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Surface immobilizable chelator for label-free electrical detection of pyrophosphate†

David J. Liu,*^a Grace M. Credo,^a Xing Su,^a Kai Wu,^a Hsiao C. Lim,^a Oguz H. Elibol,^{ab} Rashid Bashir^c and Madoo Varma*^a

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A new pyrophosphate (PPI) chelator was designed for surface-sensitive electrical detection of biomolecular reactions. This article describes the synthesis of the PPI-selective receptor, its surface immobilization and application to label-free electrical detection on a silicon-based field-effect transistor (FET) sensor.

Nucleotide incorporation reactions, catalyzed by DNA and RNA polymerases, are critically important in the biological processes of living systems. Their common byproduct, pyrophosphate (PPI), is a negatively charged small molecule typically detected using optical techniques, such as chemiluminescence.¹ Label-free electrical monitoring of biochemical reactions offers several advantages including increased portability and improved integration.² The former advantage is due to the elimination of bulky optical measurement components and the latter is due to the ability to fabricate many individually addressable electronic devices at micro- or nanoscale. Scalable semiconductor manufacturing techniques can be adapted to produce dense, highly reproducible sensor arrays to process samples and signals in a highly parallel fashion. We are developing an electrical signal detection platform capable of detecting DNA synthesis reactions by making use of the intrinsic physicochemical properties of PPI. Here we report the synthesis of a PPI-selective receptor, its surface immobilization and application to label-free electrical detection on a field-effect transistor (FET) device.

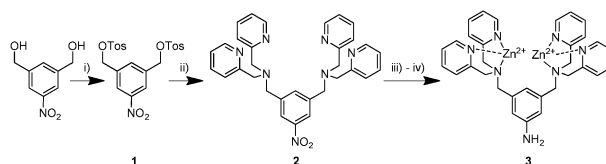
Various optical PPI detection technologies have been developed. Among these, luciferase-based PPI detection has been used for bacterial detection and DNA sequencing applications.¹ Non-enzymatic PPI detection technologies have also been reported.^{3,4} These include fluorescence- and absorption-based detection using PPI chelators, which can

detect submicromolar PPI in bulk solution. One class of chelators is designed such that they can bind to indicator dyes, where PPI is detected either colorimetrically⁵ or fluorescently⁶ when dye molecules are displaced from chelator by PPI binding.

Extending chelation-based sensing to surface-sensitive electrical detection requires a chelator compatible with surface immobilization and selective to the target analyte. Surface capture of PPI signaling molecules is expected to enhance the sensitivity of field-effect devices to PPI in a process that we call “*signal immobilization*”. The negatively-charged PPI molecules are expected to decrease the number of positively charged carriers in a p-type field-effect transistor (FET) sensor functionalized with such a chelator, resulting in a decrease in threshold voltage. In order to test this “*signal immobilization*” concept, we designed a new chelator with three functional components: a binding site, a linker, and a handle. The binding site selectively captures PPI from solution, while the linker between the binding site and handle provides steric flexibility. Finally, the handle ensures that the chelator can be selectively attached to a chemically compatible surface.

The selected PPI chelator was based on di-(2-picoly) amine (DPA), which has demonstrated strong binding affinity to PPI⁶ and is relatively straightforward to synthesize. The hydroxyl groups of 5-nitro-1,3-bishydroxymethylbenzene were first tosylated to accelerate substitution with DPA (Scheme 1). After DPA substitution, the nitro group was reduced to an amine by catalytic hydrogenation. The addition of zinc nitrate produces a functional complex with two Zn²⁺ coordination sites per chelator molecule.

Basic functionality of the synthesized chelator was verified in solution. Selective binding studies were performed using a coumarin-based fluorescent dye⁶ or a colorimetric dye, pyrocatechol violet (PV).⁵ In the case of the fluorescent dye,



Scheme 1 Synthesis of an immobilizable PPI chelator. (i) TosCl; (ii) di-(2-picoly)amine; (iii) H₂, Pd/C; (iv) Zn(NO₃)₂.

^a Integrated Biosystems Lab, Intel Labs, Intel Corporation, 2200 Mission College Blvd., Santa Clara, CA 95054, USA. E-mail: djqliu@gmail.com, madoo.varma@intel.com; Fax: 408-765-2393; Tel: 408-653-9341

^b Birck Nanotechnology Center, Purdue University, West Lafayette, IN 47907, USA

^c Departments of Electrical and Computer Engineering and Bioengineering, Micro and Nanotechnology Laboratory, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

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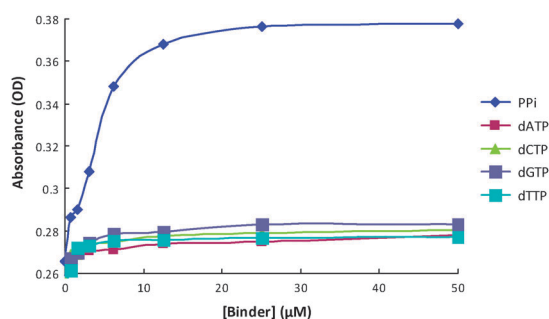
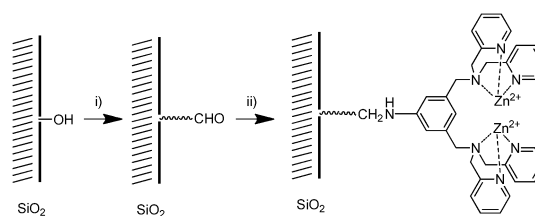


Fig. 1 Competitive displacement assay of immobilizable chelator in solution with PPI (◆), nucleotides dATP (■), dCTP (▲), dGTP (■), or dTTP (■). 1 : 1 mixtures of chelator and pyrocatechol violet (PV) were treated with the different binders with concentrations ranging from 0–50 μM . Absorption was monitored at 444 nm.

binding to the chelator caused fluorescence quenching. As more chelator was added, the fluorescence intensity decreased, reaching a plateau near 10 μM . When the PV was used, binding to the chelator caused a color change from blue (free dye, λ_{max} 444 nm) to yellow (complex, λ_{max} 624 nm). The strong absorption at 624 nm indicates formation of a chelator-dye complex. This change was also visible to the naked eye. The dose response curve corresponds to a binding constant of $1.7 \times 10^6 \text{ M}^{-1}$, close to literature values for a similar chelator.⁶

We further characterized the chelator with a colorimetric competitive assay. In this assay, PV dye is first treated with an equimolar amount of chelator to form a 1 : 1 chelator-dye weak complex. During PPI titration the PV dye is released into solution because PPI has a higher binding constant. This process is monitored by UV-Vis spectroscopy. At 444 nm the absorption increased with PPI concentration, consistent with dye displacement by PPI (Fig. 1). Similarly the fluorescent dye was also used to study PPI displacement. In this case, PPI released the fluorescent dye from the binding site resulting in increase of its fluorescence intensity at 480 nm when excited at 347 nm. These results also indicated that the addition of an amine handle to the chelator did not affect its PPI binding properties. Both fluorescence and absorption data indicate that the new chelator shows strong selectivity to PPI over interfering components including nucleotides (such as dATP) and phosphate (Fig. 1 and the ESI†).

The amine-containing PPI chelator can be immobilized onto many different surfaces provided a proper linker strategy is devised. To demonstrate that the chelator can be immobilized to silicon-derived surfaces, we introduced an aldehyde group to a clean silicon oxide surface by modification with 4-(triethoxysilyl)butyraldehyde. Compound **3** was covalently attached to surface by reductive amination using sodium triacetoxyborohydride (Scheme 2). To ensure the chelator was indeed immobilized, changes in thin film thickness and surface properties were characterized by ellipsometry, atomic force microscope (AFM) and surface-sensitive mass spectrometry (TOF-SIMS). Monolayer thicknesses and sample topography were consistent with step-by-step surface modification of silicon surfaces (ESI†). TOF-SIMS measurements of modified surfaces yielded the expected mass of the immobilized chelator (Fig. 2a) while the chelator was not detected on a blank substrate (no reaction, green line) and a negative control



Scheme 2 Surface immobilization of immobilizable PPI chelator. (i) 4-(Triethoxysilyl)butyraldehyde; (ii) **3**, borate (pH 8), NaBH_4Ac_3 .

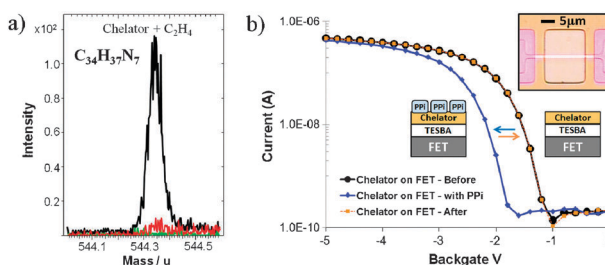


Fig. 2 Surface characterization of chelator on modified silicon and electrical detection of pyrophosphate with a chelator-modified FET device. (a) Confirmation of chelator molecule by TOF SIMS compared to blank and process control samples (green, red lines); (b) Label-free detection of 25 μM PPI (◆) by a chelator-modified FET device in pH 8 Tris buffer with Zn^{2+} , compared to overlapping response in same buffer before PPI exposure (●) and after rinsing PPI with dilute acid (■). The inset is an optical microscopy image of the FET device used for these measurements.

substrate exposed to chelator, but missing the aldehyde modification (process control, red line).

To demonstrate the effect of “*signal immobilization*” on PPI sensitivity, we explored pyrophosphate sensing using a silicon-on-insulator field effect transistor (SOI-FET) device.⁷ SOI-FET devices can sense changes in surface charge and have been used as a surface sensitive biosensors.^{2,8–10} Due to the ability to tune both top gate and back gate voltages, SOI-FET devices can offer better sensitivity compared to bulk FET devices.¹¹ The chelator-immobilized FET device was exposed to 25 μM PPI in Tris buffer (pH 8). The drain current *versus* gate potential obtained in accumulation mode of the p-type device shifted towards more negative potentials after exposure to the PPI-containing solution (Fig. 2b), consistent with a field-effect caused by the binding of a positively charged molecule to chelator on the p-type sensor surface. While this response was not in the expected direction based on charge alone, the negatively charged PPI was not directly interacting with the FET sensor surface. We hypothesize that the unexpected direction of the sensor response may be caused by a surface dipole effect in which the surface dipole of the chelator is changed only when the immobilized chelator-zinc complex binds to PPI at the surface.^{9,12} A control buffer solution without PPI containing the same concentration of zinc does not yield the same effect as exposure to PPI, as shown in Fig. 2b (‘before’ and ‘after’). In addition, chelator-modified FET devices were relatively pH insensitive, with similar IV curves upon exposure to both pH 8 Tris buffer and pH 3 aqueous acetic acid (0.1 M).

We collected IV characteristics from several devices on the same chip to characterize PPI selectivity. The devices were

probed serially after the addition of each solvent. For example, upon exposure to buffer, four different devices were characterized in order to avoid drawing conclusions from single, anomalous devices. Then, the next buffer was introduced to the chamber and the next set of measurements were collected. Upon exposure to PPI solution, a similar threshold voltage shift at 1 nA (-0.5 ± 0.1 V compared to initial buffer) was observed on four different chelator-modified SOI-FET devices on the same chip and this response was reversible after a 0.1 M acetic acid rinse and second incubation in buffer ($+0.4 \pm 0.1$ V). While there were slight variations in curve shape and threshold voltage position from sensor to sensor, the IV curve shapes for individual sensors for Tris buffer before, during PPI exposure, and Tris buffer after were similar, as shown in Fig. 2b.

FET devices modified with PEG blocking molecules instead of PPI-sensitive chelator were not responsive to 25 μ M PPI in Tris buffer solution with Zn^{2+} (ESI $^+$). In addition, unmodified FET devices exhibited no response to the same level of PPI in solution. These observations strongly indicate that PPI capture by the chelator on the sensor surface causes changes in surface charge distribution and thus enables selective electrical detection of PPI. These results also suggested that a chelator-modified FET device could detect PPI generated from polymerase reactions in solution. Preliminary data from our lab indicate this indeed is the case and results will be published elsewhere.¹³

In summary, we have synthesized and characterized a new type of pyrophosphate chelator and demonstrated electronic detection of PPI by immobilizing it to a field-effect sensor. Because PPI is a common product of many important biological reactions,¹⁴ the concept of “*signal immobilization*” of PPI presented in this work can be applied to develop electronic biosensors for broad biomedical applications from DNA sequencing to microbe detection. This concept could

also be expanded to other surface-sensitive detection technologies for PPI or its analogues, such as on-surface optical fluorescent or colorimetric detection, waveguide-based detection, and surface plasmon resonance.

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