensor arrays with varying nanowire properties that enabled us to study the influence of these parameters on device sensitivity. Our findings suggest that each of these
parameters are critical in determining nano-FET biosensor performance and provides several design strategies for optimizing sensitivity. Specifically, we found that single-nanowire devices exhibit improved biomolecular sensitivity, as do smaller nanowire diameters and low nanowire doping densities. Our results also suggest that the spacing between individual nano-FET sensors in a sensor array can influence device sensitivity.

This work is the first to (1) show how osteocytes respond to different oscillatory fluid flow conditions, (2) examine the relationship between nano-FET biosensor sensitivity and nanowire number, (3) experimentally quantify the effect of nanowire diameter and doping density on nano-FET biosensor sensitivity, and (4) demonstrate a novel nanomanipulation approach for controlling nanowire number and diameter in the manufacturing of nano-FET biosensors.

5.2 Future Work

5.2.1 Identification of osteocytic soluble factors

The current study investigated the mRNA expression of RANKL, OPG, and COX-2. Future work will involve characterization of the secreted soluble factors RANKL, OPG, and PGE2 within conditioned medium from mechanically-stimulated osteocytes. Further, our study was unable to identify changes in the Sost gene. Therefore, future studies, using primary osteocytes, will investigate the effect of different oscillatory fluid flow conditions on Sost and sclerostin expression.
5.2.2 Improved nano-FET biosensor function

While our current sensor system exhibits consistent device response, further work will aim at improving the re-usability of the sensors through novel co-functionalization schemes such that a consistent and complete signal drop can be observed upon removal of the analyte solution. This process is necessary for the future investigation of intercellular communication between osteocytes and osteoblasts and osteoclasts.

5.2.3 Regulation of osteoblast and osteoclast activity by mechanically stimulated osteocytes

Investigation of how osteocytes regulate osteoblast and osteoclast populations in real time requires the development of a microfluidic co-culture system with embedded nano-FET biosensors. The goal of this work will be to develop such a system and to subsequently investigate the effects of mechanically stimulated osteocytes on osteoclastogenesis and osteoblast matrix production through released soluble factors. The embedded nano-FET biosensor system will enable dynamic measurement of the soluble signal gradients set up by mechanically stimulated osteocytes, and relate this information to the observed activity of co-cultured osteoblast and osteoclasts.