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Enzymatic/Immunoassay Dual Biomarker Sensing Chip: Towards Decentralized Insulin/Glucose Detection

Eva Vargas[+], Hazhir Teymourian[+], Farshad Tehrani[+], Ece Eksin, Esther Sánchez-Tirado, Paul Warren, Arzum Erdem, Eyal Dassau, and Joseph Wang*

Abstract: Performing different bioassay formats, based on enzyme and antibody recognition reactions, with a single detection chip remains an unmet challenge due to the completely different requirements of such bioassays. Here we describe a dual marker biosensor chip, integrating enzyme and antibody-based assays for simultaneous electrochemical measurements of insulin (I) and glucose (G). Such simultaneous G/I sensing capability has been realized by addressing key fabrication and operational challenges associated with the completely different operational requirements and surface chemistries. The insulin immunosensor relies on a peroxidase-labeled sandwich immunoassay while glucose is monitored enzymatically in the presence of a mediated glucose-oxidase (GOx) reaction. The dual biomarker diabetes chip offers selective and reproducible detection of picomolar insulin and millimolar glucose concentrations in a single microliter sample droplet within less than 30 min, including direct measurements in whole blood and saliva samples. The resulting integrated enzymatic-immunoassay biosensor chip opens up a new realm in point-of-care multiplexed biomarker detection.

A major goal in disease screening, diagnosis and control has been the development of bioassay platforms capable of simultaneous measurements of different types of analytes in a single assay.[1] Major advances toward multiplexed biomarker detection chips based on either immunoassay[2] or enzymatic bioassays[3] have thus been reported. However, the combination of both enzymatic and immunoassay sensing systems - each with its own fundamentally distinct sensing format and fabrication requirements - into a single disposable chip platform is yet to be addressed.

Here we present the successful implementation of a dual marker biosensor, integrating enzymatic and immunosensing electrochemical detection protocols into a single sensing chip. The new concept is demonstrated toward the reliable decentralized detection of insulin (I) and glucose (G), as two key diabetes biomarkers. Point-of-care tandem measurements of glucose and insulin in the same microliter sample droplet is of tremendous importance to personalized diabetes management towards improved estimates of insulin sensitivity and enhanced regulation of glucose levels.[4] Despite major advances in diabetes management, including self-testing glucose strips, the ability to rapidly measure insulin and to concurrently monitor both glucose and insulin represents an unmet challenge that can transform clinical care.

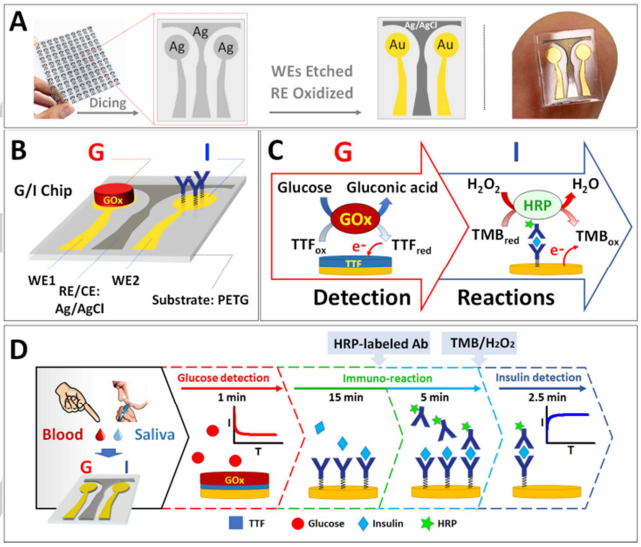

Figure 1. The concept of dual G/I biosensor chip. (A) Image of the G/I sensing chip array on PETG substrate and schematics of a sensor chip showing the two Au WEs for the glucose and insulin biosensors, with an Ag/AgCl electrode acting as a joint RE/CE. (B) The localized detection of G and I on a single sensor chip, showing the immobilized GOx and insulin antibody bioreceptors. (C) Recognition and redox processes involved in the G/I sensing. Glucose detection is performed amperometrically at +0.2V on Au WE1 through the TTF-mediated biocatalytic (GOx) oxidation of glucose. Insulin is detected on Au WE2 at -0.1V via a sandwich immunoreaction assay by measuring the current signal of H_2O_2 reduction, catalyzed by the captured HRP tag and mediated by TMB. (D) Glucose is measured, followed by 15 min sample incubation and subsequent 5 min incubation of HRP-labeled D-Ab. Finally, insulin is monitored after washing the surface and adding TMB/H_2O_2 probe.

The integration of the fundamentally different enzymatic and immunochemical assays onto a single chip device, towards simultaneous detection of insulin and glucose, faces major fabrication, operational and sensing challenges, all of which have been addressed successfully in our work. These include (i) the huge difference between the physiological concentrations of insulin (pM) and glucose (mM); (ii) the different surface chemistries required for enzymatic and immunosensors; (iii) the completely different assay formats and time scales; (iv) the need for compact low-cost chip footprint capable of simultaneous bioassays of microliter (~10µL) samples; (v) ultra low (pM)

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insulin concentration in bodily fluids which requires ultrasensitive immunoassay format; (vi) potential cross-talk and cross-reactivity between the neighboring biocatalytic and bioaffinity sensors; and (viii) short analysis time to prevent changes in biomarker levels, avoid surface biofouling and provide timely information. The dual G/I sensor chip is designed in a three-electrode system, comprising of thin-film sputtered silver and gold electrodes on a plastic PETG substrate (Figure 1), with a joint Ag/AgCl reference/counter electrode (RE) and two Au working electrodes (WEs) as transducers for glucose and insulin detection. Figure 1A shows an array of fabricated sensor chips. The fabrication process is detailed in Figure S1. The localized detection of the insulin and glucose targets is shown in Figure 1B, where different recognition and surface chemistries are exploited for each analyte. The glucose biosensor is fabricated through a layer-by-layer deposition protocol (described in the SI) where the glucose oxidase (GOx) enzyme is immobilized within a permeable biopolymer chitosan film and onto the TTF redox mediator layer on WE1 (Figure 1B). The insulin sensor relies on a sandwich immunoassay format using horseradish peroxidase (HRP) as enzymatic label and 3,3',5,5'-tetramethylbenzidine (TMB)/H_2O_2 as the mediator/substrate detection system. The insulin capture antibody (C-Ab) is covalently attached to carboxylic groups of the mixed self-assembled monolayer (SAM) on WE2 via EDC/NHS coupling, along with surface blocking with ethanolamine. This is followed by two-step incubation of insulin and HRP-labeled detection antibody (D-Ab) for 15 and 5 min, respectively, to form a sandwich immunocomplex of insulin and to capture the D-Ab. Figure 1C illustrates the mechanisms involved in each sensing platform. The GOx-based biocatalytic detection of glucose relies on the electron-carrying mediator TTF, and the reoxidation of the reduced form of TTF to yield current response proportional to the glucose concentration. In the case of insulin immunosensor, the Ab-modified electrode is incubated in different insulin concentrations, followed by incubation with the D-Ab, and introduction of the TMB/H_2O_2 detection solution. HRP thus catalyzes the reduction of H_2O_2 coupled to the oxidation of TMB into its oxidized form. The corresponding H_2O_2 reduction signal is thus directly proportional to the amount of the captured HRP tag and hence to the insulin concentration.

The resulting electrochemical chip holds considerable promise for the simultaneous dual-marker G/I detection in different body fluids samples within less than 30 min (Figure 1D). The ability of the G/I chip to detect each biomarker in such fluids was demonstrated using complex untreated whole blood and human saliva sample. A single sample droplet (10µl) was cast over the sensor chip to cover the entire electrode area. Glucose is thus analyzed amperometrically on WE1 within the initial 60 s, while the insulin recognition requires additional 15 min incubation for binding with the anti-insulin antibody. Subsequently, to complete the sandwich immunoreaction, a 5min incubation with D-Ab is performed, followed by amperometric detection of the H_2O_2/TMB redox probe. Such measurement sequence and assay protocol, assuring minimal cross-talk between dual G/I measurements, was carefully optimized and will be discussed in the text.

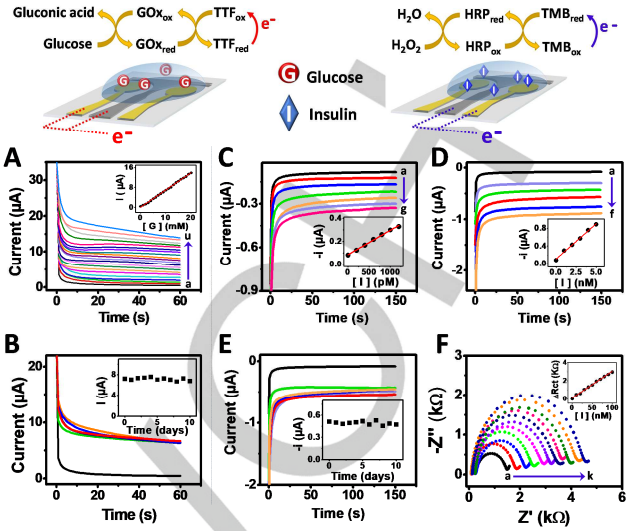

Figure 2. The electrochemical sensing performance of G/I chip. (A) Amperometric response of enzymatic G sensor to increasing concentrations of glucose from 0 (a) to 20mM (u) with 1mM increments. (B) Reproducibility of the G sensor for 10 mM glucose detection. Inset is the stability of G sensor, showing 10mM glucose signal monitoring during 10 days from fabrication. (C) Amperometric response of the I immunosensor to increasing insulin concentrations from 0 (a) to 1200pM (g) with 200pM increments. (D) Amperometric response of the I immunosensor to increasing insulin concentrations from 0 (a) to 5nM (f) with 1nM increments. (E) Reproducibility of the I immunoassay for 3nM insulin. Inset is the stability of I immunosensor, corresponding to signals for 3nM insulin during 10 days from fabrication. (F) Nyquist curves of the I immunosensor upon increasing the I concentration over the range 0-100nM (a-k) with 10nM increments.

The electrochemical sensing performance and operational characteristics of the dual biosensor chip were evaluated (Figure 2) after optimizing the experimental conditions for each sensor (detailed in Table S1). The analytical performance of the G/I chip was assessed first by constructing calibration plots for both analytes in PBS. The enzymatic G sensor was characterized first from its amperometric response for millimolar glucose solutions (Figure 2A), whereas the insulin immunosensor was evaluated both in the pM and nM concentration ranges (Figures 2C and 2D, respectively). These data yield limits of detection (LODs) of 0.2mM glucose and 41pM insulin. The analytical performance of the insulin sensor was examined also by label-free EIS immunosensing approach using Nyquist curves for increasing insulin concentrations between 10 and 100nM (Figure 2F), which results in a LOD of 8.9nM insulin. Such label-free immunosensing approach results in approximately 200-fold lower sensitivity compared to the HRP-labeled sandwich based amperometric immunosensor. The latter thus meets the demands of highly sensitive pM insulin detection in body fluids.

The reproducibility of the amperometric sensor response was assessed by testing 5 different glucose and insulin biosensors constructed using the same fabrication protocol. Chronoamperometric measurements recorded for 10mM glucose (Figure 2B) and 3nM insulin (Figure 2E) yielded RSD values of 2.0% and 8.9% respectively, confirming the reliability of the chip fabrication and operation. Additionally, the storage stability of the G and I sensors was evaluated by monitoring the amperometric response for 10mM glucose and 3nM insulin. Both

sensors were thus stored at 4ºC and monitored during 10 days. The highly stable glucose and insulin response observed over these 10-day periods (Figure 2B and 2E, respectively) demonstrate that such storage has no apparent effect on the sensitivity.

The dual G/I chip is fabricated through optimal control of the modification protocols (see fabrication details in Figure S2). The primary fabrication challenge that we addressed is the fundamentally different surface (receptor-immobilization) chemistries required for the adjacent G and I sensors on the same single chip. As shown in Figure S3A, initial results showed that the presence of SAM, necessary for the I immunosensor, suppresses the response of the adjacent G sensor. Further experiments showed that SAM on RE should be removed in order to preserve an optimal current response. This may be ascribed to an increasingly sluggish oxygen reduction as the cathodic half-reaction on RE surface in the presence of SAM. In contrast, TTF and GOx enzyme can be co-immobilized on the SAM-modified G WE without compromising the enzymatic activity and detection, with the glucose signal independent of the presence of SAM on WE surface.[5] During the fabrication development procedures, various methods were examined for removing the SAM from the metallic surface of the sensor (see Figures S3B and S3C). These included oxygen plasma treatment, chemical reduction with $NaBH_4$ and chemical oxidation by using NH_4OH:H_2O_2.[6] All treatments were suitable for removing SAM; however, because of the nature of oxygen plasma and $NaBH_4$ treatment methods which require sealing I WE by masking, the most practical and safe SAM removal protocol was found to be oxidizing the SAM with NH_4OH:H_2O_2. It is also worth noting that Ag RE conversion into Ag/AgCl is performed after removing the SAM as the opposite order led to destruction of the conductive nature of electrode, which may be attributed to an irreversible partial transition of AgCl to Ag_2O. Another challenge relates to spreading of acetone/ethanolic solution of TTF during immobilization by drop casting to neighboring electrodes. This was addressed through optimizing the spatial separation of the electrodes to 2mm to avoid spreading of TTF to the I sensing electrode.

In order to demonstrate the applicability of the fabricated G/I chips toward assays of microliter sample mixtures, different G-I mixtures of varied concentrations of G and I in the ranges of 2-18mM and 0.5-3nM, respectively, were tested. For this purpose, a two-step incubation protocol was adopted for the insulin immunoassay. Figure 3 (A-D) displays the signals obtained for glucose (red curves) and insulin (blue curves) on 4 different chips exposed to these different G/I mixtures of varying concentrations. Well-defined amperometric signals were obtained for all cases, indicating minimal cross-talk and successful G/I measurements using disposable single dual-analyte chips in the same µL sample drop (Figures 3E and 3F). Such protocol offers also high reproducibility, as demonstrated in Figure 3G for six different chips with a 12mM glucose/3nM insulin sample mixture (RSD values of 5.0% and 8.3% for G and I sensors, respectively).

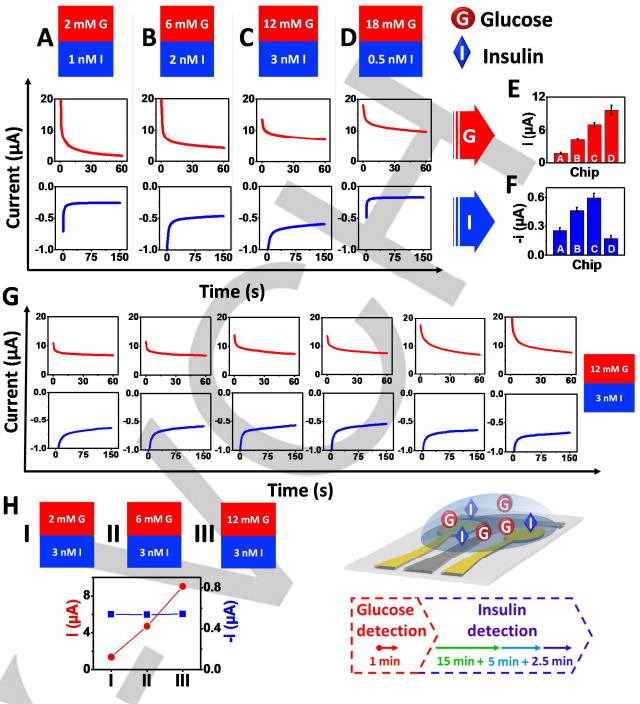

Figure 3. Cross-talk evaluation of the G/I chip in G/I mixture solutions. Amperometric response of the G/I sensor chip to various combinations of G/I concentrations: (A) 2mM G/1nM I; (B) 6mM G/2nM I; (C) 12mM G/3nM I; (D) 18mM G/0.5nM I. Red and blue curves represent the amperometric response of the G and I sensors, respectively. Glucose amperometric measurements were carried out during the first minute, while insulin was detected after 15min incubation with the same sample followed by 5min incubation with HRP-labeled D-Ab. (E,F) Amperometric response of G (E) and I (F) in sample mixtures. (G) Reproducibility of dual analyte measurements at 6 G/I chips. (H) Validation of the dual G/I chips using measurements of three different sample mixtures of increasing G concentrations and a fixed insulin level (I,II,III) using the two-step incubation protocol.

We compared the two-step *vs.* one-step immunoassay incubation protocols towards G/I mixture analysis; the results are shown in Figures 3H and S4. In the one-step protocol, the D-Ab is introduced immediately after glucose measurement and incubated along with insulin in the sample during 15min. For each protocol, the G/I signals were recorded for different chips when exposed to a fixed (3nM) insulin concentration and increasing glucose levels (2, 6, and 12mM). The two-step protocol resulted in similar insulin response along with increasing glucose signals. Surprisingly, the one-step method resulted in decreased current values for insulin upon increasing the glucose concentrations. While H_2O_2 is not directly involved in our mediated glucose detection mechanism, it can still be produced in the presence of GOx enzyme with glucose and oxygen in the solution,[7] and affect the activity of the captured HRP enzyme tag of D-Ab during the 15min incubation step,[8] ultimately leading to a false-negative insulin response. This mechanism was further confirmed by a two-step protocol in which D-Ab was incubated separately on the chip. In this case, increasing G level in the mixture did not impact the insulin signal (Figure 3H).

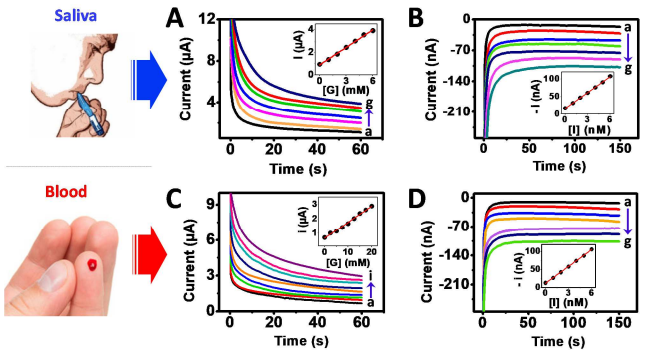

Figure 4. Electrochemical sensing performance of G/I chip using blood and saliva samples. (A) Amperometric response of the G sensor to increasing concentrations of glucose from 0 (a) to 6mM (g) with 1mM increments in real human saliva. (B) Amperometric response of I immunosensor to increasing concentrations of insulin from 0 (a) to 6nM (g) with 1nM increments in real human saliva. (C) Amperometric response of G sensor to increasing concentrations of glucose from 0 (a) to 20mM (i) with 2.5mM increments in whole blood (D) Amperometric response of the I immunosensor for increasing insulin concentrations from 0 (a) to 6nM (g) with 1nM increments in whole blood.

The ability to detect multiple biomarkers in as-received body fluids, without any sample treatment can have a profound impact on decentralized clinical screening. The newly developed G and I sensors were tested individually using untreated whole blood and raw saliva samples. The saliva collected by a Salivette® and the finger-stick capillary blood sample were directly pipetted on the electrode surface for analysis. The saliva sample was spiked with increasing 1mM glucose and 1nM insulin concentrations, displayed a well-defined response (Figures 4A and 4B, respectively), with of LOD 209μM G and 340pM I. Similarly, using the capillary blood, the chip offers convenient measurements of the increasing 2.5mM glucose and 1.0nM insulin concentrations (Figures 4C and 4D), with LOD values of 674μM G and 300pM I. Despite the lower sensitivity observed with blood and saliva samples, compared to PBS (owing to matrix effects), the G/I sensors demonstrated a very attractive biosensing performance, leading to a first demonstration of strips capable of G/I measurements in body fluids.

In conclusion, we presented the first example of a single electrochemical chip incorporating fundamentally different enzymatic and immunoassays of microliter sample mixtures and demonstrated its analytical attractive performance toward simultaneous point-of-care detection of key diabetes biomarkers (insulin and glucose). Such single-chip G/I sensing platform has been realized by judiciously addressing key operational and fabrication challenges associated with the different surface chemistries and procedures of these two different bioassays, and the significantly different (>million fold) G and I concentration levels. While the new G/I microchip offers considerable promise toward the management of diabetes, the new device requires further development, critical evaluation and extensive validation for widespread implementation. Such decentralized diabetes assays would benefit also from the electrochemical nature of the chip and from the new low-cost non-lithography simple microchip fabrication approach. Ultimately, such coupling of enzymatic and immunoassays of metabolites and protein biomarkers onto single microchip sensing devices opens up new possibilities for point-of-care screening and diagnosis of various diseases and disorders and for variety of environmental and biodefense applications.

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Conflict of interest

The authors declare no competing financial interests.

Keywords: body fluids analysis• diabetes chip• dual-marker detection• electrochemical biosensor• enzymatic-immunoassay integration

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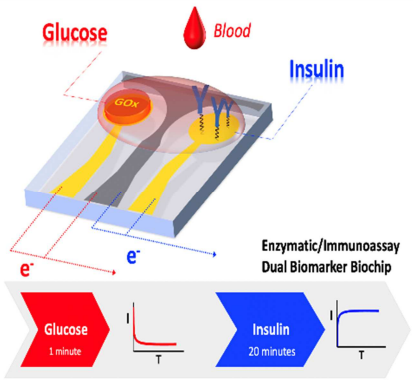

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