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# Oirented Antibody Immobilization Vs Random Covalent Binding: Improving Reproducibility for Label-Free Electrochemical Immunosensing of Multi-Allergens on Silicon Chips

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***Abstract*— The development of compact biosensing devices capable of sensitive and selective concomitant detection of two or more analytes in a single sample has enormous potential for point-of-care and -use applications. Silicon microfabrication technologies outperform other fabrication processes in terms of reproducibility, miniaturization capability, and design adjustability. In this work, we present a novel miniaturized fully integrated multiplexed silicon-based sensing platform for rapid and label-free detection of milk and egg allergens in water. After modifying the microelectrodes with gold nanostructures and chitosan-gold nanocomposite hydrogel layer, we compared random covalent binding with boronic acid-assisted oriented strategy for antibody immobilization to the surface. The Boronic affinity-based approach exhibited a high reproducible and reliable immuno-response towards casein as a major milk allergen. The stepwise surface modifications characterized using various analytical techniques, confirming the successful attachment of antibodies to the surface. We then successfully adapted the biosensor surface for ovalbumin detection, an egg allergen, demonstrating multiplexed allergen detection and universal immobilization strategy for any IgG type antibodies, opening a wide range of potential applications in portable immunosensing for various target analytes beyond food allergens.**

**Keywords—*silicon microfabrication, multiplexed electrochemical immunosensing, label free detection, food allergens, site-selective orientations***

## I. INTRODUCTION

The susceptibility of people to food allergens is on the rise, posing a major concern for public health, food and beverage industry and food safety authority sectors. Casein and ovalbumin, comprising 80% of bovine milk and 58% of egg white proteins, are key markers for detecting milk and egg contamination, both common food allergens in the food industry. Current quantitative methods for detecting food allergens such as polymerase chain reaction (PCR), liquid chromatography-tandem mass spectrometry (LC-MS/MS), and enzyme-linked immunosorbent assay (ELISA) are laboratory-based, typically slow, labour-intensive and costly[1]–[3]. In contract, label-free electrochemical immunosensing for allergens detection proposes high sensitivity, better selectivity, fast analysis, low cost, simple use, capability for portability, miniaturisation and multiplexing. Silicon microfabrication has revolutionised large-scale, mass production of highly reproducible multi-array platforms. Combined with portable electronics, these compact devices are particularly valuable for diagnostics beyond laboratories, including point-of-care (POC) and point-of-use (POU) applications for rapid detection [4]–[6].

The strategy for antibody immobilization on a solid electrode surface greatly affects immunosensor performance. Traditional methods like physical adsorption and direct covalent binding are simple but often lead to random antibody orientation, causing issues like denaturation and blocked Fab regions [7]. In contrast, oriented immobilization strategies, such as Fc-binding proteins (Protein A/G) and targeting specific groups (carbohydrate moieties, sulfhydryl, aldehyde, histidine), or biotin/(strept)avidin systems, ensure proper antibody orientation for antigen detection[8], [9] . The boronic acid (BA)-assisted strategy offers a promising alternative, forming stable cyclic boronate esters with diols in the Fc region, improving sensitivity, reducing steric hindrance, and being cost-effective and applicable to all IgG type antibodies [10]–[12].

Despite their limitations, random immobilizations can still produce stable immunosensors on larger macroelectrodes, where favorable orientations may occur by chance. Several studies have reported successful label-free detection of casein and ovalbumin using random approaches in both single [13]–[16] and multiplexed detection setups [17], [18]. However, when dealing with microelectrodes, random immobilization becomes a significant challenge, affecting the stability and reproducibility of the sensor. Thus, it is necessary to investigate suitable strategy for antibody attachment on microelectrodes.

Herein, we aimed to explore for the first time the application of a fully integrated silicon chip multi-sensing platform to develop a robust portable electrochemical immuno-device. We first modified the microelectrodes with gold nanostructured particles, followed by electrodeposition of a chitosan-gold nanocomposite to provide functional groups for covalent binding. We then assessed two immobilization methods, covalent random and boronic acid-assisted approaches, to determine the most reliable and reproducible method for detecting casein as a model target milk allergen. After thorough testing, we concluded that the boronic acid-assisted immobilization strategy was the most suitable and compatible for antibody attachment onto the microelectrode surface on silicon chip. Characterization studies confirmed the immobilization pathways, and we successfully expanded the method to immobilize anti-ovalbumin for ovalbumin detection, demonstrating the platform's potential for multiplexed analysis.

## II. MATERIALS & METHODS

### *A. Materials*

Rabbit polyclonal anti-casein and anti-ovalbumin antibodies was purchase from Abcam. Chitosan, bovine

serum albumin (BSA), potassium ferrocyanide/ferricyanide, phosphate buffer saline tablets (PBS, 0.01 M, pH7.4), gold (III) chloride trihydrate, α-Casein from bovine milk, Ovalbumin from chicken egg white, NHS, EDC and 4-carboxyphenylboronic acid (CPBA), alizarin red-s (ARS) and ethanolamine were obtained from Sigma Aldrich. Superblock, goat anti-rabbit IgG conjugated with cyanine5 were purchased from Thermofisher.

### B. *Silicon Chip multiplexed platfrom microfabrication*

The chips were fabricated using photolithography. The process began with the thermal growth of a silicon dioxide passivation layer. Three single band electrodes were patterned using metal mask-1, followed by titanium-gold deposition and lift-off. After patterning the leads, connection tracks, and counter/reference electrodes using mask-2, a layer of titanium, gold, and platinum was then deposited, followed by another lift-off. Finally, a silicon nitride ($Si_3N_4$) passivation layer was applied, and windows were etched to expose the electrodes and contact points before dicing [4], [19], [20].

### C. *Electrochemical measurments for immunoassay*

Differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS) studies were performed using an Multi AutoLab M101 potentiostat in PBS pH 7.4 solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$ redox probe . Both DPVs and EIS were recorded before and after incubation with different concentrations of casein and ovalbumin. We used individual sensors for each concentration and calculated standard deviations from at least three chips, each containing three immunosensors.

### D. *On-Chip multi- immunosensors development*

Prior to electrode modifications, the chip was cleaned by acetone and deionised water. To increase the surface area, all three microelectrodes were modified with gold nanostructures by applying a potential 0.0 V for 3.0 s in 50 mM $HAuCl_4$ dissolved in 0.1 M KCl solution. Then, chitosan-gold nanocomposite (CS-Gold) was electrodeposited by applying a potential of −1.4 V for 30 s in the acetate buffer pH 5 solution containing chitosan 0.04% (v/v) and 50 ppm $HAuCl_4$ as mentioned previously [19], [21].

Here, after chitosan modification, two immobilization strategies were explored to find out the suitable immobilization strategies compatible to our electrode design on chip. (i) Random immobilization was achieved using NHS/EDC coupling chemistry by casting 5 µl of an activated antibody mixture (3 µL anti-casein 200 µg/mL, 15 µLEDC 2.5 mM, 12 µL NHS 50 mM) onto the electrodes and incubating overnight at 4°C. (ii) For boronic acid assisted immobilization strategy, we first covalently modified chitosan with 4-carboxylphenylboronic acid (CS-CPBA) using a solution containing CPBA:NHS: EDC (4:1:1 molar ratio) in PBS (pH 7.4. After casting 5 µL of this mixture on each chip and shaking in a humidity chamber for 1 hour, the CPBA-modified chitosan was washed with PB buffer (pH 7.0). Then, 5 µL of antibody (30 µg/ml) was dropped to each chip and incubated for 1 hour at room temperature. Different blocking agents (BSA 0.1%, superblock), alone or in combination with different concentration of ethanolamine ( ETA 0.1% and 6%), were tested to prevent non-specific binding. The biosensors then were stored in the fridge until use.

## III. Results and Discussion

### A. *Sensing platfrom charestrisations*

Fig.1A shows a fully integrated compact silicon chip comprising three single-band gold microelectrodes (1 µm width and 178 µm length), enabling multi analyte detection,

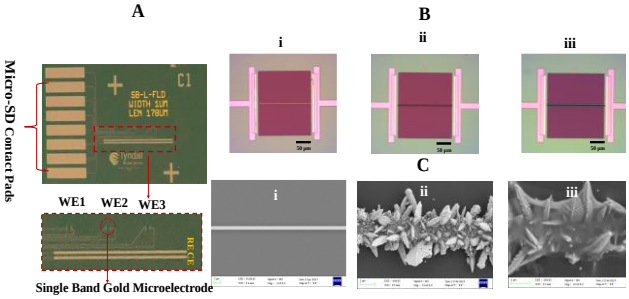



Fig.1 (A) fully integrated silicon chip; (B) optical and (C) SEM images of (i) bare, (ii) gold nanostructured and (iii) chitosan-gold nanocomposite modified single band gold microelectrode.

along with a platinum counter and pseudo reference electrodes, can be connected to the potentiostat via micro-SD-connectors. Optical images (Fig.1B) and SEM images (Fig.1C) illustrate the step-by-step electrochemical modification of microelectrodes with gold nanostructures (ii) and chitosan-gold nanocomposites (iii). Both characterizations confirm the formation of a thin and uniform layer of chitosan hydrogel over the gold nanostructures modified electrode.

### B. *Random immobilization*

Fig. 2 shows the initial study of random immobilization by examining the DPV currents of immunosensors on three chips before and after incubation with casein. The results revealed notable variations in both on -chip and between-chip responses, attributed to the statistically random alignment of antibodies at the microelectrode surface. To address the irreproducibility, we explored boronic acid-assisted immobilization to achieve an efficient, reliable, reproducible, and cost-effective method.

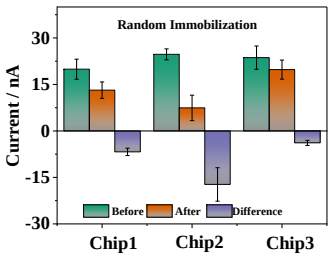



Fig. 2. The performance of three chips with three immunosensors prepared by random immobilization approach toward 10 µg $mL^{-1}$ casein.

### C. *Fluorescence characterizations*

Fig.3A shows fluorescence images of the CS-CPBA modified electrode before (i) and after (ii) incubation with alizarin red S (ARS). Boronic acids have a strong affinity for cis-diol-containing compounds like ARS forms a fluorescent boronate ester complex, increasing fluorescence emission [27]. The significant fluorescence observed on the CS-CPBA modified electrode surface confirms successful functionalization with boronic acid. In contrast, bare CS electrodes (Fig.3B) showed no fluorescence, indicating

selective ARS binding to CPBA. Fluorescence microscopy (Fig.3C) further validated effective immobilization of Cyanine 5-conjugated anti-IgG antibodies on the CS-CPBA modified electrodes, confirming high-density antibody attachment.

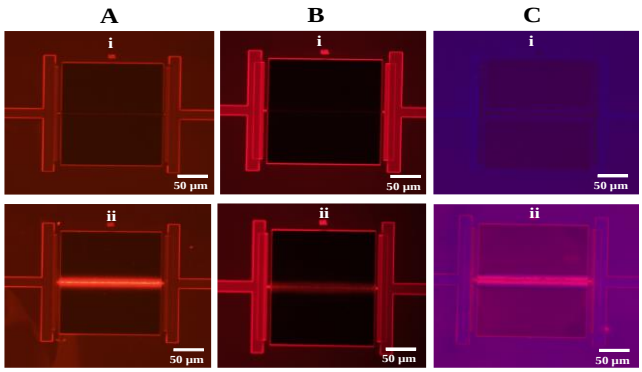



Fig.3. Fluorescence images of microelectrodes modified with (A) CS-CPBA, (B) CS before (i) and after (ii) incubation with ARS; and (C) CS-CPBA before (i) and after (ii) immobilization of Cyanine 5 conjugated anti IgG.

### *D. Analytical features of the immunosensor*

The capability of the CS-CPBA modified microelectrodes for casein determination in phosphate buffer was examined by DPV and EIS studies as shown in Fig. 4. The current in DPV exhibited a decrease proportional to the concentration of casein (Fig. 4A). Fig. 4B illustrates the calibration graphs from two independent experiments, prepared using 0.1% BSA blocking agent for 30 minutes, demonstrating a linear relationship between the decrease in current and the log [casein] within the range of 1 to 100 µg mL$^{-1}$ with a detection limit (LOD, 3σ/S) 0.1 µg mL$^{-1}$ of casein. These results indicate highly reproducible calibration curves and successful detection of casein in PBS solution.

We then conducted further studies on the effect of blocking protocols on the immunosensors' performance. Fig. 4C presents the slope of various calibrations. The result revealed that the sensitivity of immunosensor toward casein enhanced when the surface was blocked with superblock and ETA 6% (CAL-4). Also, the immunosensors showed linear response with the log [casein] in the range of 1 to 200 µg mL$^{-1}$ with a LOD 0.036 µg mL$^{-1}$ of casein. As a result, the sensitivity increased, leading to improvements in both the linear range and limit of detection.

We also measured EIS signal of biosensors before and after incubation with different concentrations of casein. As depicted in Fig. 4D, charge transfer resistance (Rct) of the immunosensors by increasing the concentration of casein increased due to antigen-antibody interactions. The ΔRct have a linear relationship with the log [casein] in the range of 5 to 200 µg mL$^{-1}$, showing a good agreement with the DPV results.

In addition, we assessed on-chip and between-chip reproducibility of the casein immunosensors as illustrated in Fig.6, indicating significant enhancement in the reproducibility of the immunosensor preparation and in the reliability of the immunosensor. Also, the improvement in both sensitivity and reproducibility may be attributed to the mildly alkaline pH (8.5) of ETA 6%, as well as the smaller size of ETA compared to BSA or superblock proteins, which helps reduce non-specific binding more effectively. Additionally, the pH may positively favor the binding between the boronic acid functional group and the diol moiety in the Fc region of the antibody, while minimizing secondary interactions such as hydrophobic and charge transfer interactions. This reduces, and potentially eliminates, partially random orientations [17].

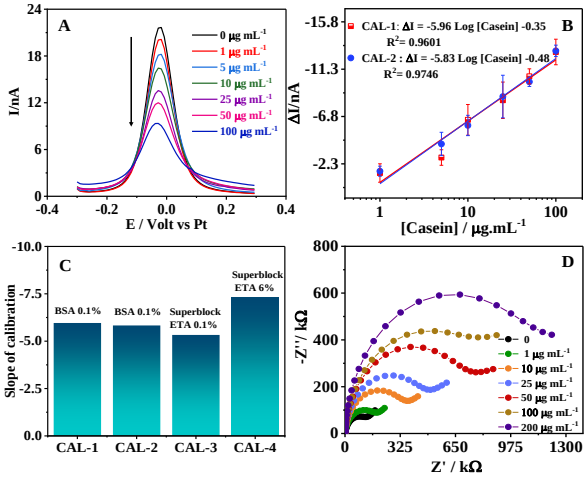



Fig.4 (A) DPVs, (B) corresponding calibration curves of the immunosensor prepared with BSA blocking protocol, (C) Comparison of calibration slopes using different blocking protocols; (D) (A) Nyquist plots of the immunosensors using superblock-ETA 6% blocking protocol toward different concentrations of casein.

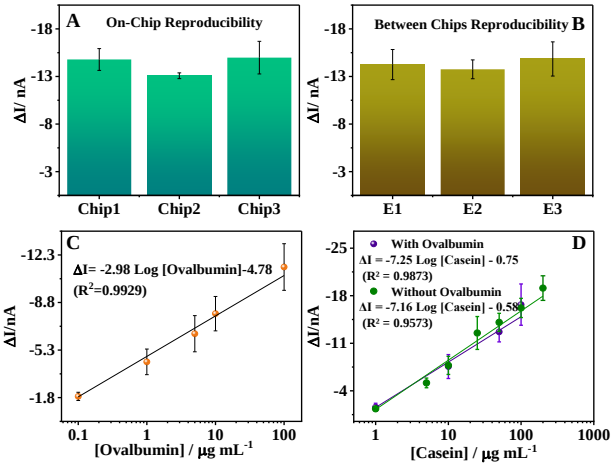



Fig. 5 (A) on-chip and (B) between chips reproducibility of the biosensors toward casein 50 µg mL$^{-1}$; (C) Calibration curves for Ovalbumin detection (D) overlayed two calibration curves of the casein immunosensors toward casein in the absence and presence of 10 µg mL$^{-1}$ Ovalbumin.

After successfully developing a biosensor for casein using a boronic acid-assisted approach, we adapted this platform to enable multi-analyte analysis. To achieve this, we replaced the anti-casein antibody with an anti-ovalbumin antibody and conducted ovalbumin detection. Fig. 5C shows the calibration curve for the ovalbumin assay, which exhibited a good linear range from 0.1 to 100 µg mL$^{-1}$ with a LOD of 0.07 µg mL$^{-1}$. This study clearly demonstrates that the sensing platform can be applied to any anti-IgG isotype antibodies, as they possess sugar moieties in their Fc regions. Fig. 5D presents calibration curves for the casein assay in the absence and presence of 10 µg mL$^{-1}$ ovalbumin, indicating successful and interference-free detection of casein in the presence of ovalbumin.

We also evaluated the capability of the biosensor for detecting casein in tap water as a real sample. For this, we prepared all casein solutions using tap water instead of PBS

and compared the results. Both assays exhibited good correlation (recovery ranging from 95–108 %).

## IV. CONCLUSIONS

In this work, we successfully developed a compact multi-immunosensing silicon platform for two allergens. We evaluated two immobilization techniques and found that the boronic acid-assisted approach was the most reliable and reproducible for attaching antibodies to the microelectrode surfaces. This resulted in reproducible and sensitive detection of casein, a model target allergen. Additionally, the platform demonstrated strong potential for multiplexed analysis of multiple allergens. The developed biorecognition layer is compatible with any IgG isotype antibody, making it adaptable to a wide range of target analytes. We also plan to integrate this sensing device with portable electronics for real-time detection and remote monitoring.

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