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Original scientific paper

Label-free electrochemical immunosensor for porcine gelatin using a boron-doped diamond electrode *via* diazonium salt electrografting

Irkham[1,2,✉], Fadli Taufik Abdillah[1], Muhammad Ihda H. L. Zein[3], Adisyahputra[1,4], Nazwa Alya Zahra[1], Salma Nur Zakiyyah[1] and Yeni Wahyuni Hartati[1,2,]

[1]*Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Bandung 45363, Indonesia*
[2]*Study Center of Sensor and Green Chemistry, Faculty of Mathematics and Natural Science, Universitas Padjadjaran, Bandung 40132, Indonesia*
[3]*Department of Chemistry "Giacomo Ciamician", Alma Mater Studiorum - University of Bologna, Bologna 40126, Italy*
[4]*Department of Chemistry, Faculty of Science and Engineering, Universitas Bangka Belitung, Merawang Bangka, Indonesia*

Corresponding author: ✉irkham@unpad.ac.id; ✉yeni.w.hartati@unpad.ac.id; Tel.: +62-812-2132-349

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Abstract

Porcine gelatin is widely used in the food and pharmaceutical industries due to its favorable functional properties and low cost. However, its presence in consumer products raises serious concerns for individuals with dietary restrictions based on religious, ethical, or health considerations. In this study, a label-free electrochemical immunosensor was developed using a boron-doped diamond electrode modified with aryl diazonium salt for the selective and sensitive detection of porcine gelatin. The diazonium electrografting enabled stable covalent immobilization of anti-porcine gelatin antibodies via protein A, preserving antibody orientation and activity. Experimental parameters were optimized using the Box-Behnken design, yielding ideal conditions of 500× antibody concentration, 60 min antibody incubation, and 15 min gelatin incubation. Detection was performed using differential pulse voltammetry with $[Fe(CN)_6]^{3-/4-}$ as a redox probe, allowing label-free monitoring of antibody-antigen interactions based on changes in current. The immunosensor demonstrated excellent analytical performance, with a detection limit of 142.15 pg mL^{-1}. Specificity testing confirmed that the sensor responds exclusively to porcine gelatin, showing no cross-reactivity with bovine gelatin. These results demonstrate that the proposed immunosensor provides a rapid, highly sensitive, and specific platform for porcine gelatin detection, offering great potential for food authentication and halal verification.

Keywords
Modified diamond electrode; diazonium derived film; pork skin gelatin; food authentication

Introduction

Gelatin is a widely used raw material in various global industries, particularly in the food and pharmaceutical sectors. In the food industry, it functions as a gelling agent, stabilizer, and thickener in products such as bread, jelly, and ice cream. Meanwhile, the pharmaceutical industry utilizes gelatin primarily as the shell of drug capsules and in other formulations [1]. In recent years, the issue of food fraud, especially the adulteration of food products with undeclared or prohibited ingredients, has become increasingly concerning. Such practices not only compromise product integrity but also pose health risks to the general public [2,3]. According to the U.S. Food and Drug Administration (FDA), a food product is considered adulterated if it contains harmful substances, includes undeclared components, or has been modified to increase value falsely [4]. A major concern in this context is the use of gelatin from non-declared animal sources, such as porcine gelatin. This is particularly problematic for individuals with dietary restrictions based on religious, ethical, or medical grounds. Despite these concerns, porcine gelatin remains widely used in the industry due to its cost-effectiveness and favorable physical properties [5-7]. Various parts of the porcine body, including meat, fat, blood, and skin, are processed into ingredients used in foods, cosmetics, and pharmaceuticals. Porcine gelatin is typically extracted from skin and bone by-products through partial hydrolysis of collagen using acid or base treatments, resulting in a mixture of polypeptides [8,9].

To differentiate porcine gelatin from other sources, such as bovine, several analytical techniques have been employed. These include Fourier transform infrared (FTIR) spectroscopy [10,11], high-performance liquid chromatography (HPLC) [12,13], lateral flow immunoassay (LFA) [14,15], polymerase chain reaction (PCR) [16,17], and enzyme-linked immunosorbent assay (ELISA) [18,19]. While effective, these methods often involve time-consuming protocols and lack the ability to offer rapid, on-site analysis. Recent advancements have introduced electrochemical biosensors as promising alternatives. For example, electrochemiluminescence sensors based on carbon nano-structures have achieved detection limits as low as 1 pg mL^{-1} [20], and surface plasmon resonance-based biosensors have also been applied for gelatin identification [21].

In this study, an electrochemical immunosensor was developed using a boron-doped diamond (BDD) electrode as a novel sensing platform for porcine gelatin detection. BDD electrodes, known for their wide potential window, chemical stability, and low background current, have gained significant attention in electroanalytical applications over the past five years [22,23]. Due to the inert nature of the diamond surface, surface modification is essential for biomolecule immobilization. Here, aryl diazonium salt was used to covalently graft functional groups onto the BDD surface, allowing for stable attachment of Protein A and subsequent immobilization of anti-porcine gelatin antibodies [24]. The optimization of experimental parameters was performed using the Box-Behnken design, which enables efficient modeling of linear and quadratic effects with minimal sample usage. Importantly, the developed immunosensor operates on a label-free detection principle, eliminating the need for additional labels such as enzymes, nanoparticles, or fluorescent tags. Instead, the system relies on direct measurement of changes in electrochemical signals resulting from specific antibody - antigen interactions on the modified BDD surface. This approach simplifies the detection process, reduces cost and complexity, and makes the sensor highly suitable for rapid, on-site applications in food authentication and halal verification.

Experimental

Chemicals and materials

The equipment used in this study were an autoclave sterilizer (Hirayama Autoclave HVE-50, Japan), hot plate (IKA C-MAG HS 7, Germany), magnetic stirrer (Eppendorf, Germany), micro pipette (Eppendorf, Germany), weighing balance (Mettler Toledo AL204, USA), Zimmer and Peacock potentiostat connected to a computer using PSTrace 5 software (USA), centrifuge (Eppendorf, Germany), boron-doped diamond electrode (Japan), micro tubes (Eppendorf, Germany), and micro pipette tips. The materials used in this study were demineralized water (PT Ikapharmindo Putramas Indonesia), anti-porcine gelatin (MyBioSource, USA), hydrochloric acid (HCl) (Sigma-Aldrich, USA, *p.a.*), bovine serum albumin (BSA), MiliQ water (Laboratorium Sentral UNPAD, Sumedang), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide (EDC/NHS) (Sigma-Aldrich, USA), diazonium salt (Sigma-Aldrich, USA), porcine gelatin (Sigma-Aldrich, USA), bovine gelatin (Sigma-Aldrich, USA), potassium ferricyanide ($K_3[Fe(CN)_6]$) (Sigma Aldrich, USA), potassium chloride (KCl) (Merck, USA, *p.a.*), sodium hydroxide (NaOH) (Merck, USA, *p.a.*), methanol (Sigma-Aldrich, USA), phosphate buffer saline (PBS) pH 7.4 (Merck, USA, *p.a*), and protein A-agarose (Sigma-Aldrich, USA).

Preparation of modified electrode

Diazonium salt modified electrode was prepared by electro-grafting with 20 mg of 4-amino-benzoic (ABA) in 2 mL of 37 % HCl, then the solution was cooled at temperature of 0 °C and stirred for 15 min. Next, 2 mL of demineralized water was added to the mixture and stirred to dissolve 4-ABA chloride. Then, 25 mg $NaNO_2$ was dissolved in 3 mL of water. Then, the electro-grafting was carried out in quiescent solution by 11 consecutive cyclic scans in the potential range from 0 to +0.8 V at a scan rate of 100 mV s^{-1}. After that, the electrode was rinsed with water. Protein A modified electrode was fabricated by according to the reference [25]. The electrode was immersed in 50 µL of protein A and EDC/NHS in the ratio (0.5 mg mL^{-1} of protein A, 4.8 mg mL^{-1} of EDC and 2.87 mg mL^{-1} of NHS), followed by incubation for 1 hour at 4°C and rinsed again with water. The electrode was immersed in 50 µL of porcine anti-gelatin with a concentration of 0.1 g mL^{-1}. It was then incubated at 4 °C with varying incubation times. Next, 50 µL of 1 % BSA solution was added and incubated again for 30 min at room temperature. The surface was rinsed with PBS solution at pH 7.4. After the porcine anti-gelatin immobilized on the electrode surface, 50 µL of standard porcine gelatin in 0.1 M PBS was then applied to the electrode and incubated at room temperature for varying times of 5 to 15 min. Each step of electrode modification was characterized using differential pulse voltammetry (DPV) with a redox system of 40 µL 0.01 M ($K_3[Fe(CN)_6]$) in 0.1 M KCl, over a potential range of -0.3 to +0.7 V at a scan rate of 8 mV s^{-1} [26,27].

Optimization of parameters

Factors such as antibody concentration, antibody incubation time and gelatin incubation time were selected to be optimized in the experiment [28,29]. Each factor is designed at three different levels, the lowest (-1), medium (0), and highest (+1) level, as shown in Table 1. In order to get a high current difference between blanko and target (ΔI) value Table 1 anticipates a low current response. The response of the measurement results from the experiment was then processed and determined the optimum value of each of these factors using the Box-Behnken experimental design with Minitab19 software.

***Table 1**. Optimization of factors affecting experimental conditions using Box-Behnken design*

Parameter	Level			Reference
	-1	0	+1	
Anti-porcine gelatin concentration (dilution)	1 : 1500	1 : 1000	1 : 500	[28]
Incubation time of anti-porcine gelatin, h	8	16	24	[29]
Incubation time of porcine gelatin, min	5	10	15	[29]

Results and discussion

Electrode modification and characterization

The immobilization of biomolecules on boron-doped diamond (BDD) electrodes requires surface activation due to the inherently inert nature of diamond, which lacks functional groups for direct covalent bonding with proteins or other biological molecules. This chemical inertness, while advantageous for long-term electrochemical stability, presents a challenge for direct functionalization, necessitating the use of surface modification strategies to introduce reactive sites. To address this, chemical linkers such as hydroxyl, amine, or carboxyl groups are typically introduced to facilitate covalent attachment of biomolecules *via* coupling agents [30,31]. Among various strategies, the electrografting of aryl diazonium salts has gained significant attention due to its simplicity, strong surface binding, and ability to form robust, stable layers on carbon-based materials. In this study, aryl diazonium salts were electrografted onto the BDD surface to enable the immobilization of biological recognition elements. This approach not only provides a chemically stable interface but also allows for precise control over the surface functionality, which is crucial for maintaining biomolecular activity and sensor performance. In this study, aryl diazonium salts were electrografted onto the BDD surface to introduce reactive sites capable of stable biomolecule attachment. This surface modification was carried out *via* voltammetric electroreduction of 4-aminobenzoic acid (4-ABA) in acidic conditions, which produced diazonium ions that generate diazo radicals upon single-electron reduction. These radicals, containing a benzoic acid group, covalently bond to the carbon atoms on the BDD surface, forming a robust organic layer, the illustration can be seen in Figure 1. The process, monitored using cyclic voltammetry, showed a distinct reduction peak at approximately 0.2 V within a potential range of -1.2 to +0.6 V (Figure 2A), with diminishing peaks in subsequent cycles (Figure 2B), indicating progressive coverage of the electrode surface by the diazonium-derived film.

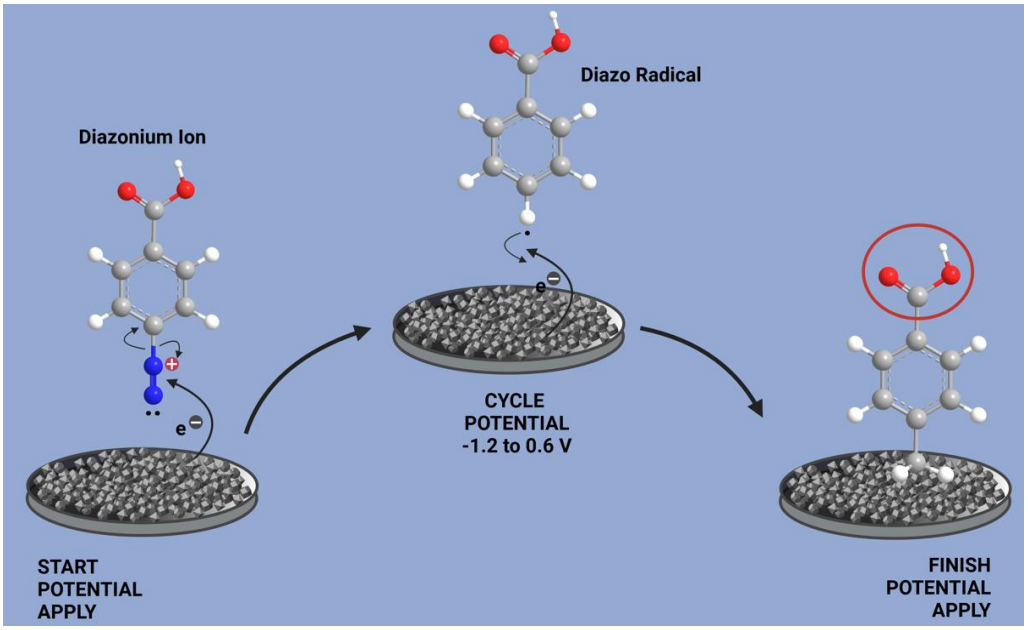

***Figure 1.** The mechanism of BDD electrode modification using diazonium salts*

Following diazonium modification, protein A was immobilized using EDC/NHS coupling chemistry, which activates carboxyl groups to facilitate stable amide bond formation with the electrode-bound layer [31,32]. Protein A plays a critical role in orienting antibodies *via* specific interaction with the Fc region, thereby leaving the Fab region accessible for target binding [33,34].

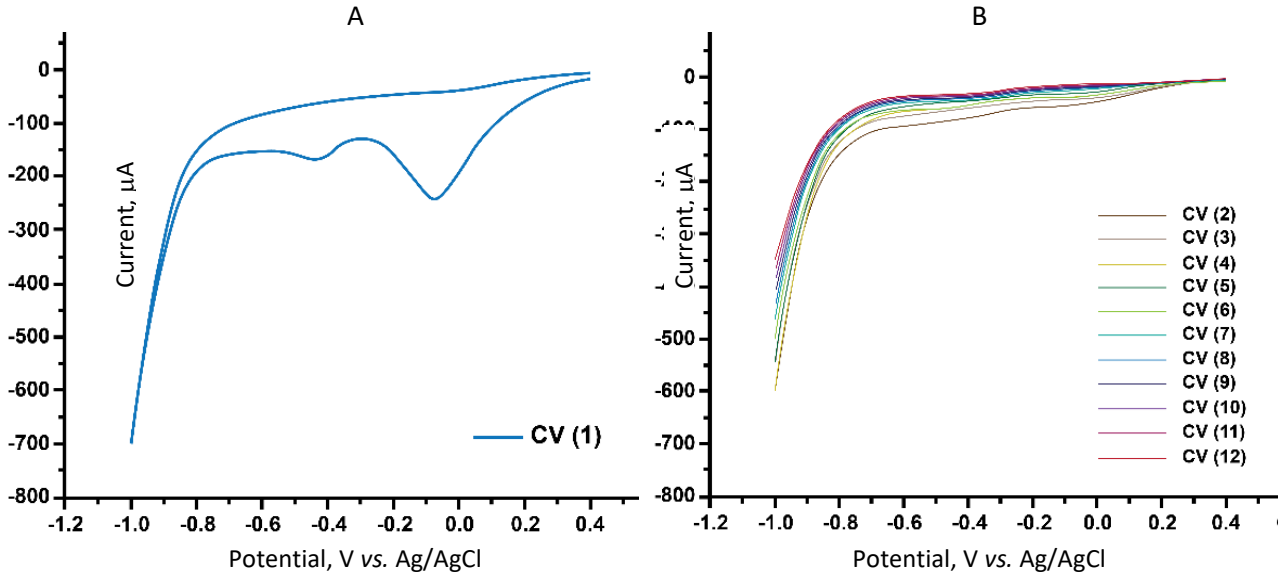

Figure 2. *(A) Cyclic voltammogram the first cycle of diazonium salt compounds over a potential range of -1.2 to 0.6 V with scan rate of 0.1 V s^{-1} and (B) the second to twelve cycle CVs of the same solution in the diazonium salt*

To minimize non-specific adsorption, BSA was introduced to block unreacted sites on the electrode surface, before then anti-porcine gelatin immobilized at the top of protein A. Importantly, BSA does not interfere with antibody-antigen interactions but prevents unwanted binding of other species, improving sensor selectivity. The final step involved applying a porcine gelatin solution (50 ng mL^{-1}) using the drop-casting technique, followed by incubation at 5 °C for 15 min to allow specific antigen-antibody binding, where the antibody's paratope recognizes and binds to the gelatin epitope [1,35]. These modification stages are illustrated in Figure 3.

The success of each functionalization step was further evaluated using differential pulse voltammetry (DPV) with $[Fe(CN)_6]^{3-/4-}$ serving as the redox probe. This technique was chosen due to its high sensitivity in detecting surface modifications that influence electron transfer processes. The DPV profiles (Figure 4 and Table 2) showed a progressive decrease in peak current following each stage of electrode modification, which is indicative of increasing surface coverage by non-conductive biomolecules. As the BDD surface became increasingly occupied by the diazonium layer, Protein A, antibodies, and finally the target antigen, electron transfer between the redox probe and the electrode was progressively hindered, leading to a measurable reduction in current. Notably, a substantial current drop was observed after the addition of porcine gelatin, confirming that the immobilized anti-porcine gelatin antibody successfully captured its target. This specific antibody-antigen binding event introduces steric and electrostatic barriers that further impede the diffusion of the redox probe to the electrode surface. The clear and reproducible current change validates the integrity and biological activity of the immobilized recognition elements. These findings demonstrate that when modified with aryl diazonium salts, the BDD electrode forms a stable and functional platform capable of supporting sensitive biosensing reactions. Importantly, this system operates under a label-free detection mechanism, meaning no secondary antibodies, enzymatic tags, or nanomaterial-based amplifiers are required.

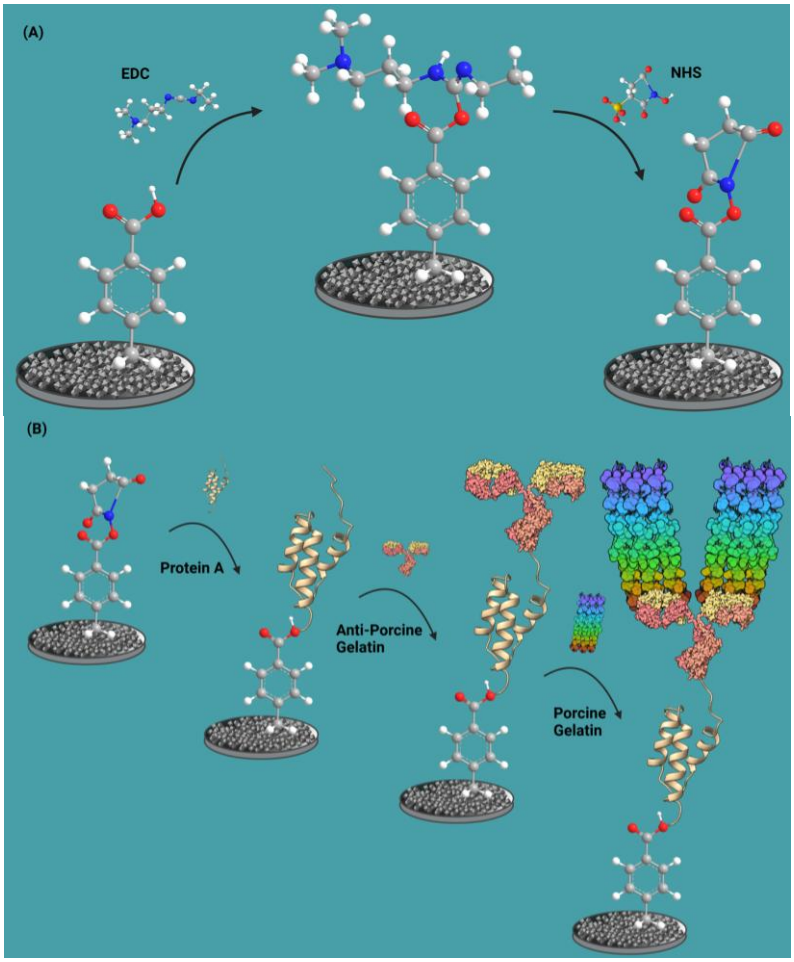

Figure 3. *Illustration of an electrochemical immunosensor detecting porcine gelatin: (A) linker attached mechanism, (B) biomolecule attached mechanism*

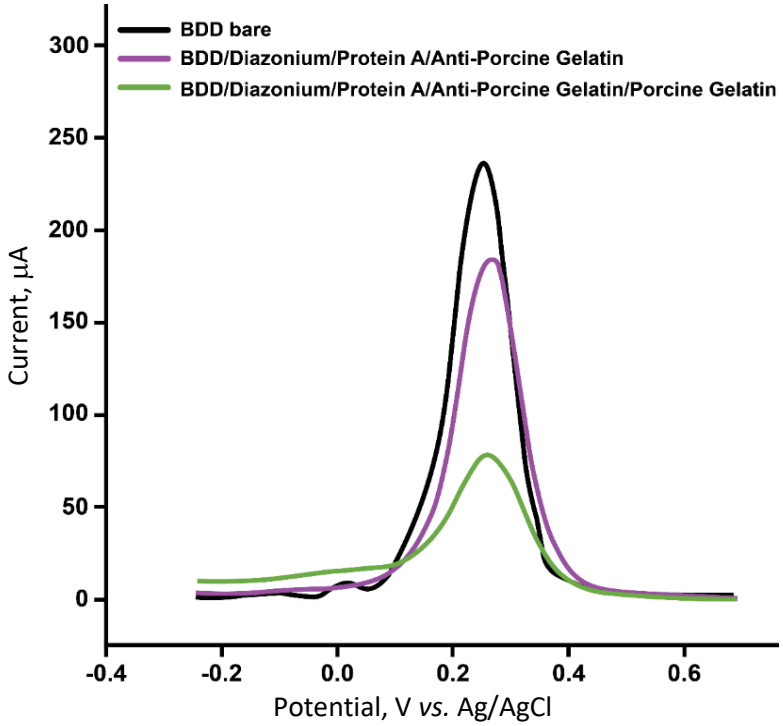

Figure 4. *BDD characterization results using differential pulse voltammetry: BDD bare (black line), BDD/diazonium/protein A/anti-gelatin (purple line), BDD/diazonium/protein A/anti-gelatin/gelatin (green line) with a redox system of 40 µL 0.01 M ($K_3[Fe(CN)_6]$) in 0.1 M KCl, over a potential range of -0.3 to +0.7 V at a scan rate of 0.008 V s^{-1}*

Table 2. *Peak current response at each Immunosensor stages performed*

BDD modification	Current, mA
BDD bare	0.237
BDD/diazonium salt/protein A/anti-porcine gelatin	0.186
BDD/diazonium salt/protein A/anti-porcine gelatin/porcine gelatin	0.073

The detection signal arises exclusively from intrinsic electrochemical changes caused by the molecular interaction between the antibody and the antigen on the electrode surface. This not only reduces complexity, time, and cost but also minimizes potential sources of variability, making the method highly attractive for real-time and point-of-care applications. The ability to detect specific binding events through direct current suppression highlights the robustness, simplicity, and high selectivity of the developed immunosensor.

Determination of optimum conditions

The optimum conditions for this experiment were determined using the Box-Behnken experimental design with the aid of Minitab19 software. Three key factors were selected based on a literature review of similar biosensor optimization studies [29,36], namely, antibody concentration, antibody incubation time (hours), and gelatin incubation time (min). Each factor was evaluated at three levels: low (-1), medium (0), and high (+1), to ensure a balanced and statistically meaningful design. The quadratic model employed generated a total of 15 experimental trials, comprising 12 factorial points and 3 center points. These center points serve as internal replicates to assess the consistency and reproducibility of the data. To minimize experimental bias, all trials were randomized. The resulting measurement data (Table 3) were analyzed using analysis of variance (ANOVA) in Minitab19 to identify the significance and contribution of each factor. As presented in Table 4, the P-values for all three variables—antibody concentration, antibody incubation time, and porcine gelatin incubation time—were found to be greater than 0.05, indicating that none of the factors had a statistically significant effect on the current response within the tested range. Nonetheless, the optimization process provided valuable insights for identifying practical and efficient experimental conditions for the immunosensor system.

Table 3. *Factors, levels, and current response from the analysis of the experimental optimization conditions*

Anti-porcine gelatin concentration (dilution)	Antibody incubation time, h	Antibody incubation time, min	Current, mA
1:1000	24	5	0.063
1:500	16	15	0.01
1:1000	16	10	0.039
1:1000	8	15	0.018
1:500	16	5	0.082
1:1500	16	15	0.012
1:1000	24	15	0.056
1:1000	16	10	0.027
1:1500	16	5	0.069
1:1000	16	10	0.008
1:500	8	10	0.026
1:1500	8	10	0.006
1:1500	24	10	0.007
1:1000	8	5	0.049
1:500	24	10	0.106

***Table 4**. P-values from the ANOVA results for each factor*

Parameters	*P*-value
Antibody concentration	0.146
Anti-porcine gelatin incubation time	0.124
Porcine gelatin incubation time	0.117
Lack of fit	0.228

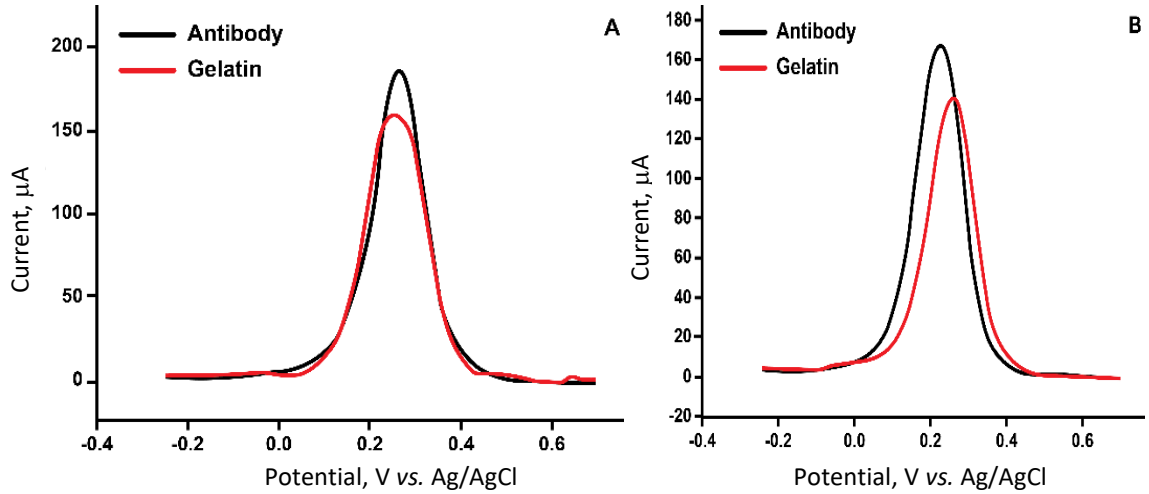

***Figure 5.** Characterization of BDD under optimal conditions using differential pulse voltammetry, and antibody incubation of (A) 60 min, (B) 8 hours, with a redox system of 40 µL 0.01 M ($K_3[Fe(CN)_6]$) in 0.1 M KCl, over a potential range of -0.3 to +0.7 V at a scan rate of 0.008 V/s*

Table 4 shows that the *P*-value for the lack of fit is 0.228, which is greater than the threshold of 0.05, indicating that the linear model generated is appropriate and does not significantly deviate from the experimental data. The lack-of-fit test is crucial in confirming whether the model adequately describes the relationship between the experimental factors and the response. Based on the Box-Behnken design analysis presented in Table 3, the optimum conditions were identified as follows: 500× antibody concentration, 8 hours of porcine anti-gelatin incubation, and 15 min of porcine gelatin incubation. However, given that an 8-hour incubation period is relatively long and conflicts to develop a rapid detection system, especially considering the target gelatin incubation time is only 15 min, the antibody incubation time was reduced to 60 min, while the gelatin concentration used was 0.5 ng/mL. The differential pulse voltammetry (DPV) results, shown in Figures 5A and 5B, confirmed that 60 min of antibody incubation was sufficient to produce a measurable distinction between the antibody and target binding events, supporting the feasibility of a more time-efficient detection protocol.

Analytical parameter

After determining the optimum experimental conditions, a calibration curve was constructed to evaluate the analytical performance of the developed porcine gelatin immunosensor, particularly its limit of detection (LoD). Porcine gelatin was tested at varying concentrations of 0.5, 5.0, 50.0, 500.0 and 5000.0 ng mL^{-1} using the optimized immunosensor platform. The electrochemical response was recorded *via* differential pulse voltammetry (DPV), focusing on the current difference between the signal generated after antibody immobilization and the signal after target (porcine gelatin) binding. The resulting data were plotted to produce a calibration curve (Figure 6), which yielded a linear regression equation of $y = 0.0355x + 0.0671$, with a correlation coefficient (R) of 0.9854 and a coefficient of determination (R^2) of 0.9711, indicating excellent linearity over the tested concentration range. In the calibration curve (Figure 6), the intercept ($a = 0.0671$) represents the potential influence of the sample matrix; a higher intercept suggests more pronounced matrix effects. Meanwhile, the slope ($b = 0.0355$) corresponds to the sensor's sensitivity, where a steeper slope reflects a more

responsive system. The strong linear relationship confirms the validity of the regression model, as evidenced by the high *R* value, which is close to 1. Based on calibration data, the immunosensor achieved a limit of detection of 142.15 pg mL^{-1}, demonstrating its high sensitivity for porcine gelatin detection using the BDD-modified electrode. Notably, although the LoD is slightly higher than that of a previously reported electrochemiluminescence biosensor (1 pg mL^{-1}) using carbon nanostructured materials [20], the method presented in this study offers significant advantages in terms of simplicity, cost-effectiveness, and the label-free detection approach. Unlike electrochemiluminescence (ECL) systems that require complex electrode fabrication and signal amplification strategies, this platform relies solely on direct electrochemical changes induced by the antigen-antibody interaction. Additional comparisons with other reported biosensing methods for porcine gelatin detection are summarized in Table 5, highlighting the competitiveness of this approach.

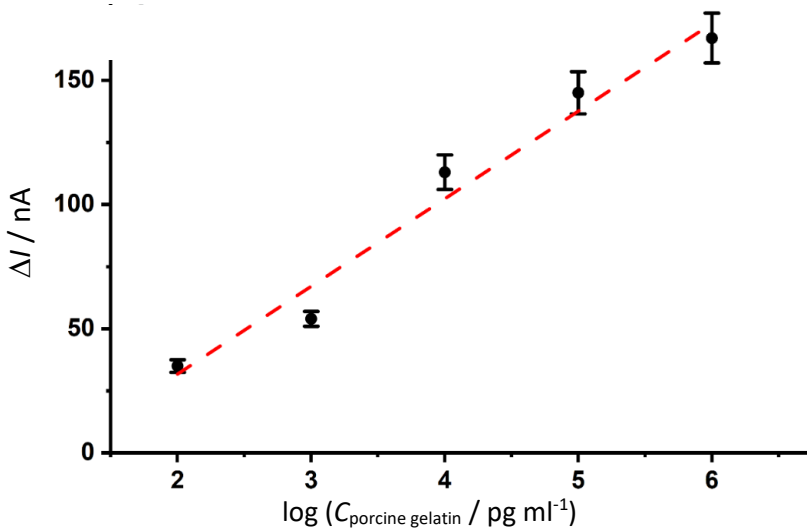

Figure 6*. Calibration curve of immunosensor with variation of concentration. The plot was ΔI of blank minus variation of the porcine gelatin concentration (50 to 5000000 pg/mL) versus logarithm of the concentration divided by 5*

Table 5. *Performance comparison of biosensors for detecting porcine gelatin*

No.	Technique	Electrode	Modification	LoD, pg mL^{-1}	Ref.
1	Electrochemical	Boron-doped diamond	Diazonium salt/anti-gelatin	142.15	This work
2	ECL	CNF-SPE	CNHs/NAF/anti-gelatin	1.00	[20]
3	Electrochemical	GCE	MWCNTs-CS-AuNus/anti-gelatin	23.079	[37]

With the optimal conditions established, a specificity test was conducted to evaluate the immunosensor's ability to selectively detect porcine gelatin in the presence of non-target substances. In this test, bovine gelatin was used as a negative control to assess whether it would elicit a response from the immobilized anti-porcine gelatin antibody [36]. The experiment was performed under identical conditions as the standard detection protocol, with bovine gelatin substituted as the target analyte. As shown in Figure 7, there was no significant decrease in the DPV current peak following the addition of bovine gelatin (± 7.3 %), indicating that it did not bind to the antibody-functionalized BDD electrode surface. This observation confirms the high specificity of the developed immunosensor, as the anti-porcine gelatin antibody exhibited no cross-reactivity toward bovine gelatin. The sensor's performance is attributed to the strong affinity and selectivity of antibody-antigen interactions, where the paratope of the antibody precisely recognizes the epitope of porcine gelatin. These results demonstrate that the immunosensor can discriminate between closely related proteins, making it highly reliable for food authentication and halal verification applications.

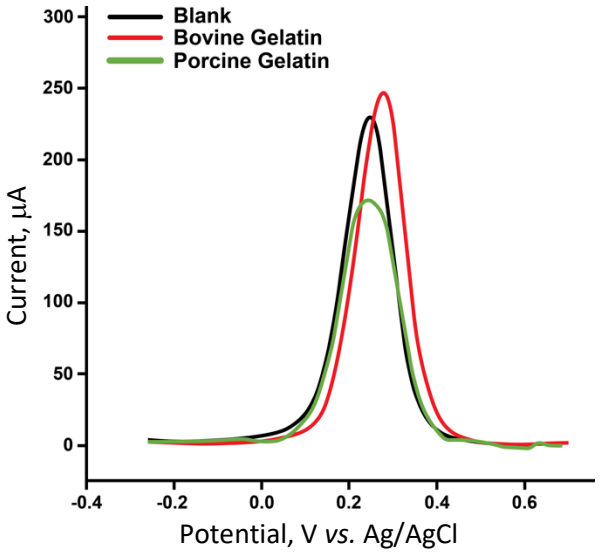

Figure 7. *Differential pulse voltammograms of blank versus target bovine gelatin and porcine gelatin for specificity test (500000 pg/mL) with a redox system of 40 µL 0.01 M ($K_3[Fe(CN)_6]$) in 0.1 M KCl, over a potential range of -0.3 to +0.7 V at a scan rate of 0.008 V/s*

Conclusion

In this study, a highly sensitive label-free electrochemical immunosensor was successfully developed for the detection of porcine gelatin using a BDD electrode modified *via* electrografting of aryl diazonium salt. The diazonium modification provided a stable and covalent platform for immobilizing Protein A, subsequently enabling the oriented attachment of anti-porcine gelatin antibodies. This surface chemistry strategy not only enhanced the binding efficiency of antibodies but also preserved their biological activity, leading to improved sensitivity and specificity of the sensor. The immunosensor's operational parameters were systematically optimized using the Box-Behnken experimental design, identifying the ideal conditions as 500× antibody concentration, 60 min of antibody incubation, and 15 min of porcine gelatin incubation. Under these conditions, the sensor exhibited excellent analytical performance, achieving a low detection limit of 0.569 ng/L. DPV measurements revealed a significant decrease in current upon target binding, confirming successful antigen-antibody interaction and effective sensor response. A specificity test using bovine gelatin demonstrated that the sensor selectively detects porcine gelatin, with no significant current change observed for the non-target protein. This confirms the high specificity of the anti-porcine gelatin antibody and the robustness of the immobilization strategy. Compared to previous biosensing approaches, especially those relying on optical methods such as colorimetry, the proposed electrochemical platform offers superior sensitivity, simpler sample preparation, and faster response time. Overall, the developed immunosensor holds significant potential for practical applications in food authentication, particularly in ensuring halal compliance and detecting prohibited or undeclared animal-derived ingredients in processed products. The use of BDD electrodes combined with diazonium-based surface functionalization offers a promising approach for future biosensor development targeting various biomolecules in complex matrices.

Conflict of Interest*: The authors have no conflict of interest.*

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