Research Proposal

Generating Functional Components in Microfluidic Chemical Systems: From Enzyme/Substrate Interaction to Electrochemistry

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Oral Presentation:
October 8th 2002

I. Introduction

Developments in microfluidics (sometimes referred to as lab-on-a-chip or micro-total analytical system (μTAS)) during the past decade or so can be divided into three categories. The first one is device fabrication. Following the first-generation chips based on Si and glass [1,2], polymer-based materials have started to attract more and more attention. The most often used polymer for microfluidics is poly(dimethylsiloxane) (PDMS), which was first described by Whitesides *et al*. [3,4] The second category relates to important discoveries about the properties of fluids flowing in micron-scale channels. [5,6] These findings can be interpreted by fluidic dynamics and surface tension theory. [7,8] Although there are new discoveries still being made in the first two categories, these fields are becoming relatively less active as compared to the last one, the integration and realization of diversified types of chemistry in the microfluidic format.

While the first-generation chip-based analysis mainly dealt with the transformation of electrophoresis-based separation from capillary to microchip, today's micro total analytical systems have started to show impact on the analysis of many important compounds using different types of chemistry [1,2]. In particular, the development of microfluidics-based analytical tools, such as sensors, presently dominates the field due to the obvious advantages of economy, disposability, small sample size, and high throughput. [1,2,9,10] Among these applications, specific chemical interactions between compounds, i.e., DNA base pairing, enzyme/substrate reaction, and antigen/antibody binding, have been intensively studied. In terms of detection, fluorescence-based techniques are most often used.

This research proposal is designed to address the possibilities of enriching the functionality of microfluidic chemical systems. Specifically, two types of classical chemistry, enzyme/substrate reactions and electrochemical reactions, will be investigated in the microfluidic format. The high efficiency and specificity of enzymatic catalysis allow substrates to be detected quickly and specifically. In addition, the tiny sample consumption, as well as the parallel processibility and multiplexing associated with microfluidics, address the high cost of many enzymes. Electrochemical detection methods have a number of desirable intrinsic characteristics that make them well suited for microfluidics. These include low cost, simple miniaturization, and low power consumption. The experiments described in this proposal generally follow the following steps. First, device fabrication methods will be developed to immobilize or place functional components within a microfluidic device. Second, the fabricated devices will be characterized and used for chemical and biochemical detection and sensing. Third, the performance of these devices will be evaluated and generalized for a wider range of analysis tasks.

II. Background

1. Microfabrication: Lithography and Soft-Lithography. Several attractive advantages of using poly(dimethyl siloxane) (PDMS) to fabricate microchannels and other microstructures have made it one of the most popular building materials in the field of microfluidics in research laboratories. [4,11] Features with sub-micron size can be obtained when high-quality masks and master molds are used. Generally, PDMS is chemically inert and can be bonded to many planar substrates including commonly used transparency film and microscope cover slides. The bonding can be either reversible or irreversible, thus offering flexible combinations for optimum device configuration. In addition, PDMS is transparent down to 300 nm and therefore compatible with most fluorescence-based and photonic measuring techniques. Finally, PDMS is biocompatible and its surface can be easily modified.

A significant disadvantage of PDMS is that it is useful mainly for aqueous media. When exposed to organic solvents, such as hexane and dichloromethane, PDMS dissolves or deforms. [12] Even though this drawback can be partially solved by modifying the PDMS surface with self-assembled monolayers (SAMs), the treatment procedure often is not perfect. Therefore, when nonaqueous media must be used in the microfluidic devices, silicon or glass wafers are often a better choice. While these materials share some advantages with PDMS, such as biocompatibility, they are relatively hard to fabricate for the following reasons.

- Microstructure patterning/transferring. For PDMS-based soft lithography, this step can be simply done by casting a prepolymer solution on a pre-fabricated reusable master structure of photoresist or silicon. For silicon and glass, a tedious etching step is required.
- Hole drilling. For PDMS, holes with diameter as small as 150 μm can be easily prepared with disposable-syringe needle. Because both silicon and glass are hard materials, hole fabrication is normally slow and the yield can be low. Sometimes specific tools, such as sand drilling, are required.
- Bonding. A microfluidic device is generally formed by bonding a mold containing microchannels with a planar substrate. In the soft lithography format, this often means bonding between the patterned PDMS mold and a glass cover slide. For devices constructed from glass or silicon, successful bonding relies on more complex procedures such as hydrogen bonding, anodic bonding, and adhesion layer-assisted bonding. These procedures often involve high temperature processing, which limits the types of materials that can be placed within the channels prior to bonding.

2. Immobilization of Biological Reagents. Biological materials can be immobilized onto surfaces or within a scaffold using one or more of the following approaches: physisorption, chemisorption, covalent binding, encapsulation, or crosslinking. [13-15] For many applications biological materials must be positioned with 10-1000 μm spatial resolution. The length scale of photolithography is well matched to this range so it is not surprising that it has been used extensively for reagent placement. Typically, photolithography is

used in one of two modes. In the first case, a pattern of a photopolymerizable material that either increases or decreases the interaction energy between the biomaterial of interest and the surface is transferred to a surface from a mask. [16,17] In the second case, the biological material of interest is polymerized directly, or in the presence of a polymerizable encapsulant, in the desired configuration. [18,19] Another family of methods based on soft lithography can be used to directly place biomaterials on surfaces or, in a two-step process, restrict immobilization to locations previously patterned. [4,20,21] There are several related methods in this family, including: microcontact printing, microfluidic patterning, and laminar flow patterning. Immobilization of biological reagents by jet-printing and robotic spotting have also been pursued because of their promise of high efficiency and automation.

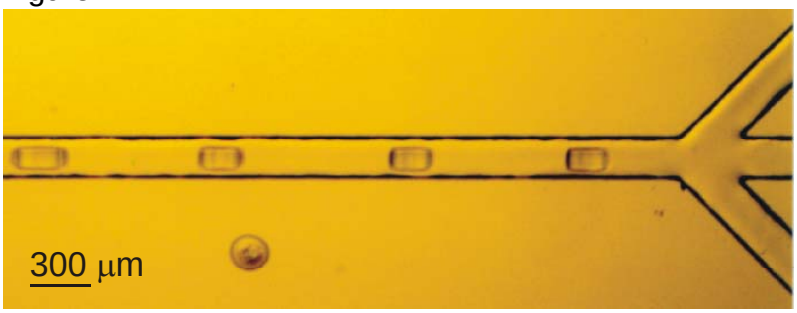
Bio-immobilization within microchannels presents some special considerations related to their small size and also post-immobilization processing of the device (chemical and thermal treatments associated with sealing the channel, for example). Some successful strategies that have been reported include direct immobilization of reagents onto the inside wall of the channel via conjugation reactions [22,23] and microbead immobilization [24]. These methods allow straightforward placement of biomaterials within a channel, but it is not possible to prepare spatially distinct micropatches containing different reagents after the channels are sealed without addition to the device of extra fluidic channels.

3. Ultra-Sensitive Spectroscopic and Electrochemical Detection. Ultra-low concentration (≤ picomolar level) detection (ULCD) and single-molecule detection (SMD) have attracted broad attention in recent years. [25-27] While conventional detection techniques rely on populations of molecules to obtain average information about one kind of molecule, ULCD and SMD aim to identify the heterogeneity between the same kind of molecules coexisting in the same environment. There are two types of approaches that have been intensively employed to achieve ULCD and SMD, namely, fluorescence-based techniques and scanning microscopy-based techniques. In terms of instrumentation, these two approaches generally require expensive and highly sophisticated machines, which in turn, need to be operated by well-trained professionals. Bard [28,29] and others [30] have demonstrated an alternative approach to SMD by using electrochemical techniques. Technically, these achievements were made possible by their use of highly efficient electrical circuits (pA sensitivity), cleverly designed ultramicroelectrodes (5-nm diameter), and low-volume electrochemical cells (~10^{-18} cm^3). Even though detection of single electrochemical events is not achievable, the measurable current in Bard's experiment derived from redox recycling of a single molecule. [28] In terms of absolute detection limit, electrochemical detection is generally several orders lower than fluorescence-based techniques. For example, the concentration of the electrochemical probe used in Bard's SMD report was in the millimolar range.

III. Preliminary Results

1. Hydrogel-Based Microreactors as a Functional Component of Microfluidic Systems. We used a simple two-step method for fabricating poly(ethylene glycol) (PEG) hydrogel-based microreactors and microsensors within microfluidic channels. The intrachannel micropatches contain either a dye, which can report the pH of a solution within a fluidic channel, and/or enzymes that are able to selectively catalyze specific reactions. Analytes present within the microfluidic channel are able to diffuse into the micropatches, encounter the enzymes and undergo conversion to products, and then the products interact with the co-encapsulated dye to signal the presence of the original substrate. The micropatches are prepared by photopolymerizing the PEG precursor within the channel of a microfluidic system consisting of a poly(dimethylsiloxane) (PDMS) mold and a glass plate. Exposure takes place through a slit mask oriented perpendicular to the channel, so the size of the resulting micropatch is defined by the channel dimensions and the width of the slit mask (Figure 1). Following polymerization, the mold is removed, leaving behind the micropatch(es) atop the glass substrate. The final microfluidic device is assembled by irreversibly binding the hydrogel-patterned glass slide to a second PDMS mold that contains a larger channel. Multiple micropatches containing the same or different enzymes can be fabricated within a single channel (Figure 2). The viability of this approach is demonstrated by sensing glucose using micropatches co-polymerized with glucose oxidase, horseradish peroxidase, and a pH-sensitive dye.

Figure 1

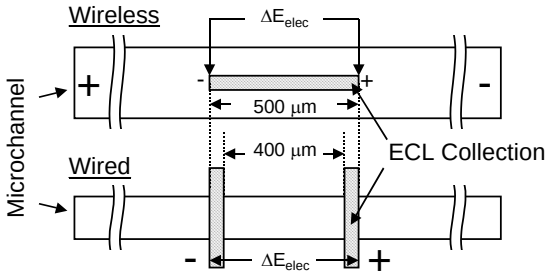


2. **Electrochemical Sensing in Microfluidic Systems Using Electrogenerated Chemiluminescence as a Photonic Reporter of Redox Reactions.** We recently reported a microfluidics-based sensing system that relies on electrochemical detection and electrogenerated chemiluminescent (ECL) reporting. The important result is that the ECL reporting reaction is chemically decoupled from the electrochemical sensing reaction. That is, the electrochemical sensing reaction does not participate directly in the ECL process, but

Figure 2

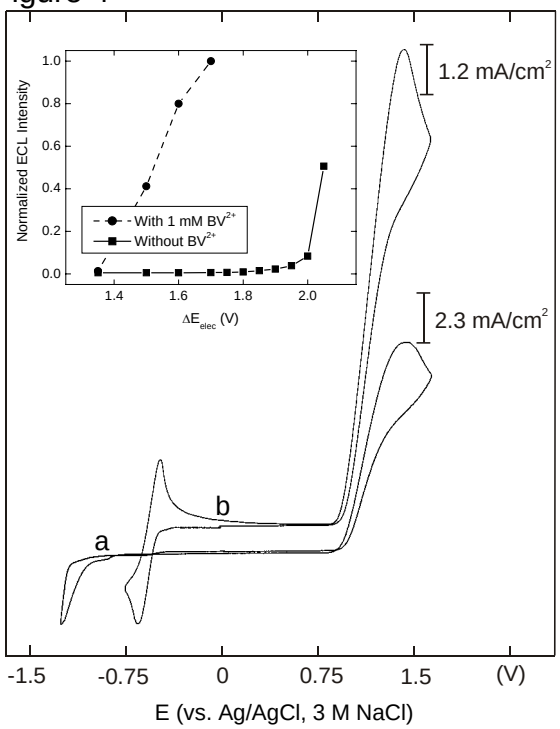
because of the charge balance between the two electrodes, the sensing and ECL reactions are electrically coupled. This provides a convenient and sensitive means for direct photonic readout of electrochemical reactions that do not directly participate in an ECL reaction. The approach can be implemented in either a two-electrode or bipolar one-electrode (wireless) configuration (Figure 3). We found that the minimum potential bias required to trigger the anode and cathode reactions must be at least equal to the onset potential of the two processes. This effect is shown in Figure 4 where different potential biases are required to turn on the reactions between ECL reaction and hydrogen reduction or viologen reduction. By manipulating the placement and dimensions of the conductors, the photonic response can be enhanced with respect to the faradaic electrochemical process of interest. The system is used to electrochemically detect benzyl viologen present in solution at 1 nM and report its presence via $Ru(bpy)_3^{2+}$ (bpy=bipyridine) luminescence. Lower detection limits are envisioned when the system is optimized (part of the proposed research).

Figure 3



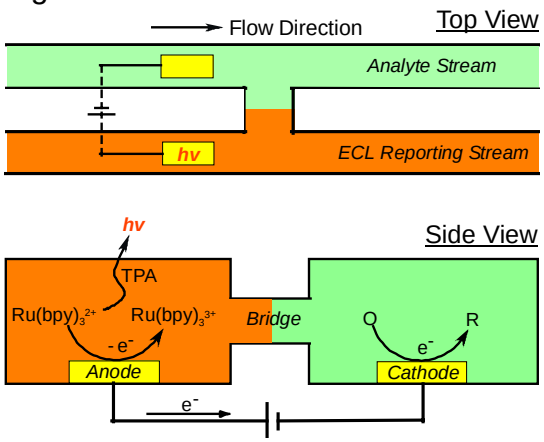
3. **A Two-Channel Microfluidic Sensor that Uses Anodic Electrogenerated Chemiluminescence as Photonic Reporter of Cathodic Redox Reactions.** This project focused on the development of a new two-electrode redox sensing system for microfluidic systems. The sensing mechanism relies on electrochemical detection at one electrode and electrogenerated chemiluminescent (ECL) reporting at the other electrode. Each indium tin oxide (ITO) electrode is located in a separate channel, and the two channels that connected downstream to maintain electrochemical contact (Figure 5). Under these conditions the electrochemically generated products at the sensing electrode cannot quench ECL at

Figure 4



the reporting electrode. Because both electrochemical reactions are coupled electrically, charge balance allows direct correlation of the emitted light to the concentration of the sensed species when the corresponding potential bias is applied between the two electrodes. The system was used to electrochemically detect $Fe(CN)_6^{3-}$, $Ru(NH_3)_6^{3+}$ and benzyl viologen (BV^{2+}) in solution and report their presence via $Ru(bpy)_3^{2+}$ (bpy=bipyridine) luminescence. Each target analyte turns on the ECL at a different potential bias, which is related to its standard redox potential. As a result, quantitative analysis of redox species can be achieved by recording the steady-state ECL.

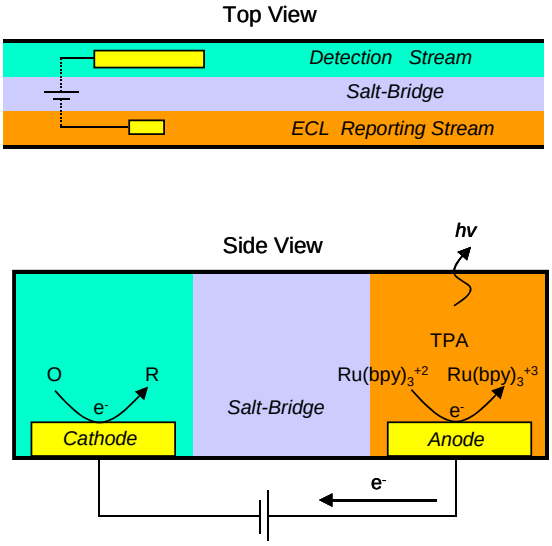
Figure 5



IV. Proposed Research

1. A Microfluidic Sensing System Based on Electrochemiluminescence Reporting: The Signal Enhancement and Detection Limit. As in any one-, two-, or three-electrode electrochemical cell, the number of electrons transferred into the anode and out of the cathode must be equal at all times. [31] As a result, the current intensity, and thus the ECL signal, can be enhanced if the reaction occurring at the other electrode can be modified to generate more electrochemical events. This observation leads to at least two experimental strategies for achieving ECL enhancement. First, the relative area of the two electrodes can be changed. Because the number of electrons transferred at all times must be the same for the two electrodes, the smaller electrode will have a higher current density and thus yield a more intense ECL signal. The second way to achieve ECL enhancement signal is to

Figure 6



Note: Even not indicated in the figure, the mixing between streams due to interdiffusion as the fluids progress downstream is envisioned.

increase the supply of the redox target to the detection electrode. The resulting increase in the rate of electrochemical reduction (faradaic current) must be compensated at the ECL reporting electrode. As a result, ECL enhancement will be observed. The design for the microfluidic system that will be used to test these two strategies is summarized in Figure 6.

In addition to modifying the dimension of the microchannel and microelectrode, sensitive detection devices, such as photomultiplier tube (PMT), will be employed to achieve the low detection limits.

2. **Cathodic ECL Sensing of Anodically Responsive Redox Compounds.** This project represents a natural extension of our preliminary report of using an anodic ECL reaction (i.e., $Ru(bpy)_3^{2+}$/TPA system) to detect redox compounds that can undergo reduction reactions. The completion of this part of the proposed project address the need for using this ECL reporting approach for detecting redox-active targets that can be oxidized (but not reduced). On the basis of a similar reaction cascade (with a final step of annihilation of Ru^{3+} and Ru^{+} to give Ru^{2+*}), $Ru(bpy)_3^{2+}$ can also generate cathodic ECL if a suitable oxidant coexists in solution, e.g., persulfate. [32,33] Our preliminary studies indicate that this system exhibits ECL at an onset potential of around –1.6 V when a mixture of $Ru(bpy)_3^{2+}$ and $Na_2S_2O_8$ was probed using a glassy carbon working electrode. One potential problem associated with ITO-based materials is that they often show no response for organic compounds such as redox-active neurotransmitters, due to their sluggish electron transfer on ITO. Alternatively, Au electrodes promote catalytic hydrogen reduction, which limits their usefulness in aqueous solutions. On the other hand, carbon-based electrodes generally provide a relatively broader working potential range and are the most responsive for organic redox compounds. [34]

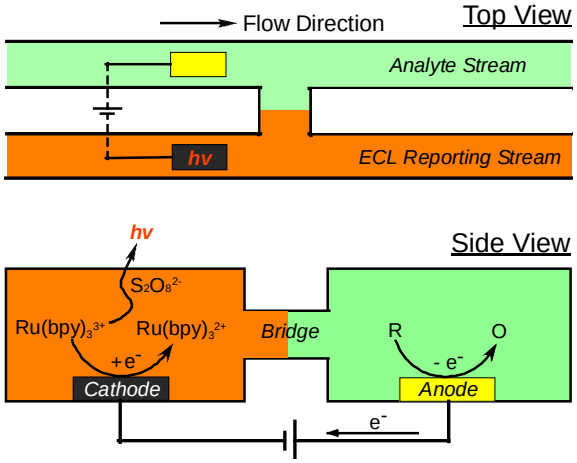
   One of the difficulties in these experiments is how to integrate carbon microelectrodes into microfluidic channels. Screen-printing can generate carbon electrodes with micron-size features, but the resulting electrodes are too thick to be sealed into a microfluidic device using our current technology. [35] Other choices include carbon fiber and carbon paste. [36] Another approach is to modify the surface properties of the electrode through electrodeposition. The deposited material will function as a conductor as well as a protector of the adhesion materials. The sensing strategy of this approach, which is similar to the anodic ECL process, is shown in Figure 7.

3. **Photonic Voltammetry Based on a Microfluidic System with ECL Reporting.** There has been considerable interest in extracting new types of information (other than current, charge, and potential) from electrochemical systems in recent years. [37] Successful examples include the coupling of various spectroscopic techniques, such as UV-vis absorption, Raman, IR, and EPR spectroscopies, with electrochemical techniques. [38] We have already shown that, when the potential bias applied between two electrodes exceeds a critical value (E_{onset}), a pair of reduction/oxidation reactions will be triggered at the two electrodes. To

a good first approximation, E_{onset} is equal to the difference in the formal potentials of the two redox reactions. This relationship between the applied potential and the formal potentials of the reactions under investigation offers us a ruler to measure the potential-ECL intensity profile and thus, use it to study various redox reactions. Specifically, if there are several redox species that undergo reduction at different potentials coexisting in the solution, they can be detected one-by-one by monitoring the ECL intensity while the potential bias is scanned.

In a series of papers, Bard and his colleagues have shown a directly correlated profile between the applied potential and the ECL emission when $Ru(bpy)_3^{2+}$ ECL system was investigated. [32, 39] Experimentally, this profile was obtained by measuring ECL from a three-electrode cell using a photo multiplier tube (PMT). They found that the shapes of the current-vs.-potential and photon-flux-vs.-potential curves differed, presumably because of the very complicated ECL reaction cascade. In other words, not every electron-transfer event results in photon emission. [40] In this sense, the amount of chemical information that the potential-dependent ECL intensity can provide is limited.

Figure 7



We envision that by using the two-electrode ECL reporting system described previously, it will be possible to construct potential-ECL intensity profiles for redox species other than ECL emitting species and coreactants that can couple with the ECL reaction used. There should be a one-to-one correspondence between the potential bias applied between the two electrodes and the luminescence intensity emitting from the reporter electrode. The prerequisite for this photonic voltammetry to be valid is that the concentration of ECL species used must be much higher than that of the compounds of interest. This requirement is important for two reasons. First, it ensures that the applied potential will be applied nearly exclusively to the electrochemical reaction of the analytes rather than to the reporter redox process(es). That is, a high concentration of ECL cocktail will poise the reporter electrode at a particular potential. Second, a high concentration of ECL reporters guarantees that the ECL reaction proceeds by a single mechanism, thereby allowing quantification of analytes with high fidelity.

In comparison to the conventional three-electrode CV measurement, our photonic voltammetry has the following advantages and disadvantages. First, it

allows analytes having extremely low concentration (for example, ~ nM - pM) to be probed due the inherently high sensitivity and low background noise of ECL. Therefore, the restriction of using micromolar to millimolar-level analytes in a conventional three-electrode system is eliminated. Second, the indicating solution (ECL species) can be conveniently separated from the analyte solution via a salt bridge. Therefore, any *potential* interference due to the coexisting redox species (e.g., ECL compounds) to the investigated reaction(s) will be prevented. Third, our system works without an external reference electrode. Instead, the extent of the reaction of the interested species is referred to its coupling, ECL reaction. Finally, this method will be able to probe only reductions or only oxidations, but not both. For example, when an anodic ECL reaction only reductions can be studied.

V. Summary

The primary goal of this proposal is to integrate different types of chemistry into microfluidic systems thus enriching their function and performance. The most important part of this proposal is a demonstration that it is possible to fabricate an electrochemical sensor using two electrodes undergoing *two* reactions: one detects the analyte of interest and the other reports its presence photonically. This differs from most two-electrode electrochemical detection strategies in which only current at a single electrode is recorded. After realizing the anode and cathode reactions are coupled by charge balance, it is straightforward to understand the significance of using the highly sensitive ECL reaction to probe other electrochemical reactions. The ability to implement this idea is largely dependent on the use of microfluidic systems in which precise control of the size and positioning of the functional components is possible. Our work in an interesting way shows the beauty of simplicity and essentially resembles the Chinese *yin-yang* philosophy. [41] Even though the three-electrode electrochemical system is well developed and will remain an important mainstream technique, our method can be used as a convenient alternative means for chip-based electrochemical studies (partially because of difficulties of fabricating on-chip reference electrodes). Again, by taking advantage of the technical merits of the microfluidic system, various electrochemical techniques, such as potential scanning and signal enhancement, based on our two-electrode principle can be realized.

VI. References

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