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# Synthesis of robust electrochemical substrate and fabrication of immobilization free biosensors for rapid sensing of salicylate and β-hydroxybutyrate in whole blood

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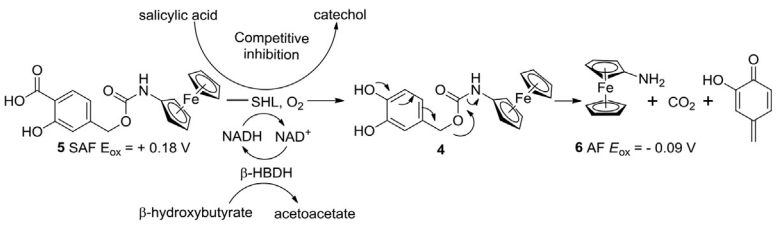
## HIGHLIGHTS

- Design, synthesis and characterization of electrochemical redox substrate, SAF.
- A solution-based rapid sensitive assay for salicylate hydroxylase.
- Biosensors fabrication for rapid sensing of salicylate and β-hydroxybutyrate in whole blood.
- Method to screen salicylate poisoning and diabetic ketoacidosis.

## GRAPHICAL ABSTRACT

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## ABSTRACT

An electrochemical latent redox probe, SAF **5** was designed, synthesized and characterized. A rapid and sensitive solution-based assay was demonstrated for salicylate hydroxylase (SHL). In presence of NADH at aerobic conditions, SHL catalyzed the decarboxylative hydroxylation of SAF and released a redox reporter amino ferrocene (AF **6**). The release of AF **6** was monitored at interference free potential region (−50 mV vs. Ag|AgCl) using differential pulse voltammetry as signal read-out. The current signal generated by this process is highly specific, and insensitive to other biological interfering compounds. Next, the SAF incorporated SHL assay was extended to fabricate immobilization-free biosensors for rapid sensing of salicylic acid (SA) and β-hydroxybutyrate (β-HB) in whole blood. The described method rapidly detects SA in a linear range of 35–560 μM with detection limit of 5.0 μM. For β-HB determination, the linear range was 10–600 μM and detection limit was 2.0 μM. Besides, the assay protocols are simple, fast, reliable, selective, sensitive and advantageous over existing methods. The whole blood assay did not required cumbersome steps such as, enzyme immobilization, pre-treatments and holds great practical potential in clinical diagnosis.



## 1. Introduction

Salicylate hydroxylase (SHL), a flavin adenine dinucleotide (FAD) dependent monooxygenase binds to salicylate and NADH to form a reduced enzyme-substrate complex, which upon binding with

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oxygen produces catechol, $CO_2$, and $H_2O$ [1,2]. SHL was used as a signal generator in electrochemical detection of NADH with high performance characteristics [3]. Recently, the co-immobilization of $NAD(P)^+$-dependent dehydrogenase with SHL in front of Clark-type oxygen electrode has been investigated in the development of dehydrogenase-based biosensor that can detect several important analytes [4,5]. We are interested to design a similar SHL incorporated dehydrogenase-based biosensor for multiple analytes, but without using Clark electrode. The Clark electrode often encountering issues such as, instability in readings, uniform signal drift, changes in sensor response time, and formation of gas bubbles in electrolyte [6]. Generally, enzyme immobilized biosensors have poor long-term storage stability, more likely to undergo enzyme denaturation, and exhibit poor reproducibility [7].

Latent electrochemical probes, a class of highly selective and stable small molecules equipped with unique triggering groups and masked electroactive reporters. These probes selectively interact with the analyte of interest via user-designated biochemical events to unmask their inherent reporter signal [8] In the current work, we exploited the design and synthesis of robust latent electrochemical probe, SAF **5** that can be used to assay SHL. The chemical structure of SAF **5** consisted of salicylate moiety linked to a reporter AF **6** (amino ferrocene) through a carbamate linkage. In presence of NADH and under aerobic conditions, SHL catalyzes the decarboxylative hydroxylation of SAF followed by a quinone-methide-type rearrangement reaction ejects AF **6**. AF **6** able to report its redox signals at low overpotential region of the voltammogram [9]. Next, a general type of immobilization-free biosensors for salicylic acid (SA) and β-hydroxybutyrate (β-HB) have been developed by co-incubation of $NAD^+$-dependent dehydrogenase with SHL in presence of SAF.

SA, a main metabolite of aspirin is responsible for the pharmacological activities of aspirin. The regular uptake of aspirin in low dose is useful in preventing or delaying neurodegenerative disorders such as, Parkinson's disease [10,11]. However, its excess concentration in blood (>2.2 mM) is toxic, while > 4.3 mM in blood is lethal [12]. The salicylate toxicity affects multiple organ systems including the central nervous system, the gastrointestinal system and the pulmonary system [13]. Therefore, rapid and accurate determination of SA in blood is highly important in hospitals. Here, we developed a rapid and simple SA assay protocol using SAF **5,** SHL and NADH and this method is successful in quantifying SA in human blood (Scheme 1).

The determination of β-HB is of high significance, as it is a biomarker for glycemic control in diabetes. The development of diagnostic device for the early detection of β-HB offers an opportunity for the screening of diabetic ketoacidosis (DKA) [14]. In a healthy individual, the blood concentration of β-HB is less than 0.5 mM, however it exceeds to higher levels (4.3–6.0 mM) in patients with DKA [15]. The common β-HB detection method involves two steps; (1) Oxidation of β-HB to acetoacetate by β-hydroxybutyrate dehydrogenase (β-HBDH) with simultaneous conversion of NAD + to NADH, and (2) the detection of as-produced NADH via mediated electron transfer [14]. Here, SHL coupled co-immobilization of $NAD(P)^+$-dependent dehydrogenase is the usual method, while immobilization-free solution based assay procedure has never been reported in the literature for β-HB assay. Nevertheless, the solution-based enzymatic assay procedure is, (1) fast since the enzymatic reaction can be quickly performed via enzyme-substrate-analyte incubation at physiological conditions, (2) easy to be performed by lab technician as the procedure requires simple mixing of solutions, and (3) it is less likely to get denaturized since fresh solutions of enzyme can be quickly prepared on the spot.

The main objective of this work is to develop a novel substrate for SHL assay and utilize it to construct an immobilization free biosensor for rapid sensing of SA (Scheme 1) and β-HB (Scheme 2). The synthesis of SAF **5** is straightforward in facile steps. The described method eliminates time-consuming steps such as, enzyme immobilization, pre-treatments, derivatization and electrode modifications.

## 2. Experimental

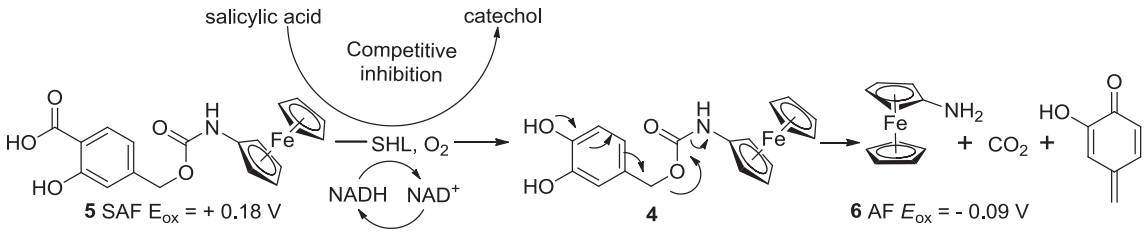
### 2.1. Chemicals and instrumentation

All experiments were performed in a solution mixture of 100 mM Tris-HEPES buffer (Tris-H buffer) pH 8.0, while a small amount (0.1%) of triton x-100 was included in the mixture. The stock solution of SAF (**5**) was prepared in DMSO. All assays were performed in triplicate, and the results reported are the average of at least three experiments.

Three electrode cell comprising glassy carbon electrode (GCE) as a working electrode (area = 0.071 $cm^2$), Pt wire as a counter electrode, and Ag|AgCl (saturated KCl) as a reference electrode was adopted to perform electrochemical experiments. The optimized cyclic voltammogram (CV) parameters: potential range = −0.30–0.40 V and scan rate = 50 mV $s^{-1}$. The optimized differential pulse voltammogram (DPV) parameters: −0.30 to 0.05 V, amplitude = 0.05 V, sampling width = 0.0167 s and pulse period = 0.5 s. Additional details of chemicals and instrumentations are given in Supporting information section S1.

### 2.2. Electrochemical assay procedure

For SHL assay, the solution of SAF (0.2 mM) in 100 mM Tris-H (pH 8.0) containing SHL (1 U/mL) with or without NADH (0.4 mM) was incubated at 37 °C for 10 min. After incubation, the solution was transferred to an electrochemical cell to perform electrochemical experiments. For SA assay, the solution of SAF (1 mM) in 100 mM Tris-H (pH 8.0) containing SHL (1 U/mL), NADH (0.4 mM) and various concentration of SA was incubated at 37 °C for 10 min. For β-HB assay, the solution mixture containing SAF (1 mM), SHL (1 U/mL), HBDH (8 U/mL), $NAD^+$ (1 mM), and different concentrations of β-HB in 100 mM Tris-H buffer (PH 8.0) was incubated for 30 min at 37 °C.




**Scheme 1.** The chemical structural of SAF (**5**), the AF (**6**) releasing mechanism, and illustration of SHL coupled biosensor for the determination of salicylic acid.