Nanostructured Microelectrodes for High-Activity Electrochemistry in Biosensing and CO₂ Recycling

by

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Electrochemical biosensors are promising candidates for point-of-care (POC) diagnostics, since they require neither costly instrumentation nor expert operators. However, for them to become widely used in a clinical setting, biosensors must be enhanced in sensitivity without increasing costs or compromising the speed of detection. The goal of this thesis is to realize this vision by promoting high-activity redox reactions via rational design of the size, shape and structure of electrodes.

Incorporating nanostructures on the surface of microelectrodes greatly increases their surface area and reaction rates, while maintaining efficient diffusion of analytes to electrode surfaces. Here, by coupling optimized electrode morphology with an enzyme-based assay, I report on a new class of ultra-sensitive biosensors. I showcase the applicability of these sensors for POC diagnostics by incorporating them on a platform designed to isolate rare cells in biological samples. The integrated device is the first example of an electrochemical sensing platform capable of detecting cancer cells with clinically-relevant sensitivity and specificity.

Decreasing the cost of POC diagnostic devices is vitally important for their adoption in low-resource settings. To that end, I advance a strategy to decrease the costs associated with the fabrication of nanostructured microelectrodes (NMEs). I present an image-reversal soft lithography (IRSL) technique based on an elastomeric stamping protocol, eliminating the need for costly photolithographic fabrication steps. The distinct morphology of the sensors made
by IRSL improves access to the surface of the electrodes leading to enhanced performance in the detection of biomolecules. Overall, the strategies presented in this thesis can guide the development of low-cost, sensitive, and rapid diagnostic systems.

Outside of biosensing, NMEs have potentially important applications in CO$_2$ recycling. Realizing efficient CO$_2$ conversion to chemical fuels is an important step towards reducing the atmospheric concentration of CO$_2$ and decreasing the carbon footprint of energy. Recently, our group reported highly-efficient electroreduction of CO$_2$ to CO using nano-sharp Au structures. Here, I improve upon the performance of these structures by increasing the density of honed needle-like features on the surface of NMEs and demonstrate a 15-fold improvement in the reaction rates relative to the previously reported best results.
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List of Abbreviations

POC: point-of-care
PCR: polymerase chain reaction
CTC: circulating tumor cell
NME: nanostructured microelectrode
ELISA: enzyme-linked immunosorbent assay
WE: working electrode
CE: counter electrode
RE: reference electrode
CV: cyclic voltammetry
DPV: differential pulse voltammetry
CA: chronoamperometry
VV: velocity valley
EpCAM: epithelial cell adhesion molecule
EC-ELISA: electrochemical enzyme-linked immunosorbent assay
CK: cytokeratin
ALP: alkaline phosphatase
p-APP: p-aminophenyl phosphate
p-AP: p-aminophynol
PDMS: poly(dimethylsiloxane)
FBS: fetal bovine serum
IRSL: image-reversal soft lithography
SAM: self-assembled monolayer
NC: non-complementary
eSHHA: electrochemical steric hinderance hybridization assay
MCH: 6-mercaptophexanol
OER: oxygen evolution reaction
CO2RR: CO₂ reduction reaction
ECSA: electrochemical surface area
RHE: reversible hydrogen electrode
FIRC: field induced reagent concentration
FID: flame ionization detector
SEM: scanning electron microscopy
TEM: transmission electron microscopy
Chapter 1

Introduction

Electrochemical reactions range from corrosion of metals to photosynthesis of plants. Centuries of scientific investigations have been carried out to understand and control natural electrochemical reactions, as well as to design electrochemical systems.

Today, industries such as healthcare, energy, and the food industry rely extensively on electrochemical systems. For example, electrochemistry is used to detect biomolecules like glucose, to store energy in super-capacitors, to generate energy using fuel cells, and to assess the quality and safety of food products such as milk. Because of their robustness, ease of use and cost-effectiveness, electrochemical methods are in high demand. With the success of existing electrochemical devices such as batteries and glucose sensors, efforts are underway to utilize electrochemistry to overcome many other challenges. However, I show herein that this will be possible only if the rate and efficiency of electrochemical reactions are improved.

In this chapter, I discuss the shortcomings of present-day clinical diagnostic methods and the potential of electrochemical systems in addressing these issues.
1.1 Point-of-Care Diagnostics

With today’s technologies, healthcare systems suffer from long turnaround times in providing molecular diagnostic information on patients. This impedes efficient disease management and imposes high costs on patients and the system [1]. The development of point-of-care (POC) diagnostics could alleviate these issues [2].

Since even low concentrations of pathogens can exist in infectious diseases, POC diagnostics must be highly sensitive to be clinically applicable. Currently, to overcome sensitivity requirements, a pre-amplification step is commonly carried out prior to any diagnostics. This amplification step underlies long test times. Gold standard amplification methods are culture for bacterial samples [3] and polymerase chain reaction (PCR) for detecting specific nucleic acid sequences [4]. To achieve detectable levels of bacteria, multiple days of culture are required. Also, PCR requires approximately 5 hours and a highly skilled operator to prepare samples. Developing sensitive, rapid, and automated detection methods for POC applications without the need for amplification is a highly sought goal.

1.2 Electrochemical Biosensors for Point-of-Care Diagnostics

Electrochemical biosensors are promising candidates for POC diagnostics as they are robust, easy to use and fast. They do not require bulky and costly instrumentation, and since they have electronic readout, signal analysis can be automated to eliminate the need for a skilled operator to interpret the results.

The glucose meter is an example of a commercially available electrochemical biosensor. A handheld device, it electrochemically detects the level of glucose in blood. To produce electrochemical signals, glucose molecules are oxidized by an enzyme immobilized on the electrode. As a result of this reaction, hydrogen peroxide is produced, which is subsequently oxidized. The intensity of the generated current indicates the concentration of glucose in blood [5].
Glucose + O$_2$ $\rightarrow$ gluconic acid + H$_2$O$_2$

H$_2$O$_2$ $\rightarrow$ O$_2$ + 2H$^+$ + 2e$^-$

Glucose meters are a widely used, commercially available electrochemical POC diagnostic devices. Other diagnostics need better sensitivity and speed to become practical [6]. The limit of detection of glucose meters is in the range of micro- to millimolar, which is low enough for their application. However, for disease diagnostics, a 9 to 12 orders of magnitude enhancement in sensitivity relative to that limit of detection is required. To develop clinically applicable POC diagnostics, it is essential to enhance the sensitivity of biosensors, without compromise to cost and speed.

In this thesis, I aim to increase the sensitivity of electrochemical diagnostics while decreasing turnaround times and lowering the cost to fabricate the sensors. By engineering the interface between electrodes and sample solutions, I demonstrate improved efficiencies and reaction rates, enabling sensitive detection of analytes over short time scales. I apply these sensors to detect specific nucleic acid sequences and rare cancer cells in whole blood samples. Finally, I present a cost-effective fabrication technique for the manufacture of these biosensors.

1.3 Electrochemical Detection of Rare Cancer Cells

A variety of electrochemical assays have been designed to read out biological analytes such as DNA, RNA, proteins, metabolites, and cells [7–11]. An emerging application of biosensors is the detection of rare cancer cells in blood [12–14].

Circulating tumor cells (CTCs) are cancer cells disseminated from a tumor into the bloodstream. CTCs can provide information about the stage of disease, tumor phenotype and effectiveness of therapy [15–17]. Early detection of CTCs in the bloodstream at very low concentrations could provide a pathway to early diagnosis of cancer and, as a result, more effective disease management. However, CTCs are very rare in whole blood, with billions of healthy blood cells present in a sample that may contain only a few CTCs.
Chapter 1. Introduction

The rarity of CTCs in the blood imposes demanding requirements for sensitivity and specificity for CTC isolation and detection. To this end, systems have been developed that use pre-enrichment of CTCs and/or dilution of blood samples to facilitate isolation and analysis [18–21]. Some CTC detection techniques use fluorescence imaging, and this requires both costly instrumentation and skill on part of the operator [18–22]. Alternative readout approaches include quartz crystal microbalance [23], micro NMR spectroscopy [24], micro-Hall detectors [25], surface enhanced Raman spectroscopy [26], lateral flow [27] and electrical impedance spectroscopy [28].

Electrochemical sensing techniques have been investigated for CTC detection in light of their simplicity and resultant low cost [13, 29–37]. However, to date, reported electrochemical techniques fail to meet sensitivity specifications for clinical applications. Whereas detection limits would ideally be below 2 cells mL\(^{-1}\), prior electrochemical reports exhibit detection limits that exceed 100 cells mL\(^{-1}\). The ability to process multi-mL blood samples in clinically-actionable times is a further requirement yet to be fulfilled by electrochemical CTC technologies. Finally, electrochemical CTC sensors have yet to be validated in the detection of target cells in whole blood. Herein I seek to develop a platform for high-throughput cancer cell capturing and detection in whole blood samples with clinically-relevant sensitivity and specificity.

1.4 Thesis Overview

In summary, electrochemical systems are promising candidates to overcome challenges in disease diagnosis. To increase the applicability of electrochemical devices, the rate and efficiency of electrochemical reactions need to be increased. Such enhancements can be realized by manipulating the physical, chemical or structural properties of electrodes and electrolytes, and by understanding and exploiting the interplay between them.

This thesis focuses on bringing about high-activity electrochemistry by engineering the prop-
properties of the solid-liquid interface from physical and structural points of view (Figure 1.1).

I manipulated the morphology of electrodes of electrochemical systems to enhance the availability and mass transport of reactants in the electrolyte solution. I began by applying these enhancements to highly-sensitive biosensors for POC diagnostics. However, in the course of this research, I also came to appreciate the benefits of controlling the morphology of electrodes for CO$_2$ reduction. In the later chapters, I discuss the rapidly-growing need for efficient CO$_2$ recycling and demonstrate the enhanced reaction rates via engineering the solid-liquid interface of CO$_2$ electroreduction systems.

Figure 1.1: This thesis focuses on enhancing electrochemistry at the interface between the structural and physical properties of the electrode-electrolyte interface.

In Chapter 2, I present an overview of the electrochemical techniques used in this thesis. Also, I introduce nanostructured microelectrodes (NMEs) as platforms for ultrasensitive electrochemical biosensing.

I demonstrate in Chapter 3 a highly sensitive method for the detection of rare cancer cells. The enhanced performance of microelectrodes is combined with an electrochemical enzyme-linked immunosorbent assay (ELISA) to improve the limit of detection.

In chapter 4, I discuss a simple, cost-effective method for fabrication of NMEs that enables
the application of electrochemical POC diagnostics in low-resource settings. Additionally, I investigate the performance of the electrodes developed via this method in electrocatalytic and blocking bioassays that exploit their distinctive morphology.

In chapter 5, I give an overview of CO$_2$ recycling as a method for combating global warming. I demonstrate that, by engineering the nucleation and growth of NME structures, we can tune the tip sharpness and the tip density of NMEs’ needle-like features. The optimized structures are then applied as catalysts to CO$_2$ electroreduction and we demonstrate that high densities of sharp needles enhance the conversion rate of CO$_2$ to CO.

In Chapter 6, I highlight the findings of this thesis, discuss their importance to the field and propose future directions to increase the applicability of electrochemical systems to POC diagnostics and CO$_2$ recycling.
Chapter 2

Background

2.1 Electrochemistry

Electrochemistry dates back to 18th century, when Luigi Galvani discovered a relationship between electric current and chemical reactions [38]. Electrochemistry studies the connection between chemical reactions and changes in the electrical energy of a system. During electrochemical reactions, either an electric charge drives a chemical change, or a spontaneous chemical reaction induces a change in electrical energy.

The first devices designed based on electrochemical reactions were batteries; however, today electrochemistry has many other applications, ranging from biosensing to electrocatalysis. Regardless of their application, the efficiency of electrochemical reactions relies on the characteristics of the solid-liquid interface of the electrode and the electrolyte solution in which the chemical reactions occur.

The solid-liquid interface characteristics are determined by the properties of the electrode and electrolyte, as well as the interplay between them. Key properties include the conductivity of the electrode and electrolyte, the affinity and availability of the electrolyte’s chemical compounds for the electrode, the pH of the electrolyte, and the structural and morphological properties of the electrode. These parameters collectively define the efficiency and the rate of electrochemical reactions.
The focus of this thesis is on promoting high-activity electrochemistry by engineering the geometry and morphology of electrodes. These enhancements were applied to biosensing and CO$_2$ reduction. In the following sections, I will first introduce the electrochemical techniques that were used in this thesis. Second, I will give an overview of the application of electrochemistry in biosensing. Finally, I will discuss the effect of electrodes' morphology on the sensitivity of bioassays.

### 2.2 Electrochemical Techniques

Most electrochemical systems are comprised of three electrodes: the working electrode, reference electrode and counter electrode. On the working electrode the electrochemical reaction occurs; it is the interface for charge transfer to the electrolyte ions. The reference electrode, at a known potential, is used to gauge the potential of the working electrode. To ensure the potential stability of the reference electrode, current must not pass through it. Therefore, in most systems, a counter electrode is used to complete the circuit (Figure 2.1).

![Figure 2.1: Schematic of a three-electrode system showing the working electrode (WE), counter electrode (CE) and reference electrode (RE).](image)

The electrochemical measurements of this thesis were performed using three-electrode systems via cyclic voltammetry, differential pulse voltammetry, and chronoamperometry.
All three of these techniques are classified as amperometric methods, as they involve measurement of a current that is generated as a result of a redox reaction in response to an applied voltage over time. Depending on the type of the technique, voltage application and current measurement regimes may vary.

In cyclic voltammetry (CV), the voltage is increased linearly with time (forward scan) until it reaches a defined maximum. Then it is cycled back to the initial voltage, decreasing at the same rate as the forward scan. The current is simultaneously measured and plotted against the applied voltage (Figure 2.2a).

As the voltage approaches the redox potential, the redox reaction starts to occur. By increasing the voltage, the rate of the redox reaction, and thus the current increases (Figure 2.2b, A to B). However, at some point the reaction rate exceeds the rate of analyte replenishment, and the current starts to decrease (Figure 2.2b, B to C). After the potential switch, in the case of reversible reactions, a reverse-polarity current is generated. Based on the same principle, we observe a similar peak in current in the reverse scan.

Figure 2.2: Cyclic voltammetry (CV). (A) The voltage is linearly ramped up and down over time. (B) Occurrence of the redox reaction results in formation of a peak in current.

CV can provide various insights into the state of the system such as the concentration of analytes, their redox potential and the rate of the reactions. Additionally, CV scans can be used
to determine the surface area of an electrode when the surface adsorbents undergo a redox reaction or an adsorption/desorption cycle.

In differential pulse voltammetry (DPV), the voltage is linearly increased over time and a small pulse is superimposed on it. The current is measured immediately before and after application of the pulse, and the difference between these two current values is plotted against the voltage (Figure 2.3). The main advantage of DPV is that the background current is eliminated when two current values are subtracted from each other. The technique offers higher sensitivity and better peak resolution than CV.

Over relatively short measurement times, the current in CA can be modeled by Cottrell equation (eq. 2.1).

\[ i(t) = \frac{nFA\sqrt{DC^\infty}}{\sqrt{\pi t}} \] (2.1)
where \( i \) is the current (A), \( n \) is the number of electrons, \( F \) is the Faraday constant (C/mol), \( A \) is the area of electrode (cm\(^2\)), \( D \) is the diffusion constant (cm\(^2\)/s), \( C^\infty \) is the concentration of ions in bulk solution (mol/cm\(^3\)), and \( t \) is the time after a change in equilibrium (s).

The technique is used to identify the electrochemical surface area of electrodes and the diffusion constant of analytes. Additionally, CA is used to electroplate structures onto electrodes. It can also implement real-time monitoring of electrochemical systems for extended periods, especially after they reach the steady-state.

### 2.3 Electrochemistry in Biosensing

Integrated circuits for the analysis of biomolecules have the potential to enable rapid and convenient POC diagnostics [39,40]. Spectroscopic [41–44], electronic [45–48], and mechanical [49–51] sensing strategies have allowed rapid advances in POC detection, as have electrochemical biosensors [40]. Nearly 100 years after Luigi Galvani’s early formalisms of electrochemistry, the first electrochemical sensing device, the pH meter, was developed [52]. This was followed by development of the first electrochemical biosensing device, the glucose oxidase sensing electrode, by Leland Clark in 1962 [53].

Since then, electrochemical systems have been extensively studied for biosensing applications. The applicability of electrochemical systems has been demonstrated in the detection of analytes in biological samples ranging from cancer cells [7, 12–14] to nucleic acids and proteins [8–11]. Electrochemical biosensors are promising candidates for point-of-care applications, since they have miniaturization potential, short sample-to-response times, and minimal reliance on costly instrumentation [54].

Since many biomolecules do not exhibit electrochemical activity, complementary reporter systems are needed to detect them electrochemically. Reporter species are electrochemically active molecules that undergo a redox reaction when target biomolecules are present in the system.
Chapter 2. Background

The presence of the target molecules leads to the production of the reporter species in the electrolyte. Alternatively, the target molecules carry and place the reporter molecules onto the electrode, as they themselves get immobilized on the electrode’s surface. Immobilization of the target molecules is commonly carried out by their hybridization with complementary probe molecules. Therefore, to develop highly-sensitive electrochemical assays, the electrodes should have high cross sections of interaction with the solution. The probe molecules should also be highly accessible to the target molecule, to increase the hybridization efficiency.

For electrochemical systems to become clinically applicable, especially for POC diagnostics, sensitivity must be enhanced. This can be achieved by increasing signal-to-noise ratios. Noise levels can be decreased by using techniques such as DPV and by improving the characteristics of the solid-liquid interface.

One approach to optimize the solid-liquid interface properties of electrochemical systems is to use nanostructured microelectrodes. Micron-sized electrodes enhance the mass transport of analytes to electrodes. To enable efficient mass transport of analytes without compromising the signal strength, microelectrodes can be nanostructured.

In following, I will discuss in detail how signal-to-noise ratios can be improved by controlling the geometry and morphology of electrodes.

2.3.1 Microelectrodes

The size of electrodes is one of the factors that influence the characteristics of the solid-liquid interface. Micron-sized electrodes are desirable, since they promote more energetically efficient electrochemistry and enable faster and more accurate electrochemical analyses. Utilization of microelectrodes instead of macroelectrodes changes the diffusional regime from linear to radial (Figure 2.4). Radial diffusion occurs at the edge of electrodes. Therefore, as the ratio of electrodes perimeter to area is increased in smaller electrodes, the radial diffusion regime is promoted over linear diffusion. The radial diffusional regime provides a higher flux of analytes
to the electrode, decreasing the thickness of the double-layer [55], a region at the electrode-electrolyte interface in which counterions are separated from each other.

Figure 2.4: Diffusion near macro and micro-sized electrodes. (A) Linear diffusion is observed at macroelectrodes. (B) Radial diffusion is observed at microelectrodes.

The double-layer, or Helmholtz layer, forms as a result of the electrostatic forces between the charged surface of the electrodes and the ions in the solution. The Helmholtz layer extends into the solution as the diffusion of ions to the electrode’s surface creates a gradient of their concentration. The extension of the double-layer is called the diffuse layer. By improving the flux of ions to the electrode, one can lower the thickness of the diffuse layer (Figure 2.5).

Figure 2.5: Schematic of double-layer formation showing the adsorbed counterions on the electrode’s surface (Helmholtz layer) and the extended region where ions diffuse towards the electrode (diffuse layer). The figure is adapted from Wikipedia [56].

The double-layer in electrochemical systems acts as a capacitor that stores electrical energy. The
current that contributes to formation of the double-layer is called charging, or non-Faradaic, current and is mainly responsible for the generation of a background current. The decreased thickness of the microelectrode’s double-layer is favorable because it decreases the capacitance and the amount of charge that is stored in the layer, resulting in lower background currents.

Although microelectrodes minimize the energy loss and lower the noise of the system, they also exhibit lower signal intensity, due to their reduced surface areas (refer to eq. 2.1). Therefore their use may not necessarily result in higher sensitivities [57, 58]. Moreover, microelectrodes exhibit reduced cross sections of interaction with the electrolyte ions that leads to lower reaction rates. These issues can be addressed by changing the surface morphology of microelectrodes.

### 2.3.2 Nanostructured Microelectrodes

Nanostructuruing of microelectrodes not only retains the radial diffusion of analytes offered by microelectrodes, but also increases the electrodes’ surface area, increasing the signal intensity. Overall signal-to-noise ratios and sensitivities can be improved by the morphological enhancement of microelectrodes [59, 60]. Additionally, nanostructured microelectrodes provide higher cross sections for interaction of ions with the electrodes, which improves reaction rates [61–63].

To date, multiple methods have been developed to nanostructure microelectrodes, including in-situ formation of the nanostructured materials [64, 65], thermal wrinkling of electrodes [66], DNA assembly [67], and hierarchical growth [60].

High sensitivities can be achieved in the case of electrochemical detectors by enhancing the efficiency with which analyte molecules bind specifically to molecular probes displayed on the biosensor surface. The use of nanometer-sized electrodes, while advantageous for the display of probe molecules on their nanoscale-curved surfaces, comes at the expense of speed of detection [57, 58]. Diffusive mass transport of analyte molecules to individual nanoscale sensors is limited to the point that the detection of analyte molecules at lower than picomolar concentrations is not feasible within practical time scales.
Fortunately, it has recently been proven that macroscopically-sized (microns and larger) electrodes, particularly those that have been finely nanostructured, can overcome the limitations of diffusive transport that previously impeded the performance of nanosized sensors. These nanostructured microelectrodes have achieved impressive limits of detection in the femtomolar range over time scales of seconds to minutes [60,68–70].

A facile method for nanostructuring of microelectrodes is electrodepositing noble metals onto them, resulting in nanostructured microelectrodes (NMEs) [59].

![Figure 2.6: SEM image of a typical NME, showing the microscale and nanoscale features.](image)

NMEs have microscale and nanoscale features that create different scales of roughness (Figure 2.6). The micron-sized branches of NMEs protrude into the electrolyte, significantly increasing the sensor footprints, and the probability of interaction with the electrolyte. At the same time, the nano-sized features on the branches provide anchoring sites for immobilization of the probe molecules at highly accessible positions, improving hybridization efficiency.

Because of the enhanced properties of NMEs, outstanding sensitivities were achieved by using them for detection of small molecules [60,71], proteins [72] and RNA sequences [7,73].
2.4 Conclusion

Microelectrodes promote radial diffusional regimes and enhance the mass transport of analytes to electrodes. Nanostructured microelectrodes maintain the enhanced mass transport and increase the surface area, therefore enhancing the probability of interaction of analytes with electrodes. In following chapters, I demonstrate that such morphological enhancements improve electrochemical reaction rates and increase sensitivity of electrochemical biosensors. I showcase higher sensitivity of microelectrodes and nanostructured microelectrodes in detection of very low concentrations of cells, proteins, and nucleic acid sequences based on various electrochemical assays.
Chapter 3

In Situ Electrochemical ELISA for Specific Identification of Captured Cancer Cells

In this chapter, I discuss a simple system that enables isolation and detection of circulating tumor cells. The detection technique is based on an enzymatic reaction that produces an electrochemically active species specifically in the presence of cancer cells. The enzymatic reaction is highly active; however, to obtain high sensitivities, electrochemical reduction of products must also be carried out efficiently. Therefore, to ensure enhanced availability and mass transport of the active species to electrodes, microelectrodes are used in the system.

By developing a sensitive electrochemical ELISA method integrated with microelectrodes within a microfluidic cell capture system, I was able to detect reliably very low levels of cancer cells in whole blood. The system demonstrates clinically-relevant specificity and sensitivity and is a prototype of a convenient, point-of-need device for cancer cell counting.

with permission, copyright 2015, American Chemical Society. As the first author of this paper, I contributed to all experimental design, device fabrication and characterization, data interpretation, and writing. The COMSOL simulation was performed by R. M. Mohamadi.

3.1 Cancer Cell Capture

The microfabricated system herein uses both microscale and nanoscale phenomena to isolate and detect cancer cells on-chip (Figure 3.1). The integrated circuit is comprised of patterned microstructures that facilitate cell capture. They are printed on top of a glass substrate that is overlaid with patterned gold structures used for electrochemical readout (Figure 3.1A). The capture method improves upon the previously-reported velocity valley (VV) chip [74]. Surface expression of the epithelial cell adhesion molecule (EpCAM) is commonly used to target and capture cancer cells [75–78]. Here, cells are specifically labeled with magnetic nanobeads conjugated with the anti-EpCAM antibody and then introduced into the capture chip sandwiched between two arrays of magnets. The amplitude of the magnetic field generated is insufficient - in an open channel - to overcome the drag force acting on the magnetic-particle-labelled cells. The chip therefore fails to capture cells. However, when X-shaped structures are included within the channel, they decrease the local velocity of the fluid, thereby decreasing the drag force (Figure 3.1B). As a result, cells having high magnetic particle labeling (corresponding to high surface biomarker expression) are robustly captured (Figure 3.1C).
Chapter 3. Microelectrodes for Rare Cancer Cell Detection

Figure 3.1: (A) Schematic of the chip design showing the capturing well with X-shaped posts and on-chip electrodes. (B) COMSOL simulation of flow velocity gradient showing the low velocity regions around X-shaped posts (velocity valleys). (C) Immunofluorescent image of a captured cancer cell on chip. (D) Image of a chip with 8 independent sensors and a zoomed in view of one sensing chamber, showing the on-chip reference, counter, and working electrodes, along with the X-shaped posts. Small dimensions of wells allow further multiplexing and parallel detection of cancer cells on one chip. (E) Cancer cells are tagged with magnetic nanoparticles functionalized with anti-EpCAM antibody. These cells get captured in the low flow regions created by velocity valleys. Afterward, they are functionalized on-chip with biotinylated anti-CK18 and streptavidin-alkaline phosphatase conjugates in two subsequent steps. (F) Alkaline phosphatase molecules convert p-aminophenyl phosphate to p-aminophenol. The electroactive p-aminophenol gets oxidized at the potential of -30 mV against Au reference electrode. The generated signal is indicative of the presence of a cell and its intensity is proportional to the number of captured cells.
3.2 Electrochemical Enzyme-Linked Immunosorbent Assay

In situ on-chip detection herein is based on an electrochemical enzyme-linked immunosorbent assay (EC-ELISA). Cancer cell detection is carried out on-chip, in-line with cell capture. Captured cells are tagged with an antibody targeted to a universal epithelial cancer marker (cytokeratin, CK). The anti-CK18 antibody is labeled with alkaline phosphatase (ALP) based on biotin-streptavidin binding (Figure 3.1E). In this assay, ALP is used to convert p-aminophenyl phosphate (p-APP) to an electrochemically active reagent (p-aminophenol) through an enzymatic reaction. p-Aminophenol (p-AP) is then oxidized electrochemically and the signal is read as a change in the current (Figure 3.1F). The intensity of the electrochemical signal therefore depends on the number of cells captured in that chamber, enabling the enumeration of cells. Moreover, the enzyme molecules are not consumed in this reaction, leading to the amplified production of p-AP. The catalytic nature of the reaction enables detection of a low numbers of cells and makes the detection method sensitive. It should be noted that all of the binding steps were performed on-chip and under a continuous flow of reagents. However, to prevent the loss of the redox species, fluid flow was stopped during p-APP incubation and during the electrochemical sampling period. During the incubation time, the generation and diffusion of redox species are high enough that electrochemical signals are independent of the position of the captured cancer cells relative to the electrodes.

3.3 Integrated Isolation and Detection of Cancer Cells

The integrated chip provides a simple method of both isolating and enumerating cancer cells. The approach provides a remarkably low cancer cell detection limit, allowing sensing of a single cell. I showcase the successful specific detection of cancer cells introduced into whole blood samples, demonstrating the high specificity of the method. The use of a microfluidic chip allows processing of a large volume of sample, important for clinical applications in view of the rarity of cancer cells in patient samples. Moreover, each rare cell capture/sensing well is ~0.2 square centimeters (comparable to a single well in a regular 96 well ELISA plate), permitting the fabrication of multiple wells in a small surface (Figure 3.1D). Each well was designed to analyze 350 µL of sample per hour. The parallel use of multiple wells enables high throughput
analysis of samples, critical for clinical applications. The miniaturized capture well, together with the simple integrated read-out presented here, open a new possibilities for combined speed, specificity, sensitivity, and convenience in high throughput rare cell analysis. The integrated approach enables automation, so that minimal sample preparation and technical skills required to implement the new assay.

The performance of the in situ ELISA approach was evaluated by monitoring the electrochemical response of various concentrations of the electrochemical reporter group paminophenol (p-AP) in TBS buffer. Differential pulse voltammetry (DPV) reveals an oxidation peak centered at -0.03 V vs. on-chip gold electrodes, which corresponds to the oxidation potential of p-AP. The intensity of the signals grows as the concentration of the analyte is increased, and no signal was detected in the absence of electroactive species (DPV of TBS shows no signal in this potential window) (Figure 3.2). I observed that the lowest reproducibly detectable concentration of p-AP is \( \sim 1 \mu M \). The incubation time for the enzymatic reaction and the concentration of the added enzyme were optimized to stay above this limit for detection of target cells.

I validated the impressive sensitivity of the chip in the detection of rare cancer cells by sensing different concentrations of VCaP cells. These cells are an important epithelial cancer cell model that express the surface antigen used for capture, EpCAM. The cells were captured on the VV chip and analyzed using in situ ELISA. The results demonstrate the capability of the chip to detect as few as one captured cancer cell (Figure 3.3A).
Figure 3.2: (A) DPV results show the oxidation peak of p-AP at the potential of -30 mV against on-chip electrodes. No signal was detected from TBS buffer in this potential window. (B) Quantitation of oxidation peak amplitudes produced at -30 mV against on-chip electrodes with different concentrations of p-AP in TBS buffer. The measurements were carried out in the potential window of -200 mV to 200 mV with potential step of 5 mV, pulse amplitude of 50 mV, pulse width 50 ms, and a pulse period of 100 ms.

The number of cells captured was independently verified by performing immunostaining and fluorescence microscopy on each chip and correlating the captured cell numbers with obtained electrochemical signals. The logarithmic relation between intensity of collected signals and the number of captured cells enables the quantitative enumeration of rare cells (Figure 3.3B). The results were highly reproducible, as evidenced by the run-to-run low error values shown in Figure 3.3. The error bars account for both inaccuracy of the detection method and imprecision of the number of spiked cells. Data normalization was accomplished by dividing the signal intensities by the maximum value of each corresponding graph.

The logarithmic relation observed in Figure 3.3B is due to the catalytic behavior of the enzyme and the resultant p-AP production amplification, whereas in the case of figure 3.2 there is no enzymatic reaction involved and p-AP solutions were ready-made, therefore the signal intensity increases linearly with increasing the concentration of the solutions.
Figure 3.3: (A) Signals from electrochemical ELISA were collected for detection of VCaP cells. Afterwards, the number of captured cells was verified using immunostaining and fluorescence microscopy. (B) A logarithmic relation correlates signal intensity to the number of captured cells.

I sought next to assess the performance of the chip when it is challenged with an abundance of nonspecific blood cells. I investigated, by attempting the capture and detection of VCaP cells spiked in whole blood, whether it would have clinically-relevant specificity (Figure 3.4).

To study the capture efficiency of the chip, I tested different concentrations of cancer cells in blood. The number of captured cancer cells was extracted from immunofluorescence images. To distinguish between cancer cells and white blood cells, three stains targeting (1) cell nuclei, (2) CD45 (a surface marker specific for WBC) and (3) CK18 (a surface marker specific for cancer cells) were used (Figure 3.4C). The capture efficiency for the intended cancer cells was on average 85% (Figure 3.4A). In addition to cancer cells, an average of about 80 white blood cells were captured non-specifically in the chips. This level of non-specific capture is much lower than what has been observed in other cancer cell isolation techniques.

It is noteworthy that higher levels of error were observed when higher numbers of cancer cells were present in samples. This trend relates to the design and mechanism of function of the VV chip. We have previously reported [79] detailed calculations and simulation data for the spatial distribution of linear velocities of cells in the presence of X-shaped capture structures used in the VV chip. At the flow rate employed, only 24% of the chip area has linear velocities lower
than the threshold required for effective capture of typical magnetically-labelled cells employed in this work. Moreover, I have observed that the capture efficiency decreases as the number of captured cancer cells per well exceeds 20. It is on this basis that I explain lower reproducibility and larger error bars in the capture of more than 20 cells. In future work, this could be addressed by increasing the well size to serve applications in which higher captured-cell numbers are of interest.

As shown Figure 3.4B, I achieved detection of as few as two cells in each mL of whole blood. The system therefore remains highly sensitive even in the presence of an abundance of nonspecific cells. Nevertheless, the complexity of blood media resulted in a higher background signal relative to what was observed when detecting cancer cells in buffer. Signal intensities are decreased for blood samples, attributed to non-specific partial coverage of the surface of electrodes by white blood cells (compare Figure 3.4B and C). The signal intensity and the number of cells were correlated using the equation displayed in Figure 3.4C. This fit allowed quantification of the number of cells present in blood samples from the amplitude of the electrochemical current. As seen in Figure 3.4A, the calculated number of cells is in good agreement with the number of captured cells counted in immunofluorescent microscopy images (a representative image of cancer cell is displayed in Figure 3.4D).
Figure 3.4: (A) Immunofluorescence microscopy was used to investigate the capture efficiency for VCaP cells in whole blood and the nonspecific capture of WBCs. The calculated number of cells from EC-ELISA measurements are also displayed on the graph. (B) Different concentrations of VCaP cells spiked in whole blood were detected using electrochemical ELISA. (C) A logarithmic relation correlates signal intensity to the number of spiked cells. A cutoff threshold of -3.25 was established as the minimum current required for a positive result. (D) Representative immunofluorescence images of a VCaP cell and a WBC. The scale bar is 10 µm and all the images are to the scale.
The remarkably high sensitivity of this system compared to the other electrochemical techniques is due to the enzymatic nature of the assay. Enzyme molecules specifically bound to the target cells amplify the number of electroactive species generated per cell. As a result, higher signal-to-noise ratio is achieved, and this translates to higher sensitivity of the immunosensor.

\section*{3.4 Conclusion}

In summary, I validated a chip-based system that enables capture and enumeration of very low concentrations of cancer cells. This method requires only simple instrumentation and has the potential to become cost-effective, via automation, compared to conventional immunostaining approaches. Because it can be tailored to employ different capture or labeling antibodies, the system can readily be adapted to different cancer cell types. It offers a significant advantage over commercially-available systems that require much higher levels of manual processing time and data interpretation. The level of sensitivity and the ability to analyze whole blood samples indicate excellent performance compared to previously-reported electrochemical cancer cell capture and sensing solutions.

\section*{3.5 Methods}

\textbf{Chip fabrication:} Electrodes were patterned on glass substrates (obtained from Telic Co. (Valencia, CA)) using standard contact lithography. The substrates were pre-coated with 5 nm Cr followed by 50 nm Au and a top-coat of positive photoresist (AZ1600). The patterns were transferred to the substrate by selectively exposing the photoresist with UV lamp 900 W for 12 s and further developing it for 40 s in MF312 developer. The remaining photoresist served as a protective layer in the subsequent gold and chrome wet etching steps. Afterwards, the remaining photoresist was stripped away with AZ300T stripper for 2 min 30 s to expose the patterned gold structures. SU-8 2002 was then spun cast at 4500 rpm for 40 s, exposed for 12 s and developed for 1 min to create a passivating layer with only open apertures on the electrodes. For fabricating the channel and microposts a second layer of SU-8 3050 was spun cast at 1500 rpm for 40 s, exposed selectively for 23 s and developed for 8 min. Finally the microchip was washed with acetone, IPA and water and then plasma etched to achieve a clean electrode surface. The
Microfluidic channel was later capped with a layer of poly(dimethylsiloxane) (PDMS) (Dow Chemical, MI) and the silicone tubing were inserted in place for inlet and outlet connections.

**Immunolabeling for Electrochemical Measurements:** To prevent non-specific adsorption on PDMS the microfluidic chip was conditioned with PBS-1 % Pluronic F68 Sigma (St. Louis, MO) overnight and washed with PBS pH=7.4 before use. The cells were spiked in 1 mL of blood and were initially incubated with 10 µL of anti-EpCAM-magnetic nanobeads (MACS-the initial concentration is not disclosed by the manufacturer) for 20 min and then they were introduced into the chip at 0.35 ml/h flow rate using a syringe pump. After capturing the cells they were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in PBS. The primary antibody solution was prepared by addition of 2 µL of biotin-mouse monoclonal anti-cytokeratin 18 (Lifespan-1 mg/mL) in 98 µL of PBS-1% BSA. The solution was added to the chip for 30 min. Subsequently secondary streptavidin-alkaline phosphatase conjugate (Invitrogen- 2 mg/mL) was diluted 1:10\(^5\) and introduced for another 30 min. Finally, prior to the electrochemical scan the cells were incubated for 10 min in 1 mM p-APP in TBS (50 mM Tris-HCl, 10 mM NaCl, 10 mM MgCl\(_2\), pH 9.0) for the enzymatic reaction to occur. It should be noted that in order to minimize the non-specific adsorption of reagents, both primary and secondary labels were diluted in PBS- 1% BSA and after each step a washing step was carried out with 0.1% Triton X-100.

**Immunostaining and Fluorescence Microscopy:** To verify the number of captured cells and to distinguish cancer cells from white blood cells, immunostaining was carried out on the chip. After the isolation step, first the red blood cells were rinsed with PBS-EDTA. Then the cells were fixed and permeabilized with PBS-4% formaldehyde and PBS-0.2% Triton X-100, respectively. Subsequently an antibody cocktail was flowed in the chip for 1 hour, which consisted of 3 µL of anti-CD45 Alexa Flour 488 from (Invitrogen MHCD4520- 0.4 mg/mL) and 3 µL of anti-CK APC (Genetex GTX80205- 0.1 mg/mL) in 94 µL of PBS-1% BSA. Finally, nuclei of the cells were stained with 10% DAPI ProLong Gold reagent (Invitrogen, CA) for 10 min. After each staining step the excess solution was rinsed with with PBS- 0.1% Tween 20.
After immunostaining chips were scanned using a 10X objective and a Nikon Eclipse Ti microscope equipped with an automated stage controller and a cooled CCD (Hamamatsu, Japan). Images were acquired with NIS Element software. Red, green and blue fluorescence images were recorded. The captured images were then analyzed in NIS Elements and target cells were enumerated.

**Electrochemical measurement.** All electrochemical measurements were done using a BASi EC Epsilon Potentiostat (Bioanalytical Systems, Inc., West Lafayette, IN) with three-electrode system. All of the reference, counter and working electrodes were on-chip gold electrodes. Differential pulse voltammetry (DPV) measurements were carried out for signal collection in a potential window of -200 mV to 200 mV with a step of 5 mV, pulse amplitude of 50 mV, pulse width 50 ms, and a pulse period of 100 ms.

**Cell culture:** The VCaP cell line (ATCC) was maintained in DMEM (ATCC) supplemented with 10% fetal bovine serum (FBS) (ATCC) at 37 °C in a humidified incubator under % CO2. After harvesting the cells, they were centrifuged at 1800 rpm for 5 min, the supernatant was discarded and the pellet was re-suspended in PBS buffer.

**Flow simulations:** Numerical simulations obtained using COMSOL Multiphysics and Matlab (Mathworks).

**Statistical analysis:** All data were analyzed with Students t-test, and represent the means ±SEM of at least triplicate samples. P <0.05 was considered statistically significant.
Chapter 4

Image-Reversal Soft Lithography: Fabrication of Ultrasensitive Biomolecular Detectors

As discussed earlier, microelectrodes can be nanostructured to increase the probability of interaction of electrolyte ions with electrodes and to display probe molecules in more accessible positions for analytes. In this chapter, I present the image-reversal soft lithography technique, as a cost-effective new method to fabricate NMEs.

The Kelley laboratory has developed NMEs by electrodepositing noble metals onto conductive surfaces. The structures enable highly sensitive bioassays. However, the templates used for NME deposition are fabricated through costly lithography techniques, and this limits the applicability of the NME-based biosensors in low-resource settings.

The present work began as an effort to eliminate costly lithographic steps previously employed in the precise definition of apertures from which NMEs are grown. My premise was that reducing the complexity of manufacture could help advance the goal enabling cost-effective point-of-care applications. Important related advances in this area include the recent achievement of nanostructured probe anchors based on DNA assembly [67, 80], thermal wrinkling of electrode
substrates [66, 81], in situ formation of hierarchical structures [64, 65] and in situ growth of an array of nanostructures [68, 82].

My approach to NME placement and manufacture described was based on soft lithography techniques. Soft lithography utilizes a stamp to transfer a pattern of molecules to metal surfaces. It has been previously implemented for different applications ranging from selective etching of metals [83, 84] to transferring a pattern of biomolecules to a substrate [85, 86]. Here I developed an image-reversal soft lithography (IRSL) technique that replaces high-resolution photolithographic steps previously required in the microelectrode fabrication process. I show that sufficiently controlled bottom-up nanostructured electrodes can be fabricated when top-down definition is provided via a coarse stamping technique.

Remarkably, I found in the course of my fabrication-related studies that not only equivalent - but enhanced - performance was achieved in certain assays when IRSL-defined electrodes were employed. Specifically, I found that a newly-reported blocking assay achieves superior performance compared to the reference case when concave NMEs defined by IRSL were used. The recessed growth geometry of these electrodes improved signal-to-background ratios when devices were challenged with a specific protein analyte.

Section 4.1 to 4.5 contain material from: Safaei, T. S., Das, J., Mahshid, S. S., Aldridge, P. M., Sargent, E. H., & Kelley, S. O. (2016). Image-Reversal Soft Lithography: Fabrication of Ultrasensitive Biomolecular Detectors. *Advanced Healthcare Materials, 5*(8), 893-899. Figures are reprinted with permission, copyright 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. As the first author of this paper, I contributed to all experimental design, device fabrication and characterization, data interpretation, and writing. J. Das helped with the electrocatalytic assay experiments and S. S. Mahshid helped with the eSHHA experiments. The COMSOL simulations (not included in this thesis) were performed by P. Aldridge.
4.1 Image-Reversal Soft Lithography

I begin by discussing the image-reversal soft lithography technique I developed in the present work. A poly(dimethylsiloxane) (PDMS) stamp is made by replica molding using a silicon master, enabling fabrication of a pattern of apertures on PDMS (Figure 4.1A). The stamp is used to transfer a self-assembled monolayer (SAM) of hexadecanethiol onto a gold substrate only in areas in which full contact is achieved, i.e. everywhere but in the intended apertures (Figure 4.1B). I then dispense molten alkane onto the substrate and notice that the thiol layer repels the liquid alkane: as a result, the alkane is retained as droplets and is present only in the areas corresponding to the stamped apertures (Figure 4.1C). By spin-coating a resist layer on to the substrate containing solid alkane droplets and then melting the alkane, I thereby form apertures in the resist (Figure 4.1D). Electrochemical deposition of gold occurs therefore only in the openings, while all other areas of the chip remain insulated and passivated (Figure 4.1E).

Using the IRSL technique, I fabricated apertures as small as 20 µm in diameter (Figure 4.2). The thickness of the resist layer was controlled to be 50 µm. It is difficult to open small apertures reproducibly using thicker resist layers, as they may cover the solid alkane droplets and fail to produce opened apertures upon melting the droplets. Thinner resist layers fail to provide consistently complete passivation, and even small residues of undesired leftover alkane can cause holes in the resist layer. Although small apertures can be made successfully using this technique, similar to conventional photolithography, fabrication yield decreases when sizes decrease below 20 µm. Above this diameter, the growth mechanism changes such that NMEs are mainly formed on the apertures edge while the central region exhibits recessed growth. This behavior is attributed to fast depletion of plating species in the center of the aperture, and this becomes more dramatic still as the NME’s growth continues [87]. The morphology of NMEs made in large apertures is consequently different from that of smaller holes (Figure 4.2). The NMEs deposited in the smaller apertures exhibit a much more compact configuration of nanostructures especially in the central regions of electrodes [88].
Figure 4.1: Schematic presentation of fabrication steps of NMEs using IRSL technique. (A) A patterned PDMS stamp is made by replica molding. (B) The stamp is covered with a thiol layer (light blue). (C) After drying, SAM of thiol is formed all over the stamp but not on the holes. (D) Upon bringing the stamp into contact with the substrate, a patterned thiol SAM is transferred on to the gold layer on glass. (E) Molten alkane (brown) are only retained as droplets on bare gold regions. (F) Subsequently SU-8 resist (gray) is spin coated on the substrate which now has solidified alkane droplets. (E) Alkane droplets are melted again to open the apertures in the SU8 layer. (H) Ultimately, everywhere else being passivated by the resist, NMEs are electroplated on the exposed regions of gold.

4.2 Coarsely-Templated Nanostructured Microelectrodes

I posited that, in light of this new NME architecture, the outer regions of the NMEs would benefit from more efficient mass transport through convergent diffusion of electrochemically active species. On this basis, I expect the edge of NMEs to be more active in electrochemical current generation and collection. The NMEs made in the larger apertures, on the other hand, exhibit a less compact morphology due to the recessed central regions. Therefore, I hypothesized that higher current densities could be generated with these types of NMEs, since their nanostructures are more accessible and their morphology can result in more efficient mass transport regimes.

Cyclic voltammetry (CV) of typical NMEs made using IRSL was carried out in mild sulfuric acid
Figure 4.2: SEM images of NMEs grown using (A) photolithography on 10 \( \mu \text{m} \) aperture and by stamping technique on (B) 20 \( \mu \text{m} \) and (C) 40 \( \mu \text{m} \) apertures. The images exhibit recessed growth area in the central region of NMEs made in larger apertures, resulting in less compact positioning of nanostructures.

solution. As displayed in Figure 4.3A, gold reduction and oxidation occurs at the expected potentials (0.5 and 1.2 V, respectively), while no additional peak is observed, confirming the compositional metallic purity of the electroplated NMEs. Moreover, CV measurements in 1 mM hexamineruthenium (III) chloride demonstrate the occurrence of redox reactions with no significant increase in the background signal obtained from the NME with recessed growth area (Figure 4.3B).

Figure 4.3: (A) Acid scan of NMEs in 50 mM H\(_2\)SO\(_4\), demonstrate absence of any impurity. (B) Cyclic voltammetry scans in 1 mM ruthenium hexamine demonstrate electrochemical detection of redox species using NMEs with similar active surface area made on 10 \( \mu \text{m} \) and 40 \( \mu \text{m} \) apertures. The results prove that the NMEs with recessed growth areas do not exhibit significant increase in the background signals.
4.2.1 Performance in the Electrocatalytic Bioassays

Further evaluation of the NMEs’ performance in the detection of biological samples was carried out based on electrochemical detection of specific nucleic acid sequences using the electrocatalytic Ru-Fe system [59,60,89]. The protruding structures of electroplated gold provide anchors for effective binding of PNA probes to the electrode using thiolated linkers. Negatively-charged phosphate present in the backbone of target nucleic acids attracts positively-charged redox ions such as, in the present case, $[\text{Ru}(\text{NH}_3)_6]^{3+}$. Upon target and probe hybridization, the $[\text{Ru}(\text{NH}_3)_6]^{3+}$ ions are attracted to the electrode surfaces and thereby reduced given their proximity to the electrodes. The amount of attracted $[\text{Ru}(\text{NH}_3)_6]^{3+}$ ions is directly controlled by the number of phosphate ions. As a result, a larger reduction current is generated in the presence of a higher concentration of target nucleic acids. Moreover, in the Ru-Fe systems, Ru reduction turnover and signal gain are amplified due to simultaneous reduction of $[\text{Fe(CN)}_6]^{3+}$, which reoxidizes Ru$^{2+}$ species and makes them available for additional redox cycles (Figure 4.4).

Figure 4.4: Schematic of a Ru-Fe electrocatalytic system, showing increased attraction of Ru$^{3+}$ (red circles) to NMEs upon target (blue strands) hybridization with PNA probe (grey strands) that results in an increase in Ru reduction signal. The signal gain indicates the presence of specific target molecules. Fe molecules (green circles) readily reoxidize Ru$^{2+}$ ions, so they will be available for another redox cycle, which amplifies the signal gain and enhances the systems sensitivity.

Using the electrocatalytic system and an electrode made via the IRSL method having target aperture diameter of 40 µm, I demonstrated successful detection of synthetic oligonucleotides with clinically relevant levels of sensitivity and specificity. An impressively low limit of detection
is achieved as a result of the coupling of nanostructured microelectrodes features with the reporter system. Specifically, a statistically-significant difference is seen between 1 fM concentration of target-containing solution compared to the non-complementary reference case (Figure 4.5A). Moreover, the same amplitude of electrical current is achieved by lithographically-defined NMEs made in 5 µm apertures when fully three times higher analyte concentrations are provided. The background signals collected in the presence of non-complementary targets are at the same low level in each case.

Figure 4.5: (A) A specific nucleic acid sequence was successfully detected in the femtomolar range with Ru-Fe electrocatalytic system using NMEs fabricated by IRSL. The obtained signal from detection of a non-complementary (NC) sequence attest to the specificity of the technique. (B) Higher number of turnover of Ru ions is obtained with NME made on larger apertures.

The enhanced performance of the coarsely-templated NMEs is attributed to their higher accessibility. This characteristic can be further demonstrated by studying the number of catalytic cycles of Ru$^{3+}$ regeneration. A higher number of Ru$^{3+}$ turnovers achieved from NMEs made on larger apertures (Figure 4.5B) is indicative of the more efficient mass transport of electroactive species to the electrodes’ surfaces.
4.2.2 Performance in Blocking Bioassays

I further hypothesized that the concave geometry of the new NMEs based on recessed growth could make these electrodes favorable for improved implementation of blocking assays. I focused on one such recently-reported assay, the electrochemical steric hindrance hybridization assay (eSHHA) [90]. In eSHHA, the reporter molecules (in this case methylene blue) are bound to one end of secondary oligonucleotides. These molecules generate electrochemical signals when they are placed on the electrodes’ surfaces via hybridization of their carrier oligonucleotides with probe strands. The target molecules are specifically attached to the carrier oligonucleotides using an appropriate recognition element, one that previously was bound to the other end of the secondary oligonucleotides. The large size of target molecules provides steric hindrance, and this prevents the successful hybridization of secondary oligonucleotides containing methylene blue with probes. As a result, signals generated in presence of target proteins are lower compared to the reference current that is collected in the absence of the target molecule. The key figure of merit, the relative gain reduction ($\frac{(I_{\text{target}} - I_{\text{reference}})}{I_{\text{reference}}}$), is proportional to the concentration of target proteins (Figure 4.6A). Here I used digoxigenin as the recognition element and anti-digoxigenin antibodies as the target molecules.

I employed eSHHA in the detection of proteins and found that NMEs made in larger apertures exhibited higher values of gain reduction compared to those ones made in small, conventional lithographical NME apertures. The constraining geometry of NMEs with recessed growth (Figure 4.6B) enhances blocking effects (Figure 4.7), with the distinctive morphology of such NMEs impeding the diffusion of large molecules to the central regions during the relatively short measurement times.
Figure 4.6: Schematic representation of an electrochemical steric hindrance hybridization assay and its performance on different types of NMEs. (A) Capturing DNA probes (grey strands) are immobilized on the surface of electrode. They are complementary to secondary carrier DNA strand (blue strands). The carrier strand is dual labeled with a small recognition element (digoxigenin) and a signaling redox tag of methylene blue [black circle (●)]. The recognition element is specific to the target molecule (anti-digoxigenin antibody). In presence of large target proteins, steric hindrance therefore impedes carrier strands from hybridizing to the probes. As a result, the signal generated in presence of anti-digoxigenin molecules are less than the reference signals obtained with no target molecule. (B) NMEs with recessed growth area provide a constraining geometry resulting in blocking of the probes trapped in the central regions, thus producing larger gain reductions. NMEs without recessed growth have higher ability to accommodate large molecules on their surface.
Figure 4.7: Gain reduction percentage is higher for the NMEs made on larger apertures, indicating their enhanced blocking effects.

4.3 Conclusion

In summary, I demonstrated a fabrication method for nanostructured microelectrodes on coarse templates, which allows for cost-effective, lithography-free fabrication of bioanalytical integrated circuits. Additionally, using coarser templates, I formed structures that exhibited a new geometry that enhances performance in both electrocatalytic and blocking assays. I further demonstrated that it is possible to achieve very low limits of detection and clinically-relevant levels of sensitivity in electrochemical biosensing while employing these coarse fabrication techniques. I developed image reversal soft lithography as a simple alternative to lithographic fabrication of NMEs. Future directions include demonstration of alignment techniques for multi-level IRSL, as well as even more cost-effective fabrication employing lower-cost polymers.

More broadly, the enhanced electrochemical performance of electrodes made on coarser templates raises the possibility of using simpler, cost-effective fabrication techniques (such as inkjet printing) to generate biosensor integrated circuits. Emerging techniques of biosensor fabrication will thus enable automated and high-throughput fabrication of even more sensitive electrochemical biosensors. It will contribute to increasing the range of economically feasible point-of-care devices.
4.4 Methods

Photolithographic Fabrication: Glass chips were purchased form Telic (Valencia, CA). They were precoated using 5 nm Cr and 50 nm Au and a layer of AZ1600 positive photoresist. The substrates were selectively exposed using 900 W UV for 12 s and developed in MF312 for 40 s. The patterning of electrodes as realized by wet etching of Au and Cr in the unprotected areas. Negative photoresist (SU-8 2002) was then spin-cast on the chips at 4500 rpm, for 40 s. It was exposed for 12 s and developed for 1 min to form the apertures having 10 $\mu$m diameter.

Image Reversal Soft Lithography: Using SU-8 3050, 70 $\mu$m-tall posts of various diameters was fabricated on silicon (spin-coated at 1500 rpm for 40 s, exposed for 23 s and developed in SU-8 developer for 8 min). The silicon wafer served as the master for molding poly(dimethylsiloxane) (PDMS) (Dow Chemical, MI) to fabricate a stamp to define the holes. The stamp was then dipped in 3 mM 1-hexadecanethiol (Sigma, MO) solution in IPA and allowed to dry completely under a light stream of nitrogen gas.

SAM of thiol was formed on the substrate upon full contact with the stamp. Molten alkane (paraffin wax, Sigma, MO) was poured on the substrate which was kept heated at 95$^\circ$C. The thiol layer repelled the alkane, permitting it to be wicked off of the chip. Only small droplets of alkane remained, and only in the the areas corresponding to the stamp holes. The substrate was then cooled and spin-coated with SU-8 3010 at 1500 rpm for 40 s. When the chip was then heated (15 s at 95$^\circ$C), the resist layer solidified while alkane droplets melted and could be washed away using IPA and water, opening up well-defined apertures in the resist.

Fabrication of the Nanostructured Microelectrodes: Chips were cleaned using isopropyl alcohol and DI water, and dried using a flow of nitrogen. Electrodeposition was carried out at room temperature. Apertures exposing gold formed the working electrode; these were contacted using exposed bond pads. The Au sensor was made using a deposition solution containing 50
mM solution of HAuCl₄ and 0.5 M HCl using DC potential amperometry at 0 mV for 100 s. Ensuingly, the Au sensors were coated with a thin layer of Pd to form nanostructures by replating in a solution containing 5 mM PdCl₂ and 0.5 M HClO₄ at -250 mV for 10 s.

**PNA Probe Design for Ru-Fe Assay:** PNA probe (Cys-AEEA-5’-TTG TGG TAC TGC CTG ATA GGG-3’) was obtained from PNA Bio Inc., Canada. Synthetic complementary target (5’-CCC TAT CAG GCA GTA CAA CAA-3’) and non-complementary target (5’-TAG CTA CAG AGA AAT C-3’) were obtained from ACGT, Canada. Probe and DNA sequences were quantified by measuring absorbance at 260 nm using a NanoDrop.

**Functionalization and Hybridization of Electrodes for Ru-Fe Assay:** Aqueous solution containing 2 µM of PNA probe was mixed with 20 µM of aqueous TCEP. The mixture was left for one hour to cleave disulphide bonds. After 18 µM of 6-mercaptohexanol (MCH) were mixed into this solution, 50 µl of the resultant solution were pipetted onto the chips and incubated overnight in a dark humidity chamber at room temperature to provide probe immobilization. The integrated circuits were then washed twice for 5 min using 1 X PBS at room temperature. After washing, the chips were challenged with different concentrations of targets, in each case for 30 min and at room temperature. After hybridization, the chips were washed twice for 5 min with 1 X PBS at room temperature and the electrochemical scans were acquired.

**Electrochemical Measurements for Ru-Fe Assay:** All electrochemical measurements were performed using the BASi EC Epsilon potentiostat in the standard 3-electrode configuration with Ag/AgCl reference and Pt wire counter electrodes. To study the effective surface area of the NMEs, cyclic voltammetry scans were recorded in 50 mM H₂SO₄ solution in the potential window of 0 to 1.5 V with 100 mV/s scan rate. Ru and Fe scans were carried out in 1 mM Ru(NH₃)Cl₃ and 2 mM K₄Fe(CN)₆ in 1 X PBS solution between -0.5 to 0 V and 0 to 0.5 V, respectively. Ru solution was purged with nitrogen for 15 min before the tests to remove the oxygen content.

To measure the amount of hybridized nucleic acid, electrochemical signals were measured in
0.1 X PBS with 10 µM [Ru(NH₃)₆]Cl₃ and 4 mM K₃[Fe(CN)₆]. DPV signals were obtained with a potential step of 5 mV, pulse amplitude of 50 mV, pulse width of 50 ms, and a pulse period of 100 ms. Signal changes that corresponded to target hybridization were calculated based on background-subtracted currents: \( \delta I = (I_{after} - I_{before}) \) (where \( I_{after} \) = current after target hybridization and \( I_{before} \) = current before target hybridization, i.e., current with only probe). All current values obtained from electrodes made on smaller apertures were rescaled to normalize to the surface of area of electrodes. This factor is the ratio of geometric surface area of the electrodes made in larger aperture to the geometric surface area of electrodes made in smaller apertures. The error bars represent the standard error of measurements.

**Ru Turnover Experiment**: 50 µl of PBS solution containing 1 µM of thiolated ssDNA (5’-CCG CGA GAC TGC TAG C 3’, obtained from Integrated DNA Technologies (IDT), USA), 20 µM of TCEP, and 50 mM of MgCl₂ was pipette on to the chips and incubated overnight in a dark humidity chamber at room temperature for DNA immobilization. The chips were then washed twice for 5 min with 1 X PBS at room temperature.

To obtain turnover numbers, cyclic voltammetry was obtained at ssDNA-modified NME at different scan rates in 0.1 X PBS solutions containing only 10 µM [Ru(NH₃)₆]³⁺ and then solutions containing 10 µM [Ru(NH₃)₆]³⁺ and 4 mM [Fe(CN)₆]³-. A ratio of catalyzed to uncatalyzed background subtracted cathodic peak currents was representing turnover.

**Target Solution Preparation and Probe Design for eSHHA**: Polyclonal anti-Dig (Roche Diagnostics, Indianapolis, IN) was dissolved in 50 mM NaH₂PO₄ and 150 mM NaCl (pH 7). The solution was then used to prepare different concentrations of target solutions. Probe DNA strands (5’-HS-(CH₂)₆- AAGG AAA GGG AAG AAG) and signaling/carrier DNA strands (5’-Digoxigenin-CTT CTT CCC TTT CCTT-MB(Methylene Blue)) were obtained from Biosearch Technologies Inc., Novato, CA.

**Functionalization of Electrodes for eSHHA**: DNA probes (0.1 mM) were activated by 1 µM TCEP solution for 1 h to remove disulfide bonds. Further the probe solution was diluted
in PBS to get final probe concentration of 100 nM. Electrodes were then incubated in 50 µL of probe solution overnight. Subsequently, the chips were incubated in 3 mM MCH in buffer for 3 h to passivate the remaining electrodes area. Electrodes were thoroughly washed with PBS prior to testing.

**Electrochemical Measurements for eSHHA:** The EmStatMUX potentiostat multiplexer (Palmsens Instruments, Netherland) was used for electrochemical of eSHHA with standard three-electrode configuration against Ag/AgCl reference electrode. The square wave voltammetry technique was used to obtain the electrochemical signals. The potential was swept from 0.2 to -0.4 V in increments of 0.001 V, with a pulse amplitude of 50 mV at frequency of 60 Hz. All measurements were taken immediately following addition of target solution to 100 nM of carrier strands without any prior incubation period. As in the case of Ru-Fe data, results are normalized to the geometric area of electrodes and the error bars represent the standard error values of each measurement.

**Electron Microscopy:** Scanning electron images of NMEs were taken by FEI-Quanta 250 FEG at 15 KV accelerating voltage.
Chapter 5

High-Density Nanosharp Microstructures Enable Efficient CO$_2$ Electroreduction

5.1 Translating Advances in Nanostructured Biosensors to CO$_2$ Reduction Electrochemistry

I reported in previous chapters that microstructured and nanostructured gold electrodes aid in the efficient sensing of biological analytes. The improvements relate in significant part to increased interaction between molecules in solution and the solid-state electrodes fabricated using bottom-up nanochemistry.

I became interested in whether these concepts could be applied in an important and rapidly-advancing parallel field in interdisciplinary materials chemistry: the electrical reduction of the greenhouse gas CO$_2$ to upgraded carbon fuels and feedstocks using renewable electricity.

In this chapter, I exploit many of the same materials, and also a number of the concepts in sharp-tipped nanoneedle electrochemistry, to this parallel domain of CO$_2$ electroreduction.
Section 5.5 to 5.11 contain material from: Saberi Safaei, T., Mepham, A. H., Zheng, X., Pang, Y., Dinh, C. T., Liu, M., Sinton, D., Kelley, S. O., & Sargent, E. H. (2016). High-Density Nanosharp Microstructures Enable Efficient CO$_2$ Electrocatalysis. *Nano Letters*, 16(11), 7224-7228. Figures are reprinted with permission, copyright 2016, American Chemical Society. As the first author of this paper, I contributed to all experimental design, device fabrication and characterization, data interpretation, and writing. A. H. Mepham performed the MATLAB simulations of the effect of nucleation and growth on the morphology of Au structures. X. Zheng helped with the TEM characterizations. The COMSOL simulations were performed with the guidance of Y. Pang. C. Dinh provided guidance on evaluation of the electrocatalysis performance of catalysts. M. Liu helped with the growth of nanoneedles and provided insights into principles of the FIRC mechanism.

### 5.2 CO$_2$ Reduction: A Method for Combating Global Warming

Global warming threatens human life on Earth. Human activities account for greenhouse gas-driven climate change [91]. CO$_2$ accounts for 90% of greenhouse gas emissions and its concentration in the atmosphere is steadily increasing at a rate of 2 ppm/year [92]. In September 2016, the atmospheric concentration of CO$_2$ reached 402 ppm (Figure 5.1). This is mainly due to continued growth of global energy demand and a heavy reliance on fossil fuels as the energy source: combustion of fossil fuels is the dominant source of CO$_2$ production. Climate scientists have predicted that in order to reduce the rate of the global warming back to its preindustrial rate, atmospheric CO$_2$ concentration should be lowered to 350 ppm [93]. It is important to reduce CO$_2$ pollution by reducing emissions, supplying energy from other (renewable) sources, and by reducing atmospheric CO$_2$.

CO$_2$ sequestration has been used to mitigate greenhouse gas emissions. However, it is purely a cost on CO$_2$ emitting operations, and fails to add value based on CO$_2$ as a feedstock. An alternative approach is to convert CO$_2$ to carbon-based fuels or chemicals. If CO$_2$ reduction is powered using renewable energy, it mitigates CO$_2$ emissions and also provides a means to store...
Chapter 5. Nanostructured Microelectrodes for CO$_2$ Recycling

Figure 5.1: Global concentration of CO$_2$ is increasing at the rate of 2 ppm/year. The figure is reproduced from the NASA website [94].

intermittent energy sources.

Solar energy, the most abundant renewable source of energy, can be directly used for the conversion of CO$_2$ to other carbon-based products. Alternatively, it can be converted to electrical energy and be used as the energy input for CO$_2$ electrochemical reduction. Using today’s high-efficiency solar cells, the latter approach offers a promising path to efficient CO$_2$ reduction that we explore herein.

5.3 CO$_2$ Electrocatalysis

Electrochemical conversion of CO$_2$ to higher carbon products is comprised of two half-reactions, the oxygen evolution reaction (OER) and carbon dioxide reduction reaction (CO2RR):

\[
2\text{H}_2\text{O} \rightarrow \text{O}_2 + 4\text{H}^+ + 4\text{e}^-
\]

\[
x\text{CO}_2 + y\text{H}^+ + \text{ne}^- \rightarrow \text{C}_x\text{H}_y\text{O}_z + \text{H}_2\text{O}
\]

\[
\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{C}_x\text{H}_y\text{O}_z
\]

Each half-reaction requires high energy inputs to proceed. At thermodynamic equilibrium, this energy is stored in the carbon bonds of the final products. Each reaction also has a high kinetic barrier. In practice, a potential greater than the equilibrium potential is therefore needed for
both OER and CO2RR. To minimize the amount of the overpotential, catalyst materials are incorporated into electroreduction systems.

5.4 Heterogeneous Catalysts for CO2 Electroreduction

The selectivity and catalytic behavior of molecular [95, 96] and metallic [97] compounds have been studied for CO2RR. Catalysts are classified into heterogeneous and homogeneous catalysts. Heterogeneous catalysts (such as metals) are in a different phase than the reactants; whereas homogeneous catalysts (such as molecular catalysts) are dissolved in the solution that contains the reactants. Metallic catalysts are commonly used also as the electrode for charge transfer.

Transition metals [98, 99] are among the most promising CO2RR catalysts that have been reported to date. Pb [99–102], Hg [99,100], In [99,100], Cd [99,100] and Sn [99,101] are among the formate-forming metals. Au [103], Pd [104,105], Ag [106] and Ga [99] have been demonstrated in the formation of CO.

CO formation is an important step in production of carbon-based chemicals and fuels from CO2RR. CO can either be chemically converted to fuels by the established Fischer-Tropsch process; or it can be an intermediate product that is subsequently converted to other fuels or chemicals via CO electroreduction. For this to happen, the catalysts should have optimal binding energy with CO [107]. Overly high binding energy leads to electrode poisoning from adsorbed CO; and production of hydrogen from the competing hydrogen evolution reaction. Low binding energy of CO with the catalyst results in release of CO from the electrode, generating CO gas as the main product of CO2RR. To produce higher-carbon or more reduced products, intermediate CO should be stabilized on the electrode to be able to undergo further conversions. Cu and Cu alloy catalysts have demonstrated optimal binding energy to CO, resulting in the production of methane, ethylene and ethanol from CO2RR [108–111].
5.5 Nanostructured Catalysts

Though different catalysts have been studied for electroreduction of CO$_2$, the reaction still suffers from low conversion efficiencies. In an effort to enhance the activity of catalysts, morphological control [112] and nanostructuring [98,113–116] of metals have been investigated. The higher activity of most nanostructured catalysts is attributed to their high-energy planes and defects.

Earlier, I discussed how incorporating NMEs into biosensing systems can lead to sensitivity enhancements. However, the benefits offered by NMEs are not limited to biosensing assays; I hypothesized that any electrochemical system that requires improved mass transport would benefit from them. Here, I will discuss the application of NMEs in CO$_2$ electroreduction. I demonstrate that engineering the morphology of the NMEs improves the characteristics of the solid-liquid interface in CO$_2$ reduction systems.

In Sargent group, we have recently reported CO$_2$ reduction that is enhanced via local electric field concentration at the tips of sharp gold nanostructures. The high local electric field enhances CO$_2$ concentration at the catalytic active sites, lowering the activation barrier.

Here I engineer the nucleation and growth of NMEs to optimize their morphology for CO$_2$ electroreduction. I manipulate the electroplating overpotential to generate an appreciably increased density of honed nanoneedles. I increase the density of sharp tips via sequential electrodeposition steps. Following the first electrodeposition step, selective regions of the primary nanoneedles are passivated using a thiol SAM (self-assembled monolayer) and secondary nanoneedles are grown atop the uncovered high-energy planes. This method ultimately leads to an increase in the density of the nanosharp structures, which results in a new record in CO$_2$ to CO reduction, with a 15-fold improvement over the best prior reports of electrochemical surface area (ECSA)-normalized current density.
5.6 Field-Induced Reagent Concentration

Electrochemical reduction of CO₂ to CO using electricity offers a means to store the abundant but intermittent energy available from renewable energy sources [95,117,118]. However, electrochemical conversion of CO₂ to valuable products suffers from slow kinetics, the need for high overpotentials, and poor product selectivity [119,120]. For example, highly selective reduction of CO₂ to CO has been achieved with Au nanoparticles; however, the current density of the reaction was limited to a low 2 mA/cm² at an overpotential of 0.24 V [121]. Several approaches including alloying [122], shape control [118,121,123] and surface functionalization [124,125] have been used to improve the performance of CO₂ to CO catalysts. Nevertheless, current densities have, until recently, remained below 3 mA/cm² at an applied voltage of -0.35 V relative to the reversible hydrogen electrode (RHE).

We recently reported the use of field induced reagent concentration (FIRC) to achieve a high intensity of efficient CO₂ reduction [126]. The high local electric field at the sharp tips of metal nanostructures concentrates CO₂ molecules near the active surface of the catalyst [127,128]. The Au nanoneedle electrodes exhibit CO₂ reduction with a record-high geometric current density \(j_{\text{CO}}\) of 15 mA/cm² at the low potential of -0.35 V \(\eta_{\text{CO}} = 0.24\) V with near quantitative Faradaic efficiencies (95%). The electrochemical surface area (ECSA)-normalized current density at -0.4 V, which reaches a value of 0.66 mA/cm², is over 30 times higher than for conventional nanorods and nanoparticles. We demonstrated that this enhancement in CO₂ reduction reaction rate is due to the locally concentrated electric field at the sharp tips of metal nanostructures and not because of the faceting of nanoneedles.

5.7 Increasing the Sharpness of NME Needles

I began the present study by exploring the connection between the radius of curvature of nanostructured electrode tips and high local field using electrochemical simulation (Figure 5.2B). When I lowered the tip radius from 15 nm to 5 nm, for example, the electric field at the tip doubled. I focused on nanoneedles, since they allow for a higher degree of nanostructuring and accessibility to electrolyte compared to other nanostructures such as nanoparticles and
nanowires having similar tip radius of curvature.

Figure 5.2: (A) Schematic of the multi-tip sharp Au nanoneedles with increased number of active sites (higher density of tips). Finite element computation results showing the effect of nanoneedle tip radii of (B) 5 nm, (C) 10 nm and (D) 15 nm on electric field concentration (the scale bars are 5 nm). Lower tip radius generates higher electric field concentration.

I focused on bottom-up fabrication of gold microstructures using electrodeposition on an insulator-passivated gold substrate onto which I had patterned apertures 15 µm in diameter [87,129]. The modeling output demonstrates that by controlling the ratio of nucleation rate to growth rate (α) and also by controlling the growth directionality, one may form finer structures (Figure 5.3A).
Figure 5.3: Higher plating overpotentials generate finer structures. (A) Modeling outcome demonstrates growth of finer structures by increasing the nucleation/growth rate ratio and growth directionality. (B) A peak in current traces is observed at higher plating overpotentials, indicating occurrence of a high degree of nucleation.

I sought therefore to manipulate, experimentally, the overpotential with the goal of controlling these parameters [130]. Higher overpotentials promote a surge in nucleation (higher $\alpha$), evident from the peak in current observed following 15 s of deposition. Additionally, at high plating current densities, dendritic growth is favored: it overcomes the insufficient availability of reagents by concentrating the ionic replenishment at the growing tips of the structures. When I grow with a high $\alpha$, severe local reagent depletion (resulting in the subsequent dip in current) promotes highly directional growth of gold structures into the bulk electrolyte [131–133].

With lower $\alpha$ and lower plating currents, gold ions consumed in electrodeposition are efficiently replenished via diffusion. In this case, gold structures tend to grow in both the longitudinal and lateral directions and exhibit much more isotropic features (Figure 5.3B) [134].

Gold structures are electrodeposited from a solution of 450 mM HAuCl$_4$ in 0.5 mM HCl at 0, -0.2 and -0.3 V against an Ag/AgCl reference electrode. Electron microscopy images indicate that higher overpotentials promote directional growth and finer structures (Figure 5.4). As I decrease the overpotential, nanoneedles become coarser, and leaf-like structures start to appear (at 0 V). Based on the acquired TEM images, finer structures also have sharper tips. The measured tip radii of nanoneedles are in average 50, 12 and 3 nm at 0, -0.2 and -0.3 V, respectively.
51

(Figure 5.4D). It should be noted that the reported values are the mean tip curvature radii of needle-like structures with varying fineness; however, the leaf-like structures grown at 0 V are not included in calculations.

![Figure 5.4: Higher overpotentials generate gold nanoneedles with lower tip radii of curvature. Electron microscopy images of gold structures grown at (A) 0 mV, (B) -200 mV and (C) -300 mV. (D) Tip curvature radii measured from TEM images of nanoneedles deposited at different potentials.](image)

5.8 Effect of Needle Sharpness on CO₂ Electroreduction

To investigate the effect of sharpness on CO₂ reduction efficiency, I performed the reaction in CO₂-saturated 0.5 M KHCO₃ (pH 7.2) and analyzed the products using gas chromatography.
Cyclic voltammetry experiments yielded higher current densities for the sharper structures, verifying faster kinetics for the CO$_2$ reduction reaction (Figure 5.5A). The current density is monitored at 0.29 V overpotential for one hour. The value for the sharpest nanoneedles is 3 mA/cm$^2$ (normalized to ECSA), which is approximately 1.5 times higher than for the coarser nanoneedles and more than 3 times higher than the value measured for mixed leaf and needle structures (Figure 5.5B). The enhanced performance of finer needles is also apparent in CO Faradaic efficiency measurements. Sharper needles exhibit higher efficiencies for CO production at lower overpotentials (Figure 5.5C). At a potential of -0.4 V vs. reversible hydrogen electrode (RHE), the Faradaic efficiency of CO$_2$ to CO conversion decreases from $\sim$98% to 90% and 60% as the structures become duller. This is consistent with the view that a limiting factor for achieving higher efficiencies is the availability of CO$_2$ molecules at the surface of electrodes.

Figure 5.5: Sharper nanoneedles enhance the electrocatalytic performance of gold structures for CO$_2$ to CO reduction. (A) Partial display of cyclic voltammetry results demonstrate higher activity of needles with lower tip radii of curvature. (B) Faster kinetics of sharper nanoneedles are evident from higher current densities obtained at -0.4 V. (C) Faradaic efficiency for CO generation using structures deposited at different overpotentials exhibits higher efficiency of CO$_2$ to CO conversion when sharper nanoneedles are employed.

As a result, by concentrating the electric field and thereby enhancing the adsorption of CO$_2$ molecules, not only are the reaction rates improved, but higher CO selectivity is also achieved. Although increasing the sharpness of Au needles concentrates the electric field to a greater extent, this effect is very local, and the needles low tip radius limits the scale of the effective area.
(Figure 5.2). I sought therefore to increase the density of active tips, and took a hierarchical growth approach.

5.9 High-Density Nanosharp Needles for High-Rate CO$_2$ Electrocution

I grew the new hierarchical structures by partially covering nanoneedles using a thiol SAM and then introducing a secondary gold electrodeposition step. The SAM increases nucleation sites on the sidewalls of the nanoneedles. Without this passivation step, electrons are mostly accumulated at the tip of the structures, and the second growth step occurs mainly at the preexisting tips (Figure 5.6).

![Figure 5.6: The structure generated from sequential electrodeposition without the passivation step.](image)

When instead I apply the thiol layer, the features on the sidewalls that are left uncovered become the regions of electron transfer and act as new nucleation sites. Based on this approach, I transform otherwise inactive sidewalls selectively into nucleation sites for secondary structures having active tips. I observe that as each step is added in the hierarchical growth of microstructures, the number of tips is significantly increased (Figure 5.7A, B, C).
Figure 5.7: Structures with larger density of active sites exhibit enhanced electrocatalytic performances. SEM images showing multi-tip nanoneedles grown after (A) zero, (B) one, (C) two repeat(s) of sequential thiol passivation and growth steps. (D) Current densities are enhanced, as the number of passivation-growth repeats are increased. (E) Further increase of the density of multi-tip structures deposited on carbon paper results in outstanding geometric current densities.

The thiol layer (Figure 5.8) was removed prior to CO$_2$ reduction studies, by applying 1.4 V vs. Ag/AgCl electrode in 50 mM H$_2$SO$_4$ for 800 s (Figure 5.9) [135, 136]. Using these multi-tip Au nanoneedles for CO$_2$ reduction, I achieve an ECSA-normalized current density of 9 mA/cm$^2$ at 0.29 V overpotential, while maintaining the selectivity and achieving near quantitative CO faradaic efficiency. The current density is about 3 times higher than that measured using single tip Au nanoneedles and 15-fold increase over the best earlier performance (Figure 5.7D) [126].

In practical applications, the loading of catalyst on a porous electrode to improve catalytic performance is favorable. To achieve this, I implemented the multi-tip Au nanoneedles on carbon fiber. As shown in (Figure 5.10), multi-tip Au needles can be densely grown on carbon fiber with very sharp tips of 5 nm. The materials show a geometric current density of 38 mA/cm$^2$ at an overpotential of 0.29 V, which is 1.5 times higher than the best catalyst
Figure 5.8: TEM image of a nanoneedle before removal of the passivating thiol layer.

Figure 5.9: Cyclic voltammetry results on (A) bare Au nanoneedles, (B) multi-tip Au nanoneedles covered with the SAM of thiol. The thiols reduction peak height decreases while the gold peak intensifies.

reported (Figure 5.7E).

5.10 Conclusion

I demonstrated herein that sharp-tipped Au structures with smaller radii-of-curvature enhance CO\textsubscript{2} reduction performance. I grew electrodes with optimal morphologies by promoting directional growth and increasing the fineness of gold microstructures by controlling the relative nucleation to growth rate during the electrodeposition. The Au nanoneedles with the sharpest tips (i.e. 3 nm in radii of curve) exhibit CO\textsubscript{2} to CO conversion with a current density per ECSA of 3 mA/cm\textsuperscript{2}, which is 5 times higher than the previously reported performance. I then improved further the performance of Au nanostructures by increasing the density of field-
concentrating sites through multi-step growth of secondary and tertiary pointed structures on the initially grown nanoneedles. This optimization resulted in current density per ECSA of 9 mA/cm$^2$ (15-fold higher than previously reported values). Finally, I leveraged this new understanding of Au nanostructure growth to maximize performance by growing multi-tip Au nanoneedles on porous carbon fibers. Such structures demonstrated high geometric current densities of $\sim$38 mA/cm$^2$ at 0.29 V overpotential for CO$_2$ reduction, attesting to the accelerated kinetics of the reaction. From a broader perspective, catalysts that can be electrodeposited as pointed structures can potentially benefit from this approach, achieving more efficient and kinetically accelerated CO$_2$ reduction.

5.11 Methods

**COMSOL Multiphysics simulations:** The electric field generated in the vicinity of nanoneedles was simulated using the COMSOL Multiphysics finite-element-based solver. The Electrostatics module was used to solve the electric field near the nanoneedles, where the field is opposite to the gradient of the electric potential $V$: $\vec{E} = -\nabla V$. The gold electrode conductivity was taken to be $4.42 \times 10^7$ S m$^{-1}$.

We used 2D axisymmetric models to represent nanoneedle structures in 3D. Triangular meshes were used for all simulations. The element size of the meshes was set as small as 0.17 nm on the surface of the electrodes. In other parts of the model where less precision is required, e.g. in the bulk electrolyte, the maximum element size was 20 nm.
**Growth modeling:** The model was created and run in MATLAB 2013. The simulation consisted of a region of 400×400 tiles which could either be designated a value of 0 (solution), 1 (gold) or 2 (adjacent to gold surface). At the start, a collection of grains (nuclei) are randomly initiated along a line representing the aperture, with each having a position and a preferential direction of growth. The rate of growth of the tip of the grain versus the sides of the grain were being adjusted by a weighting function to account for the different directionality of various structures. Also, the relative rate of the appearance of new grains versus the growth of existing ones were controlled. Growth occurs when a 2 value tile is converted to a 1 value tile, and is a probabilistic process based on the weighting function. The results of the model demonstrate how growth directionality and nucleation to growth rate ratio affect morphology.

**Chip fabrication:** Glass substrates pre-coated with 5 nm Cr, 50 nm Au and a layer of AZ1600 positive photoresist were obtained from Telic Company (Valencia, CA). The electrode layer was fabricated by selectively exposing the substrate to UV-light and developing the positive photoresist. Gold and chrome layers were etched away in the areas that AZ1600 was removed followed by complete removal of remaining photoresist. The gold electrodes were then overcoated by spin-coating SU8-2002 at 4500 rpm for 40 s. The SU-8 layer was then exposed and developed to make an array of 15 μm diameter apertures on the gold electrodes. Chips were diced in house using a standard glass cutter.

**Preparation of gold needle:** Fabrication of gold nanoneedles was done by electroplating in a solution of 450 mM HAuCl₄ (99.99% Sigma) and 0.5 M HCl (TraceSELECT) using direct current (DC) potential amperometry method at -300, -200 and 0 mV against Ag/AgCl. Plating times were adjusted to keep the amount of deposited gold constant at different overpotentials (60 s for -300 mV, 120 s for -200 mV and 200 s for 0 mV).

**Au nanoneedles sequential electrodeposition:** For secondary nanoneedle growth, primary gold needles initially fabricated at -300 mV for 60 s were coated with 1 mM solution of MCH (99% Sigma) in ethanol. The second electrodeposition was performed at the same potential for
30 s. For the structures with the three-step sequential growth, another step of MCH coverage and 10 s gold electrodeposition was carried out.

To obtain high geometric current densities, highly dense nanoneedle structures were fabricated via sequential electrodeposition of gold on 0.1-0.3 cm$^2$ carbon paper (Toray TGP-H-060, purchased from Fuel Cell Store).

**Electrochemical surface area (ECSA) measurement:** To measure the electrochemically active surface area of gold structures, cyclic voltammetry (CV) was performed on samples in 50 mM H$_2$SO$_4$. The scans were repeated from 0 mV to 1200 mV versus Ag/AgCl, until the signals stabilized. In each cycle, oxygen molecules get adsorbed on the active surface of gold and then get reduced at 900 mV. The gold oxide reduction peak area was integrated to calculate the ECSA. It was previously reported that the amount of charge transferred during the gold reduction is 448 µC per 1 cm$^2$ ECSA (Figure 5.9).

**Electrocatalytic reduction of CO$_2$:** CO$_2$ reduction experiments were performed in CO$_2$-saturated, 0.5 M KHCO$_3$ solution (pH=7.2) using a three-electrode system with Ag/AgCl reference electrode and platinum film counter electrode. The measurements were carried on in a gas-tight H-cell compartment with Nafion117 ion exchange membrane. The setup was connected to Autolab PGSTAT302N for electrochemical measurements. CO$_2$ gas was continuously fed into the cathodic chamber of electrochemical cell at 15 s.c.c.m, while the buffer was stirred at 500 rpm. The gas products were collected at different intervals from the head space of the cathodic chamber and injected in a gas chromatograph (PerkinElmer Clarus 600) for quantification. The GC was equipped with a Molecular Sieve 5A capillary column and a packed Carboxen-1000 column. Argon (Linde, 99.999%) was used as the carrier gas. The GC columns led directly to a flame ionization detector (FID) equipped with a methanizer to quantify carbon monoxide.

**Electron microscopy characterization:** Scanning Electron Microscopy (SEM) images were acquired using a Quanta FEG 250 environmental SEM under the high vacuum mode. An
accelerating voltage of 10 kV and 2.5 nm spot size were employed. Transmission electron microscopy (TEM) was carried out on a Hitachi HF-3300 with an acceleration voltage of 200 kV.
Chapter 6

Conclusion and Perspective

The application of electrochemistry for analytical purposes has been extensively investigated because of the promise of electrochemical methods as rapid and robust detection techniques. These characteristics make electrochemical biosensors promising candidates for point-of-care diagnostics. However, for them to become clinically applicable, the sensors’ sensitivity levels have to be enhanced without compromise to speed and cost.

In the first part of this thesis, I sought to develop highly sensitive electrochemical biosensors by engineering the properties of electrode-electrolyte interfaces. Microelectrodes improve the mass transport of analytes to electrodes, thus minimizing double-layer capacitance and lowering background currents. However, this comes at the expense of reduced signal intensities and reaction rates due to lower cross sections of interaction with analytes. To overcome this tradeoff, one can increase the surface area of electrodes via nanostructuring, increasing signal intensities while keeping background currents at the reduced levels.

I combined the benefits of electrochemistry on microelectrodes with high-turnover production of electroactive species via an enzymatic reaction to detect rare cancer cells in blood samples. This work was the first report of an electrochemical technique that achieved clinically-relevant levels of sensitivity and specificity in the detection of cancer cells in whole blood. Prior to this work, the reported electrochemical methods required pre-enrichment steps and sample processing; yet their sensitivities were far worse than clinically-required levels. Other detection techniques
involved fluorescence microscopy that relies on costly instrumentation, and skilled operators. The EC-ELISA technique is rapid, and has the potential for automation and high-throughput analyses of whole blood samples.

In the important emerging domain of circulating tumor cells, several issues have yet to be addressed in the quest to develop devices suitable for clinical settings. In particular, devices that can look at multiple distinct surface markers are urgently required. This approach calls for development of multiplexed biosensors, a key next step in this field.

By improving accessibility using nanostructured microelectrodes, I reported enhanced signal-to-noise ratios and high sensitivities in detection of biomolecules. To make these NME-based sensors viable for clinical applications, it is important to reduce their fabrication costs. I designed the image-reversal soft lithography (IRSL) technique as a cost-effective alternative method for the fabrication of NMEs. IRSL is a stamp-based technique that can be used for the cost-effective fabrication of templates onto which the NMEs are electrodeposited. The resolution of the IRSL technique is lower than conventional photolithography techniques: I demonstrated that the distinct morphology of the NMEs that are made on coarser templates does not negatively impact their electrochemical performance. In fact, the concave geometry of such electrodes is beneficial for applications such as electrochemical blocking assays. It is of interest to investigate other material systems for the passivating layer, sacrificial filler and stamp to increase the resolution and decrease the cost of the material. An automated system will be highly desirable to align precisely the stamp with the substrates. This will allow for multilayer IRSL, increasing the applicability of the technique.

The mechanical and chemical stability of the biosensing assays based on NMEs need to be enhanced. Many chemical molecules used in electrochemical assays - such as enzymes and mediators - are not chemically stable. The nanostructured features of the NMEs require additional improvements in their mechanical stability under practical conditions.

In the second part of my thesis, I focused on enhancing the morphological properties of the
NMEs for CO\textsubscript{2} electroreduction. It has been demonstrated that gold electrodes lower the energy required for the conversion of CO\textsubscript{2} to CO. I sought to increase the reaction rate of CO\textsubscript{2} reduction by enhancing the characteristics of the solid-liquid interface of gold electrodes in a CO\textsubscript{2}-saturated electrolyte solution. I demonstrated that higher densities of sharper structures on gold NMEs enhance the kinetics of the reaction by improving the adsorption of CO\textsubscript{2} molecules to the electrode. With these advances, I was able to achieve unprecedented reaction rates for conversion of CO\textsubscript{2} to CO. Compared to electrochemical sensors, CO\textsubscript{2} electroreduction systems require much more development before they can be considered commercially viable. Even with my advances, they require high energy inputs, suffer from low electrochemical activities, and lack the long-term stability required for large-scale CO\textsubscript{2} recycling.

Although morphological modification of catalysts is a step forward towards scalable recycling of CO\textsubscript{2}, continued progress in this field rests on development of more selective, higher throughput, and more stable systems. Reaction rates of CO\textsubscript{2} reduction to CO should be enhanced by one to two orders of magnitude for the systems to be considered commercially viable. Additionally, high cost of gold and mechanical degradation of nanoneedles are the issues that have to be addressed for enabling large-scale CO\textsubscript{2} reduction to CO.

From a broader perspective, DFT and experimental works should be aimed at designing new catalysts for selective production of higher-carbon products with lower energy requirements. Mass transport enhancements with physical, chemical and structural modifications of electroreduction systems should be carried out to increase the activity of the reactions. In system design, stability issues and a techno-economic evaluation are needed to enable the large-scale application of CO\textsubscript{2} recycling systems.

Zooming out, this work showed the power of bottom-up nanomaterials chemistry in the realization of materials and devices for enhanced-activity electrochemistry. It illustrated the power of multi-lengthscales engineering to overcome the limitations of the diffusion of analytes/chemical precursors in solution to the heterogeneous catalyst surface. It also proved the importance of texturing on the sub-20-nm nanoscale to influence local chemical interactions and surfaces
and program electric field profiles at the interface between solid supports and electrochemical solutions. The field of nanoelectrochemistry is still in relative infancy, and these early contributions contribute to the foundations of a field with enormous potential in bioanalysis, renewable energy, and beyond.
Bibliography


