Rapid and cost-effective fabrication of biosensors for *Salmonella* detection

Ivana Kundacina, Manil Kukkar, Ivan Nastasijevic, Sasa Jankovic, Radmila Mitrovic, Vasa Radonic

University of Novi Sad, BioSense Institute, Dr Zorana Djindjica 1, 21000 Novi Sad, Serbia

Institute of Meat Hygiene and Technology, Kacanskog 13, 11040, Belgrade, Serbia

vasarad@biosense.rs

***Abstract*—Ensuring food safety is of predominant importance, and the realization of user-friendly and rapid detection methods of pathogens plays a critical role in this effort. *Salmonella Typhimurium* (*S. Typhimurium*) has the highest incidence rate among all *Salmonella* infections. In this paper, a cost-effective biosensor for the quantitative detection of *S. Typhimurium* is proposed based on a rapid manufacturing process. Gold leaf electrodes (GLEs) based impedimetric immunosensor have been developed and validated for highly sensitive and specific detection of *S. Typhimurium* with a detection limit of 10^2 cfu/mL.**

Keywords—biosensor; Salmonella Typhimurium; gold leaf electrodes; impedimetric immunosensor;

I. INTRODUCTION

Salmonella stands out as a highly recognized pathogen responsible for numerous outbreaks of foodborne illnesses [1], [2]. The World Health Organization (WHO) reports that *Salmonella* was responsible for 58 food safety incidents across 86 Member States and territories [3]. The infectious dose for *Salmonella* needed to provoke foodborne disease, which should be ingested by a generally healthy person is relatively high and it is estimated to be 10^5 cfu/g [4], but it could be significantly lower in the case of a vulnerable population (young, old, pregnant, etc.).

Recently, there has been a significant advancement in the development of novel biosensors to take over conventional methods that require special equipment, methods, and trained personnel (plating and incubation, ELISA, PCR)[5]–[7]. Novel sensing solutions propose colorimetric, fluorescence, surface-enhanced Raman scattering, and electrochemical biosensors [8]–[11]. Electrochemical biosensors have emerged as the leading contenders due to their suitability for field applications, remarkable sensitivity, selectivity, cost-effectiveness, labor-saving attributes, and ease of accessibility [12], [13]. However, their commercialization has remained relatively limited due to the challenge of the fabrication of gold electrodes, which are the most used substrate for electrochemical sensor realization [14].

Traditional methods of fabrication of gold electrodes like photolithography and shadow mask lithography are labour-intensive and require specialized cleanroom facilities [15]. Other technologies like electroplating and vacuum deposition are expensive and generate waste that has to be additionally treated, while inkjet and screen-printing are affordable alternatives but can result in non-uniform films and low reproducibility [16]. Screen-printed electrodes (SPEs) are the most commercially available and the number of biosensors proposed in the literature so far use SPEs from different manufacturers like Dropsens [17], Palmsens [18], Nanoshell [19], etc. However, this technology is limited for substrate selection since it needs to handle the high temperature in postprocessing and therefore they are often not suitable for integration into PoC devices. Gold leaf sheets are rarely used due to fragility [20]. Few studies employed pure gold leaf for glucose and DNAse sensing [20], [21] while our previous study shows the advanced potential of GLEs for enhanced sensitivity of bacteria detection [22]. Previous research studies realized gold leaf electrodes based on the lamination process on polyvinyl chloride (PVC) foils [20], [21]. In comparison with previously described technologies, beyond its simplicity, the lamination process does not require the use of harmful chemicals like metal ion-free (MIF) developers, acetone, photoresist, gold etchant, etc., making it a rapid and simple solution for electrode fabrication.

In this paper, we introduce the custom-made, single-use GLEs fabricated in-house, utilizing 24-karat gold leaves and showcase their analytical capabilities in identifying *Salmonella* cells. The study affirms that gold-leaf electrodes possess favorable attributes that make them suitable for developing cost-effective biosensing solutions. This is evidenced by their successful utilization in achieving highly sensitive immunodetection.

II. ELECTRODE FABRICATION AND CHARACTERIZATION

A. Fabrication of GLEs

A schematic diagram in Fig. 1 illustrates the fabrication process of a GLE with a 2.5 mm radius of working electrode. The electrode fabrication involves the lamination of four supporting layers- 125 µm thick PVC (ImageLast Micron Laminating Pouch, Fellowes Brands) foils with two layers of 24-karat gold leaves (Pozlata Dimitrijevic, Serbia). The PVC foil contains glue, which is released at 180 ˚C during the hot lamination (Laminator PDA3 330C (PINGDA)).

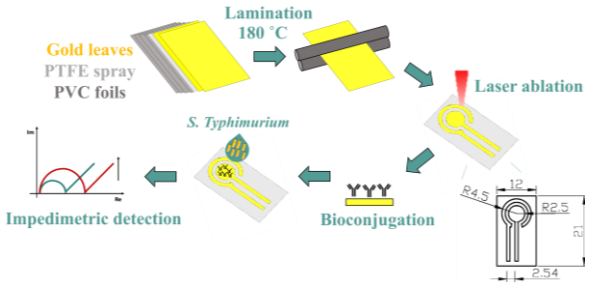

Fig. 1 Schematic diagram of the fabrication process of gold leaf electrodes based impedimetric immunosensor with sensor dimensions in mm.

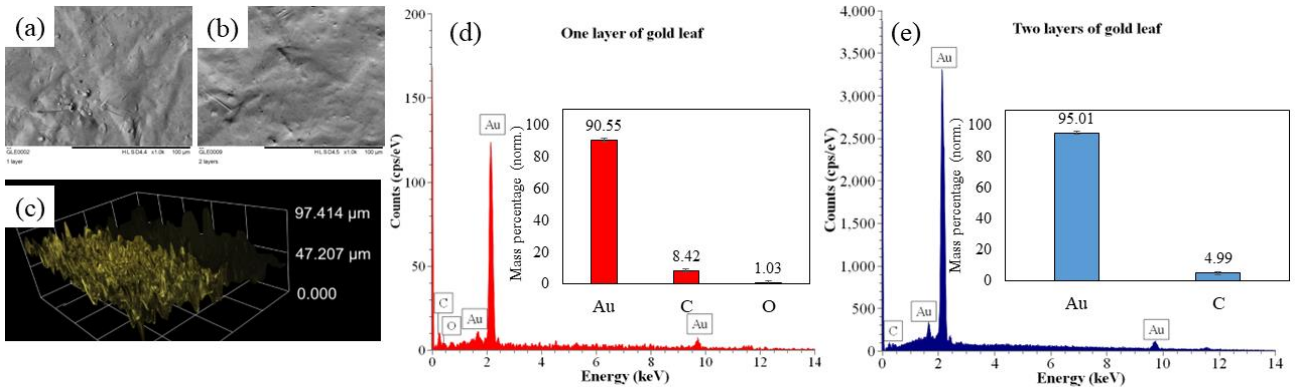

Fig. 2 (a) SEM image of one-layer GLE (b) SEM image of two-layer GLE (c) 3D profile of two-layer GLE (d) EDX spectrum of one-layer GLE (e) EDX spectrum of two-layer GLE.

(Rofin-Sinar, Germany) operating in hatch mode at 26.2 A current, 65 kHz frequency, and 500 mm/s speed for laser ablation of gold. In one shot of laser ablation, 18 electrodes with dimensions 12 mm × 21 mm can be produced. The laser ablation enables realization of different geometries, while laser resolution can go down to 30 µm.

B. Impact of gold leaf layers on electrode surface quality

An optical profilometer (Huvitz, Panasis, Republic of Korea) and SEM (Hitachi TM3030, Japan) have been used for examining the influence of the number of gold leaf layers on surface roughness and element content. Figs. 2a and 2b show SEM images, while Figs. 2d and 2e show energy-dispersive X-ray spectroscopy (EDX) analyses, for GLEs realized with one and two gold layers, respectively.

The SEM image in Fig. 2a shows that the adhesive layer of the PVC foil penetrates through the thin and porous gold leaf layer, which enables the binding of an additional layer of gold leaf and enhances the surface roughness and sensitivity of the electrode. This assumption is confirmed through EDX results, presented in Fig. 2d, where a greater content of carbon and oxygen (originating probably from the glue of PVC foil) is detected in comparison with GLEs with two layers, Fig. 2e. Moreover, electrodes with two layers displayed a noticeable surface roughness in the range of dozens of microns, likely resulting from the additional layer of gold, Fig. 2c. Subsequently, the gold surface has been utilized for functionalization with antibodies for the realization of an immunosensor.

III. BIOSENSOR REALIZATION

A. Electrochemical characterization of GLEs

Initially, GLEs have been cleaned in the 0.5 M H_2SO_4 by cyclic voltammetry (CV) for 10 cycles in a potential window of -0.2 to 1.9 V vs. Ag/AgCl reference electrode (R0303, China) at a scan rate of 0.5 V/s. Fig. 3a presents a fingerprint of the gold, made in the 0.5 M H_2SO_4 where oxidation/reduction peaks can be seen, indicating the pure gold content of electrodes.

The 5 mM redox probe used in the experiments has been prepared in 10 mM phosphate buffer saline (PBS, Fisher Bioreagents, UK) as a mixture of potassium ferricyanide and potassium ferrocyanide, both purchased from Sigma Aldrich. Redox probe has been used for electrochemical impedance spectroscopy (EIS) characterization of the gold surface for five different electrodes, examining the reproducibility of the manufacturing. The results in Fig. 3b show a similar response with low variability, especially in the semi-circle part of the Nyquist diagram, which defines charge transfer resistance (R_{ct}). This parameter is followed for each functionalization step and different concentrations of bacteria, describing their binding at the gold surface.

B. EIS measurements and equivalent circuit fitting

Electrochemical characterization has been done with potentiostat PalmSens 4 (Netherlands), and PSTrace 5.8 software. EIS measurements have been performed in the frequency range from 1 Hz to 100 kHz with a direct potential set to zero, and a potential amplitude of 10 mV versus open circuit potential. The open-source program, EIS Spectrum Analyzer [23] has been used for fitting the impedance spectra with an equivalent circuit. The Nyquist plots have been fitted with the equivalent circuit presented in Fig. 3f. The proposed circuit contains a solution resistance R_{sol}, and a parallel RC circuit which described the droplet-metal interface, where the R_{ct} presents a charge transfer resistance, while the constant phase element (CPE) has been used for simulation of a real capacitor properties of double-layer capacitance.

C. Antibody immobilization on the GLE

The proposed immunosensor based on GLEs has been realized through multiple incubations of drop-cast droplets at the working electrode. A self-assembled monolayer (SAM) has been formed during overnight incubation at 4 ˚C of 1 µL of 10 mM 11-mercaptoundecanoic acid (MUA, Sigma Aldrich) prepared in ethanol. A confirmation of MUA formation on the gold surface has been shown through contact angle measurements (Krüss Scientific, Germany). The MUA contains a thiol group with a strong affinity for binding to the gold surface, and a carboxyl group, which can bind an amino group of antibodies. Activation of MUA's carboxyl groups has been done through 1 h incubation of 10 µL droplet of 50 mM N-hydroxy succinimide (NHS, Sigma-Aldrich)/50 mM N-(3-Dimethylaminopropyl)-N-ethyl carbodiimide hydrochloride (EDC, Sigma-Aldrich) prepared in PBS. Afterward, 1 h incubation of antibodies (Anti-*Salmonella* typhimurium, LPS antibody, ab8274, ThermoFisher Scientific) enables covalent binding at the gold surface and finally, to prevent non-specific binding, 50 µg/mL of bovine serum albumin (BSA, Sigma Aldrich) has been incubated for 20 min at room temperature. The schematic of the functionalization layers is presented in Fig. 2d. Each step of functionalization has been characterized via EIS, shown in section Results.

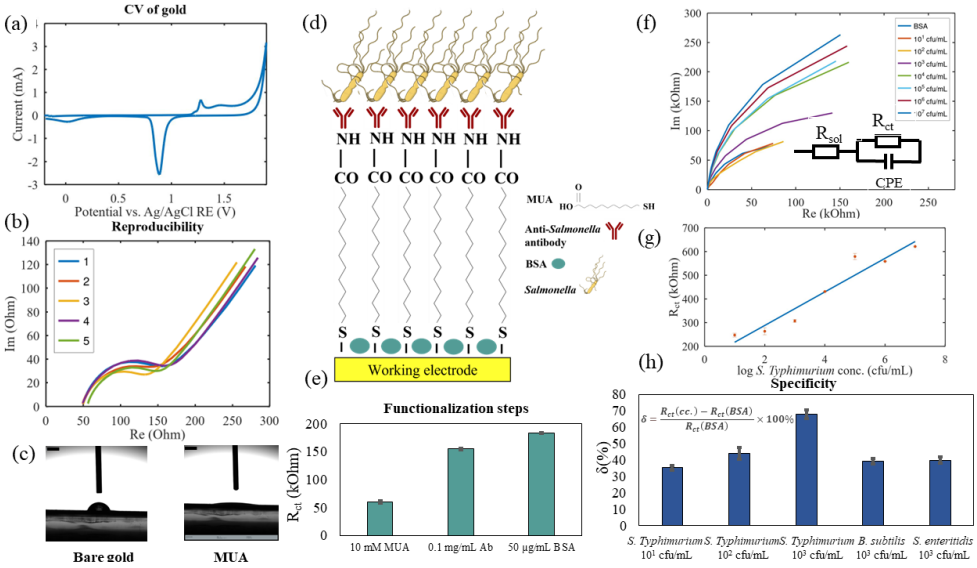

Fig. 3 (a) CV of gold in 0.5 M H_2SO_4 (b) EIS response of different electrodes illustrating reproducibility of fabrication process (c) Contact angle results for bare gold and MUA treated gold (d) Schematics of functionalization steps (e) R_{ct} response for different functionalization steps (f) EIS response for different *S. Typhimurium* concentration. Inset: Equivalent circuit used for fitting EIS results (g) Calibration curve for *S. Typhimurium* detection. (h) Relative change of the R_{ct} versus BSA functionalized electrodes for specific and non-specific bacteria.

D. Bacteria preparation

The recovery of *S. Typhimurium* ATCC 13311, PY79 *Bacillus subtilis* (*B. subtilis*) and *Salmonella enteritidis* (*S. enteritidis*) ATCC 13076 (Microbiologics, USA) from the commercial stock culture was carried out 48 h before testing to ensure the required volume of suspended culture with required serial dilutions starting from 0.5 McFarland concentrations. The preparation step included suspension of the nutrition broth (ONE Broth-Salmonella Base, Oxoid, UK) in distilled water, autoclaving at at 121 °C / 15 minutes, and cooling to room temperature, to obtain a liquid medium for *Salmonella* growth. The incubation step included treatment of the inoculated test tube overnight (37 °C/18-24 h) to allow bacterial growth.

E. Biosensor immunoassay

The previously described GLE immunosensor has been tested with *S. Typhimurium* and two non-specific bacteria, gram-negative *S. enteritidis* and gram-positive *B. subtilis*. *S. Typhimurium* detection has been tested with a series of bacterial dilutions in the range 10^1 - 10^7 cfu/mL, while specificity tests have been done with an infective concentration of *S. enteritidis* and *B. subtilis.* Each concentration of bacteria has been incubated 20 min, and afterward, rinsed with MiliQ water. EIS has been done with an 80 µl droplet of redox probe. Each measurement has been repeated with a fresh droplet of redox probe three times, and the mean value and standard deviation have been calculated.

IV. Results

The equivalent circuit model has been used for quantification of the functionalization process through comparison of R_{ct} parameter after MUA, antibodies (Ab) immobilization, and BSA steps, Fig. 3e. The results show an increasing trend of R_{ct} which proves the formation of MUA, Ab and BSA layers at the gold surface, respectively. In addition, the contact angle of the water droplet at the surface of bare gold and after SAM formation decreased from 70.7 (4)˚ to 9.6 (4)˚. This result confirms SAM formation and shows increased hydrophilic properties of the MUA, Fig. 3c. Finally, impedance results for different concentrations of *S. Typhimurium* are presented in Fig. 3f. The shape of the Nyquist plot shows a quarter circle indicating the absence of a diffusion tail at low frequencies and no charge transfer at the surface. Therefore, the results show high impedance values and R_{ct} in the range of hundreds of kOhms. The sensor has been examined with bacteria concentration up to 10^7 cfu/mL, showing a wide dynamic range of detection with no evidence of saturation on the surface. The calibration curve is presented through R_{ct} versus bacteria concentration, Fig. 3e, with an increasing quantity of *S. Typhimurium* cells, displaying the linear trend with equation $y = 70.891x + 146.29$, with an R^2 value of 0.9276.

The same approach has been used for examining the specificity of detection for *B. subtilis* and *S. enteritidis*. Due to a slight difference between R_{ct} parameter between different electrodes, a relative change in R_{ct} versus BSA functionalized GLE, δ (calculated in %), has been compared for different concentrations of specific and non-specific bacteria, Fig. 3h. The results show that the proposed sensor can successfully detect 10^2 cfu/mL of *S. Typhimurium*.

V. Conclusion

This study introduces a novel approach to the development of highly efficient electrochemical immunosensors using a GLE for the detection of *Salmonella* cells. The proposed manufacturing process is not only affordable and low-cost but also remarkably fast, with electrode production taking less than a minute. We demonstrated GLE potential for creating immunosensors capable of detecting *Salmonella*, with a wide dynamic range and potential to detect infectious doses of salmonellosis.

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